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ADENOSINE REGULATION OF DIPEPTIDYL PEPTIDASE IV EXPRESSION AND FUNCTION IN HUMAN COLORECTAL CARCINOMA CELLS

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Dipeptidyl peptidase IV (DPPIV/CD26) is a multifunctional cell-surface protein that is aberrantly expressed in many cancers and plays a key role in tumorigenesis and metastasis. Its diverse cellular roles include modulation of chemokine activity by cleaving dipeptides from the chemokine N-terminus, regulation of extracellular nucleoside metabolism by binding the ecto-enzyme adenosine deaminase (ADA), and interaction with the extracellular matrix by binding fibronectin and collagens.

The objective of this thesis was to determine whether adenosine, a purine nucleoside that is present at increased levels in the hypoxic tumor microenvironment, might affect the expression of functional DPPIV at the surface of human colorectal carcinoma cells. Adenosine persistently reduced DPPIV protein expression at the surface of HT-29 cells. This down-regulation of DPPIV occurred at adenosine concentrations comparable to those present within the extracellular fluid of colorectal tumors growing *in vivo*, and was not elicited by inosine or guanosine. Neither cellular uptake of adenosine nor its phosphorylation was necessary for the down-regulation of DPPIV.

The adenosine down-regulation of DPPIV from the surface of HT-29 cells occurred independently of classical adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3), and was instead mediated by a novel cell-surface mechanism that induced an increase in protein tyrosine phosphatase (PTP) activity. This increase in PTP activity decreased the tyrosine phosphorylation of ERK1/2 MAP kinase that, in turn, was linked to the decline in DPPIV. The down-regulation of DPPIV occurred independently of changes in the activities of protein kinases A or C, PI3K, other serine/threonine phosphatases, or the p38 or JNK MAP kinases.

The down-regulation of DPPIV protein at the cell surface was paralleled by decreases in DPPIV enzyme activity, binding of ADA, and the ability of cells to bind to extracellular matrix proteins. Adenosine, at concentrations that exist within solid tumors, therefore acts at the surface of colorectal carcinoma cells through a PTP-dependent mechanism to decrease the levels and functional activities of DPPIV. This down-regulation of DPPIV may increase the sensitivity of cancer cells to the tumor-promoting effects of adenosine and their response to chemokines and the extracellular matrix, facilitating their expansion and metastasis.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ADA	adenosine deaminase
ADA-L	large molecular weight ADA
ADA-S	small molecular weight ADA
ADCP	adenosine deaminase-complexing protein
Ado	adenosine
AK	adenosine kinase
Alloxazine	benzo[g]pteridine-2,4[1H,3H]-dione
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
bpV(phen)	bisperoxovanadium 1,10-phenanthroline
8-Br-cAMP	8-bromo-cyclic adenosine 3′,5′-cyclic monophosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cFN	cellular fibronectin
CD	cluster of differentiation antigen
CHO	Chinese hamster ovary cells
CNT	concentrative nucleoside transporter
CO_2	carbon dioxide
cpm	counts per minute

CSC	8-(3-chlorostyryl)caffeine
d	day
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
DPPIV	dipeptidyl peptidase IV
DSP	dual-specificity phosphatase
DTT	dithiothreitol
ei	NBTI-insensitive equilibrative nucleoside transporter
es	NBTI-sensitive equilibrative nucleoside transporter
ECM	extracellular matrix
EC_{50}	concentration producing half-maximal effect
EDTA	ethylenediaminetetraacetic acid
Enprofylline	3-propylxanthine
ENT	equilibrative nucleoside transporter
EPC	endothelial progenitor cell
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
Fas	fibroblast associated
g	gravitational force

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GEF	guanine nucleotide exchange factor
GIP	glucose-dependent insulinotropic polypeptide
GLP	glucagon-like peptide
h	hour
HEPES	N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]
HIF-1	hypoxia-inducible factor-1
HMVEC	human microvascular endothelial cells
HPLC	high performance liquid chromatography
HREC	human retinal endothelial cells
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
i.p.	intraperitoneal
IB-MECA	N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide
Ig	immunoglobulin
IL	interleukin
IP_3	inositol trisphosphate
JNK	c-Jun N-terminal kinase
M	mols/litre
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
min	minute

MKP	MAP kinase phosphatase
M-MLV	murine Maloney leukemia virus
MMP	matrix metalloproteinase
mpV(pic)	monoperoxo(picolinato)oxovanadate(V)
mRNA	messenger RNA
MRS1191	3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(+/-)-dihydropyridine-3,5-dicarboxylate
MRS1220	9-chloro-2-(2-furanyl)-5-[(phenylacetyl)amino][1,2,4]-triazolo[1,5-*c*]quinazoline
MRS1523	2,3-diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate
MT1-MMP	membrane type-1 matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide
n	number
NaOH	sodium hydroxide
NBTI	S(4-nitrobenzyl)-6-thioinosine
NCS	newborn calf serum
NECA	5′-*N*-(ethylcarboxamido)adenosine
NF-κB	nuclear factor-κB
NK	natural killer cell
NPY	neuropeptide Y
NSCLC	non-small cell lung carcinoma
5′-NT	5′-nucleotidase
pFN	plasma fibronectin

PBS	phosphate-buffered saline
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbal 12-myristate 13-acetate
PRL-3	phosphatase of regenerating liver-3
PTEN	phosphatase and tensin homologue deleted on chromosome 10
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PYY	peptide YY
RANTES	regulated on activation, normal T-cell expressed and secreted
RIA	radioimmunoassay
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
Rp-cAMPs	Rp diastereomer of adenosine cyclic 3′,5′-phosphorothioate
R-PIA	N^6-(L-2-phenylisopropyl)adenosine
RPTP	receptor-like protein tyrosine phosphatase
RT-PCR	reverse transcriptase-polymerase chain reaction
s.c.	subcutaneously
SAH	*S*-adenosylhomocysteine

SAHH	*S*-adenosylhomocysteine hydrolase
SCID	severe combined immunodeficiency disease
SDF-1α	stromal cell-derived factor-1α
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SHP	Src homology region 2 domain-containing phosphatase
siRNA	small-interfering RNA
Taq	*Thermophilus aquaticus* DNA polymerase
TCF/LEF	T-cell factor/lymphocyte enhancer factor
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of matrix metalloproteinases
TNF	tumor necrosis factor
TNM	tumor size, spread to the lymph nodes, and metastasis to distant sites (staging system)
TRAIL	TNF-related apoptosis-inducing ligand
uPA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor
Wnt	wingless

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CHAPTER 1

INTRODUCTION

Brief overview of dipeptidyl peptidase IV (DPPIV)

Dipeptidyl peptidase IV (DPPIV; EC 3.4.14.5) is a 110-kDa cell-surface peptidase that is normally expressed on epithelial cells of the intestine, kidney, liver, lung and prostate, fibroblasts, melanocytes, and certain leukocyte subsets (Houghton *et al.*, 1988; Dinjens *et al.*, 1989*a*; Morimoto and Schlossman, 1998). The enzyme cleaves NH_2-terminal dipeptides from polypeptides with either L-proline or L-alanine at the penultimate position, including glucagon-like peptides (Drucker, 2001) and certain chemokines and neuropeptides (De Meester *et al.*, 1999). DPPIV usually acts on such molecules to reduce their biological activities, but sometimes may enhance their action (Proost *et al.*, 2000). DPPIV also interacts with extracellular matrix molecules such as collagen (Bauvois, 1988; Hanski *et al.*, 1988) and fibronectin (Piazza *et al.*, 1989; Cheng *et al.*, 1998), in which context it plays a role in cell spreading and migration (Ghersi *et al.*, 2002). It is also the major binding protein for adenosine deaminase (ADA), and is identical to the ADA complexing protein (ADCP) or ADA binding protein (ADAbp) described in previous reports (Schrader and Pollara, 1978; Schrader *et al.*, 1990). DPPIV is also identical to the lymphocyte activation marker CD26, which has been shown to participate in the signal transduction process of T-cell activation (Morimoto and Schlossman, 1998; Dang and Morimoto, 2002).

Origins of DPPIV research

DPPIV was first described in 1966 by Hopsu-Havu and Glenner, and served primarily as a model protein for studies in membrane protein biochemistry. This involved the use of DPPIV to elucidate the mechanisms of membrane protein turnover (Kreisel *et al.*, 1980), glycosylation events (Yamashita *et al.*, 1988), and membrane polarization (Weisz *et al.*, 1992). However, the biological and functional importance of DPPIV independently originated from investigation of the adenosine deaminase (ADA) molecule. ADA catalyzes the irreversible deamination of the purine nucleoside adenosine to inosine, and is present at the cell surface, as well as in the cytoplasm of numerous cell types (Andy and Kornfeld, 1982; Daddona *et al.*, 1984; Schrader *et al.*, 1988). Studies of ADA activity were of great importance because infants deficient in this adenosine metabolizing enzyme often develop severe combined immunodeficiency disease (SCID; Giblett *et al.*, 1972), a disorder characterized by impaired development of B-cells and T-cells leading to compromised humoral and cell-mediated immunity, respectively. Determination of the structure of ADA from a variety of species provided evidence for substantial molecular heterogeneity. Multiple forms of ADA were demonstrated via ion exchange column chromatography, starch gel electrophoresis, and gel filtration methods (reviewed by Daddona and Kelley, 1980). Two major isozymic forms were identified: (1) small molecular weight ADA (ADA-S; ~ 43 kDa) and (2) large molecular weight ADA (ADA-L; ~ 280 kDa). It was subsequently found that ADA-S could be converted to ADA-L by a "factor" extracted from certain organs (Nishihara *et al.*, 1973).

ADA-S was determined to be a monomeric enzyme (Schrader *et al.*, 1976; Daddona and Kelley, 1977) while ADA-L was formed from the interaction of two

molecules of ADA-S with a single, homodimeric glycoprotein called adenosine deaminase-complexing protein (ADCP; Schrader and Bryer, 1982). The identification of ADCP began a long period of speculation about the exact physiological function of this protein. It was assumed that the major function of ADCP must be binding of ADA (Schrader *et al.*, 1983); thus, ADCP would perhaps represent the storage from of ADA and participate in the local metabolism of adenosine. ADCP was also speculated to serve as a receptor for recovery or removal of free ADA released into the circulation by cell damage (Schrader *et al.*, 1984; Schrader *et al.*, 1988). Through these early periods of research DPPIV was therefore known as ADCP, as well as the ADA binding protein (ADAbp) and the "conversion factor", given its property of converting monomeric ADA-S to ADA-L.

Convergence of the identities of DPPIV/ADCP (ADAbp/"conversion factor") and the immunological antigen CD26

In addition to the identification of DPPIV/ADCP and its significance in the field of ADA research, immunologists were independently studying a molecule termed CD26, which had been found to play a role in T cell activation (reviewed by Fleischer, 1994; Morimoto and Schlossman, 1998). Specifically, antibodies against the CD26 antigen enhanced the proliferative response of T cells to being triggered through either the T-cell receptor or CD2 pathways, while also increasing interleukin (IL)-2 production (Dang *et al.*, 1990*a*).

The idea that DPPIV/ADCP was identical to CD26 became clear in the early 1990s. Hegen *et al.* (1990) performed immunohistochemistry for CD26 and found its

tissue distribution to be very similar to that of DPPIV. Also, immunoprecipitation revealed an identical molecular mass of CD26 to DPPIV. Finally, enrichment of typical DPPIV dipeptidase activity was determined by affinity chromatography. This evidence strongly suggested that CD26 shared the same identity as DPPIV. In 1993, Kameoka *et al.* found that CD26 from T lymphocytes coprecipitated with a 43 kDa protein. Amino acid sequence analysis and immunoprecipitation showed that this molecule was ADA. They subsequently demonstrated specificity of the association of ADA with CD26 by *in vitro* binding assays. Thus, CD26 was now known to be identical to DPPIV/ADCP. At the same time, Morrison *et al.* (1993) were studying the role of ADCP as a specific marker of the malignant transformation of normal melanocytes to melanoma. They characterized ADCP by purification from human kidney and the analysis of tryptic peptides revealed complete homology to CD26.

In the current scientific community, 'DPPIV' is most commonly used in biochemical and cancer biology studies, while immunologists continue to use 'CD26' in their investigations. The nomenclature of ADCP and ADAbp has not been used since the time of these important reports.

DPPIV is located at the cell surface membrane

DPPIV was originally characterized as a soluble glycoprotein localized in the cytoplasm of tissues such as kidney, liver, and lung (Nishihara *et al.*, 1973; Van der Weyden and Kelley, 1976; Schrader and Stacy, 1979). However, later studies definitively established that DPPIV is primarily located at the cell surface membrane. Andy and Kornfeld (1982) examined the cellular localization of human ADAbp in human skin

fibroblasts and reported three pieces of evidence supporting its expression at the cell surface: (1) that binding protein "activity" was found in the membrane fractions following subcellular fractionation, (2) that an anti-ADAbp antiserum reacted with the cell surface of intact fibroblasts, and (3) the surface of intact fibroblasts specifically bound ^{125}I-labeled ADA that could be inhibited by pretreatment with the antiserum. DPPIV was also purified from the crude membrane fraction of human kidney (Schrader and Bryer, 1982) and human placenta (Trotta, 1982), and was identified at the surface of human renal carcinoma cells (Andy *et al.*, 1984) and melanocytes (Houghton *et al.*, 1988). In a very comprehensive study, Dinjens *et al.* (1989*a*) compared the quantities of ADCP in soluble and membrane fractions in various human tissues by radioimmunoassay (RIA). They found that the quantity of ADCP in the membrane fraction was consistently greater than the amount of ADCP obtained in the cytoplasm. This preferential membrane localization was confirmed by immunohistochemistry with very high ADCP levels detected in the kidney, liver, lung, prostate, and gastrointestinal tract.

DPPIV expression in normal tissues

The expression of DPPIV in normal tissues has been evaluated in several species through the use of multiple detection methods. Van der Weyden and Kelley (1976) found that ADA-L *activity* predominates in the following human tissues (listed in descending order of activity): kidney, lung, liver, spleen, small intestine, appendix, and stomach. Schrader and Stacy (1979) used an immunoassay to detect *protein* and found a similar order of expression: DPPIV levels were highest in the kidney cortex, followed by kidney medulla, lung, liver, spleen, serum, and saliva. In rabbit tissue DPPIV expression was

studied using immunohistochemistry and was detected in four exocrine glands: pancreas (pancreatic acinar cells), small intestine (Brunner's glands), salivary glands (submaxillary gland), and liver (bile canaliculi; Schrader and West, 1985). Dinjens *et al.* (1989*b*) examined the expression of DPPIV in murine tissues (mouse and rat) by immunohistochemistry and found the pattern of expression to be the same as expression in humans. DPPIV immunoreactivity was strongly detected in the kidney cortex (brush border epithelial cells), prostate (apical membrane and cytoplasm of glandular epithelial cells), small and large intestine, and liver. The identical nature of DPPIV expression between human and murine tissue was also supported by Hong *et al.* (1989) by means of immunoblot and Northern analysis of rat tissues.

Cell types that express DPPIV

DPPIV is strongly expressed by cells of the absorptive epithelia (e.g. kidney, lung, and intestinal epithelial cells; Schrader and Stacy, 1979; Dinjens *et al.*, 1989*a*). Expression of DPPIV has also been found at the surface of the following specific cell types: melanocytes (Houghton *et al.*, 1988; Morrison *et al.*, 1993), skin fibroblasts (Andy and Kornfeld, 1982; Houghton *et al.*, 1988), keratinocytes (Reinhold *et al.*, 1998), fibroblast-like synoviocytes (Riemann *et al.*, 2001), vascular endothelial cells (Cheng *et al.*, 1998; Aimes *et al.*, 2003), glomerular endothelial cells (Pala *et al.*, 2003), pancreatic islet cells (Grondin *et al.*, 1999), placental cytotrophoblasts (Imai *et al.*, 1994) and extravillous trophoblasts (Sato *et al.*, 2002), ovarian luteal cells (Fujiwara *et al.*, 1992), and mesothelial cells of the peritoneal cavity (Kajiyama *et al.*, 2002*a*). DPPIV is also expressed on a number of cells of the immune system, including T lymphocytes

(Fleischer, 1994), B lymphocytes (Bühling *et al.*, 1995; Bauvois *et al.*, 1999), and natural killer (NK) cells (Bühling *et al.*, 1994; Yamabe *et al.*, 1997).

Structure of DPPIV

The complete cDNA and derived amino acid sequence for DPPIV/CD26 were first published by Darmoul *et al.* and Tanaka *et al.* in 1992. DPPIV is a 110-kDa type II transmembrane glycoprotein of 766 amino acids belonging to the serine protease family. It is anchored to the plasma membrane by a single hydrophobic segment located at the NH_2-terminus, and has a short cytoplasmic tail of only 6 amino acids (see Figure 1.1). The majority of the DPPIV molecule is extracellular and exists as a homodimer at the cell surface. A flexible stalk links the membrane anchor to three separate extracellular domains: (1) a membrane-proximal glycosylation domain, (2) an intermediate cysteine-rich domain, and (3) a C-terminal catalytic domain (Tanaka *et al.*, 1992; Lambeir *et al.*, 1997). The predicted amino acid sequence of human DPPIV shows a high degree of conservation between different species. There is approximately 85% homology with the rat (Hong and Doyle, 1988; Tanaka *et al.*, 1992) and mouse DPPIV protein (Marguet *et al.*, 1992).

The C-terminal catalytic domain contains a catalytic site, Gly-Trp-Ser(630)-Tyr-Gly at positions 628-632, that is typical of a serine protease consensus sequence (Gly-X-Ser-X-Gly), with Ser630 being assumed to be the catalytic residue (De Meester *et al.*, 1999). Dipeptidyl peptidase IV (DPPIV) activity cleaves NH_2-terminal dipeptides from polypeptides with either L-proline or L-alanine at the penultimate position (Tanaka *et al.*,

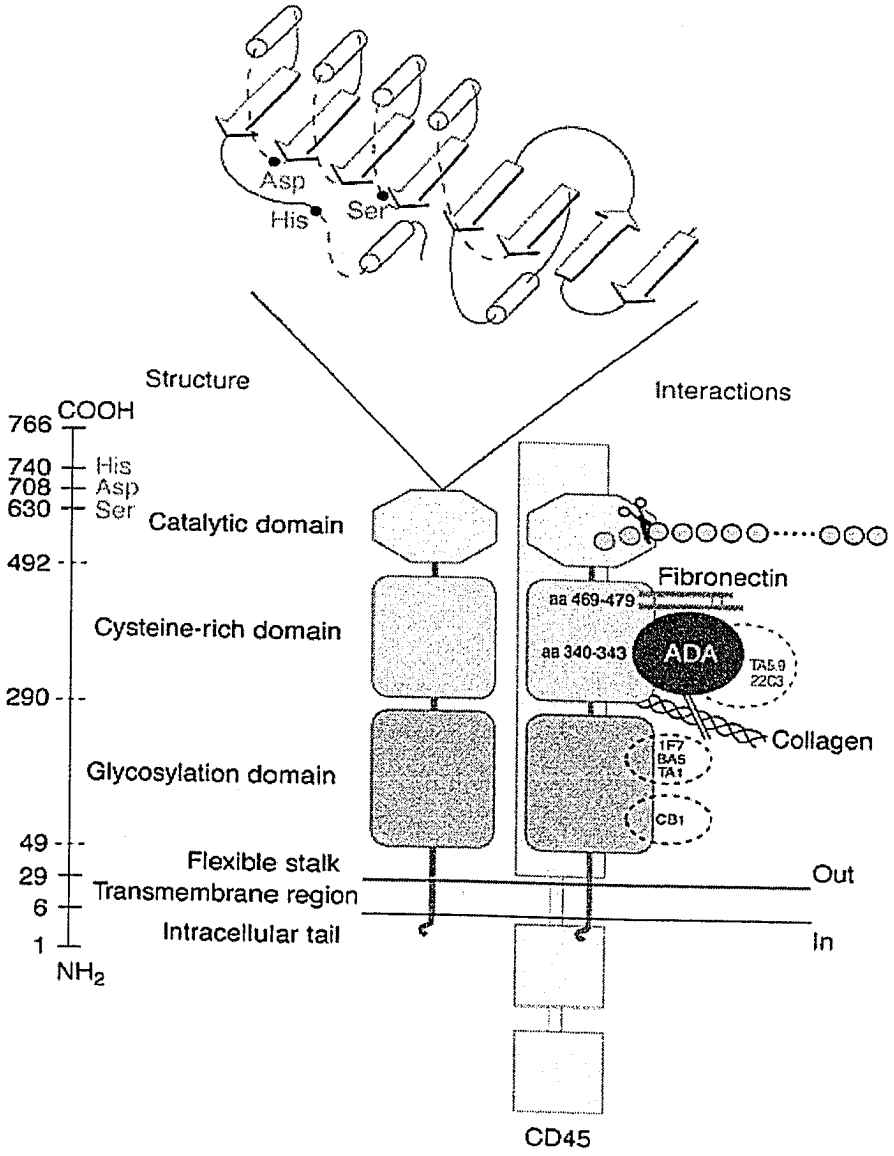

Figure 1.1 Schematic of the structure of DPPIV
(From, De Meester *et al.*, 1999)

1992). The intermediate cysteine-rich domain is responsible for protein-protein interactions of the DPPIV molecule, specifically to ADA and the ECM proteins collagen and fibronectin. Dong *et al.* (1997) identified the binding site for ADA on DPPIV and found that residues 340-343 are essential for ADA binding. Collagen binds in the cysteine-rich region between residues 238 and 495 (Löster *et al.*, 1995), whereas fibronectin binds residues 469 to 479 (Gonzalez-Gronow *et al.*, 1996). The membrane-proximal glycosylation domain of DPPIV is the target of post-translational glycosylation changes in the Golgi apparatus. Most of the available DPPIV monoclonal antibodies are directed towards this region (Hühn *et al.*, 1999).

DPPIV/CD26 also interacts with the membrane-linked tyrosine phosphatase CD45 that is solely expressed at the T lymphocyte cell surface (Torimoto *et al.*, 1991). The CD26-CD45 interaction takes place via the CD26 cytoplasmic domain, and might play a role in CD26-mediated T cell activation (Ishii *et al.*, 2001).

DPPIV dipeptidyl peptidase activity

DPPIV has a unique enzyme activity that cleaves NH_2-terminal dipeptides from polypeptides with either L-proline or L-alanine at the penultimate position (Tanaka *et al.*, 1992). A penultimate NH_2-terminal proline or alanine is commonly present in a number of chemokines (including SDF-1α/CXCL12 and RANTES), pancreatic polypeptides (NPY and PYY), and members of the glucagon family (GLP-1, GLP-2, and GIP). Several members of each of these peptide families have been identified as being subject to DPPIV-mediated cleavage *in vitro*. Furthermore, N-terminally truncated forms of these peptides are also naturally occurring and have been isolated alongside their full-length

forms (Proost *et al.*, 1998; Marguet *et al.*, 2000; Zhu *et al.*, 2003; reviewed in De Meester *et al.*, 1999). The substrates that are most readily cleaved by DPPIV dipeptidase activity are SDF-1α/CXCL12 and GLP-1 (Proost *et al.*, 1998; Marguet *et al.*, 2000; Lambeir *et al.*, 2001).

For many substrates, cleavage by DPPIV results in significant functional changes in receptor binding and/or biological activity. An initial report by Oravecz *et al.* (1997) showed that the receptor specificity of the chemokine RANTES is altered by DPPIV-mediated cleavage, thereby abolishing signaling through the CCR1 and CCR3 chemokine receptors while maintaining signaling through the CCR5 receptor. Other chemokines that exhibit altered receptor specificity and biological activity following DPPIV-mediated cleavage include macrophage-derived chemokine (MDC; Lambeir *et al.*, 2001) and LD78β (Proost *et al.*, 2000). Alternatively, the chemotactic activities of full-length SDF-1α and eotaxin are abolished following their cleavage by membrane-bound DPPIV (Shioda *et al.*, 1998; Struyf *et al.*, 1999). Truncation by DPPIV of pancreatic polypeptides (NPY and peptide YY) has also been found to alter receptor selectivity, and changes their biological activity from vasoconstriction to growth factor activity (Zukowska-Grojec *et al.*, 1998). Given the large number of potential peptide substrates for DPPIV, the functional specificity of its dipeptidase activity is most likely regulated by the site of DPPIV expression and by the availability of local substrates.

Although a large number of studies have investigated the impact of DPPIV-mediated cleavage on various peptide substrates *in vitro*, the physiological function of DPPIV activity remains to be determined. Some understanding of the physiological role of DPPIV dipeptidase activity has already been gained through the study of animal

models. Studies of DPPIV knockout mice have revealed functions for DPPIV activity in blood glucose regulation and the immune response (Marguet *et al.*, 2000; Yan *et al.*, 2003). Targeted inactivation of the DPPIV gene yields fertile and healthy mice that have normal blood glucose levels in the fasted state, but accelerated blood glucose clearance after a glucose challenge. This is thought to occur because of increased levels of intact glucagon-like peptide-1 (GLP-1), which would normally be subject to N-terminal cleavage by DPPIV (Marguet *et al.*, 2000). These findings were confirmed when pharmacological inhibition of DPPIV activity also improved glucose tolerance in diabetic animals, which occurred through augmentation of endogenous levels of intact GLP-1 (Reimer *et al.*, 2002). Studies of the immune response in DPPIV knockout mice after immunization with pokeweed mitogen revealed reduced serum levels of IgG and IgE antibodies, as well as reduced IL-2, IL-4, and delayed IFN-γ production (Yan *et al.*, 2003). Expression of DPPIV was also found to be important in the regulation of development, maturation, and migration of $CD4^+$ T cells and natural killer (NK) cells. Thus, the data from DPPIV knockout mice complements *in vitro* studies in which DPPIV activity was found to augment IL-2 production and T-cell activation following immune stimulation (Tanaka *et al.*, 1993).

DPPIV and cancer

DPPIV is frequently expressed aberrantly in solid cancers, and may influence both the genesis of the primary tumor and its subsequent capacity to spread from the initial site. In many cases, tumorigenesis is accompanied by a reduction in the expression of DPPIV, as for example in melanoma (Houghton *et al.*, 1988; Morrison *et al.*, 1993)

and prostate cancer (Dinjens *et al.*, 1990; Bogenrieder *et al.*, 1997). This reduced DPPIV expression is directly associated with carcinogenesis, since inducible gene transduction of DPPIV into melanoma cells dramatically reverses the malignant phenotype (Wesley *et al.*, 1999). Decreased levels of DPPIV have also been linked to increased invasion and metastasis (Dinjens *et al.*, 1990; Pethiyagoda *et al.*, 2001; Kajiyama *et al.*, 2002*b*).

A detailed background on the *expression of DPPIV in tumor tissue*, particularly in colorectal tumors, is provided in the Introduction section of Chapter 2. An overview of the *role of DPPIV functional activities* (ADA binding, dipeptidase activity, and ECM binding) *in tumor progression* is provided in the Introduction section of Chapter 4.

The role of DPPIV in tumor progression

The majority of studies investigating the role of DPPIV in tumor progression have taken the approach of reexpressing DPPIV or overexpressing DPPIV in carcinoma cell lines that normally express low levels or lack expression of DPPIV protein. This strategy has been applied to cell lines derived from tumors that have been reported to express reduced (or absent) levels of cell-surface DPPIV, including melanoma, prostate, non-small cell lung carcinoma (NSCLC), ovarian, and endometrial carcinomas (Wesley *et al.*, 1999; Pethiyagoda *et al.*, 2001; Kajiyama *et al.*, 2002*b*; Kajiyama *et al.*, 2003; Mizokami *et al.*, 2004; Wesley *et al.*, 2004; Wesley *et al.*, 2005). The effect of *increasing* DPPIV expression in these cell lines has been measured using common functional assays of cancer cell behavior, such as migration and invasion assays and determining changes in cell proliferation.

The impact of DPPIV reexpression in melanoma has been studied in great detail. DPPIV, at that time ADAbp, was originally identified as a phenotypic marker to discriminate between normal melanocytes and melanoma cells (Houghton *et al.*, 1988). The differential expression of DPPIV was of great significance because its pattern of expression was not associated with the stage of melanocyte differentiation; instead, it was specifically down-regulated during melanocyte transformation. It was later found that loss of DPPIV expression during the melanocyte-to-melanoma transformation occurred concomitantly with the emergence of exogenous growth factor independence and appearance of specific chromosomal abnormalities (Morrison *et al.*, 1993). To determine the role of DPPIV in regulating the malignant phenotype, Wesley *et al.* (1999) stably reexpressed DPPIV into melanoma cell lines at levels comparable to those found in normal melanocytes using the tetracycline-inducible expression system. Significantly, DPPIV reexpression induced a profound change in cell phenotype to that of normal melanocytes. In addition, the cells demonstrated reduced tumorigenicity when inoculated *s.c.* into athymic nude mice, loss of anchorage-independent growth, and a return to dependence on exogenous growth factors for survival as determined by a loss in cell viability when grown in serum-free media. In a similar study by Pethiyagoda *et al.* (2001), DPPIV reexpression into melanoma cell lines, also to levels comparable to normal epidermal melanocytes, significantly reduced cellular invasiveness in Matrigel invasion assays. The collective results of these studies have implicated a strong tumor suppressor function when DPPIV expression is maintained in normal melanocytes.

A consistent down-regulation of cell-surface DPPIV has been reported in NSCLC cell lines (Wesley *et al.*, 2004), which was found to be regulated at the level of mRNA. In

this study DPPIV was also reexpressed into NSCLC cell lines by the tetracycline-inducible system and changes in cell function were assessed. Similar to melanoma, DPPIV reexpression in NSCLC cells caused a change towards a more normal epithelial morphology. DPPIV reexpression also diminished anchorage-independent cell growth, suppressed tumorigenicity when injected *s.c.* into nude mice, restored dependence on exogenous growth factors for survival, and decreased cellular motility.

In prostate cancer, decreased DPPIV expression was recently identified in a panel of progressively more metastatic cell lines (Wesley *et al.*, 2005). Interestingly, this loss of DPPIV was associated with increased basic fibroblast growth factor (bFGF) production in metastatic prostate carcinoma cells. Tetracycline-inducible reexpression of DPPIV into prostate carcinoma cells blocked evidence of downstream signaling from bFGF, including a reduction in bFGF levels, decreased ERK1/2 activation, and decreased levels of urokinase-type plasminogen activator (uPA). These changes were accompanied by induction of apoptosis, cell cycle arrest, and decreased *in vitro* cell migration. The expression of DPPIV in normal prostate cells might therefore inhibit the malignant phenotype by restraining inappropriate activation of the bFGF signaling pathway.

Recent reports of DPPIV overexpression in ovarian carcinoma cell lines have also added to our knowledge of the role of DPPIV in tumor progression. Kajiyama *et al.* (2002*b*) initially found that cell-surface DPPIV expression in ovarian carcinoma cells is inversely correlated with invasive potential. They also observed that DPPIV-positive cell lines were of epithelial, "cobblestone" morphology as opposed to the fibroblast-like morphology of DPPIV-negative cell lines. Overexpression of DPPIV into SKOV3 ovarian carcinoma cells did not alter the growth properties of these cells, differing from

the growth inhibition observed in melanoma, NSCLC, and prostate carcinoma cells. However, DPPIV overexpression triggered significant morphological change from long-bipolar, spindle-shaped fibroblast-like cells with an unorganized growth pattern to the cobblestone-like morphology in which cell-cell adhesion was present. This was accompanied by a dramatic decrease in cell invasiveness and reduction of pro-MMP-2 activity by gelatin zymography. In an animal model, DPPIV overexpression inhibited peritoneal dissemination following *i.p.* injection as a measure of peritoneal metastasis in nude mice. This same group explored the mechanism of the anti-invasive function of DPPIV in ovarian carcinoma. They found that expression of E-cadherin was positively correlated with DPPIV expression in a series of ovarian carcinoma cell lines (Kajiyama *et al.*, 2003). Importantly, DPPIV overexpression up-regulated E-cadherin levels while reducing levels of MMP-2 and membrane type-1 matrix metalloproteinase (MT1-MMP). Levels of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and TIMP-2 were also increased upon DPPIV overexpression. These studies have suggested a role for DPPIV in preventing invasive and metastatic potential of ovarian carcinoma cells by normalizing aberrant regulation of E-cadherin and favoring reduced activity of MMPs.

A final study of DPPIV overexpression has also been reported in endometrial carcinoma by Mizokami *et al.* (2004). DPPIV overexpression reduced the basal level of cell proliferation by endometrial carcinoma cells. There was also a change in cell morphology following DPPIV overexpression indicated by increased evidence of cell-cell adhesion. Immunocytochemical staining found DPPIV localized on the surface of transfected cells, particularly at cell-cell junctions.

Adenosine

Adenosine is a purine nucleoside composed of a nitrogenous base and a pentose sugar unit (ribose) in the absence of phosphate groups. Nucleotides are mono-, di-, or triphosphate esters of nucleosides (Champe and Harvey, 1994). Purines and pyrimidine nucleotides are involved in most cellular processes and their roles range from structural, metabolic or energetic, to regulatory. Adenosine is a building block for biologically relevant molecules, including adenosine triphosphate (ATP), nucleic acids, and nicotinamide adenine dinucleotide (NAD^+). The regulatory effects of adenosine occur in multiple organ systems and tissues where it is a known regulator of inflammation, neurotransmission, cardioprotection, cerebroprotection, and pain (reviewed by Fredholm *et al.*, 2000; Klotz, 2000).

Adenosine production and metabolism

Adenosine is formed both intracellularly as well as extracellularly. Intracellular production of adenosine is mediated by the enzyme 5′-nucleotidase (5′-NT), which dephosphorylates AMP to adenosine, or by the hydrolysis of *S*-adenosylhomocysteine (SAH). Intracellular adenosine can be transported outwards to the extracellular space and extracellular adenosine can be transported into the cell via adenosine membrane transporters. This transport is mediated primarily through the action of bidirectional, sodium-independent equilibrative nucleoside transporters (ENTs; Griffith and Jarvis, 1996). To date, four equilibrative nucleoside transporters have been cloned and are termed ENT1-ENT4 (reviewed by Damaraju *et al.*, 2003). ENT1 and ENT2 are expressed at the cell surface of tissues and mediate the transport of adenosine down the

concentration gradient (Griffiths *et al.*, 1997*a*; Griffiths *et al.*, 1997*b*; Yao *et al.*, 1997). Thus, changes in the intracellular concentration of adenosine rapidly influence the extracellular levels of adenosine. ENT1 and ENT2 are subdivided based on their sensitivities (ENT1) or resistance (ENT2) to inhibition by S(4-nitrobenzyl)-6-thioinosine (NBTI; Griffith and Jarvis, 1996). However, both transporters can be inhibited by the drugs dipyridamole and dilazep (Visser *et al.*, 2002). The two isoforms also differ in their pattern of expression; ENT1 appears to be ubiquitously expressed while ENT2 is less broadly distributed in tissues (Pennycooke *et al.*, 2001). The roles of ENT3 and ENT4 in adenosine transport processes have not yet been established, although ENT3 appears to have an intracellular location on lysosomal cell membranes (Baldwin *et al.*, 2005).

Ecto-5′-nucleotidase (Ecto-5′-NT/CD73) is a membrane-bound glycoprotein that functions to produce adenosine in the extracellular space. Extracellular generation of adenosine via ecto-5′-NT depends on the availability of extracellular adenine nucleotides (e.g. AMP and ATP). The source of these extracellular adenine nucleotides is often via passive efflux resulting from local tissue injury, necrosis, hypoxia, and/or ischemia (Fowler, 1993; Wallman-Johansson and Fredholm, 1994; Braun *et al.*, 1998; Latini *et al.*, 1999). Thus, there is likely to be a significant source of extracellular nucleotides available within solid tumors owing from the frequency and severity of these processes in tumor tissue (Vaupel *et al.*, 1989).

The local production of adenosine is rapidly counteracted by its metabolism. Adenosine kinase (AK) is involved in the phosphorylation of adenosine to AMP, while adenosine deaminase (ADA) deaminates adenosine to inosine. AK and ADA have very different kinetic properties, such that the fate of adenosine is primarily determined by its

local concentration. The K_m of AK is low (~ 40 nM; Spychala *et al.*, 1996) and is therefore responsible for metabolizing adenosine at physiological concentrations, typically below 1.0 μM. In contrast, the K_m for ADA is high (~ 70 μM; Lloyd and Fredholm, 1995), which allows ADA to function as a high volume/low affinity system that becomes active at high concentrations of adenosine. Figure 1.2 summarizes the above-described mechanisms of adenosine production and metabolism.

Adenosine production during hypoxia and in solid tumors

The mechanisms regulating the elevation and accumulation of extracellular adenosine during hypoxia are largely dependent on the ratios of activities of the adenosine producing (5′-NT, ecto-5′-NT, SAHH) and adenosine metabolizing (AK, ADA) enzymes. There appears to be a coordinate alteration in adenosine-producing and metabolic activities during hypoxia that favors adenosine production. Thus, chronic hypoxia decreases the enzymatic activities of ADA and AK, while stimulating the activity of intracellular and ecto-5′-NT (Kobayashi *et al.*, 2000; Spychala *et al.*, 2000). Interestingly, a similar coordinated shift in adenosine-metabolizing enzyme levels and activities has also been observed in HL-60 lymphoid cells upon PMA-induced differentiation (Spychala *et al.*, 1997).

Elevated activity of ecto-5′-NT has been found in tumor tissues of breast (Canbolat *et al.*, 1996), pancreatic (Flocke and Mannherz, 1991), and gastric cancer (Durak *et al.*, 1994). Experimental studies have documented that the activity of ecto-5′-NT is up-regulated in response to hypoxia (Headrick and Willis, 1989; Kobayashi *et al.*, 2000). Recently, Synnestvedt *et al.* (2002) established a direct link between hypoxia and

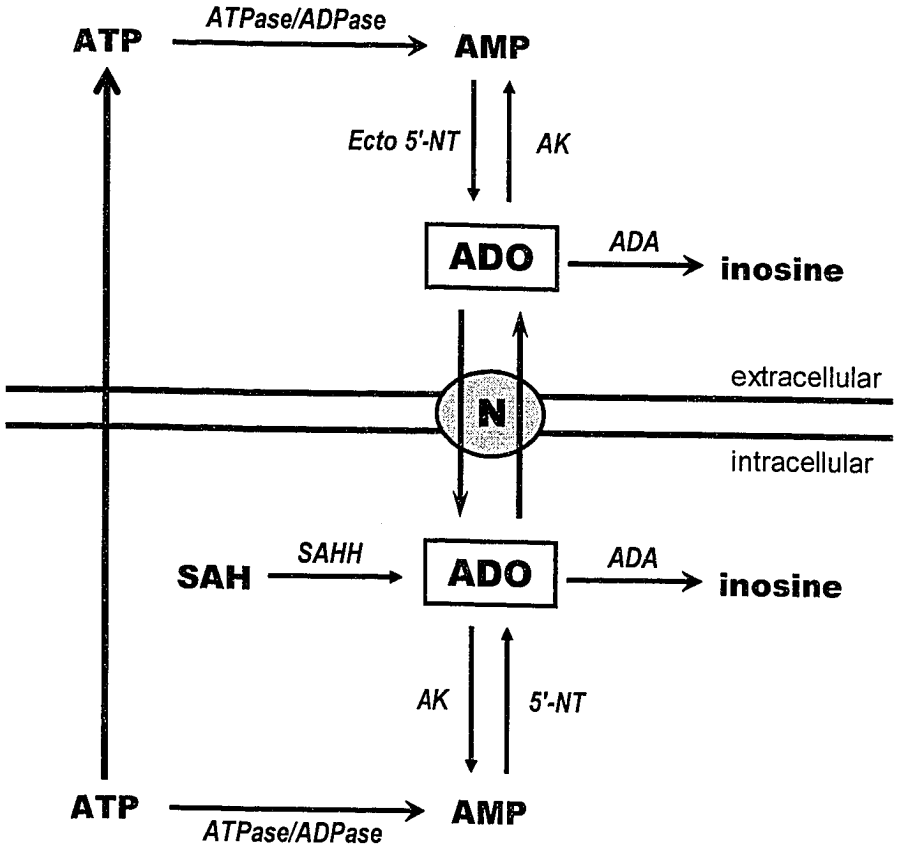

Figure 1.2 Adenosine production and metabolism.

Adenosine can be formed intracellularly in two ways. Firstly, by the conversion of *S*-adenosylhomocysteine (SAH) via SAH hydrolase (SAHH), and secondly by the conversion of adenosine monophosphate (AMP) to adenosine via 5′-nucleotidase (5′-NT). Adenosine kinase (AK) phosphorylates adenosine to AMP. 5′-NT is also present outside the cell (ecto-5′-NT), thereby allowing adenosine to be formed extracellularly from adenosine triphosphate (ATP). Adenosine is metabolized inside and outside the cell by adenosine deaminase (ADA) and ecto-ADA, respectively. Furthermore, adenosine can be transported bidirectionally via nucleoside transporters (N).

ecto-5′-NT expression by demonstrating that the expression of ecto-5′-NT is dependent on transcription by hypoxia-inducible factor-1 (HIF-1). Interestingly, adenosine itself can also induce ecto-5′-NT mRNA, surface expression and function (Narravula *et al.*, 2000). This represents an example of transcriptional induction of an enzyme by the enzymatic product.

Hypoxia decreases AK activity (Decking *et al.*, 1997), and also triggers a down-regulation of AK protein expression at the transcriptional level in PC12 pheochromocytoma cells (Kobayashi *et al.*, 2000). The hypoxia-induced inhibition of AK activity causes the amplification of small changes in free AMP into a major rise in adenosine levels. This occurs because AK is the rate-limiting enzyme in adenosine metabolism (due to its low K_m of ~ 40 nM) that phosphorylates adenosine at physiological concentrations (Lloyd and Fredholm, 1995). Down-regulation of ADA by chronic hypoxia has also been found in PC12 cells (Kobayashi *et al.*, 2000) with consequences of reduced conversion of adenosine to inosine.

Interestingly, chronic hypoxia has also been found to down-regulate expression of the nucleoside transporter ENT1 on PC12 cells (Kobayashi *et al.*, 2000). This reduction of ENT1 significantly reduced adenosine *uptake* from the extracellular medium. Although it is generally thought that nucleoside transporters work bidirectionally, many studies have suggested that they primarily mediate uptake, and not efflux, of adenosine. Accordingly, it has been shown that dipyridamole and NBTI can cause an increase in extracellular adenosine under various conditions, including hypoxia (Craig and White, 1993).

The concentration of adenosine within the extracellular fluid of hypoxic solid tumors is indeed elevated compared to normal tissue. Blay *et al.* (1997) used microdialysis to measure the levels of adenosine in the extracellular fluid of murine solid tumors grown *in vivo* and reported levels 10- to 20-fold higher than those measured in normal tissue. Adenosine levels as high as 10 μM were measured in the combined presence of inhibitors for AK (5′-iodotubercidin) and ADA (coformycin). The inhibition of ADA alone caused a much greater increase in measured adenosine levels compared to the independent inhibition of AK. This result confirms that adenosine is preferentially metabolized via ADA under hypoxic conditions where adenosine levels are elevated.

Adenosine production via signaling mechanisms

Increased generation of adenosine in solid tumors has typically been regarded as a "passive" consequence of hypoxia, ischemia, and necrosis without direct association to the process of tumorigenesis. However, recent studies have suggested that increased potential to generate adenosine may constitute an important phenotypic change acquired during tumor progression.

Spychala and Kitajewski (2004) determined that the genes encoding ecto-5′-NT and ADA contain T-cell factor/lymphocyte enhancer factor (TCF/LEF) consensus binding sites and are subject to reciprocal regulation by the Wnt/β-catenin signaling pathway. The Wnt signaling pathway has been linked to the development of a variety of human tumors, including colon, prostate, breast, and ovarian carcinomas (reviewed by Dihlmann and von Knebel Doeberitz, 2005). Wnt/β-catenin signaling induces expression of ecto-5′-NT while decreasing levels of ADA. This coordinate change was accompanied

by a 3-fold increase in the generation of extracellular adenosine. Thus, an "active" transition to an adenosine-producing cell phenotype is induced through Wnt/β-catenin signaling, and may therefore be an important event during tumorigenesis.

Ecto-5′-NT expression has also been shown to be negatively regulated by estrogen receptor (ER) signaling (Spychala *et al.*, 2004). ER-negative breast carcinoma cells were found to express high ecto-5′-NT mRNA and protein levels and produce up to 100-fold more adenosine from adenine nucleotides compared to ER-positive cells. Examination of clinical breast tumor samples also revealed a significant inverse correlation between ER and ecto-5′-NT expression. This suggests the intriguing possibility that ecto-5′-NT expression is directly linked to breast cancer progression. It also supports the emergence of an adenosine-producing cell phenotype during tumorigenesis.

Adenosine receptors

There are four known subtypes of cell-surface adenosine receptors, denoted A_1, A_{2A}, A_{2B}, and A_3. They are seven-transmembrane, G protein-coupled receptors, and each adenosine receptor subtype is characterized by unique molecular and pharmacological properties (Fredholm *et al.*, 2001). The A_1 and A_2 receptors were identified and characterized by pharmacological methods and initially subdivided on the basis of their inhibiting and stimulating adenylyl cyclase activity, respectively (Van Calker *et al.*, 1978; Van Calker *et al.*, 1979; Londos *et al.*, 1980). Pharmacological studies revealed that A_2 receptors were heterogeneous, which led to the subdivision into A_{2A} and A_{2B} receptor subtypes. Although postulated for a number of years, only discovery by cloning

confirmed the existence of the A_{2B} and A_3 adenosine receptor subtypes (Pierce *et al.*, 1992; Zhou *et al.*, 1992; Salvatore *et al.*, 1993).

Collectively, the expression of adenosine receptors is widespread such that adenosine is capable of controlling the function of virtually every organ and tissue. The localization of adenosine receptors and their signaling mechanisms is summarized in Table 1.1. A_1 adenosine receptors are expressed strongly in certain regions of the brain, including the cortex, cerebellum, and hippocampus (Fredholm *et al.*, 2000; Fredholm *et al.*, 2001). However, expression of these receptors can also be detected in most other tissues of the body. A_1 receptors signal through $G_{i/o}$, leading to the inhibition of adenylyl cyclase, activation of K^+ channels, and modulation of Ca^{2+} mobilization and phospholipase C (PLC) activity. The signal transduction from A_1 receptors mediates multiple effects, such as the depression of neurotransmission, antinociception, bronchoconstriction, and ischemic preconditioning.

A_{2A} adenosine receptors are the higher affinity A_2 receptors. They are strongly expressed in the spleen, thymus, leukocytes, platelets, and striatal GABAergic neurons (Fredholm *et al.*, 2001). Coupling of A_{2A} receptors with G_s enhances adenylyl cyclase activity and intracellular cAMP production upon receptor binding. The A_{2A} receptor functions to regulate the inhibition of platelet aggregation, vasodilatation, and protect against ischemic damage (Klotz, 2000).

The A_{2B} adenosine receptor displays the lowest affinity for adenosine of all receptor subtypes. A_{2B} receptors are thought to be activated only at exceptionally high concentrations of adenosine, i.e. under pathophysiological rather than physiological conditions. They appear to be expressed highest in the caecum, bladder, colon, as well as

Table 1.1 G protein-coupling and tissue distribution of the four adenosine receptor subtypes.

Adenosine receptor subtype	G protein	Effects of G protein coupling	Sites of Tissue Distribution (Human)
A_1	G_i/G_o	↓ cAMP ↑ IP_3/DAG (PLC)	*High:* brain, spinal cord, eye, atria, adrenal glands *Intermediate:* skeletal muscle, liver, kidney, adipose tissue, colon, testis *Low:* lung, pancreas
A_{2A}	G_s	↑ cAMP	*High:* spleen, thymus, leukocytes, platelets, GABAergic neurons *Intermediate:* heart, lung, blood vessels *Low:* most brain regions
A_{2B}	G_s/G_q	↑ cAMP ↑ IP_3	*High:* colon, caecum, bladder *Intermediate:* lung, blood vessels, eye, mast cells *Low:* brain, kidney, liver, ovary, pituitary gland, adrenal gland
A_3	G_i/G_q	↓ cAMP ↑ IP_3/DAG (PLC)	*Intermediate:* lung, cerebellum, hippocampus, pineal gland *Low:* other brain regions, thyroid, adrenal gland, spleen, liver, kidney, heart, intestine, testis

(Adapted from Fredholm *et al.*, 2000; Fredholm *et al.*, 2001)

the lung and blood vessels (Klotz, 2000; Fredholm *et al.*, 2001). Activation of A_{2B} receptors induces G_s-mediated activation of adenylyl cyclase and increased Ca^{2+} influx, as well as G_q-mediated activation of PLC. Involvement of A_{2B} receptors has been implicated in the regulation of vascular tone and mast cell degranulation.

The most recently discovered adenosine receptor is the A_3 receptor. Data regarding the expression profile of the A_3 receptor subtype indicate low-level expression in the thyroid, brain (cerebellum and hippocampus), spleen, liver, kidney, and intestine (Fredholm *et al.*, 2001). The expression of the A_3 receptor is also highly species-dependent. A_3 receptors couple to G_i and G_q, which results in reduced cAMP levels and increased PLC activity, respectively (Fredholm *et al.*, 2001). The A_3 receptor plays a role in lung inflammation and the protection of ischemic tissue.

In addition to cell-surface adenosine receptors, an intracellular adenosine reactive site has been identified. A region on the catalytic moiety of adenylyl cyclase, termed the "P-site", has been shown to bind adenosine with low affinity (EC_{50} ~ 80 μM; Johnson *et al.*, 1991). Interaction of adenosine with the P-site on adenylyl cyclase causes inhibition of the enzyme and a subsequent decrease of intracellular cAMP levels (Legrand *et al.*, 1990).

Expression of adenosine receptors and nucleoside transporters in tumor tissue

Tumor cells, such as the A375 human melanoma cell line (Merighi *et al.*, 2001), are capable of expressing all four subtypes of adenosine receptors. While adenosine has been reported to signal through each receptor subtype to produce various biological

responses in tumor cells, much less is known about the actual expression of adenosine receptors in tumor tissues.

The A_1 adenosine receptor is expressed in colorectal carcinomas (Khoo *et al.*, 1996) as determined by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. To date, there have been no published reports examining the expression of A_{2A} and A_{2B} adenosine receptors in tumor tissue. Recent reports have suggested that A_3 adenosine receptor expression is low in normal tissues (Fishman *et al.*, 2002*a*) and elevated on the surface of tumor cells (Merighi *et al.*, 2002; Madi *et al.*, 2003; Ohana *et al.*, 2003). This has led to the suggestion that A_3 adenosine receptor overexpression might be a good candidate as a tumor cell marker. Although Fredholm *et al.* (2001) reported the lack of A_3 adenosine receptor expression in the majority of normal tissues; they cautioned that this lack of expression might be underestimated because of the poor availability of good pharmacological tools for detection, particularly radioligands.

Two recent reports have reported increased expression of the A_3 adenosine receptor in tumor tissue compared to normal tissue. Madi *et al.* (2004) found that colon and breast carcinomas express higher A_3 adenosine receptor expression (determined by RT-PCR and Western blot analysis) compared to adjacent normal tissue. Interestingly, lymph node metastases expressed higher A_3 expression than primary tumor tissue. High A_3 receptor expression was associated with elevated nuclear factor-κB (NF-κB) and cyclin D1 levels, indicating a proliferating tumor cell population. Preliminary findings of increased A_3 expression were also found in small cell lung carcinoma, pancreatic carcinoma, and melanoma. Gessi *et al.* (2004) also found increased expression of A_3 adenosine receptors in colorectal carcinomas by radioligand binding,

immunohistochemistry, and real-time RT-PCR analyses. They reported a tendency for lower A_3 expression in less advanced tumor stages (TNM stages I, II) compared to the more pronounced expression in advanced stages (TNM stages III, IV), suggesting a potential role for the receptor in tumor progression.

Levels of ENT1 protein have been studied in solid tumor tissues, although there is no clear consensus regarding its expression in tumor versus normal tissue. Goh *et al.* (1995) reported the overexpression of ENT1 in human breast, colorectal, liver, and stomach tumor tissues. However, Pennycooke *et al.* (2001) reported reduced levels of ENT1 in colorectal tumor tissues, overexpression in stomach tumors, and highly variable expression in breast tumor tissues. The same group also found very low ENT2 expression in most tumor types that were comparable to the low levels in normal tissue.

Tumor-promoting functions of adenosine

There is a wide body of evidence that supports an important role for adenosine in promoting tumor progression. Research in this area has emphasized three major functions of adenosine: (1) the role of adenosine in inhibition of anti-tumor immune responses, (2) direct effects of adenosine on the growth of cancer cells, and (3) adenosine modulation of the angiogenic process.

1. Adenosine suppresses the anti-tumor immune response

A number of studies over the last ten years have demonstrated that adenosine has the ability to suppress multiple components of the anti-tumor immune response. Significantly, the levels of adenosine found within the extracellular fluid of hypoxic solid tumors are sufficient to trigger these inhibitory activities (Blay *et al.*, 1997).

Adenosine suppresses T-cell activation (Hoskin *et al.*, 1994; Hoskin *et al.*, 2002), as indicated by decreased T-cell proliferation in response to mitogenic anti-CD3 antibody, antigen-specific proliferation, and interleukin-2 (IL-2) and interferon-γ (IFN-γ) synthesis. Adenosine also interferes with the adhesion of T lymphocytes with carcinoma cell targets (MacKenzie *et al.*, 1994) via a mechanism that involves inhibition of integrin function on the lymphocyte cell surface (MacKenzie *et al.*, 2002). In addition, adenosine has been found to inhibit granule exocytosis of natural killer cells (Williams *et al.*, 1997) and lymphocyte-mediated cytolysis of tumor cell targets (Hoskin *et al.*, 1994; Koshiba *et al.*, 1997; Hoskin *et al.*, 2002). This reduction in lymphocyte tumoricidal activity by adenosine has been demonstrated through decreased activity in cytotoxicity assays, and is associated with reduced expression of granzyme B, perforin, Fas ligand, and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL; Koshiba *et al.*, 1997; Hoskin *et al.*, 2002).

2. Adenosine regulates the growth of tumor cells

The role of adenosine in modulating the proliferation of tumor cells is unclear and conflicting. The effect on cell growth seems to depend on the concentration of adenosine and the adenosine receptor subtype that is activated. For example, Tey *et al.* (1992, 1994) identified a biphasic growth response to adenosine in which low concentrations of adenosine (~ 10 μM) inhibited A431 human epidermoid carcinoma cell growth while high concentrations (up to 100 μM) stimulated growth of the same cell type. These investigators implicated the A_1 adenosine receptor subtype for the inhibitory response and the A_2 receptor subtype for the stimulatory response. In another example, Merighi *et al.* (2002) found that adenosine promotes cell proliferation via the A_{2A} receptor but

impairs cell proliferation via A_3 adenosine receptors in A375 human melanoma cells. A_1 and A_{2B} adenosine receptors expressed on these same cells were not involved in growth regulation.

The number of studies implicating a role for adenosine in *stimulating* tumor cell proliferation is almost counterbalanced by the number of reports that claim an *inhibitory* role of adenosine. Studies from our laboratory have shown that adenosine *itself*, as opposed to the use of stable synthetic analogues of adenosine (e.g. NECA), is consistently stimulatory to the growth of human colorectal and breast carcinoma cells at concentrations that are present within the tumor extracellular environment (Mujoomdar *et al.*, 2003; Mujoomdar *et al.*, 2004). Multiple cell lines differing in origin and the degree of cellular differentiation were used for both tumor types. The stimulatory effect of adenosine involved progression through the cell cycle and a genuine increase in cell number. Adenosine-induced proliferation of human colorectal carcinoma cells was also confirmed in adenosine deprivation studies, in which treatment of cultures with ADA reduced carcinoma cell growth in a comparable panel of cell lines (Lelièvre *et al.*, 1998*a*; Lelièvre *et al.*, 1998*b*).

Inhibition of tumor cell growth by adenosine itself has been reported in certain studies (e.g. Fishman *et al.*, 1998; Fishman *et al.*, 2000), while adenosine may also trigger apoptosis in cancer cells under specific conditions (Barry and Lind, 2000; Merighi *et al.*, 2002). Other studies have demonstrated inhibition of tumor cell growth using stable analogues of adenosine that typically do not have the same relative affinity for different adenosine receptor subtypes and are often selective for specific receptor subtypes (e.g. Ishii and Green, 1973; Colquhoun and Newsholme, 1997). In particular,

the A_3-selective adenosine receptor agonist IB-MECA has been shown to inhibit the proliferation of B16-F10 mouse melanoma cells (Fishman *et al.*, 2002*b*; Madi *et al.*, 2003) and PC-3 human prostate carcinoma cells (Fishman *et al.*, 2003). The inhibition of carcinoma cell growth in these studies was reversed by antagonism of the A_3 adenosine receptor, indicating specificity of the IB-MECA effect. Lu *et al.* (2003) demonstrated that IB-MECA inhibits the growth of MCF-7 and ZR-75 while inducing apoptosis in T47D and Hs578T human breast carcinoma cell lines. *However*, it is important to note that these cell lines were not found to express the A_3 adenosine receptor subtype and instead IB-MECA down-regulated expression of the estrogen receptor α. This example of a non-specific effect by a selective adenosine receptor agonist highlights the importance of studying the role of the authentic adenosine molecule in tumor cell function.

3. The role of adenosine in angiogenesis

Angiogenesis is the process by which hypoxic solid tumors induce the development of new blood vessels from the *pre-existing* vasculature. This differs from the process of vasculogenesis which involves the *de novo* differentiation of endothelial cells from mesodermal precursors. Angiogenesis is of fundamental importance for the continued growth of the primary tumor beyond the small tumor volume (~ 1-2 mm^3) that is achieved in the absence of angiogenesis, as well as for metastasis to occur to secondary sites (reviewed by Hanahan and Folkman, 1996). The most prominent regulator of angiogenesis is vascular endothelial growth factor (VEGF), which is upregulated by tumor hypoxia (Plate *et al.*, 1992). VEGF expression is induced by the transcription factor hypoxia inducible factor-1α (HIF-1α; Liu *et al.*, 1995; Forsythe *et al.*, 1996), after which it interacts with VEGF receptors to transduce signals mediating endothelial cell

proliferation, migration, organization into functional vessels, and remodeling of the vessel network (reviewed by Veikkola and Alitalo, 1999; Hicklin and Ellis, 2005).

Adenosine appears to participate in a number of important processes required during the angiogenic process. Synthesis of VEGF in response to adenosine or adenosine analogues has been shown in human retinal endothelial cells (HRECs; Grant *et al.*, 1999) and pig cerebral microvascular endothelial cells (Fischer *et al.*, 1995). However, adenosine has also been found to decrease VEGF mRNA and protein levels in PC12 pheochromocytoma cells (Olah and Roudabush, 2000). In addition to the ability of adenosine to regulate VEGF expression in different cell types, adenosine can directly induce the proliferation of endothelial cell cultures. Ethier *et al.* (1993) initially reported that adenosine stimulates proliferation of human umbilical vein endothelial cells (HUVECs), and later found that this effect was independent of adenosine receptors (Ethier and Dobson, 1997). Another study, however, implicated the role of A_{2A} adenosine receptors in HUVEC proliferation (Sexl *et al.*, 1995). A role for A_{2B} adenosine receptors has been found in growth stimulation of HRECs (Grant *et al.*, 2001), and porcine and rat arterial endothelial cells (Dubey *et al.*, 2002). Consistent with the multiple actions of adenosine in angiogenesis, signaling via A_{2B} receptors was also found to increase HREC migration and enhanced capillary tube formation on a basement membrane matrix (Grant *et al.*, 2001).

Other effects of adenosine in tumor progression

The major tumor-promoting functions of adenosine appear to be inhibition of anti-tumor immune responses and regulation of tumor cell proliferation and angiogenesis.

Adenosine also stimulates the motility of melanoma and colon carcinoma cells (Woodhouse *et al.*, 1998; M. Mujoomdar and J. Blay, unpublished data). Furthermore, A_1 adenosine receptor activation has been found to increase secretion of matrix metalloproteinase-2 (MMP-2) from human trabecular meshwork cells of the eye (Shearer and Crosson, 2002). The stimulation of tumor cell motility and degradation of the ECM (via MMP activity) by adenosine could contribute to the early stages of invasion and subsequent metastatic spread.

Objectives

1. To determine if adenosine regulates the cell-surface expression of dipeptidyl peptidase IV (DPPIV) on human colorectal carcinoma cells.
2. To identify whether classical adenosine receptors participate in the adenosine regulation of DPPIV on human colorectal carcinoma cells.
3. To determine the specific signal transduction pathway(s) by which adenosine regulates levels of cell-surface DPPIV.
4. To explore whether adenosine regulation of DPPIV levels at the surface of human colorectal carcinoma cells is associated with changes in the functions of the protein, specifically DPPIV dipeptidase activity, adenosine deaminase (ADA) binding, and/or extracellular matrix (ECM) protein interactions.

Overall hypothesis

Adenosine, at concentrations present within the extracellular fluid of solid tumors, regulates the cell-surface expression of dipeptidyl peptidase IV (DPPIV) on human colorectal carcinoma cells. The adenosine regulation of DPPIV will be specifically mediated by adenosine receptors, and will have impact on the three major DPPIV functional activities: **(1)** DPPIV dipeptidase activity, **(2)** adenosine deaminase (ADA) binding, and **(3)** extracellular matrix (ECM) protein interactions. Adenosine regulation of these DPPIV functions at the cell-surface will bring about changes in colorectal carcinoma cell behavior.

CHAPTER 2

ADENOSINE DOWN-REGULATES THE CELL-SURFACE EXPRESSION OF DIPEPTIDYL PEPTIDASE IV ON HT-29 HUMAN COLORECTAL CARCINOMA CELLS

Portions of this chapter appeared in the following publication:

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INTRODUCTION

Background of DPPIV expression as an indicator of malignant transformation

Changes in the expression of DPPIV between normal and tumor tissue were observed early on in this field of research. In 1973, Nishihara *et al.* reported the absence of "conversion factor" (that converts ADA-S to ADA-L) in an extract of lung tumor tissue, and it was speculated that lung tissue might be deprived of the capacity to express the conversion factor during the malignant transformation. It was later found that DPPIV was not expressed at the surface of certain hepatocellular carcinoma cell lines compared to its abundant expression on hepatocyte plasma membranes (Hixson *et al.*, 1983). This altered expression of DPPIV was suggested to be associated with the abnormal tissue architecture and growth patterns exhibited by hepatocellular carcinomas. Decreased expression of cell-surface DPPIV was also observed on colon and kidney carcinoma cell lines compared to primary cells (Trotta and Balis, 1978; Herbschleb-Voogt *et al.*, 1983).

Altered expression of DPPIV in solid cancers

DPPIV is frequently expressed aberrantly in solid cancers. In most tumor types, the malignant transformation is accompanied by a reduction in the expression of DPPIV. This occurs in both malignant melanoma (Houghton *et al.*, 1988; Morrison *et al.*, 1993; Van den Oord, 1998) and metastatic prostate cancer (Dinjens *et al.*, 1990; Bogenrieder *et al.*, 1997).

The change in DPPIV expression is most striking in melanoma. Houghton *et al.* (1988) initially found that cell-surface DPPIV is *specifically* down-regulated and completely extinguished during the process of melanocyte transformation. This loss of

DPPIV was independent of the stage of cellular differentiation and was not regulated as a function of cell proliferation. The data supporting loss of DPPIV expression was very convincing – DPPIV expression was detected in 51 melanocyte cultures from fetal, newborn, and adult skin vs. the absence of DPPIV on the surface of 102 melanoma cell lines derived from both primary and metastatic lesions. A study correlating DPPIV expression with the histological stage of tumor progression of malignant melanoma confirmed that loss of DPPIV is indeed associated with tumor progression (Van den Oord, 1998). Although this study detected expression of DPPIV in a low percentage of invasive melanoma lesions, there was no DPPIV expression occurring in metastatic melanomas.

The loss of DPPIV in prostate cancer is less complete than the striking absence in melanoma. Immunohistochemical studies have revealed that prostate carcinomas exhibit different and distinct DPPIV expression patterns, classified as: (1) diffuse cytoplasmic, (2) membranous, (3) cytoplasmic and membranous, and (4) absence of DPPIV (Dinjens *et al.*, 1990). There is an inverse correlation between levels of membrane DPPIV and metastatic potential. Conversely, non-metastatic prostate tumors were found to exclusively express DPPIV at the cell surface. A more recent study by Bogenrieder *et al.* (1997) reported that DPPIV is expressed in nearly all primary prostate tumors while only 50% of metastases to the lymph node, bone, and soft tissue express DPPIV. More specifically, the metastases absent for DPPIV expression almost exclusively occurred in patients that had hormone-refractory disease as opposed to hormone-naïve prostate cancer. This strongly suggests that the loss of DPPIV is involved in the development of metastatic prostate cancer.

Aberrant expression of DPPIV, generally tending towards a reduction in expression, also occurs in ovarian (Rao *et al.*, 1990; Kajiyama *et al.*, 2003) and endometrial carcinomas (Khin *et al.*, 2003). Rao *et al.* (1990) found that ~ 30% of ovarian carcinomas are absent for DPPIV expression. Of the remaining tumors, DPPIV was expressed frequently by well-differentiated and poorly invasive, stage I carcinomas. This finding has been supported by a recent study by Kajiyama *et al.* (2003), and also occurs in the case of endometrial carcinomas (Khin *et al.*, 2003).

Hepatocellular carcinomas, similar to prostate tumors, exhibit different expression patterns of DPPIV (Stecca *et al.*, 1997). However, the direction of change in overall DPPIV expression is highly variable. Only a small percentage (~ 10%) of tumors are completely negative for DPPIV while the remaining tumors either express abnormally high levels of DPPIV or contain slightly reduced levels of DPPIV.

In contrast to the *reduced* expression of DPPIV found in melanoma, prostate, ovarian and endometrial carcinomas, thyroid carcinomas instead express *enhanced* levels of DPPIV (Kotani *et al.*, 1991; Tanaka *et al.*, 1995; Aratake *et al.*, 2002). Normal thyroid tissue and benign neoplasms do not express DPPIV as assessed by Northern blotting, DPPIV activity staining, and immunohistochemistry. DPPIV is, however, expressed on virtually all cases of differentiated thyroid carcinoma and is a strong candidate as a diagnostic marker for this malignancy.

DPPIV expression in colon cancer

The first study of DPPIV expression in colon tumors found a change in the proportion of ADA-S to ADA-L in the direction of *reduced ADA-L* compared with

normal adjacent tissue (Trotta and Balis, 1978). From this finding, it was initially proposed that there was diminished synthesis and/or biological activity of DPPIV in the malignant cell, and this reduction of DPPIV might be a property of colorectal tumors.

A series of papers in the 1980s that studied DPPIV directly argued that colorectal tumors display variable expression of DPPIV (ten Kate *et al.*, 1984; ten Kate *et al.*, 1985; ten Kate *et al.*, 1986). Colorectal tumors have been found to express decreased, unchanged, or increased amounts of DPPIV. There is also a small fraction (~ 10-15%) of tumors that do not express DPPIV. *The existence of this variable expression within colorectal tumors suggests that local influences may regulate the expression of DPPIV.* However, very little is known about the endogenous factors that might regulate the surface expression of DPPIV on tumor cells.

In this chapter, we consider whether exposure to the purine nucleoside adenosine, which is present in solid tumors at elevated concentrations, might impact upon DPPIV expression on colorectal carcinoma cells. We have found that chronic treatment with adenosine markedly down-regulates the surface expression of DPPIV protein on HT-29 human colorectal carcinoma cells.

MATERIALS AND METHODS

Materials

HT-29 human colorectal carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Media and inosine were from ICN Biomedicals (Irvine, CA). Culture vessels (Nunc) and sera were purchased from Invitrogen Canada (Burlington, Ontario, Canada). Adenosine, guanosine, 5′-iodotubercidin, dilazep, dipyridamole, S(4-nitrobenzyl)-6-thioinosine (NBTI), and calf spleen ADA were from Sigma Chemical Co. (St. Louis, MO). Coformycin was from Calbiochem (San Diego, CA). Mouse anti-human monoclonal antibody (mAb) against DPPIV/CD26 (clone M-A261) and mouse IgG_1 (clone W3/25) isotype control were from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). Rabbit anti-bovine ADA antibody was from Alpha Diagnostic International (San Antonio, TX). ^{125}I-labeled sheep anti-mouse IgG, $F(ab')_2$ fragment and ^{125}I-labeled goat anti-rabbit IgG, $F(ab')_2$ fragment were obtained from PerkinElmer Life Sciences (NEN, Boston, MA). FITC-conjugated rabbit anti-mouse IgG was from PharMingen (San Diego, CA).

Cell culture and drug treatments

HT-29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, without antibiotics) supplemented with 10% $^{v}/_{v}$ heat-inactivated newborn calf serum (NCS) and maintained as stocks in 80-cm^2 flasks at 37°C in a humidified atmosphere of 90% air/10% CO_2. Cells for use in binding assays were seeded into 48-well plates at 50,000 cells/well and allowed to adapt to culture for 48 h. Cultures were then changed to medium containing 1% NCS for another 48 h, and then treated with drugs or control

vehicle for evaluation of changes in DPPIV protein expression. Adenosine uptake inhibitors were added to cultures 15 min prior to the addition of the relevant adenosine treatment. The common solvent dimethyl sulfoxide (DMSO) produced marked down-regulation of DPPIV at concentrations above 0.5% ($^{v}/_{v}$), and led to significant changes even at lower concentrations (10-12% at a final concentration of 0.25% ($^{v}/_{v}$) DMSO). For this reason, the final DMSO concentration was always kept less than 0.1% ($^{v}/_{v}$), or drugs were dissolved in ethanol, which did not affect DPPIV levels even when added to cultures at a final concentration of 5% ($^{v}/_{v}$).

Radioantibody binding assay for DPPIV

The culture plates were placed on ice, and all subsequent washes and incubations were carried out at 4°C. Cells were washed with 250 µl of phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and then incubated with 125 µl PBS containing 1% BSA and primary antibody or isotype control. After incubation for 60 min, the wells were washed twice as before and further incubated with 125 µl PBS containing 1% BSA and 1.2 µCi/ml ^{125}I-labeled sheep anti-mouse IgG, F(ab')$_2$ fragment, for 60 min. The monolayers were then washed three times and solubilized in 400 µl of 0.5 M NaOH for 60 min at room temperature before the counting of radioactivity. Figure 2.1 shows the binding of anti-DPPIV mAb to HT-29 cells with increasing antibody concentration. The specific binding of anti-DPPIV was better than 94% at saturating antibody concentrations. Maximal specific antibody binding was reached at 2 µg/ml, which was the concentration used in all subsequent assays.

Assays to assess the involvement of DPPIV antibody epitope blocking

To exclude the possibility of interference from molecules such as ADA that might be bound to DPPIV, we used acid-stripping to remove ADA and other bound ligands from the cell surface (Blay and Brown, 1985; Saura *et al.*, 1998). Use of acetate buffer was avoided because this causes the release of intracellular ADA (Senesi *et al.*, 1988). Briefly, cell monolayers that had been treated with adenosine for 48 h were washed and then incubated for 5 min at 4°C with 200 µl of serum-free medium that had been adjusted to the required pH using concentrated hydrochloric acid. After washing twice with PBS containing 0.2% BSA, the radioantibody binding assay was carried out as usual. To assess the effect of prior ADA loading on DPPIV immunoreactivity, cell monolayers were incubated for 60 min at 4°C with 10 µg/ml ADA (determined to be a saturating concentration of exogenous ADA; see Figure 4.1) before the DPPIV binding assay.

Flow cytofluorimetric detection of DPPIV

HT-29 cells were trypsinized from 80-cm^2 culture flasks, washed and resuspended (10^6 cells) in filter-clarified PBS with 2.5% BSA and 0.2% sodium azide containing anti-DPPIV mAb (1 µg/10^6 cells) for 45 min at 4°C. The cells were washed twice with the same buffer and then incubated with FITC-secondary mAb conjugate (1 µg/10^6 cells) for 40 min at 4°C. After three further washes the cells were fixed in 1% paraformaldehyde and stored in the dark at 4°C until analyzed. Flow cytofluorimetric analysis was carried out with a FACScan (BD Immunocytometry Systems, Mountain View, CA) flow cytometer equipped with a 15-mW argon laser operating at a wavelength of 488 nm and detection at 680 nm. Ten thousand events were counted for each sample. Data were

analyzed using Lysis II software.

Semi-quantitative RT-PCR

RT-PCR was used to evaluate DPPIV mRNA regulation by adenosine. Total cellular RNA was extracted from HT-29 cells at the indicated time points using TRIzol™ reagent according to the manufacturer's instructions. Reverse transcription of total RNA to complementary DNA (cDNA) was performed by combining 5 μg of total RNA, 4 μl of 5× first strand buffer, 1 μl of dNTPs (10 mM), 2 μl of DTT (100 mM), 1 μl of oligo$(dT)_{12\text{-}18}$ primer (0.5 mg/ml), 1 μl of Maloney murine leukemia virus (M-MLV) reverse transcriptase (200U/μl), and sterile water up to 20 μl in a microcentrifuge tube. Samples were then incubated for 1 h in a 37°C water bath, followed by inactivation of the reaction for 10 min at 70°C. Samples were stored at -20°C until use.

PCR reactions were performed by combining 1 μl of cDNA sample with 5 μl 10× PCR buffer, 1 μL of dNTPs (10 mM), 1μl of $MgCl_2$ (50 mM), 0.5 μl of each primer (forward and reverse), 0.4 μl of native *Taq* DNA polymerase (5U/μl), and sterile pyrogen-free water to a total volume of 50 μl. PCR mixtures were overlaid with 100 μl of mineral oil and PCR amplification was performed in an automatic thermal cycler (MJ Research, Watertown, MA). The following primer sequences were used for the PCR (the product size is given after the reverse primer):

GAPDH (forward), 5′-TGGAAATCCCATCACCATCT-3′ and

GAPDH (reverse), 5′-GTCTTCTGGGTGGCAGTGAT-3′ (351 bp);

DPPIV (forward), 5′-CAAATTGAAGCAGCCAGACA-3′ and

DPPIV (reverse), 5′-CAGGGCTTTGGAGATCTGAG-3′ (354 bp).

The amplification protocols for GAPDH (26 cycles) and DPPIV (24 cycles) were as follows: an initial denaturation step at 95°C for 5 min, followed by denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and primer extension at 72°C for 1 min. PCR amplification was concluded with a final extension step at 72°C for 10 min. The number of PCR amplification cycles was initially determined to yield PCR products during the linear range of amplification. RT-PCR performed under these conditions provides reliable detection of 2-fold or greater differences in mRNA levels (Singer-Sam *et al.*, 1990). The amplification products were visualized on a 1.5% agarose gel containing ethidium bromide (0.2 μg/ml). Relative levels of PCR products were quantified by densitometric analysis of gel photographs and normalization to steady-state expression of GAPDH. All of the reagents used for RNA isolation and RT-PCR were from Invitrogen Canada (Burlington, Ontario, Canada).

Statistical analysis

The figures show representative results from at least three separate experiments. Data were evaluated using a two-tailed Student's *t*-test for unpaired data.

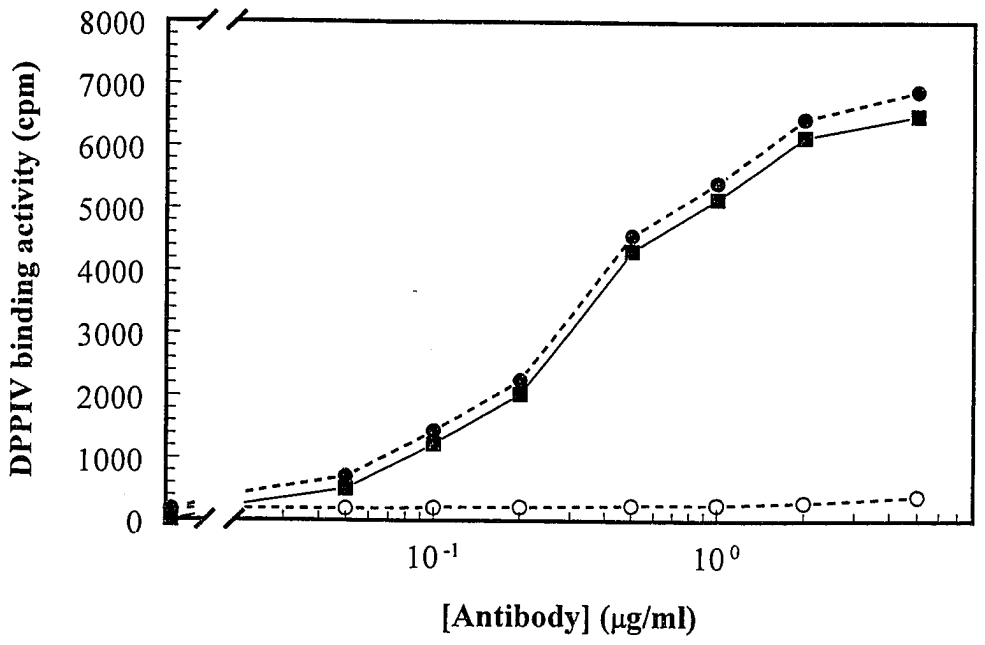

Figure 2.1 Measurement of cell-surface DPPIV on HT-29 cells by indirect radioantibody binding assay.

Confluent monolayer cultures of HT-29 cells were incubated with either anti-DPPIV mAb (total binding, •) or isotype control (non-specific binding, ○), followed by ^{125}I-labeled anti-mouse IgG, F(ab')$_2$ fragment as described in Materials and Methods. Points are mean values, $n = 4$. Standard errors fall within the symbols. The third curve (■) shows the specific binding (total binding – non-specific binding).

RESULTS

Short-term exposure to adenosine increases cell-surface DPPIV protein expression

We first investigated the effect of adenosine on the surface expression of DPPIV protein by HT-29 human colorectal carcinoma cells. Monolayer cultures of HT-29 cells were treated with a single high dose (300 μM) of adenosine and cell-surface DPPIV protein was detected on these monolayers using a radioantibody binding assay. We chose this high concentration of adenosine to allow for its rapid metabolism in culture ($t_{½}$ ~ 120 min for HT-29 cells; Mujoomdar *et al.*, 2003) while avoiding the use of inhibitors of adenosine metabolism. Treatment of HT-29 cells with 300 μM adenosine does not reduce cellular viability (E. Garcia del Busto, M. Mujoomdar and J. Blay, unpublished data), as determined by either the MTT assay (Blay and Poon, 1995) or DNA fragmentation (JAM) assay (Matzinger, 1991). Similarly, Höpfner *et al.* (2001) reported only moderate toxicity (~ 10%) of HT-29 cells in response to prolonged exposure (4 days) to 500 μM adenosine. Based on the lack of toxicity of adenosine at our starting concentration (300 μM), we did not normalize our early binding assay data to cell number. However, all binding assay results presented in Chapters 3 and 4 were normalized to cell number.

Adenosine (300 μM) produced an early significant increase in cell-surface DPPIV protein that was evident at 1 h (Figure 2.2). However, this adenosine up-regulation of DPPIV was only transient; persisting until the 4 h time point, after which time no difference was found at the 8 h time point. This short-term, modest increase in DPPIV (~ 10%) was observed when experiments were conducted using both confluent and subconfluent cell densities.

Long-term adenosine treatment down-regulates cell-surface DPPIV protein expression

Given the transient nature of the increase in DPPIV expression due to adenosine over this time period, we next investigated the effect of adenosine on HT-29 cells in response to longer term adenosine treatment. We again treated the cells with a single 300 μM dose of adenosine. The level of cell-surface DPPIV protein was significantly decreased after 12 h and maximally reduced by 48 h, at which time DPPIV expression was reduced by ~ 30% (Figure 2.3). The reduction of DPPIV by this single dose of adenosine persisted to at least 72 h. Interestingly, the baseline expression of DPPIV increased substantially (approximately 2-fold) over the 72 h time course (Figure 2.4) and the adenosine reduction of DPPIV occurred relative to this increase. This elevation of baseline DPPIV levels was not a product of increasing cell number. Under the incubation conditions used, the mean increase in cell number over 48 h was only 8.0 ± 0.36% (mean ± SE, $n = 3$).

To confirm the down-regulation of DPPIV by a single dose of adenosine (300 μM) at 48 h we performed flow cytofluorimetry to detect DPPIV on trypsinized cells in suspension. Adenosine-treated cells showed a reduction in the total proportion of DPPIV-expressing cells compared to vehicle-treated cells (~ 3-fold leftward shift in the mean cellular fluorescence after adenosine treatment; Figure 2.5). There was no change in the fluorescence profile of antibody isotype controls. The reduced antibody binding to HT-29 cells in suspension indicated that DPPIV was not being masked within the cell monolayer following adenosine treatment. This also validated our use of the radioantibody binding assay as an appropriate method to quantify the adenosine regulation of cell-surface DPPIV expression on HT-29 monolayer cultures. The radioantibody binding assay and

48 h time point were used in all further studies of the adenosine down-regulation of DPPIV protein.

The adenosine down-regulation of DPPIV is not due to masking by DPPIV-binding proteins

We next considered whether the reduction in DPPIV immunoreactivity measured in our radioantibody binding assay or flow cytofluorimetry was perhaps caused by blocking of the antibody epitope due to binding of ADA or another protein(s) to DPPIV (e.g. collagen and fibronectin). Two different methods were used to exclude this possibility. We first explored the possibility that adenosine might facilitate the binding of ADA to DPPIV at the cell surface. To test this we examined whether the adenosine down-regulation of DPPIV immunoreactivity would be reduced or even eliminated by further ADA loading of DPPIV. In this approach we exposed adenosine-pretreated (48 h) HT-29 cell monolayers to 10 μg/ml calf spleen ADA for 60 min at 4°C. This ADA incubation condition is known to saturate the ADA binding capacity of DPPIV on HT-29 cells (see Figure 4.1). ADA loading significantly interfered with the measurement of DPPIV: there was a 47.6 ± 2.7% reduction in DPPIV immunoreactivity (3 separate experiments). Because the ADA loading was performed at 4°C to prevent internalization, this decrease in DPPIV immunoreactivity was likely due to partial blocking of anti-DPPIV antibody binding to its target, rather than an authentic down-regulation of DPPIV protein. However, ADA loading did not alter the size of the DPPIV reduction in response to adenosine treatment (Figure 2.6).

We also evaluated the possibility that a protein(s) other than ADA might be interfering with DPPIV antibody binding. In this second approach we subjected the cells to acid stripping. This is a technique that is widely used to dissociate various ligands, in addition to ADA, from their cell-surface binding proteins (Blay and Brown, 1985; Saura *et al.*, 1998; Senesi *et al.*, 1988; Sarret *et al.*, 2002). Acid stripping progressively reduced the level of DPPIV immunoreactivity by 4.6%, 15.6%, and 18.3% at pH 4.2, 3.1, and 2.6 respectively. However, there was absolutely no effect on the measured decrease due to adenosine (Table 2.1). These data strongly indicated that the down-regulation of DPPIV protein observed using either the binding assay or flow cytofluorimetry represented an authentic decrease in cell-surface DPPIV protein and not blocking by DPPIV-binding protein(s).

The adenosine regulation of DPPIV protein occurs at the level of mRNA

We conducted a preliminary exploration of the molecular mechanism through which adenosine was participating to down-regulate levels of DPPIV protein at the cell surface. The expression of DPPIV mRNA was evaluated by semi-quantitative RT-PCR in response to a single 300 μM dose of adenosine. Similar to DPPIV protein, the baseline level of DPPIV mRNA increased over the 48 h time course. There was a decrease in DPPIV mRNA levels relative to control after 8 h exposure to adenosine, and which continued to be depressed until 36 h after the adenosine dose (Figure 2.7). In some experiments the adenosine reduction of mRNA persisted until 48 h. The time course of reduction of DPPIV mRNA *preceded* that of the decrease in protein expression. Changes in DPPIV mRNA following short-term adenosine treatment were also evaluated. We

identified an *increase* in DPPIV mRNA levels by 1 h; persisting until the 2 h time point, after which time no difference was found following 4 h of adenosine exposure (Figure 2.8). These data indicate that the adenosine regulation of DPPIV protein occurs at the level of transcription.

Adenosine down-regulates DPPIV at concentrations found within the tumor microenvironment

The dose-response relationship for the down-regulation of DPPIV after 48 h using a single dose of adenosine is shown in Figure 2.9. There was a dose-dependent reduction of cell-surface DPPIV that was first significant in response to a 30 μM dose of adenosine (~ 10% reduction). The maximal down-regulation of DPPIV observed was approximately 40%. The greatest effect in this approach was typically seen at 300 μM adenosine, although in some experiments the maximal effect was reached at 100 μM. The EC_{50} for down-regulation (single dose) was 43.3 ± 12.1 μM (mean ± SE, 5 separate experiments). Concentrations of adenosine as high as 100 μM are likely reached *in vivo* following an event resulting in significant cell death within the tissue (Bell *et al.*, 1998; Fredholm *et al.*, 2001). In the case of solid tumors this might occur during the course of treatment with cytotoxic chemotherapeutic agents or anti-angiogenic strategies. However, the single dosing approach we had so far used contrasts with the situation that would be more usual *in vivo*, in which adenosine production may be less dramatically raised over a period of some days (e.g. sites of inflammation) or even longer (e.g. hypoxic solid tumors; Blay *et al.*, 1997).

In hypoxic solid tumors, we would predict the adenosine signal to be more persistent *in vivo* than represented by a single dose *in vitro*, but not to reach such a high concentration. To model the exposure of tumor cells to lower and more persistent concentrations of adenosine, we adopted a strategy of extending the additions of adenosine throughout the 48 h treatment period. This would provide more consistent levels without the high initial concentration. Exposure to adenosine at 100 μM (3 doses over 48 h) produced a down-regulation indistinguishable from the single 300 μM dose (Figure 2.10). Very similar results were also obtained when we reduced the adenosine concentration to 50 μM (4 doses over 48 h), 25 μM (6 doses), and most convincingly with adenosine as low as 12.5 μM (12 doses). Equivalent down-regulation of DPPIV by adenosine was confirmed by flow cytofluorimetric analysis of trypsinized cells that were treated in a similar manner (Figure 2.11). These results were consistent with our experience that high concentrations of adenosine are necessary when given as a single dose in order to provide sufficient levels of adenosine over a prolonged time period in the face of rapid metabolism. The results also indicate that the reduction of DPPIV requires more than transient exposure of the cells to adenosine. At our chosen dosage intervals the rapid metabolism of adenosine would exclude any net accumulation (Mujoomdar *et al.*, 2003). We therefore established that the down-regulation of DPPIV on HT-29 cells can be achieved with concentrations of adenosine present within the tumor extracellular fluid (Blay *et al.*, 1997).

Chronic exposure of cells to adenosine produces a sustained decrease in cell-surface DPPIV

We had shown that treatment of HT-29 cells with a single dose of adenosine (300 μM) down-regulated DPPIV maximally at 48 h and persisted for at least 72 h (Figure 2.3). Our next goal was to explore whether longer-term continued adenosine exposure would produce an even more sustained depression of cell-surface DPPIV. The experimental strategy involved daily treatment of cells with 100 μM adenosine. This was again an attempt to expose cells to persistent levels of adenosine without net accumulation. DPPIV levels were assessed on each day (up to day 6) by the radioantibody binding assay. In the absence of adenosine addition, baseline DPPIV levels increased with time in culture (Figure 2.12) as reported earlier (see Figure 2.4). However, daily supplementation of media with adenosine (the media was not changed during the treatment time period) produced a sustained depression of DPPIV relative to the baseline increase (Figure 2.12). The decline in DPPIV continued to day 4 with repetitive dosing, and this depression was maintained throughout the culture period. The maximal effect on DPPIV was a ~ 40% decrease from control. We did not prolong this study beyond 6 days because of the reduced cell viability that occurred past this time.

Down-regulation of DPPIV is specific for adenosine and not its deamination product, inosine

It was possible that the effect of adenosine in down-regulating DPPIV was mediated by its deamination product (via metabolism by ADA), inosine, which itself has been reported to regulate a number of cellular responses (Jin *et al.*, 1997; Haskó *et al.*,

2000). To probe the specificity of the response for adenosine, we treated the cells with a single 100 μM dose of adenosine, inosine, or the alternative purine nucleoside guanosine. Neither inosine nor guanosine reduced DPPIV as was observed for adenosine (Figure 2.13). Concentrations of these nucleosides as high as 300 μM were also without effect (data not shown). To further support the specificity of the DPPIV down-regulation to adenosine, we prevented inosine production by pretreating the cells with 2.5 μM coformycin (an inhibitor of ADA). Coformycin did not abrogate the adenosine reduction of DPPIV. Instead, the presence of coformycin shifted the apparent EC_{50} for adenosine (single dose) approximately 4-fold from 43 ± 12.1 μM to 10.7 ± 4.0 μM (mean ± SE, 3 separate experiments; $P < 0.05$). This change in potency resulting from ADA inhibition is likely due to increased adenosine availability. Taken together, these data indicate that metabolism of adenosine to inosine is not required for DPPIV down-regulation to occur, and that the response is not generalized to other nucleosides.

The down-regulation of DPPIV is not dependent on cellular uptake of adenosine

The next objective was to determine the cellular location at which adenosine was acting to regulate DPPIV. For these studies we used the lowest maximally effective single dose of adenosine (30 μM) in the presence of 2.5 μM coformycin to reduce metabolism through ADA. This would permit assessment of the effects of selected pharmacological agents on the adenosine response. We used adenosine uptake inhibitors to assess the possibility that adenosine might be mediating down-regulation of DPPIV by: (1) intracellular effects on nucleoside metabolism, (2) action on the "P"-site of intracellular adenylyl cyclase (IC_{50} ~ 80 μM; Johnson *et al.*, 1991), or (3) interfering with

the transport of other nucleosides into the cell (Jarvis *et al.*, 1991). Neither dilazep (10 μM; Figure 2.14) nor NBTI or dipyridamole (each at 1 μM; Figure 2.14) were able to block the down-regulation due to adenosine. These agents were used at concentrations in excess of the IC_{50} values for nucleoside transport inhibition (Griffiths *et al.*, 1997*a*; Griffiths *et al.*, 1997*b*; Ward *et al.*, 2000). Indeed, the combination of NBTI with dipyridamole, which concurrently blocks both NBTI-sensitive and NBTI-insensitive transport (Ward and Tse, 1999), did not prevent the adenosine reduction of DPPIV. Similarly, 5′-iodotubercidin (1 μM), which inhibits the intracellular phosphorylation of adenosine through adenosine kinase, failed to alter the adenosine down-regulation of DPPIV (Figure 2.15). Thus, neither the uptake nor the intracellular phosphorylation of adenosine is necessary for this effect, and indicates that adenosine acts at a cell-surface site to down-regulate DPPIV on HT-29 cells.

Table 2.1 Effect of acid stripping on the detection of DPPIV after adenosine treatment.

	Conditions	Treatment		
		Control	Adenosine	% Decrease due to adenosine
I.	Control	5,282 ± 91	4,105 ± 29 **	22
	pH 4.2	5,041 ± 160	3,891 ± 73 **	23
II.	Control	4,531 ± 92	4,132 ± 107 *	9
	pH 3.1	3,825 ± 125	3,393 ± 69 *	11
III.	Control	4,034 ± 38	3,441 ± 55 **	15
	pH 2.6	3,295 ± 79	2,742 ± 23 **	17

HT-29 cells were treated for 48 h with adenosine (300 μM) or vehicle control. Prior to the binding assay, cells were incubated for 5 min at 4°C either at pH 7.4 ('Control'), or in buffer at the pH indicated. The data are mean values ± SE for 3-4 replicate determinations. *, significant reduction by adenosine relative to control treatment, $P < 0.05$. **, $P < 0.01$.

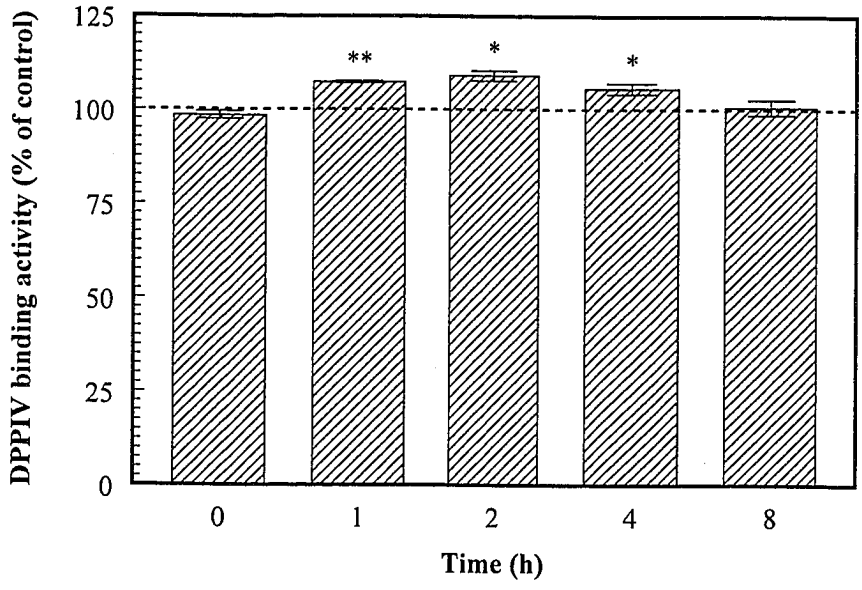

Figure 2.2 Acute up-regulation of cell-surface DPPIV protein on HT-29 cells by short-term exposure to adenosine.

The surface expression of DPPIV on HT-29 cells was measured after incubation in the absence or presence of adenosine (300 μM) for the times indicated. The data are mean values ± SE ($n = 3$). *, significant increase by adenosine, $P < 0.05$. **, $P < 0.01$.

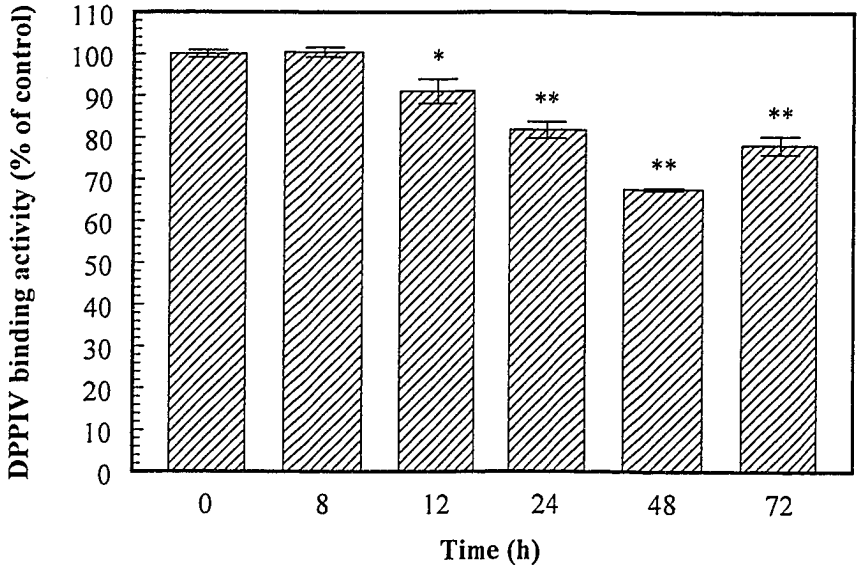

Figure 2.3 Down-regulation of DPPIV expression on HT-29 cells by long-term exposure to adenosine.

The surface expression of DPPIV on HT-29 cells was measured after incubation in the absence or presence of adenosine (300 μM) for the times indicated. The data are mean values ± SE ($n = 3$). *, significant decrease by adenosine, $P < 0.05$. **, $P < 0.01$.

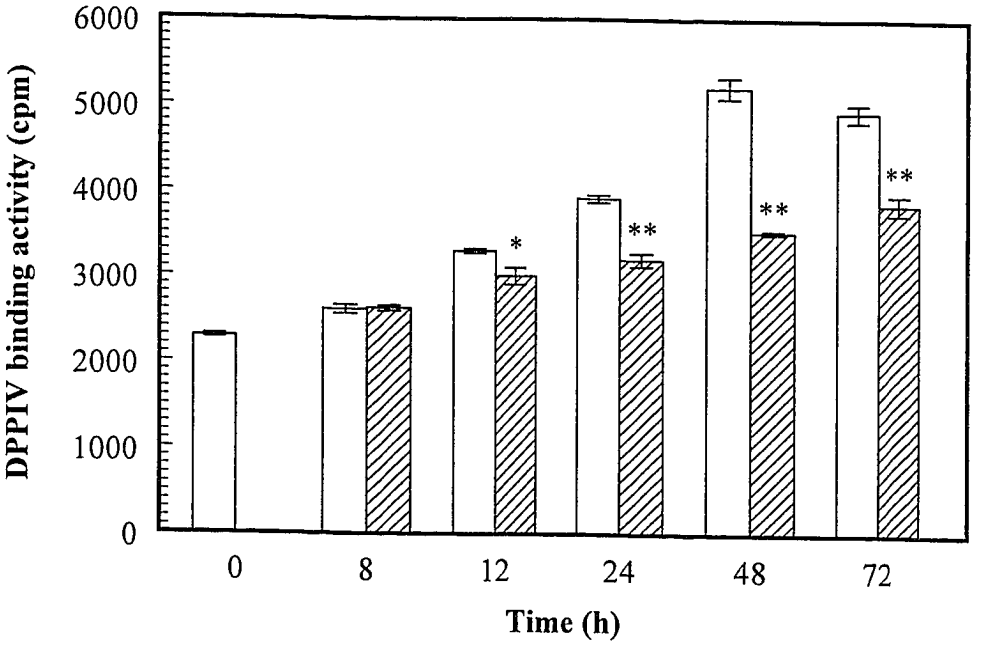

Figure 2.4 Increased baseline level of cell-surface DPPIV expression and the effect of adenosine.

The surface expression of DPPIV on HT-29 cells was measured after incubation in the absence (*clear bars*) or presence of adenosine (300 μM, *hatched bars*) for the times indicated. The data are mean values ± SE ($n = 3$). *, significant decrease by adenosine, $P < 0.05$. **, $P < 0.01$.

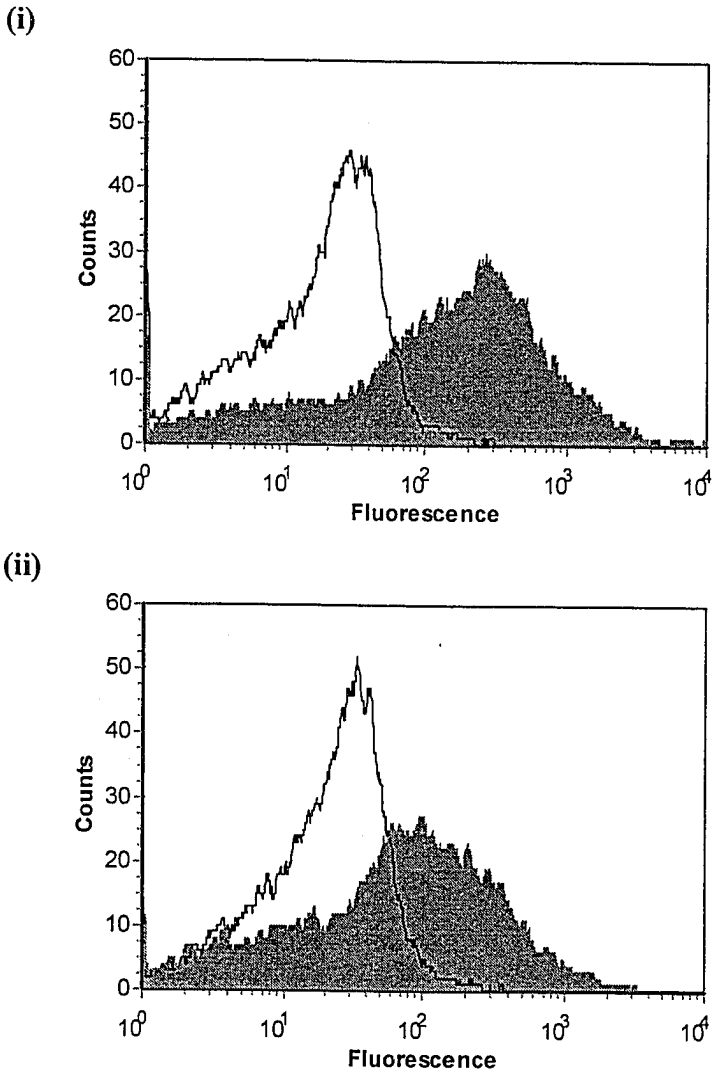

Figure 2.5 Flow cytofluorimetric analysis of DPPIV down-regulation on HT-29 cells by long-term exposure to adenosine.

HT-29 cells were incubated for 48 h in the **(i)** absence or **(ii)** presence of adenosine (300 μM) and released by trypsinization. Cytofluorimetric profiles for HT-29 cells stained with isotype control mAb (*open peaks*) and with DPPIV-specific mAb (*shaded peaks*) are shown.

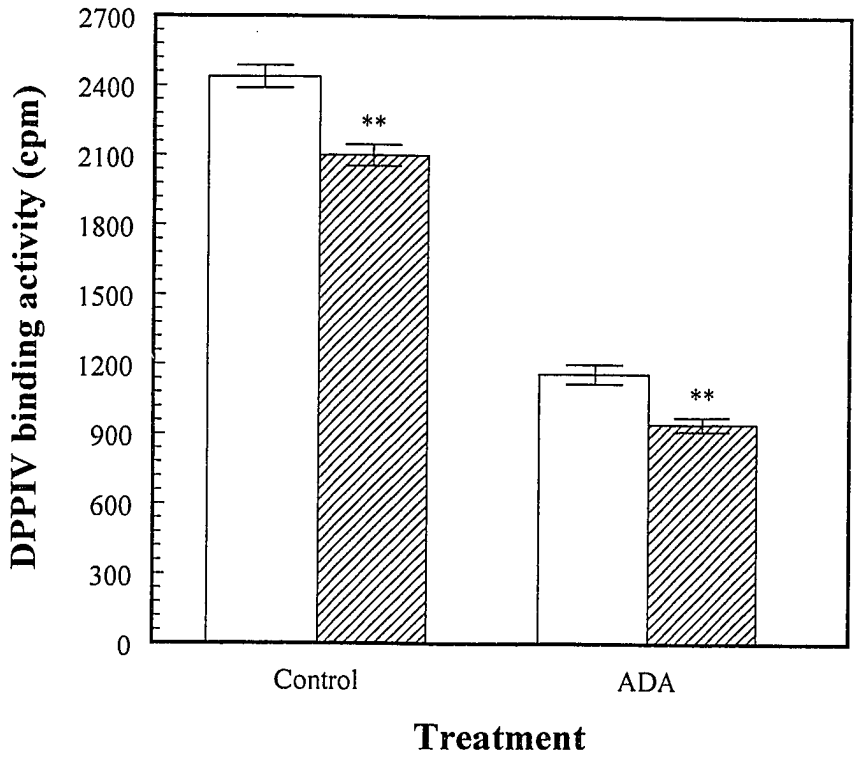

Figure 2.6 Failure of ADA loading to eliminate the decline in DPPIV due to adenosine.

HT-29 cells were incubated for 48 h in the absence (*clear bars*) or presence of adenosine (300 μM, *hatched bars*), washed, and further incubated for 60 min at 4°C with 10 μg/ml ADA prior to the binding assay for DPPIV. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

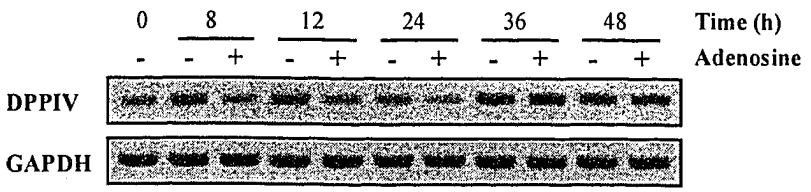

Figure 2.7 Reduction of DPPIV mRNA expression in HT-29 cells by long-term exposure to adenosine.

The expression of DPPIV mRNA by HT-29 cells was measured by semi-quantitative RT-PCR after incubation in the absence or presence of adenosine (300 μM) for the times indicated. Expression of GAPDH mRNA was also assessed at the indicated time points.

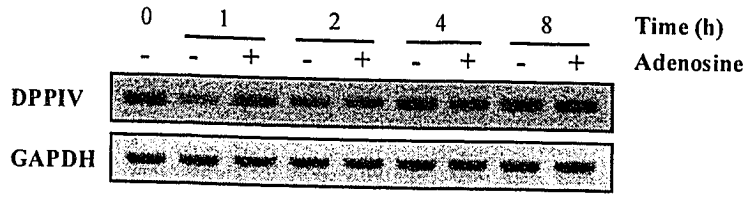

Figure 2.8 Acute up-regulation of DPPIV mRNA expression in HT-29 cells in response to short-term adenosine exposure.

The expression of DPPIV mRNA by HT-29 cells was measured by semi-quantitative RT-PCR after incubation in the absence or presence of adenosine (300 μM) for the times indicated. Expression of GAPDH mRNA was also assessed at the indicated time points.

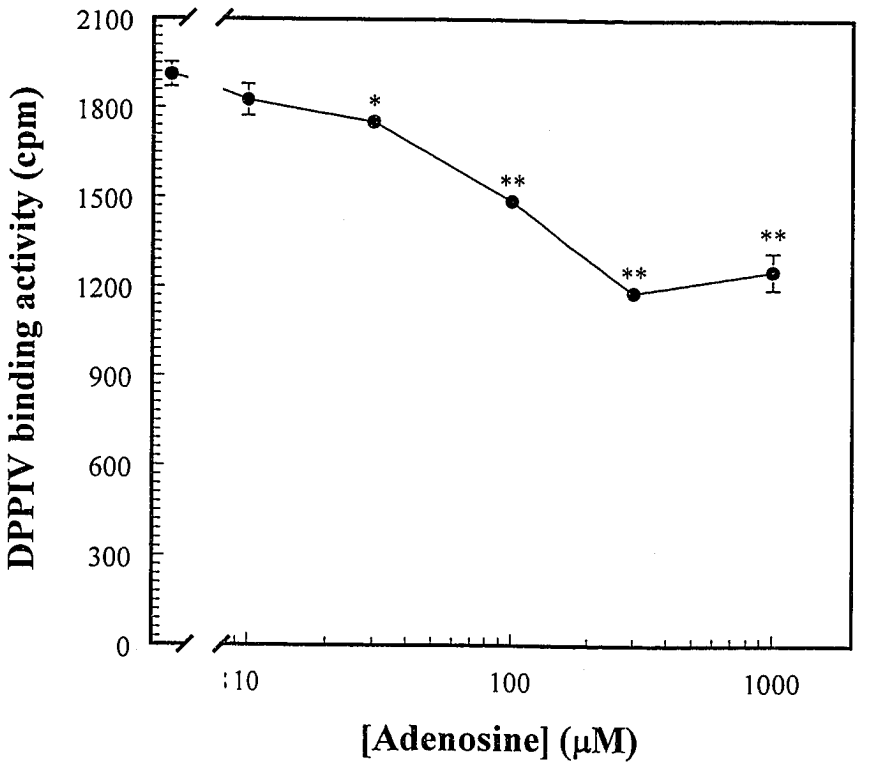

Figure 2.9 Dose-dependence of the adenosine down-regulation of DPPIV expression on HT-29 cells.

HT-29 cells were incubated at 37°C with a single dose of adenosine at the indicated concentrations. Forty-eight hours later the expression of cell-surface DPPIV was measured. The data are mean values ± SE ($n = 3$). *, significant reduction by adenosine, $P < 0.05$, **, $P < 0.01$.

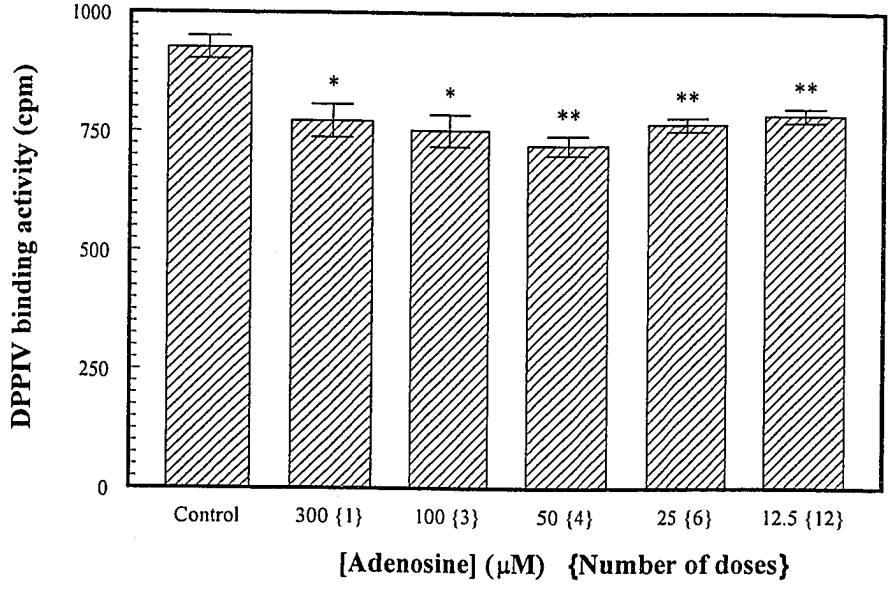

Figure 2.10 Effect of adenosine concentration and dosing frequency on the down-regulation of DPPIV on HT-29 cells.

HT-29 cells were incubated at 37°C with the indicated concentrations of adenosine (μM) and number of doses (parentheses) over the 48 h treatment period. The data are mean values ± SE ($n = 3$). *, significant reduction by adenosine, $P < 0.05$, **, $P < 0.01$.

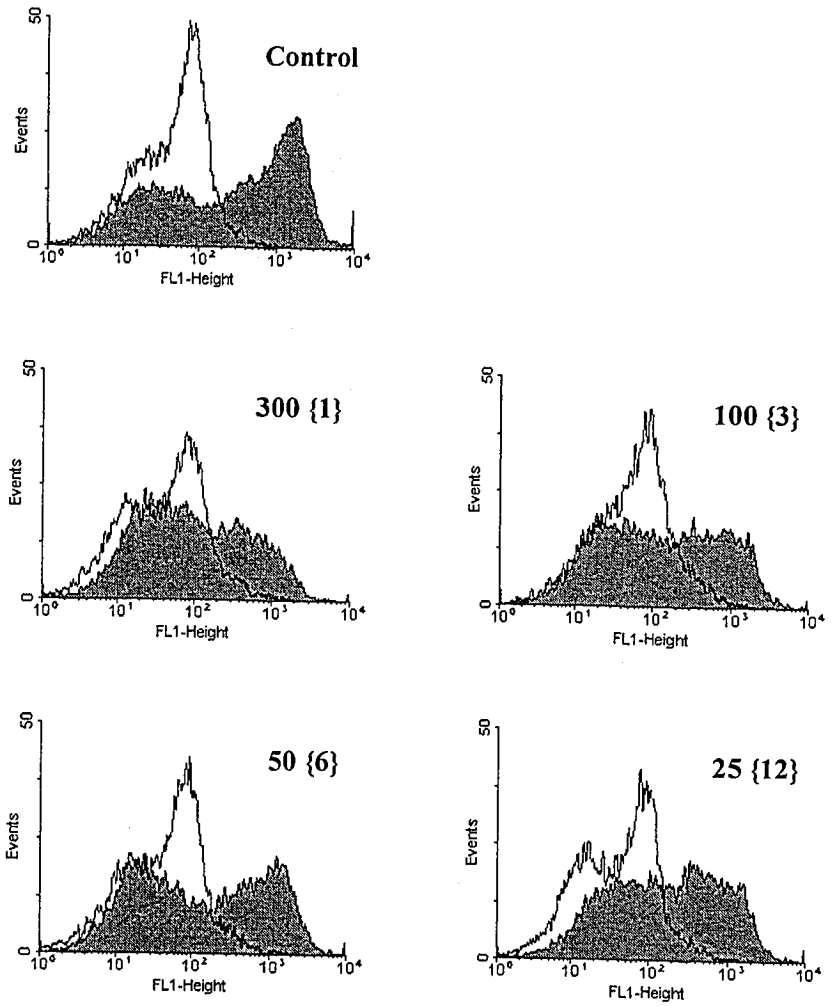

Figure 2.11 Flow cytofluorimetric analysis of DPPIV down-regulation in response to adenosine concentration and dosing frequency.

HT-29 cells were incubated for 48 h with medium alone or the indicated concentrations of adenosine (μM) and number of doses (parentheses). The cells were then released by trypsinization and processed for flow cytofluorimetry analysis. Cytofluorimetric profiles for HT-29 cells stained with isotype control mAb (*open peaks*) and with DPPIV-specific mAb (*shaded peaks*) are shown.

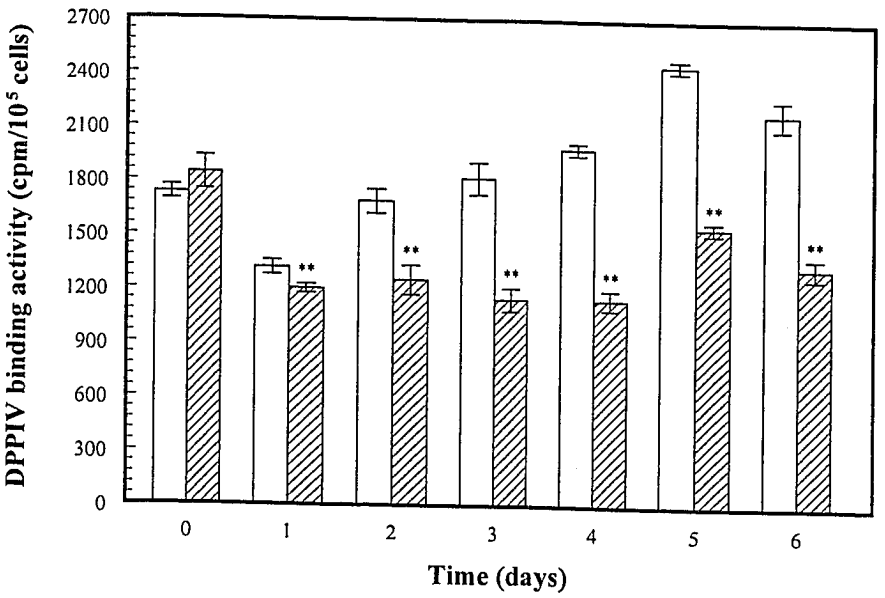

Figure 2.12 Persistent depression of cell-surface DPPIV protein with chronic exposure to adenosine.

HT-29 cultures were incubated in control media (*clear bars*) or with adenosine (100 μM, *hatched bars*) added daily. The cultures were then assayed for the presence of cell-surface DPPIV protein at the times indicated. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

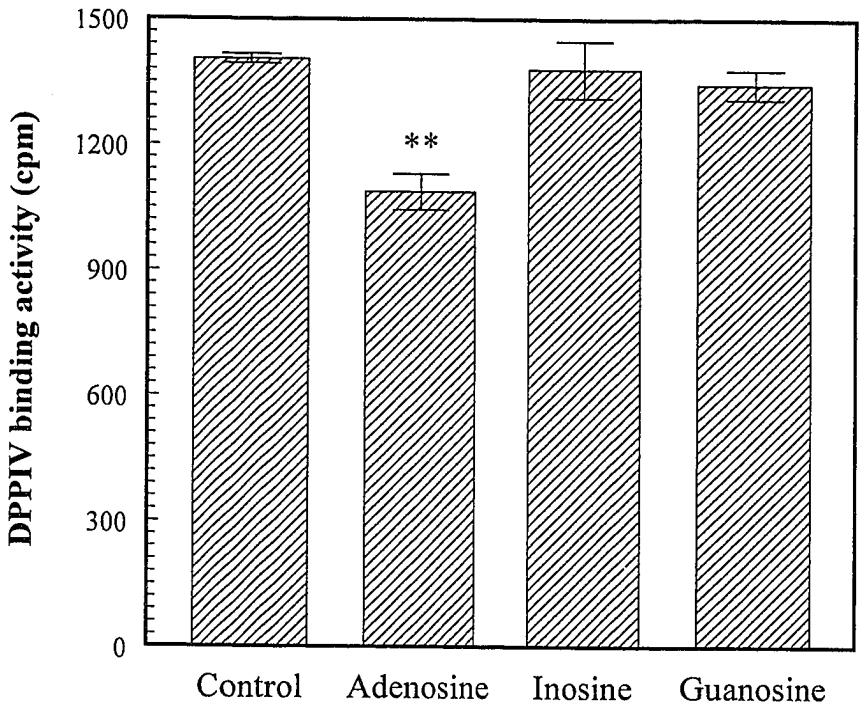

Figure 2.13 Lack of effect of inosine and guanosine on the surface expression of DPPIV.

HT-29 cells were incubated at 37°C with medium alone or in the presence of 100 μM adenosine, inosine, or guanosine. Forty-eight hours later cell-surface DPPIV was measured. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

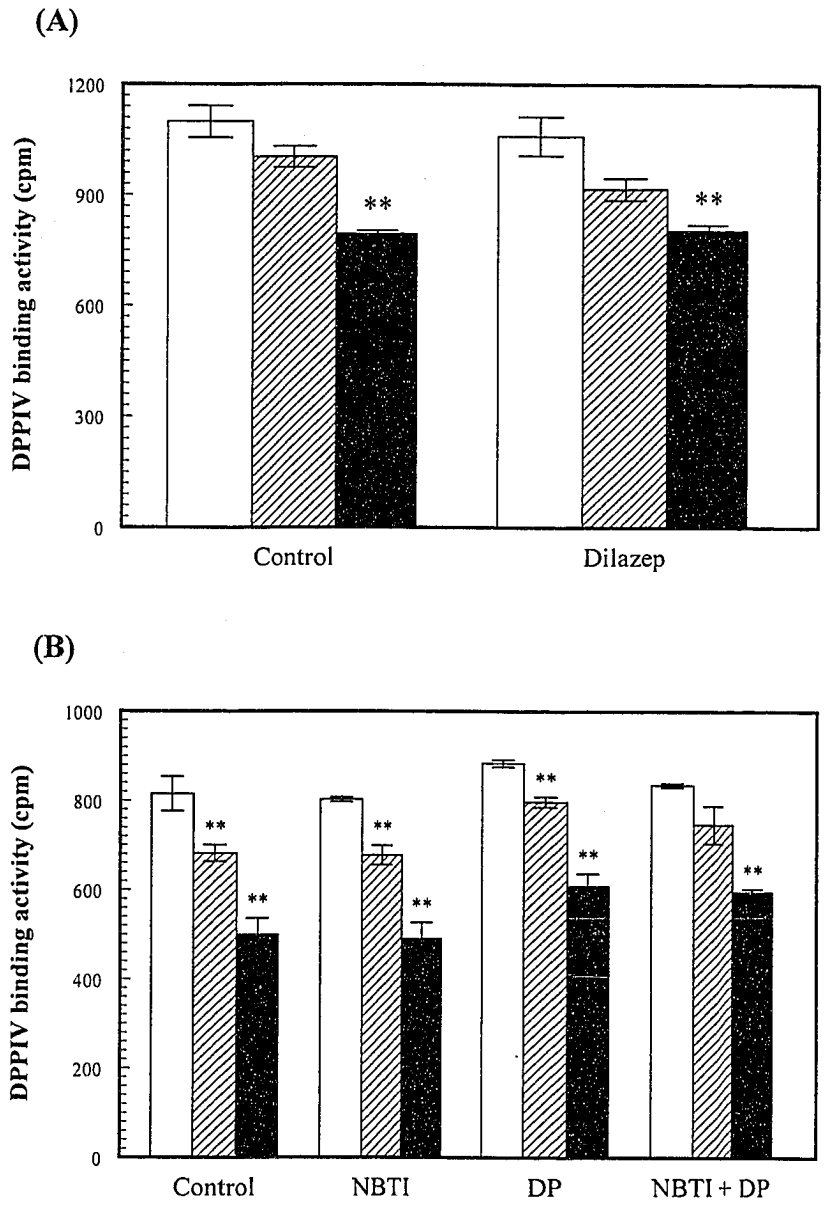

Figure 2.14 The down-regulation of DPPIV on HT-29 cells is independent of adenosine uptake.

HT-29 cells were pretreated for 30 min with control vehicle or **(A)** 10 μM dilazep or **(B)** 1 μM NBTI, 1 μM dipyridamole (DP), or 1 μM NBTI and DP. The cells were then incubated with medium alone (*clear bars*) or adenosine at 10 μM (*hatched bars*) or 30 μM (*shaded bars*), plus 2.5 μM coformycin. Forty-eight h later surface expression of DPPIV was measured. The data are mean values SE ($n = 3$). *, significant reduction by adenosine, $P < 0.05$, **, $P < 0.01$.

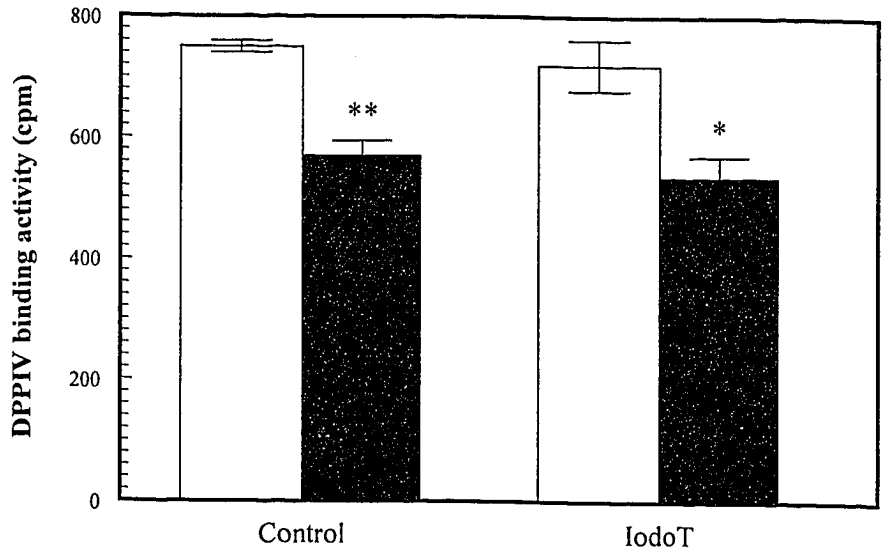

Figure 2.15 Phosphorylation of adenosine is not required for the adenosine down-regulation of DPPIV.

HT-29 cells were pretreated for 30 min with control vehicle or 1 μM iodotubercidin (IodoT). The cells were then incubated with medium alone (*clear bars*) or adenosine (30 μM, *shaded bars*), plus 2.5 μM coformycin. Forty-eight h later surface expression of DPPIV was measured. The data are mean values SE ($n = 3$). *, significant reduction by adenosine, $P < 0.05$, **, $P < 0.01$.

DISCUSSION

Several studies have reported that DPPIV is aberrantly expressed in many cancers (Houghton *et al.*, 1988; Bogenrieder *et al.*, 1997; Stecca *et al.*, 1997), and that decreased expression of DPPIV is linked to an increase in the invasive and metastatic behavior of these cells (Pethiyagoda *et al.*, 2001; Kajiyama *et al.*, 2002*b*). Colorectal carcinomas demonstrate significant intratumoral heterogeneity in DPPIV expression, suggesting that local influences of the tumor microenvironment might play a role. The results in this chapter have explored whether high levels of the purine nucleoside adenosine, comparable to those within solid tumors (Blay *et al.*, 1997), might regulate DPPIV expression on human colorectal carcinoma cells.

Adenosine regulation of cell-surface DPPIV

The amount of DPPIV protein on the surface of HT-29 cells increased with time in culture. This progressive increase in baseline DPPIV is consistent with the known ability of these cells to show increased expression of DPPIV protein during acquisition of a more differentiated phenotype in response to time in culture, achievement of confluent density, and depletion of glucose (Chantret *et al.*, 1988; Darmoul *et al.*, 1992; Howell *et al.*, 1992). Relative to this background, we consistently observed an initial, transient small (~ 10%) increase in DPPIV protein in response to a single high (300 μM) dose of adenosine. This increase was subsequently replaced by a prolonged (maximum reached at 48 h) and more profound (typically ~ 40% under optimized conditions) decrease in cell-surface DPPIV expression. The down-regulation of DPPIV was dose-dependent, with an EC_{50} for adenosine (single dose) of ~ 40 μM and a plateau at 100-300 μM. This down-

regulatory effect of adenosine was demonstrated by the radioantibody binding assay on monolayer HT-29 cell cultures and confirmed by flow cytofluorimetric analysis on trypsin-released cell suspensions. Our use of the flow cytofluorimetry assay to quantify levels of cell-surface DPPIV was justified based on the known resistance of DPPIV protein to mild, and even prolonged, trypsinization (E.Y. Tan and J. Blay, unpublished observations; Hanski *et al.*, 1985; Hong *et al.*, 1989).

The down-regulation of immunoreactive DPPIV was not an artifact of epitope blocking. Our rationale for considering this possibility was because of the major function of DPPIV as the ADA binding protein (Schrader *et al.*, 1990), and its alternative capacity to bind the ECM proteins collagen and fibronectin (Bauvois, 1988; Piazza *et al.*, 1989). Furthermore, the addition of exogenous ADA to DPPIV-expressing cells (e.g. T lymphocytes) has been shown to reduce the immunoreactive detection of DPPIV (De Meester *et al.*, 1994). The adenosine down-regulation of DPPIV persisted when HT-29 cells were loaded with a saturating concentration of exogenous ADA prior to the binding assay. Consistent with De Meester *et al.* (1994), exogenous ADA significantly blocked DPPIV immunoreactivity measured both by binding assay and flow cytofluorimetry (E.Y. Tan and J. Blay, unpublished data). Acid stripping (from pH 4.2 to pH 2.6) to remove surface ligands prior to the binding assay also did not eliminate the reduction in DPPIV due to adenosine. The failure of acid stripping additionally supports that the decrease in DPPIV immunoreactivity is not because of increased binding of ADA. Van der Weyden and Kelley (1976) found that ADA binding to DPPIV is optimal from pH 5.0 to 8.0. However, ADA binding is effectively disrupted at pH less than 4.0, which was within the pH range assessed in our assays.

Our investigation of the molecular mechanism of the adenosine effect pointed to the regulation of DPPIV protein at the level of mRNA. Both the acute, transient increase followed by reduction of DPPIV protein was preceded by corresponding changes in the mRNA signal. It remains to be determined whether the adenosine effect on DPPIV mRNA levels is due to changes in transcription, mRNA stability, or a combination of both processes. The adenosine regulation of DPPIV by this molecular mechanism is consistent with a number of other studies demonstrating regulation of DPPIV (in a number of different cell types) at the transcriptional level in response to different soluble mediators (Kehlen *et al.*, 1998; Nemoto *et al.*, 1999; Bauvois *et al.*, 2000; Kajiyama *et al.*, 2002*a*).

The degree of cell-surface DPPIV down-regulation produced by a single dose of adenosine could be matched by a dosing approach involving a more persistent exposure to adenosine at lower concentrations, comparable to those found within the extracellular fluid of solid tumor tissues (~ 10 μM; Blay *et al.*, 1997). This was convincingly shown by the identical down-regulatory response of 12.5 μM and 300 μM adenosine given twelve times or once, respectively, over 48 hours. Furthermore, daily exposure to moderate levels of adenosine, which would preclude any accumulation of the nucleoside, (based on the half-life of metabolism; Mujoomdar *et al.*, 2003) produced a sustained decrease in DPPIV protein at the cell surface. We therefore believe that the concentrations of adenosine that are present within the tumor have the capacity to down-regulate DPPIV levels *in situ*.

Specificity of DPPIV down-regulation for adenosine

We examined whether the effect of adenosine might be mediated by its metabolite inosine. The irreversible hydrolytic deamination of adenosine to inosine is catalyzed by ADA, and HT-29 cells express this particular adenosine-metabolizing enzyme at the cell surface (see Figure 4.7). Thus, the addition of adenosine to the culture medium of HT-29 cells would be expected to lead to elevated levels of inosine. The nucleoside inosine is indeed present at increased extracellular levels in response to hypoxia and ischemia (Wang *et al.*, 1994; Rego *et al.*, 1997; Bell *et al.*, 1998), and recent studies have established a role for elevated concentrations of inosine (typically greater than 50 μM) in a number of cell regulatory roles. Inosine inhibits pro-inflammatory cytokine and chemokine production (Haskó *et al.*, 2000), stimulates mast cell degranulation (Jin *et al.*, 1997) and dendritic cell chemotaxis (Idzko *et al.*, 2004), and has been shown to have positive effects on neuronal growth after injury to the central nervous system (Benowitz *et al.*, 1999; Chen *et al.*, 2002). Furthermore, inosine also reduces production of the inflammatory cytokine IL-8 in HT-29 cells (Marton *et al.*, 2001). Inosine has no definitively known unique receptor(s); however, it has been shown to either utilize A_3 adenosine receptors (binding in the range of 10-50 μM; Jin *et al.*, 1997; Tilley *et al.*, 2000), a combination of adenosine receptors (Haskó *et al.*, 2000; Gomez and Sitkovsky, 2003), or act directly upon an intracellular target (Benowitz *et al.*, 1998). The existence of a novel, not yet identified, G protein-coupled receptor for inosine has also been proposed (Idzko *et al.*, 2004).

Inosine itself at the same concentration as adenosine had no effect on DPPIV regulation. Furthermore, blocking inosine production with the ADA inhibitor coformycin

did not abrogate the adenosine response. Instead, coformycin *enhanced* the potency (~ 4-fold) of the DPPIV down-regulatory response to adenosine given as a single dose. Therefore, the down-regulation of DPPIV on HT-29 cells is not regulated by the metabolism of adenosine to inosine via ADA.

Hypoxia and ischemia also increase the extracellular concentration of the purine nucleoside guanosine (Ciccarelli *et al.*, 1999). Similar to inosine, guanosine has only recently become recognized as an extracellular signaling molecule. Cell regulatory effects specific for guanosine include the induction of necrosis of aortic endothelial cells (Han and Wyche, 1994) and the synthesis and release of neurotrophic factors from astrocytes (Middlemiss *et al.*, 1995). Guanosine has no identified cell surface receptor, although a recent report proposed the existence of a putative G_i protein-coupled guanosine receptor involved in protecting astrocytes against staurosporine-induced apoptosis (Di Iorio *et al.*, 2004). Certain effects of this nucleoside also appear to occur via a mechanism involving guanosine-evoked accumulation of extracellular adenosine, which then activates adenosine receptors (Ciccarelli *et al.*, 2000; Chen *et al.*, 2001; Di Iorio *et al.*, 2002). We therefore tested the effect of guanosine in our cultures but found no role for this nucleoside in DPPIV regulation. Our data therefore point to a specific role for adenosine rather than an effect generalized for nucleosides.

Location of adenosine action

We were interested in identifying the cellular location at which adenosine was acting to modulate cell-surface DPPIV levels on HT-29 cells. Adenosine uptake via nucleoside transporters is responsible for a minority of its regulatory effects. An

intracellular site of action has, however, been reported as a mechanism for the property of adenosine in causing apoptosis of endothelial cells (Dawicki *et al.*, 1997; Rounds *et al.*, 1998) and several different cancer cell lines (Barry and Lind, 2000; Schrier *et al.*, 2001; Saitoh *et al.*, 2004). These studies were all characterized by the requirement for high concentrations of adenosine (> 100 μM, and commonly in the millimolar range) to initiate apoptosis, while the adenosine effect could be prevented by blocking adenosine uptake with the inhibitor dipyridamole. Several of these studies also implicated the requirement for intracellular phosphorylation of adenosine to AMP (via adenosine kinase) for the apoptotic effect to occur (Barry and Lind, 2000; Schrier *et al.*, 2001). Interestingly, Saitoh *et al.* (2004) reported that intracellular conversion of adenosine to AMP is followed by the activation of AMP-activated protein kinase (AMPK), which subsequently mediates apoptosis. In addition to this uptake and phosphorylation mechanism, adenosine is also capable of interacting with a "P-site" on the catalytic site of adenylyl cyclase, resulting in inhibition of the enzyme and reduced intracellular cAMP levels (Legrand *et al.*, 1990; Johnson *et al.*, 1991).

The nucleoside uptake inhibitors NBTI, dilazep, and dipyridamole each failed to reverse the effect of adenosine at concentrations sufficient to block uptake by both NBTI-sensitive (*es*; ENT1-mediated) and NBTI-insensitive (*ei*; ENT2-mediated) Na^+-independent equilibrative transporters. The combination of NBTI and dipyridamole together, which rigorously eliminates uptake by equilibrative transport (Ward and Tse, 1999), was also without effect. Although Na^+-dependent concentrative nucleoside transporters (CNT1 and CNT2) are known to be expressed in certain cell types (Griffith and Jarvis, 1996), they are likely absent from human colorectal carcinoma cell lines.

Ward and Tse (1999) found that the T84 and Caco-2 colorectal carcinoma cell lines do not express mRNA transcripts for CNT1 and CNT2. From a functional perspective there was also no difference in nucleoside uptake in the presence or absence of Na^+ (Mun *et al.*, 1998; Ward and Tse, 1999). The addition of 5′-iodotubercidin, which inhibits the phosphorylation of intracellular adenosine by adenosine kinase, also failed to prevent the adenosine effect. Thus, neither uptake nor the intracellular phosphorylation of adenosine is required for adenosine to exert its effect on DPPIV. We conclude that the mechanism of action of adenosine in the regulation of DPPIV expression on HT-29 cells is mediated at an extracellular site.

Relevance of DPPIV regulation by adenosine and other regulatory factors

The ability of adenosine to modulate cell-surface DPPIV expression could perhaps contribute to the variability in DPPIV expression in tumor tissues *in vivo*. Significant intratumoral variability in DPPIV immunoreactivity has been demonstrated in colorectal adenocarcinoma tissues, with distinct areas of either high or low cell-surface expression (ten Kate *et al.*, 1985; ten Kate *et al.*, 1986). These latter regions of reduced DPPIV expression in colorectal adenocarcinoma tumors might represent areas of DPPIV down-regulation due to increased adenosine production resulting from local chronic hypoxia. Chronic hypoxia occurs in tumor cells situated at a substantial distance from functional tumor blood vessels (> 100-150 μm), beyond which there is limited oxygen delivery due to the high oxygen consumption of the intervening cells (reviewed by Vaupel *et al.*, 1989). Substantial heterogeneity indeed exists in the expression of chronic hypoxia within human colorectal tumors: in human colon adenocarcinoma xenografts, the

overall fraction of hypoxic cells has been found to vary between 5 and 22% and may even exceed 80% (Leith *et al.*, 1991). These areas of chronic tissue hypoxia should contain high levels of adenosine (Blay *et al.*, 1997), and on the basis of our results, would lead to a local, persistent reduction in cell-surface DPPIV expression.

DPPIV might also be subject to regulation by transient (also acute or "perfusion limited") hypoxia, which is a more dynamic form of hypoxia occurring in tumor cells that are proximal to tumor blood vessels. Transient hypoxia is caused by intermittent blockages in the vessels or collapse of tumor vessels, leading to temporary cessation of blood flow, thereby decreasing oxygen delivery and availability (reviewed by Brown, 1999). Recent work has succeeded in quantifying the levels of transient hypoxia in human tumor xenografts through the use of novel techniques (Brurberg *et al.*, 2003; Bennewith and Durand, 2004). At least 8% of cells have been found to be transiently hypoxic in human colon adenocarcinoma xenografts (Bennewith and Durand, 2004), suggesting the possibility that transient hypoxia occurring within the tumor might initiate acute adenosine production that, in turn, would down-regulate DPPIV levels. This reduction of DPPIV by transient hypoxia likely would not persist, but might also contribute to the variable pattern of DPPIV expression observed within colorectal adenocarcinoma tumors.

We have proposed a role for the tumor metabolite adenosine as a potential endogenous regulator of DPPIV cell-surface expression in human colorectal adenocarcinoma tissues. However, other factors and influences are likely to contribute to the regulation of DPPIV in these tumors. One such influence is the direct regulation of DPPIV by hypoxia itself. Sato *et al.* (2002) reported that long-term exposure of

choriocarcinoma cell lines to hypoxic conditions (48 h at 1% O_2) caused an increase in DPPIV expression and protein. Although hypoxia *increased* DPPIV in these cell lines, the participation of DPPIV in functional changes to carcinoma cells likely depends on the particular tumor type. DPPIV in colorectal tumors might also be regulated by cytokines secreted from tumor-infiltrating lymphocytes (Barth *et al.*, 1996; Kanai *et al.*, 2000). T cell-derived cytokines such as IL-4, IL-13, IFN-γ, and TNF-α have been shown to regulate DPPIV expression in human renal carcinoma cells (Riemann *et al.*, 1995; Kehlen *et al.*, 1998). Thus, DPPIV is also likely regulated by the cytokine milieu present within colorectal tumors.

CONCLUSION

The major finding of this part of the study is the characterization of a novel property for the purine nucleoside adenosine in tumor cell regulation. Specifically, we have shown that adenosine chronically down-regulates the surface expression of DPPIV on human colorectal carcinoma cells. This down-regulation of DPPIV occurs at concentrations comparable to those present within the extracellular fluid of colorectal tumors growing *in vivo*, and is not elicited by the nucleosides inosine and guanosine. Neither cellular uptake of adenosine nor its phosphorylation to AMP is necessary for the down-regulation of DPPIV. Adenosine therefore regulates DPPIV via a mechanism that is initiated at the surface of HT-29 colorectal carcinoma cells. Finally, the adenosine regulation of DPPIV protein is dependent, at least in part, by changes in transcription.

CHAPTER 3

ADENOSINE DOWN-REGULATION OF DIPEPTIDYL PEPTIDASE IV ON HT-29 HUMAN COLORECTAL CARCINOMA CELLS OCCURS THROUGH STIMULATION OF PROTEIN TYROSINE PHOSPHATASE ACTIVITY AND REDUCED PHOSPHORYLATION OF ERK1/2

Portions of this chapter will appear in the following manuscript:

Tan, E.Y., Zhang, H., Hoskin, D.W., and Blay, J. (Submitted) Adenosine down-regulates dipeptidyl peptidase IV on HT-29 human colon cancer cells by stimulating protein tyrosine phosphatase(s) and reducing the activity of ERK1/2.

INTRODUCTION

Adenosine receptor signaling

The predominant effect of adenosine receptor ligation is either the activation (A_{2A}, A_{2B}) or inhibition (A_1, A_3) of adenylyl cyclase, resulting in alterations in cAMP concentrations within the cell (Fredholm *et al.*, 2001). Cyclic AMP activates the cAMP-dependent protein kinase (PKA), which promotes signal transduction by the phosphorylation of various proteins. A signaling pathway triggered less frequently by adenosine receptors is the activation of phospholipase C (PLC), which leads to the generation of diacylglycerol (DAG) and inositol trisphosphate (IP_3) through the metabolism of phosphatidylinositol-4,5-bisphosphate (PIP_2). DAG and IP_3 signal through protein kinase C (PKC) and the elevation of cytosolic calcium levels, respectively (Fredholm *et al.*, 2001; Klinger *et al.*, 2002).

Adenosine receptors are also coupled to additional signal transduction pathways that bypass the usual regulation of adenylyl cyclase activity. For example, A_1 adenosine receptors mediate the activation of several types of K^+ channels in cardiac myocytes through a mechanism independent of cAMP, but requiring the involvement of $G\beta\gamma$ subunits (Leaney and Tinker, 2000). The A_{2A} adenosine receptor activates Ca^{2+}-independent isoforms of PKC in PC12 cells in a manner that is not mimicked by cAMP (Lai *et al.*, 1997). In addition, activation of A_{2A} receptors on neutrophils is known to inhibit chemokine actions via mechanisms that do not exclusively depend on increases in cAMP (Fredholm *et al.*, 1996). Other examples of adenosine receptor effects not necessarily dependent on adenylyl cyclase/cAMP involve the activation of mitogen-activated protein (MAP) kinases.

The MAPK protein family consists of the extracellular signal-regulated kinases (ERK) and the stress-activated protein kinases (SAPK) p38 and c-jun N-terminal kinase (JNK). These serine/threonine kinases play an essential role in processes such as cell proliferation, survival, differentiation, and death (reviewed in Camps *et al.*, 1999; English *et al.*, 1999). Importantly, a growing number of unconventional adenosine signaling pathways have been identified through studies of the adenosine activation of MAP kinases. Faure *et al.* (1994) showed that the A_1 adenosine receptor transiently expressed in COS-7 cells mediates ERK1/2 activation via the release of Gβγ subunits. The A_{2A} receptor stimulates the proliferation of endothelial cells through an ERK1/2-dependent pathway requiring Ras and MAP kinase kinase (MEK), but is independent of G_s, cAMP, Ca^{2+}, and PKC (Sexl *et al.*, 1995; Sexl *et al.*, 1997). Furthermore, the A_{2A} receptor-mediated activation of ERK1/2 in transfected HEK293 cells is independent of G_s and cAMP, but involves Ras and its guanine nucleotide exchange factor (GEF) Sos (Seidel *et al.*, 1999). Grant *et al.* (2001) demonstrated cAMP-independent ERK1/2 activation through A_{2B} adenosine receptors expressed by human retinal endothelial cells (HRECs). Finally, A_3 adenosine receptors have also been reported to activate ERK1/2 through an unconventional pathway. In this case, A_3 receptors expressed in CHO cells initiate a pathway that involves Gβγ subunit release, followed by the sequential activation of PI3K, Ras, and MEK to induce ERK1/2 phosphorylation and activation (Schulte and Fredholm, 2002). The signaling from A_3 to ERK1/2 was also found to be independent of Ca^{2+} and PKC.

In the present study, we investigated the involvement of adenosine receptors as the cell-surface mechanism on HT-29 cells initiating the adenosine-mediated down-regulation of DPPIV. We also sought to identify the signal transduction pathway downstream of adenosine action leading to the down-regulation of DPPIV.

MATERIALS AND METHODS

Materials

HT-29 human colorectal carcinoma cells, media, culture vessels and sera, adenosine, coformycin, mAb against DPPIV/CD26 and mouse IgG_1 isotype control, and ^{125}I-labeled sheep anti-mouse IgG were obtained as in Chapter 2. R-PIA, CSC, enprofylline, MRS1191, MRS1220, MRS1523, forskolin, 8-Br-cAMP, the Rp diastereomer of adenosine cyclic 3′,5′-phosphorothioate (Rp-cAMPs), PMA, ionomycin, GF109203X, genistein, herbimycin A, serine/threonine phosphatase inhibitor cocktail, protein tyrosine phosphatase inhibitor cocktail, sodium orthovanadate, and phenylarsine oxide were from Sigma Chemical Co. (St. Louis, MO). NECA, DPCPX, alloxazine, Calphostin C, LY294002, and wortmannin were obtained from Research Biochemicals International (Natick, MA). PD98059, SB203580, SP600125, bpV(phen), mpV(pic), and sodium stibogluconate were from Calbiochem (San Diego, CA). Mouse anti-phospho-ERK1/2 mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-ERK1/2 mAb was from Upstate Biotechnology (Lake Placid, NY). Sheep anti-mouse IgG-horseradish peroxidase (HRP) and sheep anti-rabbit IgG-HRP were purchased from Chemicon International (Temecula, CA). Bradford protein assay and nitrocellulose were from Bio-Rad Laboratories (Hercules, CA). Protease inhibitor cocktail tablets (complete, EDTA-free containing aprotinin, bestatin, leupeptin, PMSF, and inhibitors of calpains and trypsin) were obtained from Roche Biomedicals (Laval, PQ, Canada). The enhanced chemiluminescence (ECL) Western blot detection system was from Pierce Chemical Co. (Rockford, IL).

Cell culture

HT-29 cells were cultured as before (Chapter 2). Cells for use in binding assays and ERK1/2 immunoblotting were seeded into 48-well and 6-well plates at 25,000-50,000 and 200,000 cells per well, respectively, and allowed to adapt to culture for 48 h. Cultures were then changed to medium containing 1% NCS for a further 48 h, and then treated with drugs or control vehicle for evaluation of changes in DPPIV surface protein expression or ERK1/2 phosphorylation. Adenosine receptor antagonists and inhibitors of signal transduction were added to cell cultures 30 min prior to the addition of the relevant adenosine treatment.

Radioantibody binding assay for DPPIV

Monolayer cultures of HT-29 cells in 48-well plates were assayed for cell-surface DPPIV protein as described in Chapter 2. Briefly, cultures were incubated with anti-DPPIV antibody or isotype control, washed, and the bound antibody was measured using ^{125}I-labeled sheep anti-mouse IgG as tracer. The figures show representative results from at least three separate experiments. Data were evaluated using a two-tailed Student's *t*-test for unpaired data.

ERK1/2 immunoblot analysis

Total cellular protein was isolated from HT-29 cells grown to 60-75% confluence in 6-well plates. Cells were rinsed twice with ice-cold PBS and dissolved in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF, 1× protease inhibitor mix] for 45

min at 4°C. The cell lysates were clarified by centrifugation (10 min at 12,000 × *g*) and quantified by Bradford protein assay according to the manufacturer's instructions. Twenty micrograms of protein extract per lane were separated by SDS-PAGE using 10% gels and electroblotted to nitrocellulose. Blots were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature. Blots were then probed overnight at 4°C with anti-phospho-ERK1/2 antibody at a 1 μg/ml concentration followed by incubation with sheep anti-mouse IgG-HRP-conjugated secondary Ab for 1 h at room temperature. Protein expression was detected using an enhanced chemiluminescence (ECL) Western blot detection system. To confirm equal sample loading, the blots were stripped in stripping solution (62.5 mM Tris pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 15 min at 50°C, blocked, and re-probed with anti-ERK1/2 (non-phosphorylated) Ab followed by sheep anti-rabbit IgG-HRP secondary Ab.

Statistical analysis

The figures show representative results from at least three separate experiments. Data were evaluated using a two-tailed Student's *t*-test for unpaired data.

RESULTS

The down-regulation of DPPIV by adenosine occurs through a mechanism independent of adenosine receptors

In chapter 2, we established that the location of adenosine action to down-regulate DPPIV on HT-29 cells was at the cell surface. The ability of adenosine to produce maximal down-regulation of DPPIV at sustained concentrations in the low micromolar range is also consistent with action at an extracellular site. We hypothesized that adenosine was acting through a conventional cell-surface adenosine receptor. The four adenosine receptor subtypes are A_1, A_{2A}, A_{2B}, and A_3, which are G protein-coupled receptors that signal primarily through coupling to adenylyl cyclase (Fredholm *et al.*, 2001). HT-29 human colorectal carcinoma cells have been shown in our laboratory to express all four receptor subtypes (M. Mujoomdar and J. Blay, unpublished data).

Selective adenosine receptor antagonists were used to attempt to identify the receptor pathway. For this approach the cells were pretreated with adenosine receptor antagonists followed by the lowest maximally effective dose of adenosine (30 μM) in the presence of 2.5 μM coformycin to reduce adenosine metabolism through ADA. We were unable to block the adenosine down-regulation of DPPIV in any of these experiments. The results are summarized in Table 3.1. There was absolutely no loss of adenosine-evoked DPPIV down-regulation after pretreatment and in the continued presence of the A_1 receptor antagonist DPCPX (30 μM); the A_{2A} receptor antagonist CSC (30 μM); two A_{2B} receptor antagonists alloxazine and enprofylline (each at 100 μM); and three different A_3 adenosine receptor antagonists, MRS1191, MRS1220 (each at 30 μM), and MRS1523 (10 μM, data not shown). The down-regulation of DPPIV was also not

blocked when the concentration of adenosine was lowered to 10 μM in the presence of coformycin (see Figure 3.1 for a representative example). This lack of antagonism was evident even though the agents were used at up to 30-fold the concentrations we have found necessary to block other responses of HT-29 and other cells to adenosine (C.L. Richard, E.Y. Tan, and J. Blay, submitted manuscript; Zhang *et al.*, 2004).

Given that HT-29 cells express all four adenosine receptor subtypes, it was possible that the adenosine response might occur via concurrent stimulation of more than one subtype. We therefore tested whether simultaneous inhibition of all four receptor subtypes might abrogate the adenosine down-regulation of DPPIV. HT-29 cells were treated with the combination of DPCPX (1 μM), CSC (1 μM), alloxazine (5 μM), and MRS1523 (1 μM). These concentrations were chosen to reflect their relative inhibitory potency in other systems, and were the highest combinatorial addition that could be used without causing excessive toxicity (cell death due to antagonists ranged from 1.6 to 8.7% in these experiments). Figure 3.2 shows the result of this approach. There was absolutely no inhibition of the adenosine response (10 μM with coformycin), even though this same combination was able to block the adenosine regulation of the CXCR4 chemokine receptor in these cells (C.L. Richard, E.Y. Tan, and J. Blay, submitted manuscript). Consistent with the failure of adenosine receptor antagonists to block the adenosine response, we found no effect of the broadly-selective *agonists* NECA and R-PIA (Klotz *et al.*, 2000; Fredholm *et al.*, 2001) on DPPIV expression, at concentrations ranging from 10 to 100 μM (data not shown). Collectively, these data indicate that adenosine down-regulates DPPIV by a mechanism not involving conventional adenosine receptor subtypes.

The negative findings obtained following our extensive studies with adenosine receptor antagonists and receptor-selective analogues were unexpected. We next evaluated the effect of manipulating the cAMP signaling network, which is the principal transduction pathway that is triggered by conventional adenosine receptors (Fredholm *et al.*, 2001). Activation of cAMP-dependent targets (which would parallel signaling through A_{2A} and A_{2B} receptors) did indeed alter DPPIV levels, but in the *opposite* way to adenosine. Direct activation of adenylyl cyclase with forskolin (50 μM) and treatment with the cell-permeable cAMP analogue 8-Br-cAMP (1 mM) led to an *increase* in cell-surface DPPIV protein (Figure 3.3). Furthermore, the PKA inhibitor Rp-cAMPs (50 μM; Rothermel *et al.*, 1984) had no effect on the adenosine regulation of DPPIV on HT-29 cells (Figure 3.3). These experiments excluded changes in adenylyl cyclase regulation and modulation of PKA activity from the DPPIV down-regulatory response. This supported our conclusion that the adenosine down-regulation of DPPIV occurs through a mechanism independent of known adenosine receptors.

The adenosine down-regulation of DPPIV occurs independently of PKC and PI3K signaling pathways

Our strategy now shifted to an investigation of other signal transduction pathways that have either been shown to be alternative routes of adenosine signaling, or might be anticipated to be involved in the regulation of a surface adhesion molecule and peptidase such as DPPIV.

Phospholipase C activation, which has been observed in a minority of responses to adenosine (Parsons *et al.*, 2000; Rogel *et al.*, 2005), leads to the generation of

diacylglycerol (DAG) and inositol trisphosphate (IP_3), which signal through protein kinase C (PKC) and the elevation of cytosolic calcium levels, respectively (Fredholm *et al.*, 2001; Klinger *et al.*, 2002). Direct activation of PKC using the phorbol ester PMA (5 nM), or elevation of intracellular calcium with the calcium ionophore ionomycin (1 μM), did not however mimic the adenosine effect, but in contrast caused an elevation of cellular DPPIV (Figure 3.4). In addition, neither of two agents that are inhibitory for PKC, GF109203X (1 μM; Toullec *et al.*, 1991) or calphostin C (100 nM; Kobayashi *et al.*, 1989), interfered with the adenosine-evoked suppression of DPPIV (Figure 3.4). The PKC inhibitor Ro-31-8220 (5 μM) also did not block the adenosine effect (see Appendix A). These observations excluded the involvement of the phosphoinositide cycle and PKC from the DPPIV down-regulatory response caused by adenosine.

Phosphatidylinositol 3-kinase (PI3K) is a lipid/serine kinase that has been implicated in certain adenosine effects (Takasuga *et al.*, 1999; Zhong *et al.*, 2003). We used wortmannin and LY294002, two potent and selective cell-permeable inhibitors of PI3K (Vlahos *et al.*, 1994; Nakanishi *et al.*, 1995), to investigate the possible role of this pathway in the adenosine-mediated down-regulation of DPPIV. However, neither LY294002 (2 μM) nor wortmannin (100 nM) impeded the adenosine response (Figure 3.5), excluding PI3K as the proximal step in adenosine signaling in this context.

Protein tyrosine kinase phosphorylation modulates the adenosine down-regulation of DPPIV

We next explored whether adenosine might be activating protein tyrosine kinase (PTK) signaling pathways. Two broad-spectrum PTK inhibitors were used in these

studies. The first inhibitor we tested was genistein, a potent inhibitor of most cellular PTKs (Akiyama *et al.*, 1987). Genistein at 20 μM (Figure 3.6) and 50 μM (data not shown) did not block the adenosine response. However, the presence of genistein consistently *enhanced* the down-regulation of DPPIV due to adenosine. This enhancement of the adenosine effect was substantial with genistein at a concentration of 20 μM (mean enhancement of adenosine effect 45.5%, 6 separate experiments), but less marked at 50 μM genistein (mean enhancement of adenosine effect 16.2%, 6 separate experiments). We also tested herbimycin A, a PTK inhibitor that differs from genistein by its selectivity for *src*-like PTKs (June *et al.*, 1990). Herbimycin A at 1 μM also did not block the adenosine effect (Figure 3.6). However, it did dramatically increase the baseline level of DPPIV (~ 60% in Figure 3.6), although it did not accentuate the adenosine effect in the same manner as genistein.

The adenosine down-regulation of DPPIV requires increased protein tyrosine phosphatase activity

Given that the broad-spectrum PTK inhibitor genistein was able to *enhance* the down-regulatory effect of adenosine, we predicted that phosphatase pathways might be important in the adenosine regulatory mechanism; specifically, phosphatase inhibition might *reduce* or even eliminate the adenosine effect.

Recent studies have reported that certain effects of adenosine are regulated via serine/threonine protein phosphatases (Narayan *et al.*, 2000; Liu and Hoffman, 2003); while other studies have found that adenosine action may also be linked to the activation of protein tyrosine phosphatase (PTP) activity (Abe and Saito, 1998; Murphy *et al.*, 2003;

Zhang *et al.*, 2004). We therefore explored whether the down-regulatory response of DPPIV might occur through the adenosine activation of phosphatase(s). Our initial approach was an evaluation of two commercially available broadly inhibitory cocktails of agents selective against serine/threonine and tyrosine protein phosphatases. Inhibition of serine/threonine phosphatases showed no evidence of abrogating the adenosine effect at up to 0.2% $^{v}/_{v}$ cocktail (Figure 3.7), or at a higher concentration (0.5% $^{v}/_{v}$, data not shown) that caused a substantial (> 60%) loss of cell viability. However, the tyrosine phosphatase inhibitor cocktail caused a dose-dependent dampening of the adenosine response up to 0.2% $^{v}/_{v}$ cocktail (Figure 3.7) and complete abrogation of the effect at 0.5% $^{v}/_{v}$ (data not shown), although that high concentration again led to a substantial (~ 50%) loss of cell viability.

Having found that the adenosine down-regulation of DPPIV could be consistently blocked with a broad mixture of PTP inhibitors, we focused in on the possible mechanism that was initiated by adenosine. The constituents of the mix were each tested separately at concentrations equivalent to their presence in the cocktail. Significantly, the adenosine response was only inhibited by sodium orthovanadate; the inhibitors sodium molybdate, sodium tartrate, and imidazole were without effect (data not shown). We therefore next treated the cells with adenosine (30 μM with coformycin) in the presence and absence of sodium orthovanadate, a widely-acting inhibitor of tyrosine and dual-specificity phosphatases (Hunter *et al.*, 1995). Orthovanadate caused a dose-dependent reduction of the adenosine effect and a complete abrogation at 200 μM (Figure 3.8). We next tested the peroxy derivative of orthovanadate, bpV(phen), which is a more potent and selective PTP inhibitor (Posner *et al.*, 1994). A complete block of the adenosine

down-regulation of DPPIV was attained following pretreatment of the cells with 20 μM bpV(phen) (Figure 3.8). The blocks imposed by orthovanadate and bpV(phen) at concentrations of 200 μM and 20 μM, respectively, were highly reproducible.

We proceeded to test other agents with known PTP inhibitory activity. The potent PTP inhibitor mpV(pic) (Posner *et al.*, 1994) at concentrations up to 50 μM did not block, or at best produced a partial block, in multiple experiments (data not shown). Our ability to assess the inhibitory effect of phenylarsine oxide (Garcia-Morales *et al.*, 1990) on the adenosine response was compromised by its high degree of cytotoxicity ($EC_{50} \sim 1$ μM, reaching complete cell death at 5 μM) over the 48 h treatment period. However, there was no inhibition of the adenosine response in experiments using phenylarsine oxide at 0.5 μM and 1 μM (see Appendix B). Taken together, these data demonstrate the involvement of a PTP(s) in down-regulation of cell-surface DPPIV on HT-29 cells exposed to adenosine. The selectivity to certain PTP inhibitors and not to others may provide clues as to the particular PTP that is activated by adenosine.

The adenosine down-regulation of DPPIV is dependent upon ERK1/2, but not p38 or JNK MAP kinases

We next considered the potential role of mitogen-activated protein kinase (MAPK) signaling pathways in mediating the adenosine response. Our rationale for initiating this study was the knowledge that MAPK pathways are subject to regulation by PTPs (Pulido *et al.*, 1998; Camps *et al.*, 1999; English *et al.*, 1999; Munoz *et al.*, 2003). Furthermore, adenosine has been reported to have the capacity for signaling through the p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK)

MAPK signaling pathways (Harrington *et al.*, 2000; Liu and Hoffmann, 2003; Schulte and Fredholm, 2003*a*; reviewed by Schulte and Fredholm, 2003*b*). It therefore seemed possible that adenosine-activated PTP(s) might act through MAPK pathways to cause the changes in DPPIV.

Inhibition of p38 with SB203580 (5 μM; Cuenda *et al.*, 1995; Jiang *et al.*, 1996), did not block or enhance the adenosine effect (Figure 3.9). The inhibition of JNK by SP600125 (Bennett *et al.*, 2001; Han *et al.*, 2001) led to a dose-dependent increase in baseline DPPIV expression (Figure 3.9; mean increase, 70.5% at 20 μM SP600125). However, there was also no block or enhancement of the adenosine down-regulation of DPPIV. The effect of PD98059, a MAPK kinase (MEK1) inhibitor (Alessi *et al.*, 1995), was more informative. Firstly, the treatment of HT-29 cells with PD98059 (25 μM) *itself* caused a down-regulation of DPPIV cell-surface protein that was comparable to the adenosine-evoked depression (Figure 3.10). This suggested the possibility that a decrease in the active tyrosine-phosphorylated form of ERK, caused either through adenosine-stimulated PTP activity, or through PD98059-mediated inhibition of MAPKK (MEK), is linked to a decline in DPPIV. Furthermore, PD98059 in combination with adenosine produced a slightly greater but *not additive* decrease in DPPIV cell-surface protein (Figure 3.10). This result is consistent with both the effects of adenosine and ERK inhibition acting through the same process. However, it might also indicate the existence of a minor, alternative pathway regulated by adenosine to further reduce levels of DPPIV. In any case, these findings provide indirect evidence that the adenosine-mediated down-regulation of DPPIV involves the ERK1/2 pathway and is associated with a decrease in ERK1/2 activation.

Adenosine causes a decrease in phosphorylated ERK1/2 that is dependent upon an orthovanadate- and bpV(phen)-dependent PTP

Given the evidence supporting an adenosine-triggered PTP activity and involvement of ERK1/2 in the down-regulation of DPPIV, we investigated directly the effect of adenosine on ERK1/2 tyrosine phosphorylation. As shown in Figure 3.11, both ERK1 and ERK2 were constitutively activated in HT-29 cells. Short-term exposure to a single high dose of adenosine (300 μM) markedly decreased the level of tyrosine-phosphorylated ERK1 and ERK2 (Figure 3.11). The effect of adenosine was evident within 10 min, maximal by 20 min, and sustained for 60 minutes after stimulation. We prolonged the time course of adenosine (300 μM) exposure and found that the level of ERK1/2 phosphorylation returned to control levels within ~ 3 h (Figure 3.11), and there was no further change in ERK1/2 phosphorylation even at times out to 24 h. The dose-response relationship of adenosine on ERK1/2 dephosphorylation was also evaluated. Adenosine (single dose, no coformycin present) caused an observable decrease in ERK1/2 phosphorylation at 10 μM and a maximum effect at a concentration of about 100 μM (Figure 3.12). This dose-response relationship exactly parallels the concentration-dependence of the adenosine down-regulation of DPPIV following a single adenosine dose (see Figure 2.9). A reduction in overall tyrosine protein phosphorylation in the 40-50 kDa range of proteins from adenosine-treated HT-29 cells was also observed after western blotting of total cellular lysates with anti-phosphotyrosine mAb (see Appendix C), consistent with a reduction in ERK1/2 phosphorylation. These findings, together with those of Figures 3.8 and 3.10, strongly argue that the adenosine reduction of cell-surface

DPPIV in HT-29 cells occurs in close association with negative regulation of the ERK1/2 signaling pathway.

Finally, HT-29 cells were pretreated with sodium orthovanadate (200 μM) to inhibit PTPs, and then exposed to adenosine for 40 min before examining the consequences for ERK1/2 phosphorylation. The dose-dependent inhibitory effect of adenosine on ERK1/2 tyrosine phosphorylation was abrogated in the presence of sodium orthovanadate (Figure 3.13). The same abrogation of adenosine-induced ERK1/2 dephosphorylation was also observed using bpV(phen) at 20 μM (data not shown). These findings demonstrate that reduced phosphorylation and therefore activity of ERK1/2 due to adenosine is dependent on PTP(s). The inhibition of ERK1/2 phosphorylation due to adenosine is therefore sensitive to PTP inhibition by either orthovanadate or bpV(phen), which also abrogate the adenosine-induced down-regulation of DPPIV cell-surface protein.

The PTP activated by adenosine is not SHP-1, SHP-2, or PTP-1B

We conducted a preliminary pharmacological experiment in an attempt to identify the PTP target activated by adenosine to cause down-regulation of DPPIV on HT-29 cells. The novel PTP inhibitor sodium stibogluconate (Pathak and Yi, 2001) was tested for its ability to block the adenosine effect. Sodium stibogluconate has strong inhibitory activity against SHP-1, as well as SHP-2 and PTP-1B at higher concentrations. However, sodium stibogluconate (100 μM) caused no change in the decrease in DPPIV levels due to adenosine (Figure 3.14).

Table 3.1 Lack of effect of adenosine receptor antagonists on the adenosine-mediated down-regulation of DPPIV.

Adenosine receptor antagonist		Treatment	
		Control	**Adenosine**
A_1	Control	1,572 ± 14	1,167 ± 20 **
	DPCPX	1,377 ± 26	1,059 ± 31 **
A_{2A}	Control	5,196 ± 118	3,873 ± 70 **
	CSC	5,231 ± 32	4,000 ± 63 **
A_{2B}	Control	2,008 ± 18	1,507 ± 50 **
	Alloxazine	1,945 ± 31	1,536 ± 38 **
	Control	1,551 ± 18	1,189 ± 19 **
	Enprofylline	1,370 ± 37	944 ± 28 **
A_3	Control	3,565 ± 51	2,855 ± 49 **
	MRS1191	3,503 ± 46	2,924 ± 67 **
	Control	4,053 ± 93	2,920 ± 56 **
	MRS1220	4,163 ± 87	3,240 ± 38 **

HT-29 cells were pretreated for 30 min with the adenosine receptor antagonists DPCPX (A_1, 30 μM), CSC (A_{2A}, 30 μM), alloxazine or enprofylline (A_{2B}, 100 μM), or MRS1191 or MRS1220 (A_3, 30 μM) before incubation with medium alone or adenosine (30 μM plus 2.5 μM coformycin). Forty-eight h later surface expression of DPPIV was measured by radioantibody binding assay. The data are mean cpm ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

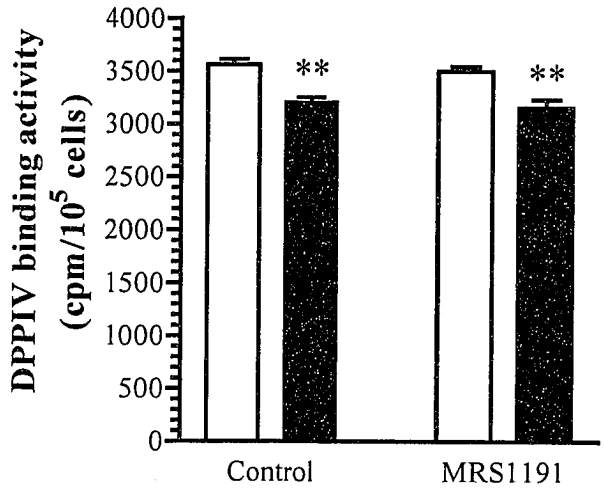

Figure 3.1 Representative example of the failure of adenosine receptor antagonists to block the adenosine down-regulation of DPPIV.

HT-29 cells were pretreated for 30 min with control vehicle or with the A_3 receptor antagonist MRS1191 (30 μM). The cells were then incubated with medium alone (*clear bars*) or adenosine at 10 μM with 2.5 μM coformycin (*filled bars*). Forty-eight h later surface expression of DPPIV was measured by radioantibody binding assay. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

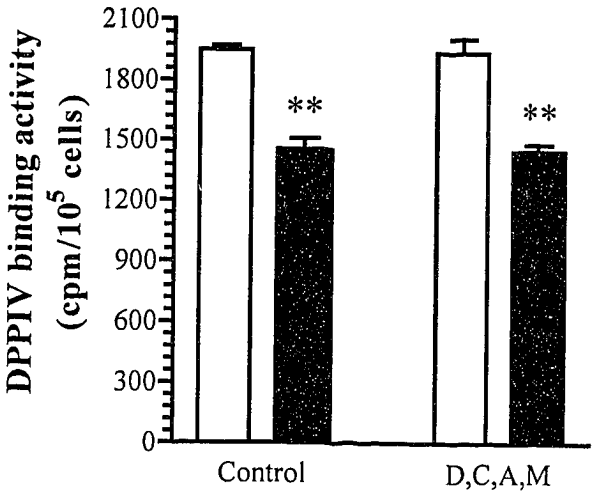

Figure 3.2 Concurrent treatment with A_1, A_{2A}, A_{2B}, and A_3 adenosine receptor antagonists does not block the decline in DPPIV due to adenosine. HT-29 cells were pretreated for 30 min with control vehicle or a combination of 1 μM DPCPX, 1 μM CSC, 5 μM alloxazine, and 1 μM MRS1523. The cells were then incubated with medium alone (*clear bars*) or adenosine at 10 μM with 2.5 μM coformycin (*filled bars*). Forty-eight h later surface expression of DPPIV was measured by radioantibody binding assay. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

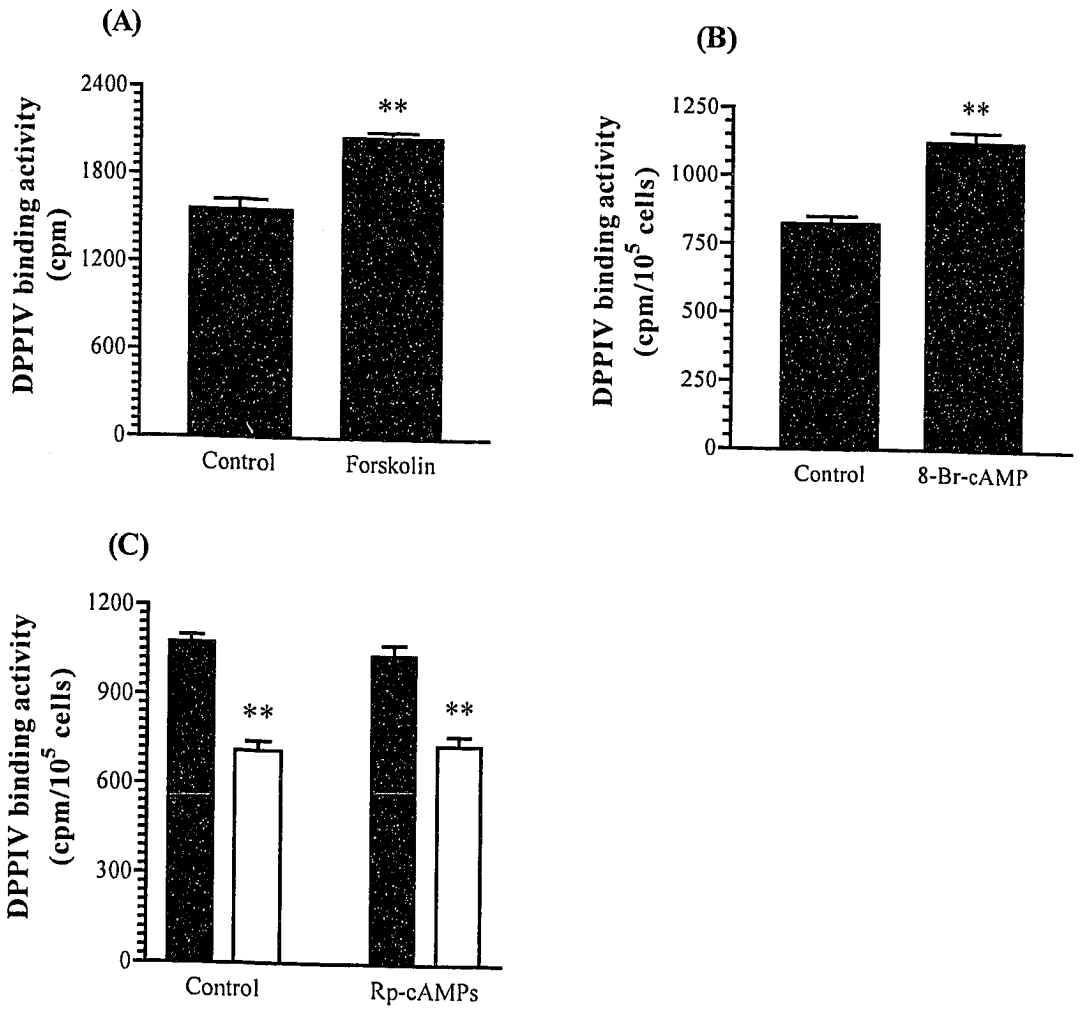

Figure 3.3 cAMP/PKA-dependent signaling pathways are not required for the adenosine-mediated down-regulation of DPPIV.

Cell-surface DPPIV levels were measured following 48 h treatment with control vehicle or **(A)** forskolin (50 μM) or **(B)** 8-Br-cAMP (1 mM). **(C)** HT-29 cells were pretreated with the PKA inhibitor Rp-cAMPs (50 μM) followed by vehicle alone (*filled bars*) or 30 μM adenosine with 2.5 μM coformycin (*clear bars*). Cell-surface DPPIV levels were measured 48 h later. The data are mean values ± SE ($n = 4$). **, significant change, $P < 0.01$.

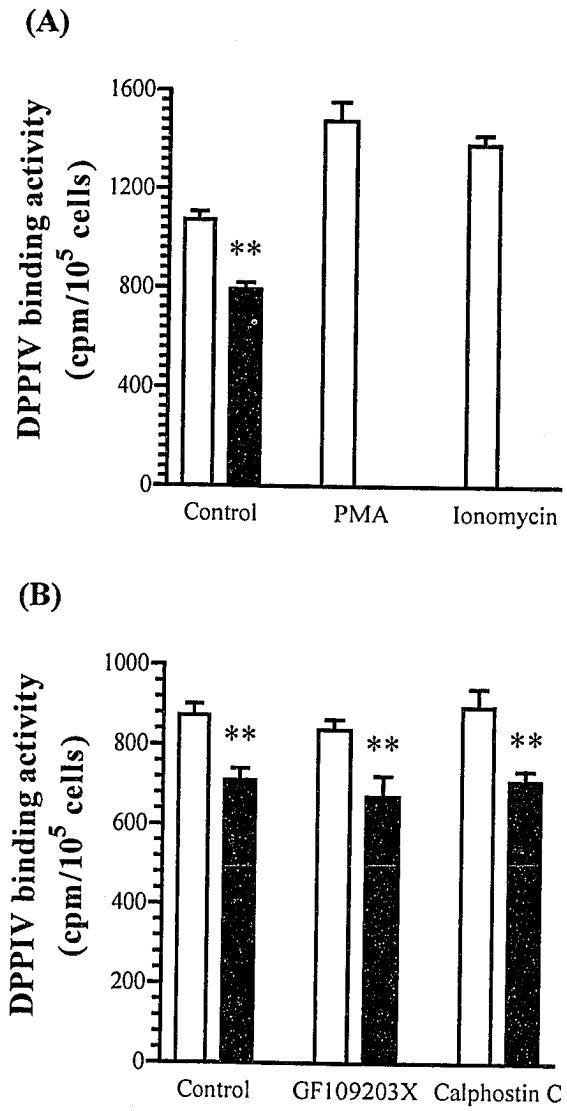

Figure 3.4 Adenosine regulation of DPPIV is independent of the PKC signaling pathway.

(A) HT-29 cells were treated with the PKC activator phorbol 12-myristate 13-acetate (PMA, 5 nM) or the calcium ionophore ionomycin (1 μM) for 48 h after which cell-surface DPPIV levels were measured by radioantibody binding assay. **(B)** Inhibition of PKC signaling does not block the adenosine effect. HT-29 cells were pretreated with the PKC inhibitors GF109203X (GF, 1 μM) or calphostin C (100 nM) for 30 min followed by control vehicle (*clear bars*) or 30 μM adenosine with 2.5 μM coformycin (*filled bars*). Forty-eight h later surface expression of DPPIV was measured by radioantibody binding assay. The data are mean values ± SE ($n = 4$). **, significant change, $P < 0.01$.

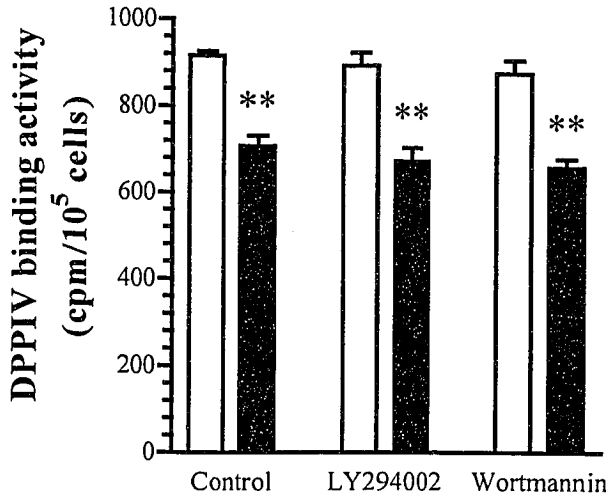

Figure 3.5 Adenosine regulation of DPPIV is not dependent on PI3K signaling. HT-29 cells were pretreated with the PI3K inhibitors LY294002 (2 μM) or wortmannin (100 nM) for 30 min followed by control vehicle (*clear bars*) or 30 μM adenosine with 2.5 μM coformycin (*filled bars*). Forty-eight h later surface expression of DPPIV was measured by radioantibody binding assay. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

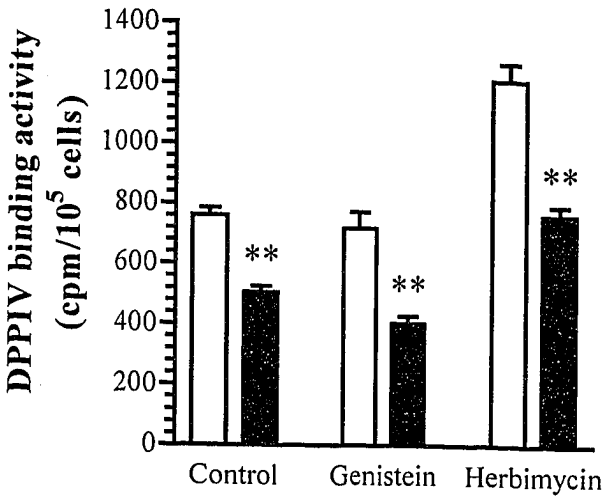

Figure 3.6 Effect of PTK signaling inhibitors on the adenosine-mediated down-regulation of DPPIV.

HT-29 cells were pretreated with the PTK inhibitors genistein (20 μM) or herbimycin A (1 μM) for 30 min followed by control vehicle (*clear bars*) or 30 μM adenosine with 2.5 μM coformycin (*filled bars*). Forty-eight h later surface expression of DPPIV was measured by radioantibody binding assay. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

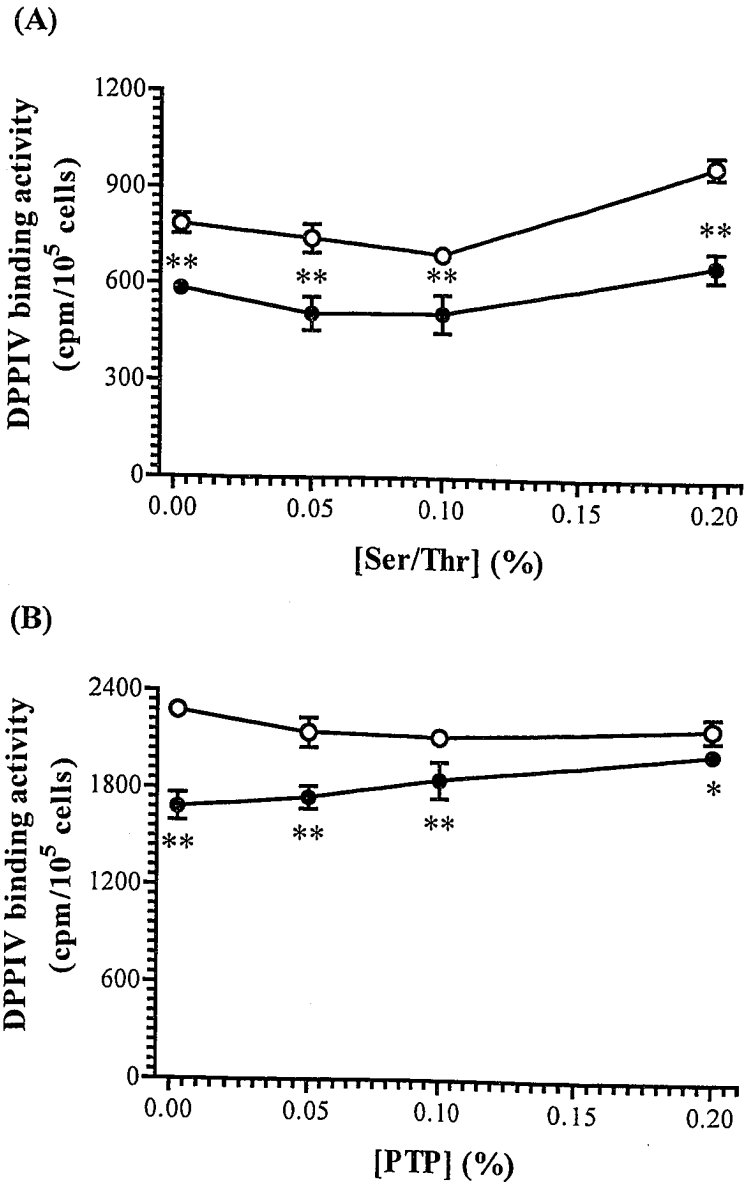

Figure 3.7 Effect of phosphatase inhibition on the adenosine-mediated down-regulation of DPPIV.

HT-29 cells were pretreated with phosphatase inhibitory cocktails against **(A)** serine/threonine (Ser/Thr) or **(B)** tyrosine protein phosphatases (PTP) at the indicated concentrations for 30 min followed by control vehicle (*clear circles*) or 30 μM adenosine with 2.5 μM coformycin (*filled circles*). Cell-surface levels of DPPIV were evaluated 48 h later by radioantibody binding assay. The data are mean values ± SE ($n = 4$). *, significant reduction by adenosine, $P < 0.05$. **, $P < 0.01$.

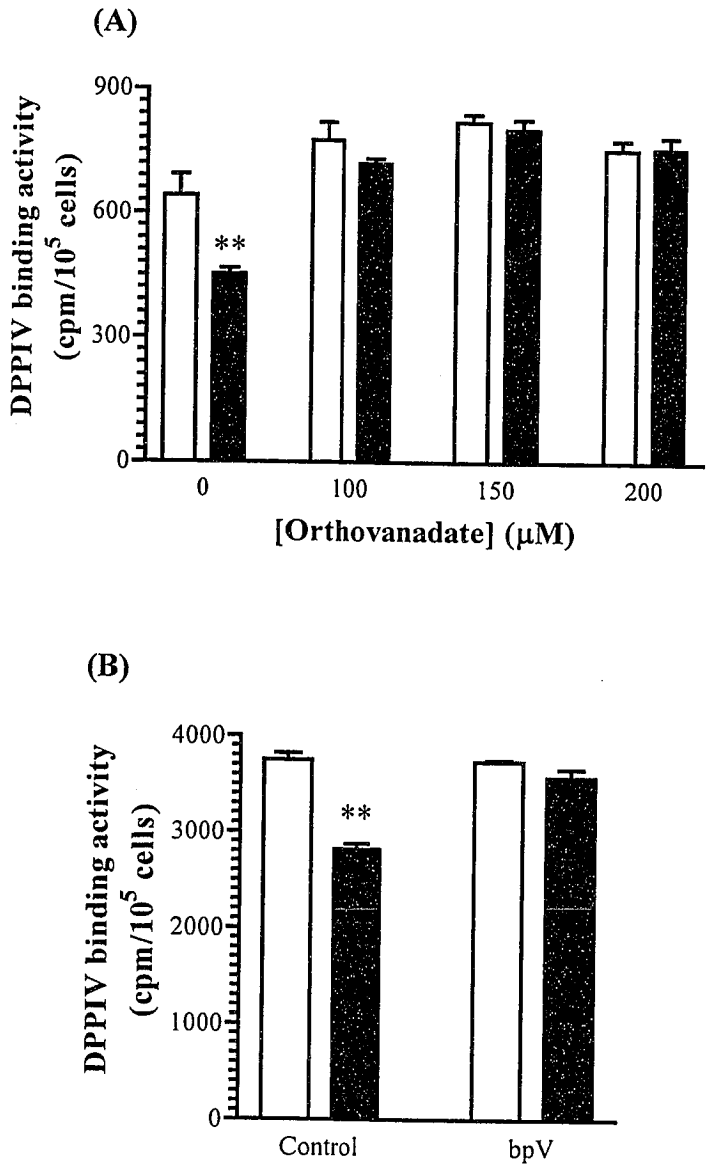

Figure 3.8 Protein tyrosine phosphatase inhibitors block the adenosine-mediated down-regulation of DPPIV on HT-29 cells.

HT-29 cells were pretreated for 30 min with **(A)** sodium orthovanadate (100, 150, 200 μM) or **(B)** bpV(phen) (20 μM). The cells were then treated with control vehicle (*clear bars*) or 30 μM adenosine with 2.5 μM coformycin (*filled bars*). Forty-eight h later surface expression of DPPIV was measured. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

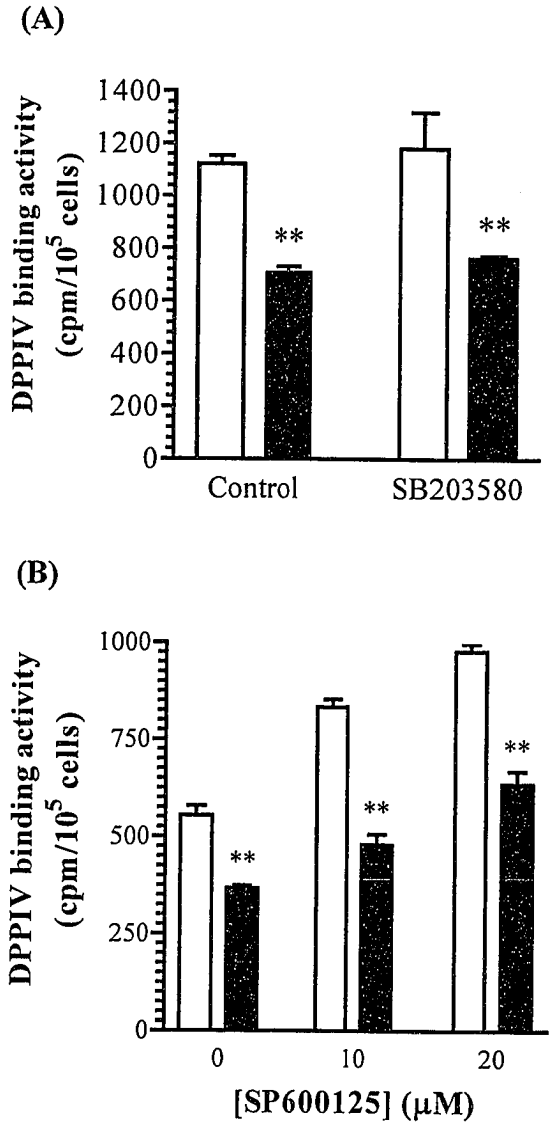

Figure 3.9 Adenosine regulation of DPPIV is independent of p38 and JNK MAPK signaling pathways.

HT-29 cells were pretreated with the **(A)** p38 inhibitor SB203580 (5 μM) or **(B)** JNK inhibitor SP600125 (10, 20 μM) for 30 min, followed by control vehicle (*clear bars*) or 30 μM adenosine with 2.5 μM coformycin (*filled bars*). Forty-eight h later surface expression of DPPIV was measured. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

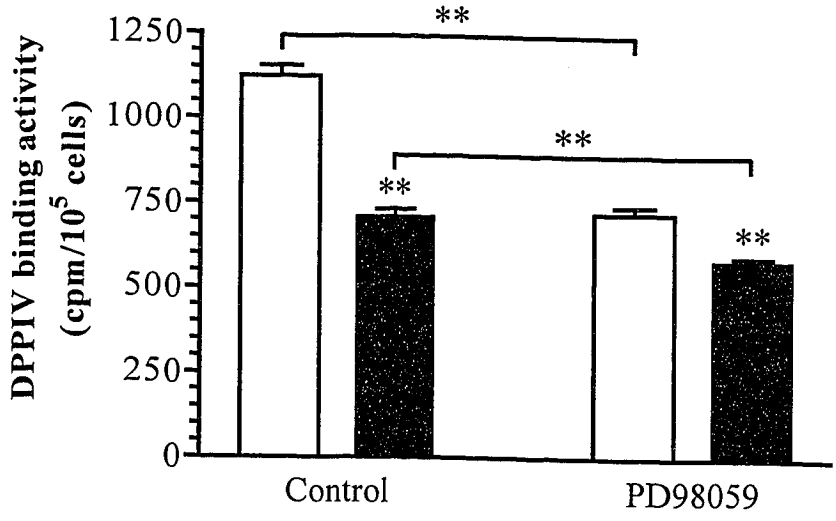

Figure 3.10 Inhibition of ERK1/2 MAPK signaling produces down-regulation of DPPIV and accentuates the adenosine effect.

HT-29 cells were pretreated with the MEK1 inhibitor PD98059 (25 μM) for 30 min, followed by control vehicle (*clear bars*) or 30 μM adenosine with 2.5 μM coformycin (*filled bars*). Forty-eight h later surface expression of DPPIV was measured. The data are mean values ± SE ($n = 4$). **, significant change, $P < 0.01$.

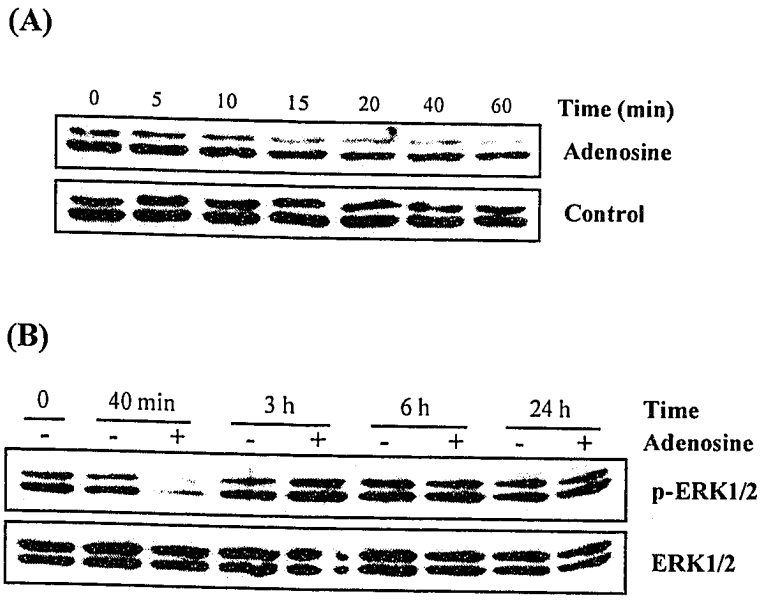

Figure 3.11 Adenosine reduces ERK1/2 tyrosine phosphorylation in HT-29 cells. **(A)** Acute reduction of ERK1/2 phosphorylation. HT-29 cells were stimulated with adenosine (300 μM) or control vehicle and then harvested at the indicated time points. Cell lysates (20 μg) were subjected to Western blot analysis for phosphorylated ERK1/2. **(B)** Longer-term effect of adenosine on ERK1/2 phosphorylation. HT-29 cells were stimulated with adenosine (300 μM) or control vehicle and then harvested at the indicated time points. Western blotting was performed for phosphorylated ERK1/2 (p-ERK1/2, *upper panel*) and total ERK1/2 (*lower panel*) protein expression.

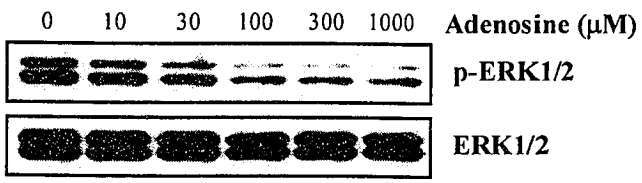

Figure 3.12 Dose-dependence of the adenosine-induced reduction of ERK1/2 phosphorylation in HT-29 cells.

HT-29 cells were stimulated with adenosine at the indicated concentrations and lysed after 45 min. Western blotting was performed to detect phosphorylated (p-ERK1/2, *upper panel*) and total ERK1/2 (*lower panel*) protein expression.

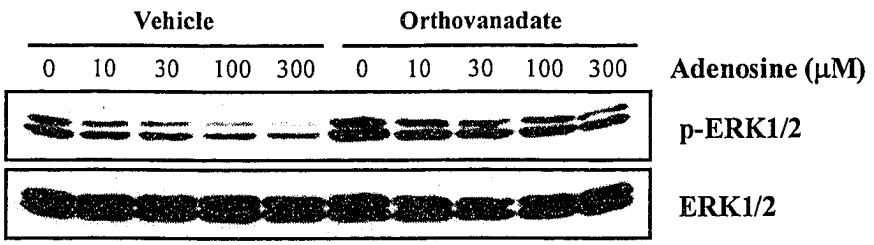

Figure 3.13 PTP inhibition blocks the adenosine-induced reduction of ERK1/2 phosphorylation in HT-29 cells.

HT-29 cells were pretreated for 15 min with control vehicle or sodium orthovanadate (200 μM). The cells were then stimulated with adenosine at the indicated concentrations for 40 min, and then harvested for Western analysis. Cell lysates (20 μg) were subjected to electrophoresis and blots were analyzed for phosphorylated ERK1/2 (p-ERK1/2, *upper panel*) and total ERK1/2 (*lower panel*).

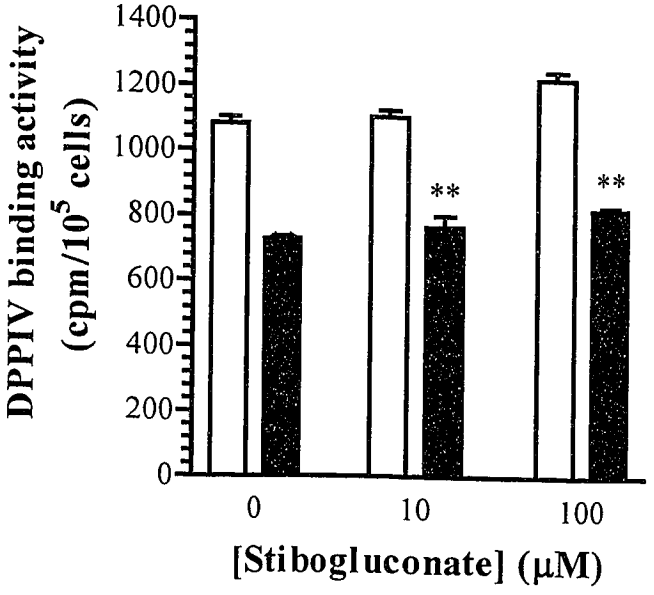

Figure 3.14 Sodium stibogluconate does not block the adenosine down-regulation of cell-surface DPPIV protein.

HT-29 cells were pretreated with the PTP inhibitor sodium stibogluconate at the indicated concentrations for 30 min, followed by control vehicle (*clear bars*) or 30 μM adenosine with 2.5 μM coformycin (*filled bars*). Forty-eight h later surface expression of DPPIV was measured. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

DISCUSSION

The objective of the experiments presented in this chapter was to understand how extracellular adenosine leads to the decrease in cell-surface DPPIV. Specifically, we sought to identify the initial events that are involved following the encounter of HT-29 cells with the adenosine signal. This involved extensive experiments to determine the involvement of cell-surface adenosine receptors and the specific signaling pathway regulated by adenosine action.

Adenosine receptors and the DPPIV down-regulation

Our expectation was that one of the well-characterized adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, or A_3) would be involved. We paid particular attention to the A_{2B} and A_3 receptor subtypes because of their higher level of expression on the HT-29 cells (M. Mujoomdar and J. Blay, unpublished data). However, numerous experiments using seven different established antagonists (at concentrations several-fold in excess of the IC_{50} values for antagonism) failed to block the adenosine down-regulation of DPPIV. This included two different A_{2B} antagonists (alloxazine and enprofylline) and three different A_3 antagonists (MRS1191, MRS1220, and MRS1523). Of these A_3 receptor antagonists, MRS1191 is most selective while MRS1220 has the highest affinity for the human A_3 receptor (Jacobson, 1998). In addition to the failure of adenosine receptor antagonists, the broadly selective *agonists* NECA (A_1, A_2, and A_3) and R-PIA (A_1 and A_2) failed to mimic the action of adenosine even at concentrations as high as 100 μM. Finally, the failure of the PKA inhibitor Rp-cAMPs to alter the adenosine-mediated

down-regulation of DPPIV shows that the major signaling route employed by all four of the adenosine receptor subtypes, the cAMP signaling pathway, is not involved.

The inability to block the adenosine response in our antagonist studies might have been complicated by the possibility of dynamic adenosine receptor expression on HT-29 cells. Studies in our laboratory have shown that the pattern of adenosine receptor expression varies depending on cell culture conditions, including cell density (M. Mujoomdar and J. Blay, unpublished data). This is consistent with data by Lelièvre *et al.* (2000) showing that adenosine receptor expression on HT-29 cells also changes as a function of cell differentiation. However, when a combination of antagonists against all four subtypes (at the highest feasible concentrations) was used, there was absolutely no decline in the adenosine-evoked reduction of DPPIV (Figure 3.2). Our experimental treatments were appropriate, and the antagonists had appropriate activity, because the identical approach using the same reagents has allowed us to completely abrogate the adenosine-mediated increase of the CXCR4 chemokine receptor in these same cells (A_2 receptors are involved in the regulation of CXCR4; C.L. Richard, E.Y. Tan, and J. Blay, submitted manuscript). We could therefore exclude the possibility of changing adenosine receptor expression profiles, as well as the possible use of more than one receptor subtype to elicit the adenosine response. This compelled us toward the conclusion that the adenosine down-regulation of DPPIV does not occur through the conventional adenosine receptors so far known.

A number of groups have found evidence and proposed the existence of 'atypical', or non-classical, adenosine receptor subtypes. This has included the possibility of a novel, lower affinity adenosine receptor (Apasov *et al.*, 2000), an atypical adenosine

A_{2A} receptor (El Yacoubi *et al.*, 2000), and atypical A_3 adenosine receptors (Ezeamuzie and Philips, 2003; Wolber and Fozard, 2005).

We established in Chapter 2 that high levels of adenosine are sensed at the cell surface, rather than evoking a change in DPPIV after entry into the cell through nucleoside transporters. The down-regulation of DPPIV is not simply a cell-surface perturbation triggered by extracellular adenosine that leads to intracellular sequestration of DPPIV (as for example occurs with Caco-2 cells treated with forskolin; Baricault *et al.*, 1995). The time course is relatively slow (maximum reached at 48 h), and we show here that there is clearly an intermediary intracellular signaling pathway that leads to the decline in DPPIV protein.

There was also the possibility that adenosine might signal via nucleotide-sensitive P_2-purinergic receptors. HT-29 cells have been found to express metabotropic P_{2Y2} receptors at the cell surface (Höpfner *et al.*, 1998; Höpfner *et al.*, 2001). However, these receptors appear to mediate growth inhibition and apoptosis of HT-29 cells in response to ATP. Furthermore, signaling downstream of these receptors is virtually absent even in the presence of very high levels of adenosine (500 μM). Therefore, the adenosine-mediated down-regulation of DPPIV is likely triggered by a novel adenosine receptor or adenosine-binding protein. Alternatively, the effect might be mediated by an already characterized membrane protein with the unreported capacity of responding to adenosine.

Other adenosine signaling pathways

We proceeded to investigate candidate signal transduction pathways that might be involved in the adenosine regulation of DPPIV. As indicated above, the cAMP pathway

was not the route of signaling for adenosine. In support of this view, direct stimulation of adenylyl cyclase with forskolin, or treatment with the stable, cell-permeable analogue 8-Br-cAMP, conversely caused an *increase* in the levels of cell-surface DPPIV. These effects are consistent with the increase in DPPIV mRNA expression observed in renal carcinoma cells in response to these same agents (Kehlen *et al.*, 1998), and show that DPPIV has the capacity to be up-regulated through cAMP in HT-29 cells. The findings indicate that either the adenosine receptors are not efficiently coupled to adenylyl cyclase in HT-29 cells, or the alternate signaling mechanism that is involved is able to overcome the effects of signaling through the established adenosine receptors.

We also found increases in DPPIV cell-surface protein using the PKC activator PMA or the calcium ionophore ionomycin. Again, this is consistent with the finding that DPPIV mRNA expression is increased in renal carcinoma cells treated with the calcium ionophore A23187 (Kehlen *et al.*, 1998). Increases in cell-surface DPPIV expression also occur in human gingival fibroblasts exposed to PMA (Nemoto *et al.*, 1999). This confirms that products of phospholipase C activation, and elevations in intracellular calcium, are able to up-regulate DPPIV. However, phospholipase C activation is clearly not the route of adenosine action because the effect is the reverse of that due to adenosine. Furthermore, the PKC inhibitors GF109203X, calphostin C, and Ro-31-8220 all failed to block the adenosine response. Similarly, we excluded the possible involvement of PI3K by the use of structurally different inhibitors.

Two protein tyrosine kinase inhibitors, genistein and herbimycin A, did not inhibit the adenosine response. We did however find that the adenosine down-regulation of DPPIV was *enhanced* by genistein, suggesting that phosphorylation pathways were

involved. This led us to our finding that the adenosine effect on DPPIV is exerted through intermediate steps that involve (i) activation of a protein tyrosine phosphatase that is sensitive to inhibition by sodium orthovanadate and bpV(phen), and (ii) reduction in the activation of ERK1/2 MAPK, but not p38 or JNK MAPKs.

Interestingly, our studies excluding the involvement of PKC and p38 MAPK pathways inadvertently supported our conclusion that adenosine is acting at a cell-surface site, as opposed to adenosine uptake. Recently, Huang *et al.* (2002) reported a novel pharmacological function for the p38 MAPK inhibitor SB203580 in preventing NBTI-sensitive (ENT1-mediated) nucleoside uptake. This inhibitory property strongly occurred at concentrations typically used to inhibit the kinase. A subsequent report by the same group also found that the PKC inhibitors GF109203X and Ro-31-8220 strongly blocked ENT1-mediated nucleoside uptake (Huang *et al.*, 2003). Importantly, we used these specific PKC inhibitors at concentrations in excess of the IC_{50} values for inhibition of nucleoside uptake.

Adenosine regulation of ERK1/2 MAPK

Regulation of ERK1/2 (p44/p42) MAPK in response to adenosine has been widely reported in different cellular systems. Human A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors expressed in CHO cells (and A_{2A} receptors expressed in HEK293 cells) all couple to the ERK1/2 MAPK pathway. However, in each case adenosine or an appropriate synthetic agonist *increases* the phosphorylation of ERK1/2 (Dickenson *et al.*, 1998; Seidel *et al.*, 1999; Schulte and Fredholm, 2000; Trincavelli *et al.*, 2002). Caution must be taken in the interpretation of such models because it is well known that

expression/over-expression of cloned receptors might result in signaling that differs from, or sometimes is opposite to, that of endogenously expressed receptors. For example, adenosine A_1 agonists exert a negative inotropic effect in wild-type mice, but exert a positive inotropic effect in mice overexpressing A_1 receptors (Neumann *et al.*, 1999). However, the stimulation of ERK1/2 through endogenous adenosine receptors (primarily A_2) has also been observed: in primary human endothelial cells (A_{2A}; Sexl *et al.*, 1997), untransfected human embryonic kidney cells (A_{2B}; Gao *et al.*, 1999), HMC-1 human mast cells (A_{2B}; Feoktistov *et al.*, 1999), BR canine mastocytoma cells (A_{2B}; Gao *et al.*, 1999), PC12 rat pheochromocytoma cells (A_{2A}; Arslan *et al.*, 2000), and XS-106 mouse dendritic cells (A_{2B} and A_3; Dickenson *et al.*, 2003).

Conversely, there are data showing that adenosine may *inhibit* the stimulation of ERK1/2 phosphorylation induced by other mediators, including thrombin (Hirano *et al.*, 1996) and nerve growth factor (Arslan *et al.*, 2000). Recently, Merighi *et al.* (2005) demonstrated inhibition of ERK1/2 phosphorylation in A375 human melanoma cells by the selective A_3 receptor agonist Cl-IB-MECA. This response to Cl-IB-MECA was indeed generated at the cell surface because of the abrogating effect of A_3 receptor antagonists. Inhibition of A_3 adenosine receptor expression using small-interfering RNA also abolished agonist effects. However, our data, to the best of our knowledge, represents the first observation of adenosine *itself* causing negative regulation of the ERK1/2 MAPK pathway. It is notable that most studies of ERK1/2 activation have used stable ligands such as NECA or agents selective for the appropriate receptor subtype, whereas we would argue based upon earlier observations that adenosine itself may elicit different cellular responses to its analogues (Mujoomdar *et al.*, 2003). Significantly,

adenosine itself has been shown to diminish activation of the p38α MAPK in endothelial cells, although ERK1/2 activity was slightly elevated in that system (Harrington *et al.*, 2000).

The dose-response relationship for adenosine inhibition of ERK1/2 phosphorylation in HT-29 cells exactly parallels that of the reduction in DPPIV cell-surface protein. This is indicative of a single affinity of ligand interaction for the two adenosine effects, which is consistent with linkage to a common receptor/cell-surface site that initiates the response. However, our results differ from other studies of ERK1/2 activation not only because we see an inhibition, rather than stimulation, of activity by adenosine, but that we have failed to assign sensing of the initial signal to any of the four well-characterized adenosine receptor subtypes. This is not for reasons of inappropriate adenosine concentration. We have shown sensitivity of the DPPIV down-regulation to adenosine as low as the 30 μM range without inhibiting its metabolism. Furthermore, we routinely treat the cells with 10-30 μM adenosine in the presence of 2.5 μM coformycin to inhibit ADA-mediated breakdown. This contrasts, for example, with the millimolar dosing used by Harrington *et al.* (2000) in their studies of p38α modulation, which leads to apoptosis.

The lack of evidence for the involvement of cAMP pathways in adenosine-induced DPPIV down-regulation is consistent with data showing that adenosine modulates ERK1/2 signaling irrespective of whether increases (A_{2A}, A_{2B}) or decreases (A_1, A_3) in cAMP would be expected to occur (Dickenson *et al.*, 1998; Seidel *et al.*, 1999; Schulte and Fredholm, 2000; Trincavelli *et al.*, 2002; Dickenson *et al.*, 2003). It is also consistent with the finding that stable cAMP analogues do not produce changes in

ERK1/2 (Sexl *et al.*, 1997; Seidel *et al.*, 1999). Moreover, the dose-response relationships for ERK1/2 phosphorylation versus cAMP accumulation in sensitive cells differ by two orders of magnitude (Schulte and Fredholm, 2000). The situation does, however, differ in adenosine receptor-transfected CHO and HEK293 cell models, in which cAMP appears to play a major causal role in ERK1/2 activation (Hirano *et al.*, 1996; Gao *et al.*, 1999).

Involvement of protein tyrosine phosphatases

We have found striking evidence for adenosine stimulation of a protein phosphatase activity that is able to produce the dephosphorylation of ERK1/2 that we have observed. A cocktail of inhibitors that blocks serine/threonine protein phosphatases, including PP1 and PP2A had no effect on the adenosine down-regulation of DPPIV. This excluded the possibility that adenosine might be acting through PP2A, which has been shown to occur in cardiomyocytes via the A_1 adenosine receptor (Liu and Hoffmann, 2002; Liu and Hoffmann, 2003). However, a protein tyrosine phosphatase inhibitor cocktail, and the individual PTP inhibitors sodium orthovanadate and bpV(phen) completely abrogated *both* the adenosine reduction of ERK1/2 activation *and* the down-regulation of cell-surface DPPIV protein. This observation parallels the findings of a recent exploration of adenosine inhibition of IL-2 signaling in T cells (Zhang *et al.*, 2004). In that work, adenosine was found to activate Src homology PTP 2 (SHP-2) to reduce the tyrosine phosphorylation of STAT5a/5b, and that this could be blocked by the PTP inhibitors orthovanadate and bpV(phen). It is noteworthy that the adenosine reduction of p38α MAPK activity in endothelial cells also appears to involve a PTP that is sensitive to vanadate inhibition (Harrington *et al.*, 2000).

The PTP target that is the focus of our adenosine action and which is inhibited by orthovanadate and bpV(phen) was not identified. Adenosine activation of protein tyrosine phosphatase activity represents a novel mechanism in the field of adenosine-induced responses. With the exception of Zhang *et al.* (2004), who implicated SHP-2 as the target of adenosine action, the remaining reports have failed to identify any specific PTP that is activated by the adenosine regulatory mechanism (Abe and Saito, 1998; Harrington *et al.*, 2000; Crist *et al.*, 2001; Murphy *et al.*, 2003). This is not surprising based on the poor understanding of the specific PTPs that are subject to inhibition by different tyrosine phosphatase inhibitors. Moreover, many of the biological functions and substrate specificities of PTPs have yet to be identified. It should be appreciated that PTPs constitute a superfamily of >75 enzymes (reviewed by Tonks and Neel, 2001), which include the transmembrane, receptor-like PTPs (RPTPs) and non-transmembrane (cytoplasmic) PTPs. The PTP superfamily also includes the dual-specificity phosphatases (DSPs), which typically dephosphorylate protein substrates that contain phosphotyrosine, phosphoserine, and phosphothreonine.

The action of adenosine in our system was not inhibited by a number of alternative PTP inhibitors, including sodium molybdate, sodium tartrate, imidazole, mpV(pic) or phenylarsine oxide. This indicates the marked selectivity for inhibition of the PTP involved. The failure of these inhibitors to have effects also excludes the involvement of various general acid and alkaline phosphatases that would be sensitive to vanadate inhibition, including alkaline phosphatases characteristic of intestinal epithelial cells (Seargeant and Stinson, 1979; Wang and Gilles-Baillien, 1992). We were able to rule out the involvement of SHP-1, SHP-2, and PTP-1B based on the failure of the PTP

inhibitor sodium stibogluconate to block the adenosine response. However, sodium stibogluconate is not inhibitory against DSPs (Pathak and Yi, 2001) as opposed to orthovanadate, which is capable of blocking this subclass of PTPs (Hunter, 1995).

Speculation on the adenosine-activated PTP

Our strategy to identify the PTP(s) activated by adenosine relies on the knowledge that the adenosine down-regulation of DPPIV could be consistently inhibited by orthovanadate and bpV(phen). It would then be logical to investigate the PTP inhibitory profile of bpV(phen) instead of orthovanadate based on its more selective nature (Posner *et al.*, 1994). Indeed, other groups have appreciated this distinction as a means to potentially identify the specific PTP involved (e.g. Roy *et al.*, 2001; Bieger *et al.*, 2002). Recent reports have begun to identify a number of PTPs that can be inhibited by bpV(phen). These bpV(phen)-sensitive PTPs include: SHP-1 (Fortin *et al.*, 2001; Barat and Tremblay, 2003), SHP-2 (Zhang *et al.*, 2004), PTP-1B (Krejsa *et al.*, 1997; Schmid *et al.*, 2004), Cdc25A (Faure *et al.*, 1995; Scrivens *et al.*, 2003), DSP4 (Scrivens *et al.*, 2003), PTEN (Schmid *et al.*, 2004), PRL-3 (Wu *et al.*, 2004), CD45 (Krejsa *et al.*, 1997), PTPβ (Schmid *et al.*, 2004), and PTPH1 (Lavoie *et al.*, 2000). The PTP CD45 is a RPTP that is exclusively expressed in T cells (Torimoto *et al.*, 1991) and its involvement can therefore be ruled out with that of SHP-1, SHP-2, and PTP-1B. However, the ability of bpV(phen) to inhibit the activity of Cdc25A, DSP4, PTEN, and PRL-3 indicates that it is not strictly a tyrosine-specific phosphatase, but also inhibits dual-specificity phosphatases. Cdc25A is a DSP overexpressed in several cancers that is required for cell cycle progression of cancer cells through G_1-S (Scrivens *et al.*, 2003). Interestingly, PRL-

3 has been implicated in colorectal carcinoma metastasis; specifically, its expression is elevated in almost all metastatic lesions derived from colorectal cancers, regardless of the site of metastasis (liver, lung, brain, or ovary; Bardelli *et al.*, 2003; Kato *et al.*, 2004).

The phosphatase activity stimulated by adenosine to down-regulate DPPIV can also be distinguished by having specificity against activated ERK MAPK. MAP kinases are activated following phosphorylation of a Thr-X-Tyr (where X represents glutamine for ERK) motif in their activation loops by a dual specificity MAPK kinase (MEK). Phosphorylation of both the threonyl and tyrosyl residue within this motif is required for activity (reviewed by Camps *et al.*, 1999; Tonks and Neel, 2001). It then follows that inactivation of MAPKs may be achieved either through the action of two distinct single specificity phosphatases acting individually on the phosphorylated residues, or through the activity of a single DSP that recognizes both residues. The DSPs that inactivate ERK are typically MAP kinase phosphatases (MKPs).

The protein Ser/Thr phosphatase PP2A has been implicated as a physiologically relevant ERK phosphatase (Zhou *et al.*, 2002). However, our data excludes the involvement of this class of phosphatase. In addition, our exclusion of the involvement of SHP-1, SHP-2, and PTP-1B is strengthened based on the lack of activity of these phosphatases against ERK (Zhou *et al.*, 2002). Furthermore, SHP-2 is instead usually coupled to an *increase* in ERK activation (Shi *et al.*, 1998; Cunnick *et al.*, 2002).

Early on it was suggested that bpV(phen) might inhibit DSPs involved in the negative regulation of ERK MAP kinase (Band and Posner, 1997; Band *et al.*, 1997), leading to the suggestion that bpV(phen) might be of value in identifying phosphatases regulating MAP kinase activity. One intriguing possibility might be MKP-1. This

particular phosphatase has been reported to be hypoxia-responsive and can therefore be regulated within the tumor microenvironment (Laderoute *et al.*, 1999). It is tempting to speculate that MKP-1 might also be activated by the high levels of adenosine in solid tumors. However, Beltman *et al.* (1996) found that the PKC inhibitor Ro-31-8220 is capable of inhibiting immediate early MKP-1 expression and activity in response to growth factors. This inhibitor did not block the adenosine response in our assays (Appendix A), and weakens the possibility of MKP-1 involvement.

Another possible adenosine-activated phosphatase is MKP-3, which has been suggested to be blocked by bpV(phen) (Wu *et al.*, 2002). This seems possible given that MKP-3 contains significant regions of homology to the Cdc25A phosphatase (Muda *et al.*, 1996) that is known to be inhibited by bpV(phen) (Faure *et al.*, 1995; Scrivens *et al.*, 2003). In addition, MKP-3 appears to be a physiologically relevant regulator of ERK1/2 kinase activity (Zhou *et al.* 2002). Other ERK-specific phosphatases that might be considered are HePTP (Pettiford and Herbst, 2000), PTP-SL (Pulido *et al.*, 1998), and STEP (Pulido *et al.*, 1998). It is not yet known whether these particular MKPs are sensitive to inhibition by bpV(phen).

CONCLUSION

We have identified a pathway leading from encounter of the cancer cell with adenosine at concentrations that exist in the tumor extracellular fluid, to the down-regulation of DPPIV protein at the cell surface. This pathway involves the activation of a PTP by adenosine through a route that is independent of the existing adenosine receptor subtypes, and does not require participation of the PKA, PLC, PKC, PI3K, or PTK pathways. Activation of the PTP by adenosine is associated with the reduced tyrosine phosphorylation and activity of ERK1/2 MAPK, which is required for the down-regulation of DPPIV to occur. The steps that are involved in initial sensing of the adenosine signal, and the identity of the phosphatase(s) involved in ERK1/2 suppression, remain to be elucidated. Understanding this pathway should allow us to block the down-regulation of DPPIV due to adenosine and its adverse consequences, leading to new ways of interfering with tumor expansion and spread. The independence of this DPPIV down-regulatory pathway from known adenosine receptors is an advantage, as the conventional signaling pathways for adenosine are involved in numerous aspects of physiologic regulation (such as blood flow), which we would prefer to spare in any pharmacologic intervention focusing on adenosine's tumor-enhancing actions.

CHAPTER 4

ADENOSINE MODULATES THE FUNCTIONAL ACTIVITIES OF DIPEPTIDYL PEPTIDASE IV ON HT-29 HUMAN COLORECTAL CARCINOMA CELLS

Portions of this chapter appeared in the following publication:

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Portions of this chapter will appear in the following manuscript:

Tan, E.Y. and Blay, J. (In preparation) Adenosine modulation of dipeptidyl peptidase IV activity, adenosine deaminase binding, and extracellular matrix binding of HT-29 human colorectal carcinoma cells.

INTRODUCTION

The role of DPPIV in regulating cancer cell behavior has been explored in numerous studies. These studies have investigated the functional consequences of the reexpression or overexpression of DPPIV in carcinoma cell lines that normally express low levels or lack expression of DPPIV protein (e.g. melanoma, prostate, NSCLC, ovarian, and endometrial carcinomas; Wesley *et al.*, 1999; Pethiyagoda *et al.*, 2001; Kajiyama *et al.*, 2002*b*; Kajiyama *et al.*, 2003; Mizokami *et al.*, 2004; Wesley *et al.*, 2004; Wesley *et al.*, 2005). Collectively, however, these studies have failed to consider the impact of the *functional activities of DPPIV* on tumor progression. Much evidence exists suggesting that the functions of DPPIV to: **(1)** bind ecto-ADA, **(2)** cleave selected peptide substrates, and **(3)** adhere to proteins of the extracellular matrix might play an important role in regulating tumor progression when cell-surface DPPIV expression is reduced (or lost) on carcinoma cells.

Functional role of ADA binding to DPPIV

The functional importance of ADA binding to the surface membrane of cells via DPPIV has been speculated since the early studies identifying ADA-L as the adenosine deaminase complexing (or binding) protein (e.g. Nishihara *et al.*, 1973; Daddona *et al.*, 1980; Schrader *et al.*, 1990). Given that the cytotoxic and cytostatic effects of adenosine on immune cells were already well established at this time, Trotta (1982) hypothesized that the association of ADA with the plasma membrane via DPPIV would be advantageous towards protecting mammalian cells against the toxic effects of elevated concentrations of adenosine in the extracellular space. This role of ecto-ADA in

protecting against adenosine toxicity was also postulated in other studies (e.g. Andy and Kornfeld, 1982; Schrader and Bryer, 1982). Notably, Kameoka *et al.* (1993) and Martín *et al.* (1995) predicted that DPPIV might be involved in regulating the extracellular concentration of ADA (from either the interior of the cell or from release after local cell death), which in turn would regulate the extracellular levels of adenosine. Both of these studies involved the regulation of ADA on the surface of T lymphocytes and its role in immune regulation. Following from that work, Dong *et al.* (1996) reported that T lymphocytes expressing ecto-ADA (through its association with DPPIV) were more resistant to the suppressive effects of adenosine on T cell proliferation and IL-2 production (i.e. the major functional indicators of T cell activation) compared with cells lacking ecto-ADA. The involvement of DPPIV in binding ecto-ADA to regulate adenosine levels was later confirmed by the same group in a study using Jurkat cells transfected with a DPPIV construct deficient in ADA-binding (Dong *et al.*, 1997). The mutant DPPIV transfectants (without ecto-ADA) were much more sensitive to the inhibitory effect of adenosine on IL-2 production than were wild-type DPPIV transfectants.

Two further theories have been proposed based on the binding of ecto-ADA to DPPIV at the surface of cells. Houghton *et al.* (1988) suggested that the specific loss of DPPIV during melanocyte transformation might allow melanoma cells to become susceptible to the "inductive" (i.e. tumor-promoting) effects of adenosine. Finally, Ginés *et al.* (2002) proposed that the ecto-ADA to DPPIV interaction might represent an alternative mechanism mediating the physical adhesion of lymphocytes to colorectal carcinoma cells. In this model, ecto-ADA would be used as a bridging molecule between

DPPIV-expressing lymphocytes and carcinoma cells (also expressing DPPIV), thereby facilitating immune cell attack.

Role of DPPIV dipeptidase activity

The enzyme activity of DPPIV cleaves NH_2-terminal dipeptides from polypeptides with either L-proline or L-alanine at the penultimate position (Tanaka *et al.*, 1992). NH_2-terminal truncation of certain chemokines by DPPIV leads to significant changes in functional activity (usually inactivation) and receptor selectivity (reviewed by De Meester *et al.*, 1999). *In vitro* experiments have shown that stromal cell-derived factor-1-alpha (SDF-1α/CXCL12) is the chemokine cleaved most efficiently by DPPIV, causing a loss of its chemotactic activity (Shioda *et al.*, 1998; Lambeir *et al.*, 2001). Recently, Busso *et al.* (2005) reported that $DPPIV^{-/-}$ mice exhibit increased levels of circulating active SDF-1α, indicating that DPPIV also regulates the *in vivo* half-life of this chemokine. This is significant given that SDF-1α has recently been reported to play an important role in the recruitment of circulating bone marrow-derived, CXCR4-positive endothelial progenitor cells (EPCs) to ischemic tissue, where the EPCs complement existing angiogenesis through the process of *vasculogenesis* (Yamaguchi *et al.*, 2003; De Falco *et al.*, 2004). This appears to occur because of the selective *in vivo* expression of SDF-1α within ischemic tissue in response to reduced oxygen tension (via hypoxia-inducible factor-1 (HIF-1)-induced expression of SDF-1α; Ceradini *et al.*, 2004). It can therefore be predicted that the hypoxic tumor microenvironment would contain elevated levels of SDF-1α, potentially stimulating vasculogenesis through the recruitment of EPCs. In turn, the reduction (or loss) of DPPIV in solid tumors would favor the

persistence of intact SDF-1α in the tumor microenvironment, thereby favoring tumor vascularization by vasculogenesis.

Chemokines can also affect tumor development by attracting immune cells with either pro- or anti-tumoral activities. The role of proteolytically modified chemokines resulting from DPPIV-mediated cleavage within the tumor microenvironment is beginning to be explored. The chemokines LD78β and RANTES are efficiently cleaved by DPPIV both *in vitro* and *in vivo* (Struyf *et al.*, 1998; Proost *et al.*, 2000), which enhances affinity of the cleaved forms to the CCR5 receptor. Increased signaling through the CCR5 receptor has been speculated to result in enhanced T-lymphocyte infiltration into solid tumors (Van Damme *et al.*, 2004). Thus, the down-regulation of DPPIV that is observed in most types of solid tumors might be predicted to result in more persistent levels of these intact chemokines, thus preventing the CCR5-mediated infiltration of lymphocytes into tumors.

Other functions of DPPIV dipeptidase activity in tumor progression might involve the regulation of angiogenesis. Neuropeptide Y (NPY) promotes vessel sprouting and adhesion, migration, proliferation, and capillary tube formation by human endothelial cells *in vitro* (Zukowska-Grojec *et al.*, 1998). NPY is also angiogenic *in vivo*, with activity comparable to bFGF and VEGF. The involvement of DPPIV in NPY function was speculated when both DPPIV and NPY were found to exhibit similar localization on the surface of endothelial cells, suggesting that DPPIV might be involved in NPY processing. Following from this research, Ghersi *et al.* (2001) established DPPIV activity as a requirement for NPY-mediated chemotaxis of HUVECs; specifically, the DPPIV-truncated form of NPY (NPY_{3-36}) binds to NPY angiogenic receptors. Furthermore, it was

speculated that the activity of DPPIV might function as a switch converting the activity of NPY from vasoconstrictive to angiogenic.

The dipeptidase activity of DPPIV might also be significant in regulating the growth-promoting effect of certain substrates. DPPIV-mediated cleavage of glucagon-like peptide-2 (GLP-2) appears to play a role in attenuating the growth-promoting effect of this peptide on human colorectal carcinoma cells (Bulut *et al.*, 2004). GLP-2 also accelerates the growth of chemically-induced colonic neoplasms in mice (Thulesen *et al.*, 2004). Significantly, a stable analogue of GLP-2 that is resistant to truncation by DPPIV increases tumor load significantly more than native GLP-2, thus supporting the role of DPPIV in restraining GLP-2-mediated cell growth.

Interaction of DPPIV with the extracellular matrix (ECM)

DPPIV has the capacity to bind the ECM proteins collagen and fibronectin. Preliminary evidence of DPPIV interacting with the ECM was shown when Walborg *et al.* (1985) identified a peptide, Hep105, which was shared by the plasma membrane of rat hepatocytes and the rat liver biomatrix. Hep105 was determined to be DPPIV, and it was also shown that rat liver biomatrix possessed significant DPPIV activity. Intriguingly, the authors speculated that DPPIV might play a role in maintenance of tissue architecture and further suggested that altered expression of DPPIV in rat hepatocellular carcinomas might be involved in the loss of proper cellular organization characteristic of these carcinomas. Hartel *et al.* (1988) studied the distribution of DPPIV expression in rat tissues by immunohistochemistry and observed that DPPIV was often attached to collagenous fibers and localized at sites of cell-ECM contacts.

The interaction of DPPIV with collagen was initially reported when an antiserum raised against mouse liver DPPIV delayed spreading of rat hepatocytes on a denatured collagen matrix (Hanski *et al.*, 1985). The involvement of DPPIV was limited to the initial spreading phase (0-2 hours), but did not have an effect on total long-term cell spreading. This finding was later extended when the same antiserum inhibited the initial spreading of hepatocytes on native type I collagen (Hanski *et al.*, 1988). Specific binding of DPPIV from solubilized rat hepatocyte membranes to native collagen fibrils was also visualized by immunohistochemistry. Biochemical studies have demonstrated that purified DPPIV from mouse fibroblasts specifically binds immobilized gelatin (denatured collagen) and type I collagen (Bauvois, 1988). Additionally, soluble DPPIV disrupts the adhesion of mouse fibroblasts to multiwell plates coated with denatured collagen and native collagen. Löster *et al.* (1995) found that DPPIV preferentially binds to collagen types I and III, with lesser binding to types II, IV, V, and VI. The binding site for collagen on the DPPIV molecule was determined to be located in the cysteine-rich domain of DPPIV. Binding to collagen has also been demonstrated by DPPIV expressed on human lymphocytes (Dang *et al.*, 1990*b*).

DPPIV purified from rat hepatocytes binds to plasma purified fibronectin, and was of higher affinity than the binding to collagen (Piazza *et al.*, 1989). Recent studies have suggested that the interaction of DPPIV with fibronectin might play a role in adhesive events occurring during tumor progression. DPPIV overexpression in ovarian carcinoma cells increases the total cell adhesion to collagen- and fibronectin-coated plates approximately 2-fold and 2.5-fold, respectively (Kikkawa *et al.*, 2003). Overexpression of DPPIV also induced adhesion to mesothelial cells, which was

improved in a dose-dependent manner by the addition of soluble fibronectin. It is speculated that ovarian carcinoma cells capture fibronectin on their cell surface through DPPIV during their transit in ascites fluid, which subsequently mediates the adhesion to mesothelial cells lining the peritoneum. A comparable model for DPPIV adhesion to fibronectin has been proposed in breast carcinoma. Cheng *et al.* (1998) have proposed that DPPIV functions as an *endothelial* adhesion molecule for rat breast carcinoma cells and mediates lung metastasis. Specifically, DPPIV expressed on the surface of lung endothelial cells binds to breast carcinoma cells that have captured and polymerized abundant fibronectin at the tumor cell surface during hematogenous circulation. Furthermore, DPPIV binding sites have been identified within fibronectin and, importantly, peptides of fibronectin containing the DPPIV binding domain block pulmonary metastasis of breast carcinoma cells in a rat model (Cheng *et al.*, 2003).

In addition to DPPIV having the capacity to bind collagen and fibronectin, it has more recently been found that DPPIV contains gelatinase activity (Bermpohl *et al.*, 1998; Ghersi *et al.*, 2002), which is the ability to cleave denatured collagen. DPPIV purified from rat kidney tissue degrades denatured type I, II, III, and V collagens as shown by gelatin zymography assay, while fibronectin is not degraded (Bermpohl *et al.*, 1998). Ghersi *et al.* (2002) found that DPPIV participates in the process of gelatin degradation by migratory fibroblasts. Interestingly, DPPIV was localized at membrane protrusions called invadopodia that are specialized structures involved in dynamic membrane motility and ECM adhesion and degradation (reviewed by Chen and Wang, 1999).

Our approach taken in this chapter to investigating the impact of adenosine on DPPIV function differs significantly from other studies. We examined changes in function by the endogenous DPPIV molecule, and the effect in response to the *quantitative* reduction in DPPIV levels triggered by adenosine. This is more likely representative of the *in vivo* situation within tumors, in which changes in DPPIV protein expression are usually not strictly qualitative (i.e. expressed vs. absent). We explored the effect of adenosine on down-regulation of DPPIV with respect to the actual functions ascribed to the molecule. To this end, we investigated the effect of adenosine on: **(1)** DPPIV dipeptidase activity, **(2)** levels of endogenous cell-surface ADA and the binding capacity for exogenous ADA, and **(3)** interactions with the extracellular matrix. A systematic study of changes in the specific functions of DPPIV in response to a potential physiological regulator has not yet been described in the literature. Results from this work demonstrate what we consider to be more representative effects of the loss/reduction of DPPIV from carcinoma cells, as opposed to changes in overall carcinoma cell function when DPPIV expression is restored in cells.

MATERIALS AND METHODS

Materials

HT-29 human colorectal carcinoma cells, media, culture vessels and sera, adenosine, mAb against DPPIV/CD26 and mouse IgG_1 isotype control, and ^{125}I-labeled sheep anti-mouse IgG were obtained as in Chapter 2. Calf spleen ADA, human cellular fibronectin (cFN), human plasma fibronectin (pFN), human placental collagen (Sigma types VI, IX, and X), RGD (Gly-Arg-Gly-Asp-Ser) peptide, control peptide (Ser-Asp-Gly-Arg-Gly), and Gly-Pro-*p*-nitroaniline *p*-toluenesulfonate salt (Gly-Pro-*p*NA), and Diprotin A (Ile-Pro-Ile) were from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-human ADA antibody and rabbit IgG isotype control were from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). Rabbit anti-bovine ADA antibody was from Alpha Diagnostic International (San Antonio, TX). Mouse anti-human mAb against α_v integrin/CD51 (clone AMF7) was purchased from Immunotech (Marseille, France). ^{125}I-labeled goat anti-rabbit IgG, $F(ab')_2$ fragment was obtained from PerkinElmer Life Sciences (NEN) (Boston, MA). ^{125}I-labeled donkey anti-rabbit Ig, $F(ab')_2$ fragment and high specific activity [^{3}H]-labeled amino acid mixture (90-157 Ci/mmol) were from Amersham Biosciences Inc. (Baie d'Urfé, Quebec, Canada).

Cell culture

HT-29 cells were cultured as before (Chapter 2). Cells for use in binding assays or for measurements of DPPIV enzyme activity (monolayer method) were seeded into 48-well plates at 25,000-50,000 cells/well and allowed to adapt to culture for 48 h. Cultures were then changed to medium containing 1% NCS for another 48 h, and then treated with

adenosine or control vehicle for evaluation of changes in DPPIV protein expression or dipeptidase activity. Cells for use in measurements of DPPIV enzyme activity on released cells and cell lysates were seeded into 10-cm diameter culture dishes at 5×10^5-10^6 cells/dish and cultured and treated with adenosine as described above.

Radioantibody binding assay for DPPIV, endogenous ADA, and integrin α_v subunit

Monolayer cultures of HT-29 cells in 48-well plates were assayed for cell-surface DPPIV protein as described in Chapter 2. The integrin α_v subunit was detected with mouse anti-α_v antibody (2 μg/ml) followed by ^{125}I-labeled sheep anti-mouse IgG. To determine levels of cell-surface endogenous ADA the cultures were incubated with rabbit anti-human ADA antibody or rabbit IgG isotype control (both at a concentration of 1 μg/ml), washed, and the bound antibody was measured using ^{125}I-labeled donkey anti-rabbit Ig ($F(ab')_2$ fragment) as tracer. The figures show representative results from at least three separate experiments. Data were evaluated using a two-tailed Student's *t*-test for unpaired data.

Assay for cellular binding of exogenous ADA

The method used to measure the cellular capacity for ecto-ADA binding was a modification of the DPPIV radioantibody binding assay, except that the cells were first loaded with saturating concentrations of bovine ADA and the bound, exogenous ADA was then detected with an antibody selective for the bovine enzyme. Monolayer cultures of HT-29 cells in 48-well plates were first treated with 10 μg/ml calf spleen ADA in medium for 60 min at 37°C. Preliminary experiments had shown this concentration of

calf spleen ADA to saturate the unfilled binding capacity of HT-29 cells (Figure 4.1). The plates were then washed and assayed for bound ADA using a rabbit anti-bovine ADA antibody (1 μg/ml) and a ^{125}I-labeled goat anti-rabbit secondary antibody ($F(ab')_2$ fragment) using the procedures described above. Data were corrected for binding in the absence of loading with exogenous ADA. The 'unloaded' background binding was never more than twice the antibody isotype control value, and likely represented a low degree of DPPIV occupancy with ADA acquired from serum; or endogenous human ADA secreted from the cells, absorbed from the medium, and subsequently detected by the weakly cross-reacting anti-ADA antibody.

Assay of cellular dipeptidyl peptidase IV enzymatic activity from monolayer cultures

DPPIV enzyme activity was measured spectrophotometrically using Gly-Pro-*p*NA as a DPPIV substrate. The culture plates were placed on ice and the wells were washed twice with 500 μl ice-cold PBS; then given 250 μl of 2 mM Gly-Pro-*p*NA in 100 mM HEPES buffer (pH 7.6) containing 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose, and 10 mg/ml BSA and incubated for 60 min at 37°C. After incubation, 100 μl of the supernatant was transferred to a 96-well flat-bottomed microtiter plate and the absorbance (due to *p*-nitroaniline release) was measured at 405 nm. The enzyme activity was calculated after subtraction of the absorbance values for cell-free controls. DPPIV dipeptidase activity was expressed as nmol *p*NA formed/10^5 cells or fmol/min/cell.

Assay of cellular DPPIV enzymatic activity from trypsinized cells

HT-29 cells were rinsed twice with ice-cold PBS and released from 10-cm culture dishes directly into trypsin. The cells were then washed and resuspended at 10^6 cells/ml in 2 mM Gly-Pro-*p*NA in 100 mM HEPES buffer (pH 7.6) containing the constituents described in the above section. Each cell suspension was pipetted into a 96-well flat-bottomed plate at 100 µl/well (10^5 cells/well) and incubated for 60 min at 37°C. After incubation, the absorbance (due to *p*-nitroaniline release) was measured at 405 nm. The enzyme activity was calculated after subtraction of the absorbance values for cell-containing (vehicle absent) controls and DPPIV dipeptidase activity was expressed as nmol *p*NA formed/10^5 cells.

Assay of cellular DPPIV enzymatic activity from HT-29 cell lysates

HT-29 cells were rinsed twice with ice-cold PBS and dissolved in RIPA buffer (see Chapter 3 for constituents) for 45 min at 4°C. The cell lysates were clarified by centrifugation (10 min at 12,000 × *g*) and combined with 2 mM Gly-Pro-*p*NA at a 1:10 ratio in a 96-well flat-bottomed plate. The plate was incubated for 60 min at 37°C and after incubation the absorbance (due to *p*-nitroaniline release) was measured at 405 nm. Bradford assay was used to determine the concentration of protein samples and the DPPIV dipeptidase enzyme activity was expressed as pmol/min/µg protein.

Assay of cell adhesion to extracellular matrix proteins

Cells for use in adhesion assays were seeded into 80-cm^2 flasks at 10^6 cells/flask and cultured and treated with adenosine as for other approaches. Prior to adhesion assays,

HT-29 cells in monolayer culture were labeled for 3 h with 1 μCi/ml [^{3}H]-labeled amino acids in amino-acid-free medium (MacKenzie *et al.*, 1994) containing 1% fetal calf serum (FCS). Four-well plates were coated overnight at 4°C with extracellular matrix proteins at a concentration of 5 μg/ml (~1 μg/cm^2) in PBS. Prior to their use in assays, plates were treated with 2% BSA in serum-free DMEM to reduce non-specific binding. Radiolabeled HT-29 cells (50,000/well to prevent cell-cell contact) in serum-free DMEM were added to the ECM-coated 4-well plates and incubated for the indicated time at 37°C. For certain experiments the HT-29 cell suspensions were pretreated with RGD or control peptide (500 μM) for 30 min at room temperature. At the end of the incubation period, plates were gently washed twice by inverting into PBS (containing Ca^{2+} and Mg^{2+}) for 10 min at 4°C to remove non-adherent cells. The bound cells were dissolved in 500 μl of 0.1 M NaOH containing 1% sodium dodecyl sulfate (SDS) and the measured radioactivity (after correction for the specific activity of labeling of the cells) was used as a measure of cell adhesion.

Preparation of endogenously-secreted HT-29 matrices

HT-29 cells were seeded into 4-well plates at 60,000-80,000 cells/well and allowed to grow to confluence for 7-8 days. Prior to the adhesion assay, the confluent monolayers were incubated with 20 mM NH_4OH for 10 min at room temperature to lyse the cells, leaving the secreted HT-29 ECM intact. The endogenous matrices were then washed three times with H_2O and twice with PBS, followed by treatment with 2% BSA in serum-free DMEM to reduce non-specific binding.

Statistical analysis

The figures show representative results from at least three separate experiments. Data were evaluated using a two-tailed Student's *t*-test for unpaired data. The data for the effect of adenosine on kinetics of DPPIV dipeptidase activity were evaluated using a *paired*, two-tailed Student's *t*-test.

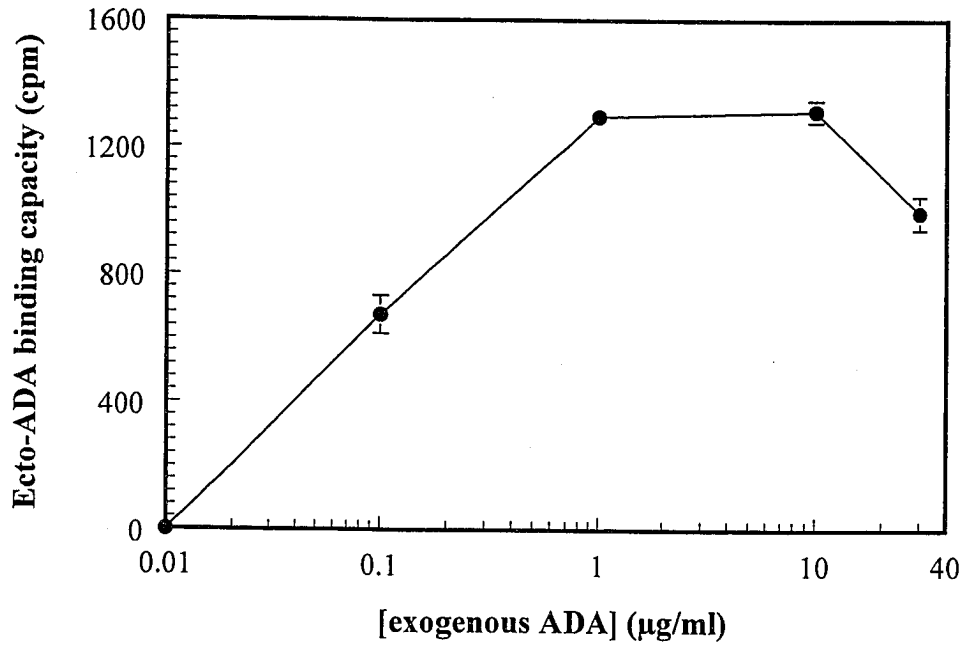


Figure 4.1 Cell surface binding capacity of HT-29 cells for exogenous ADA. Confluent monolayer cultures of HT-29 cells were incubated with exogenous calf spleen ADA at the indicated concentrations for 60 min at 4°C. The cells were then washed, and the levels of cell surface-bound ADA were measured by radioantibody binding assay. Points are mean values, $n = 3$. Some standard errors fall within the symbols.

RESULTS

Adenosine reduces the DPPIV dipeptidase activity of HT-29 cells

To evaluate the effect of adenosine on DPPIV dipeptidase activity, we measured the ability of HT-29 monolayers to cleave the model DPPIV substrate Glycine-Proline-*p*-nitroaniline (Gly-Pro-*p*NA). This is a chromogenic assay in which the dipeptidase activity of DPPIV liberates the Gly-Pro dipeptide from *p*NA, and *p*NA subsequently produces a colored product in the reaction buffer. Our objective in these experiments, and for most of the following functional experiments, was to treat the HT-29 cells in a way that would mimic the persistent exposure to adenosine that is present within solid tumors (Blay *et al.*, 1997). This treatment protocol was typically a four day exposure to adenosine involving either (i) twice daily doses of 50 μM adenosine or (ii) a daily dose of 100 μM adenosine.

HT-29 cells that were pretreated with adenosine (50 μM twice daily over 4 days) demonstrated reduced DPPIV dipeptidase activity (and reduced DPPIV protein; Figure 4.2). This was shown by the diminished production/release of *p*NA from adenosine-pretreated HT-29 monolayers. The dipeptidase activity of DPPIV in these experiments was expressed as nmol of *p*NA formed per 100,000 cells (nmol/10^5 cells). There was an approximately linear rate of *p*NA production over a 4 h time period (Figure 4.2). The reduction of dipeptidase activity was fully apparent after 60 min incubation with the Gly-Pro-*p*NA solution, and the extent of the adenosine-induced decrease was comparable after 120 and 240 min. For all future experiments we measured DPPIV dipeptidase activity after a 60 min reaction period.

We also measured the dipeptidase activity of DPPIV from adenosine-treated, trypsin-released HT-29 cells in suspension. Adenosine pretreatment (50 μM twice daily) over 4 days revealed a marked reduction of DPPIV activity from cells in suspension that could be shown over the 240 min reaction time (Figure 4.3). Similar to our use of the flow cytofluorimetry assay to confirm the down-regulation of DPPIV protein (Figure 2.5), these results also indicate that DPPIV was not being masked within the cell monolayer. In a final approach we measured the DPPIV dipeptidase activity from HT-29 protein extracts. Again, there was a reduction of dipeptidase activity (expressed as pmol/min/μg protein) in response to adenosine pretreatment (Figure 4.4). Thus, we were able to show that adenosine causes a reduction of DPPIV dipeptidase activity through the use of these three different assays. The regulation of DPPIV activity was also found to be specific for adenosine because the nucleoside inosine was without effect (data not shown).

Adenosine reduces DPPIV dipeptidase activity at concentrations found within the tumor microenvironment

Our initial experiments demonstrated a reduction of DPPIV dipeptidase activity in response to long-term exposure to adenosine. However, we still wanted to establish that adenosine could significantly reduce DPPIV activity at the concentrations present within the extracellular fluid of solid tumors. Figure 4.5 shows the dose-response relationship for the down-regulation of cell-surface DPPIV activity and protein of paired cell cultures after 4 days using twice-daily doses of adenosine. There was a dose-dependent reduction of DPPIV dipeptidase activity that was first significantly decreased in response to 5 μM

adenosine (~ 10% reduction). The maximal reduction of dipeptidase activity was approximately 15%, which occurred at 50 and 100 μM adenosine. Significantly, the dose-dependent reduction of DPPIV activity closely paralleled the adenosine-mediated down-regulation of cell-surface DPPIV protein (Figure 4.5). There was also a 15% reduction of DPPIV *protein* by 5 μM adenosine, and the maximal down-regulation of DPPIV protein was approximately 25-30% at 50 and 100 μM adenosine. Given that the adenosine-mediated reduction of cell-surface DPPIV activity and protein were both significant at 5 μM adenosine, we can conclude that these effects are both/each able to be achieved with concentrations of adenosine present within the tumor extracellular fluid.

The adenosine-mediated reduction of DPPIV dipeptidase activity corresponds to the down-regulation of DPPIV protein

Our dose-response experiments suggested that DPPIV dipeptidase activity is proportional to the level of cell-surface protein. To study more clearly the dependence of DPPIV activity on the level of protein, we performed a time course in response to persistent adenosine exposure (daily treatment with 100 μM adenosine). As reported in Chapter 2, baseline levels of DPPIV protein progressively increased with time in culture (Figure 4.6). However, daily treatment with adenosine produced a sustained depression of DPPIV protein relative to this increase. The dipeptidase activity of DPPIV also increased in control cells substantially over the 7 day culture period, the activity per cell nearly doubling at the end of this time. However, in the presence of 100 μM treatments with adenosine, there was a relative decline compared with control that closely paralleled the time course of change in immunoreactive protein (Figure 4.6). The maximum reduction

of DPPIV activity in this experiment was approximately 20%, while the maximum reduction of cell-surface protein was approximately 40%. Taken together, these results indicate that the adenosine reduction of DPPIV activity can be explained by the reduction of cell-surface DPPIV protein.

Adenosine does not alter the affinity of DPPIV enzymatic activity

We performed Lineweaver-Burke assays to determine whether adenosine treatment (50 μM twice daily over 4 days) changed the kinetic properties of DPPIV activity. Table 4.1 shows the K_m and V_{max} values of control and adenosine-treated cells from three separate experiments. There was absolutely no difference in the affinity (K_m) of the DPPIV enzyme between control (1.85 ± 0.51, $n = 3$) and adenosine-treated cells (1.87 ± 0.49, $n = 3$). However, adenosine-treated cells had a reduced number of proteolytically active DPPIV molecules (V_{max}) at the cell surface compared to control cells (13.17 ± 2.08 vs. 11.53 ± 2.07, $n = 3$). This reduction was observed even though there was a wide range of V_{max} values obtained between assays. The reduction in V_{max} by adenosine-treated cells was statistically different from control ($P = 0.0267$, Paired t-test). These results provide further evidence that adenosine reduces the net dipeptidase activity of DPPIV, while arguing against a change in the specific activity of DPPIV.

The adenosine down-regulation of DPPIV leads to a reduction in levels of endogenous cell-surface ADA and in ecto-ADA binding capacity

The next objective was to investigate the effect of long-term adenosine exposure on the expression of endogenous human ADA at the surface of HT-29 cells. Adenosine-

treated cells (100 μM daily over 4 days) expressed reduced levels of endogenous cell surface-bound ADA, and the reduction of endogenous ADA was also quantitatively comparable to the measured reduction in DPPIV protein (23.6% reduction of endogenous ADA and 29.7% reduction of DPPIV; Figure 4.7).

We then proceeded to investigate the ability of HT-29 cells to bind exogenous ADA following long-term treatment with adenosine to down-regulate DPPIV. To measure the remaining binding capacity for ecto-ADA, the cells were first incubated with a saturating concentration of calf spleen ADA (10 μg/ml incubated for 60 min at 4°C), followed by an indirect radioantibody binding assay incorporating a rabbit antibody against the bovine enzyme. We performed a prolonged time course (6 days) involving twice daily treatment of cells with 50 μM adenosine. As anticipated, the baseline level of cell-surface DPPIV increased substantially with time in culture (86.0% increase; Figure 4.8). However, the baseline level of ecto-ADA binding also increased and followed a temporal profile very similar to the DPPIV protein (49.3% baseline increase; Figure 4.8). Adenosine-treated HT-29 cells showed a reduced capacity for binding of exogenous ADA. Significantly, this reduced ecto-ADA binding capacity was quantitatively comparable to the measured reduction of DPPIV protein in paired cell cultures at each time point.

Collectively, these results confirmed our expectation that the adenosine down-regulation of DPPIV, which is identical to ADA complexing protein (ADCP; Schrader *et al.*, 1990) or ADA binding protein (ADAbp; Schrader and Pollara, 1978), is responsible for reducing the amount of cellular ecto-ADA.

The adenosine down-regulation of DPPIV diminishes the ability of cells to adhere on cellular fibronectin

DPPIV has the capacity to interact with the extracellular matrix proteins collagen and fibronectin (Bauvois, 1988; Hanski *et al.*, 1988; Piazza *et al.*, 1989; Cheng *et al.*, 1998). In particular, it appears to bind preferentially to cell-surface fibronectin even in the presence of the soluble plasma form of this protein (Cheng *et al.*, 1998). The linkage through DPPIV to cellular fibronectin (cFN) on neighboring tumor cells might be one way in which tumor cells are contained within the tumor cell population and restrained from leaving the primary site to move to a different location. We therefore investigated the capacity of HT-29 cells to bind to cFN and the effect due to adenosine treatment.

The kinetics of HT-29 cellular adhesion to cFN was determined by incubating radiolabeled HT-29 cells in microwells coated with 5 μg/ml cFN (equivalent to a coating density of ~ 1 $\mu g/cm^2$). HT-29 cells adhered rapidly to cFN between 5 and 60 min, demonstrating a half-maximal adhesion at approximately 20 min (Figure 4.9). The plateau of HT-29 adhesion to cFN was typically reached by 60 min, and the adherent fraction in most experiments was between 40 and 50%. This maximum level of adhesion remained constant for at least 6 h (Figure 4.9). Previous studies have suggested a role for DPPIV in mediating early events of cell adhesion (Hanski *et al.*, 1985; Hanski *et al.*, 1988). We therefore chose to study the effect of adenosine pretreatment on cFN adhesion by allowing the HT-29 cells to bind to cFN-coated plates for 30 min.

Adenosine-pretreated cells (single 300 μM dose over 48 h) consistently demonstrated a reduced capacity to bind cFN (Figure 4.10). This same adenosine-mediated reduction was also observed from cells treated daily with 100 μM adenosine for

three days (data not shown). The size of the reduction in cFN adhesion was comparable to the down-regulation of cell-surface DPPIV protein that we would expect from HT-29 cells treated with adenosine in this way (e.g. Figure 2.4). We also tested the effect of adenosine pretreatment on HT-29 cell adhesion to plasma fibronectin (pFN). Adenosine pretreatment indeed reduced adhesion to pFN, but the proportional decrease was not as large as the effect on cFN (Figure 4.10; 66.1% of the adenosine-induced cFN decrease, 5 separate experiments). These results indicate that adenosine reduces the early adhesion of HT-29 cells to fibronectin, and this effect is greater on the cellular form of fibronectin.

It is commonly believed that cell-surface integrin receptors, particularly $\alpha_5\beta_1$, are the primary structures responsible for the binding of cells to fibronectin (reviewed by Hynes, 1992). We therefore assessed the contribution of integrin-dependent binding in HT-29 cell adhesion to cFN and its potential for modulation by adenosine. We treated HT-29 cells with RGD peptide (500 μM), an antagonist of the major fibronectin-binding integrin receptors (Hynes, 1992). Treatment with RGD peptide decreased HT-29 binding to cFN (without adenosine pretreatment) by an average of 29.4% (5 separate experiments). This suggested that integrin fibronectin receptors play only a minor role in the binding of HT-29 cells to cFN. Furthermore, it has been demonstrated that integrin binding of HT-29 cells to fibronectin occurs through the $\alpha_v\beta_6$ integrin (Kemperman *et al.*, 1997) instead of the more usual β_1 integrins (e.g. $\alpha_5\beta_1$). We therefore evaluated the cell-surface expression of the α_v subunit on HT-29 cells using the radioantibody binding assay and found that the amount of cell-surface α_v was not altered by adenosine pretreatment (Figure 4.11). These data provide a strong argument that the effects of adenosine on cFN binding occur independently of the $\alpha_v\beta_6$ integrin, that integrins are

responsible for a minority of fibronectin binding by HT-29 cells, and is consistent with our view that down-regulation of DPPIV protein is responsible for the reduction in cFN binding.

Adenosine pretreatment reduces the adhesion of HT-29 cells to collagen

We next sought to determine whether the adenosine-mediated down-regulation of DPPIV is also associated with the reduced capacity of HT-29 cells to bind collagen. The adhesion of HT-29 cells was evaluated on Sigma collagen types IX, X and VI, which correspond to the standard Bornstein and Traub classification of types V, III, and IV, respectively. The kinetics of HT-29 cell adhesion to collagen IX (at 5 μg/ml) was similar to that of cFN binding (data not shown), and cells were allowed to bind to collagen for 30 min in these experiments. Adenosine pretreatment caused a reduction in HT-29 adhesion to each of the collagen types tested (Figure 4.12). The measured decline in collagen binding was again comparable to the level of DPPIV down-regulation that would be predicted to occur from the adenosine treatment. These data indicate that the adenosine-mediated reduction in the adhesive properties of HT-29 cells is not restricted to fibronectin, but also extends to the reduced binding of different types of collagen.

Adenosine-pretreated HT-29 cells adhere very poorly to endogenously-secreted HT-29 extracellular matrix

The above experiments established that adenosine reduced the adhesion of HT-29 cells to a number of individual ECM proteins adsorbed to tissue culture plastic. Therefore, our final objective was to assess whether adenosine-pretreatment of HT-29

cells would compromise their adhesion to a more physiologically relevant ECM substratum. We chose to test the ability of HT-29 cells to bind to the endogenous matrix ("conditioned matrix") secreted from these same cells. In these experiments, HT-29 cells were grown to confluence in four-well plates and then removed by a method sparing the secreted HT-29 matrix. We found that adenosine-pretreated cells (100 μM daily for 3 days) consistently adhered more poorly to the endogenous matrix compared to control cells (Figure 4.13). Interestingly, the measured reduction in adhesion by adenosine on the endogenous matrix was significantly greater than the adenosine effect on cFN. These results indicate that the adenosine down-regulation of DPPIV also reduces the adhesive properties of HT-29 cells to a more physiologically relevant ECM substratum.

Table 4.1 Kinetics of DPPIV dipeptidase activity and effect of long-term exposure to adenosine.

Experiment		Parameter	
		K_m	V_{max}
I.	Control	2.80	17.20
	Adenosine	2.80	15.40
II.	Control	1.70	12.00
	Adenosine	1.70	10.90
III.	Control	1.05	10.30
	Adenosine	1.12	8.30

HT-29 cells were treated with control media or with adenosine (50 μM) added twice daily for 96 h. The monolayer cultures were then assayed for DPPIV dipeptidase activity from which K_m and V_{max} values were calculated.

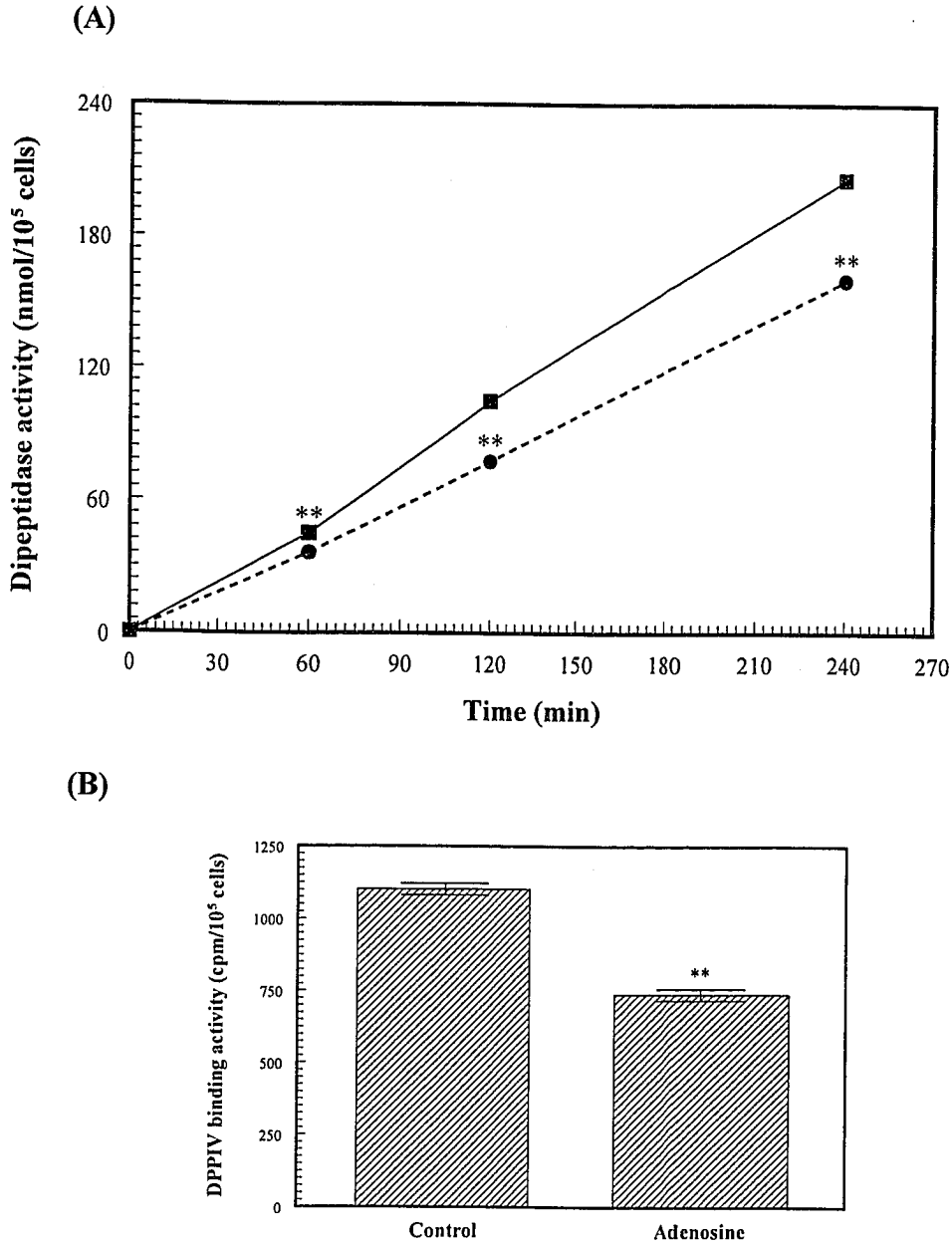


Figure 4.2 Persistent adenosine exposure reduces DPPIV dipeptidase activity from HT-29 cell monolayers.

(A) HT-29 cells were incubated with control media (■) or with adenosine (50 μM, ●) added twice daily for 96 h. The monolayer cultures were assayed for DPPIV dipeptidase activity at the indicated times. Standard errors fall within the symbols. **(B)** Decrease in cell-surface DPPIV protein following adenosine treatment in parallel cultures. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

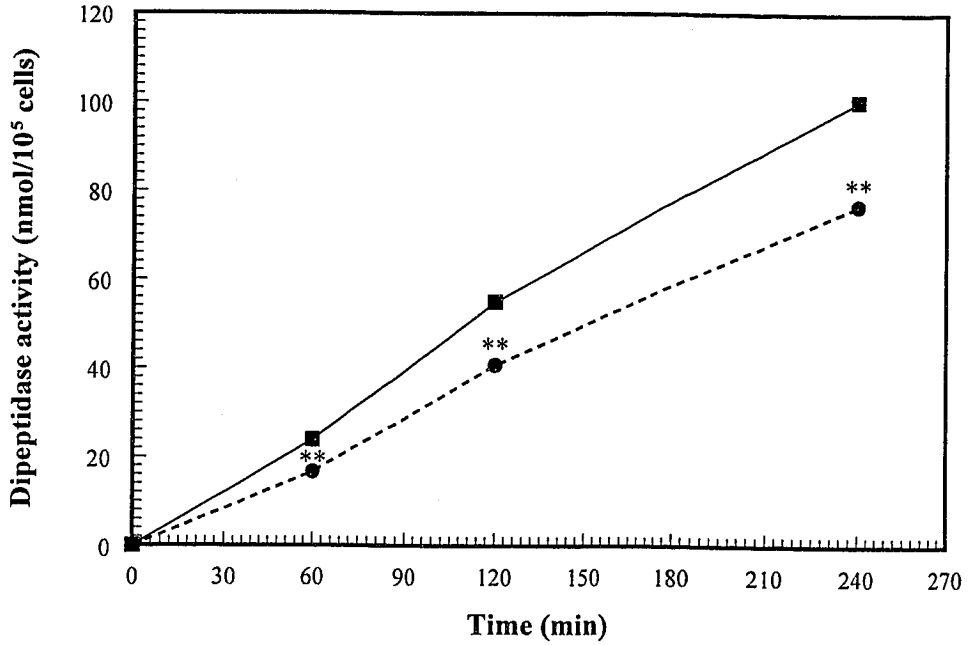


Figure 4.3 Adenosine-mediated reduction of DPPIV dipeptidase activity from trypsin-released HT-29 cells.

HT-29 cells were incubated with control media (■) or with adenosine (50 μM, ●) added twice daily for 96 h. The cultures were then released by trypsinization and assessed for DPPIV dipeptidase activity at the indicated times. The data are mean values ± SE ($n = 4$). Standard errors fall within the symbols. **, significant reduction by adenosine, $P < 0.01$.

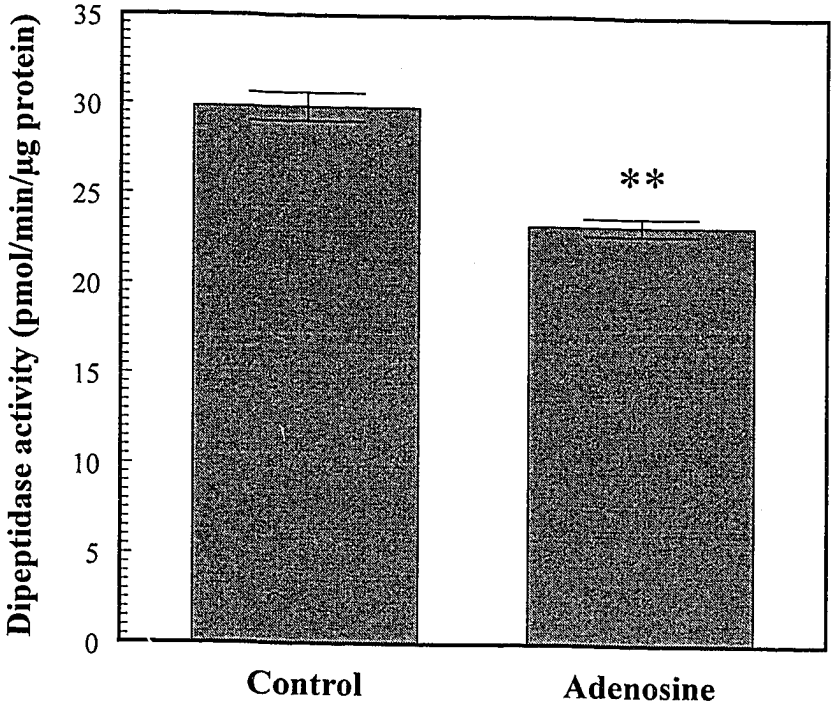


Figure 4.4 Adenosine reduces total DPPIV dipeptidase activity from HT-29 cell lysates.

HT-29 cells were incubated with control media or with adenosine (50 µM) added twice daily for 96 h. The cultures were lysed with RIPA buffer to extract cellular proteins and then assessed for DPPIV dipeptidase activity. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

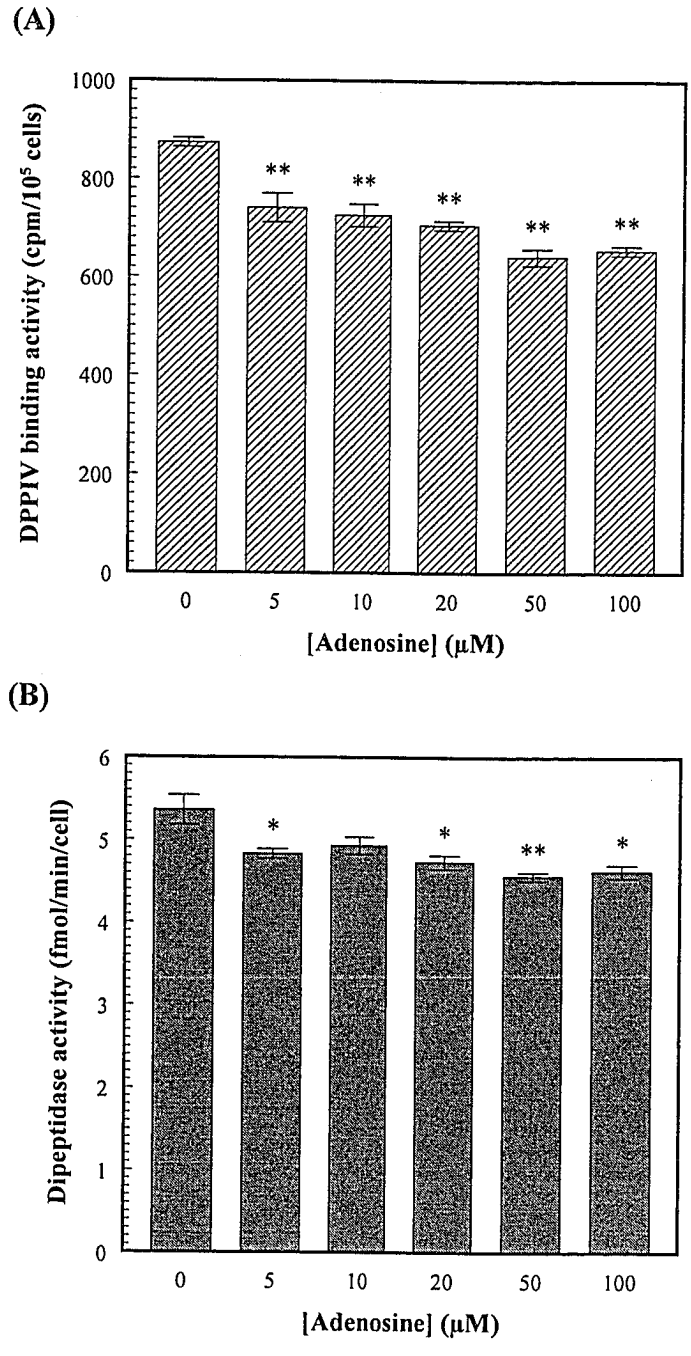


Figure 4.5 Dose-dependence of the adenosine reduction of cell-surface DPPIV protein and dipeptidase activity on HT-29 cell monolayers.

HT-29 cells were incubated with adenosine at the indicated concentrations (given twice daily) for 96 h. Parallel cultures were then assayed for **(A)** cell-surface DPPIV protein or its **(B)** associated dipeptidase activity. The data are mean values ± SE ($n = 4$). *, significant reduction by adenosine, $P < 0.05$. **, $P < 0.01$.

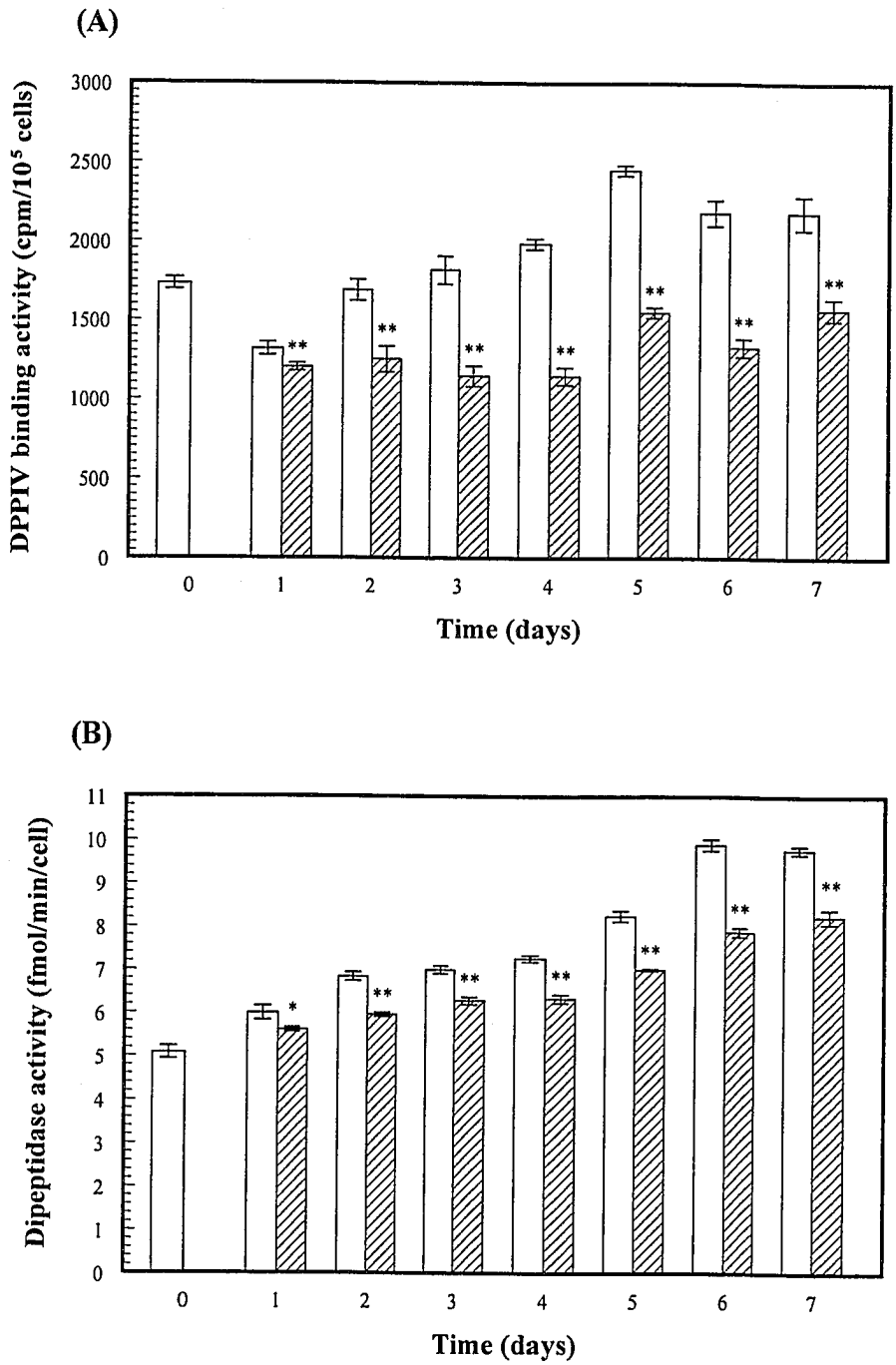


Figure 4.6 Persistent depression of cell-surface DPPIV protein and dipeptidase activity with long-term exposure to adenosine.

HT-29 cells were incubated in control media (*clear bars*) or with adenosine (100 μM, *hatched bars*) added daily. The cultures were then assayed for the presence of **(A)** cell-surface DPPIV protein or its **(B)** associated dipeptidase activity. The data are mean values ± SE ($n = 4$). *, significant reduction by adenosine, $P < 0.05$. **, $P < 0.01$.

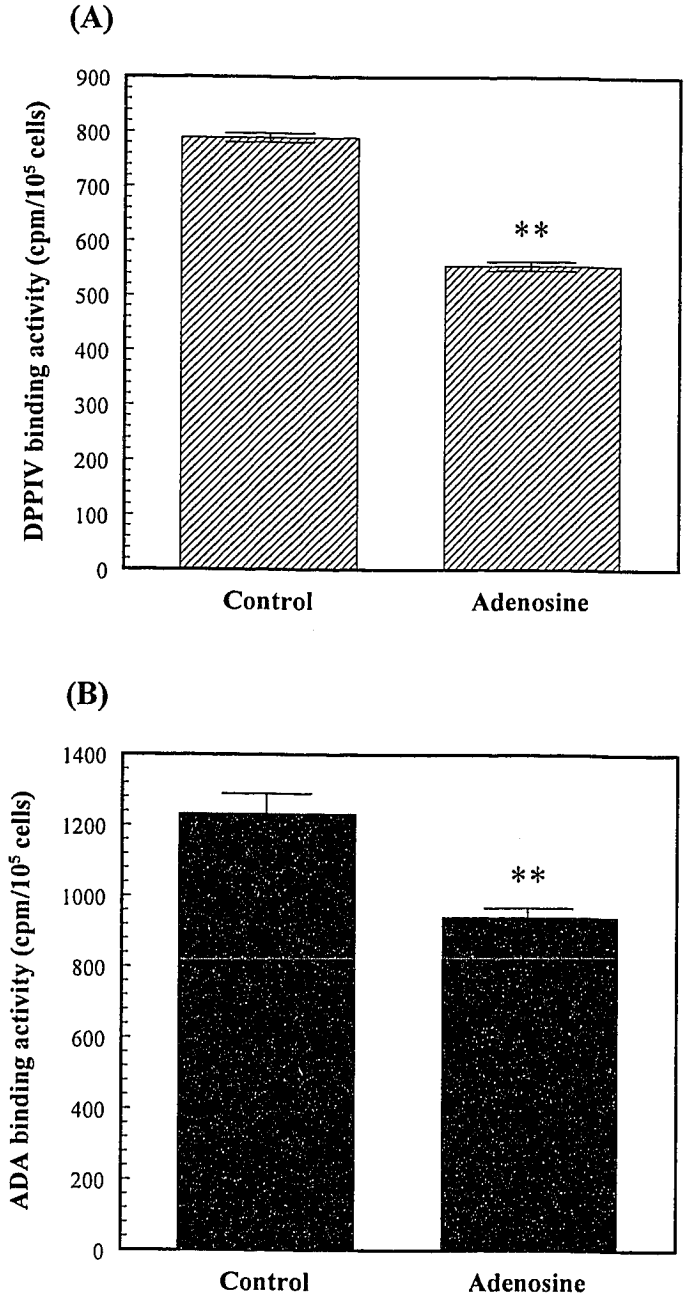


Figure 4.7 Long-term exposure to adenosine reduces levels of endogenous cell surface-bound ADA on HT-29 cells.

HT-29 cells were incubated in control media or with adenosine (100 μM) added daily. Four days later the cultures were assayed for the presence of **(A)** cell-surface DPPIV or **(B)** endogenous cell surface-bound ADA. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

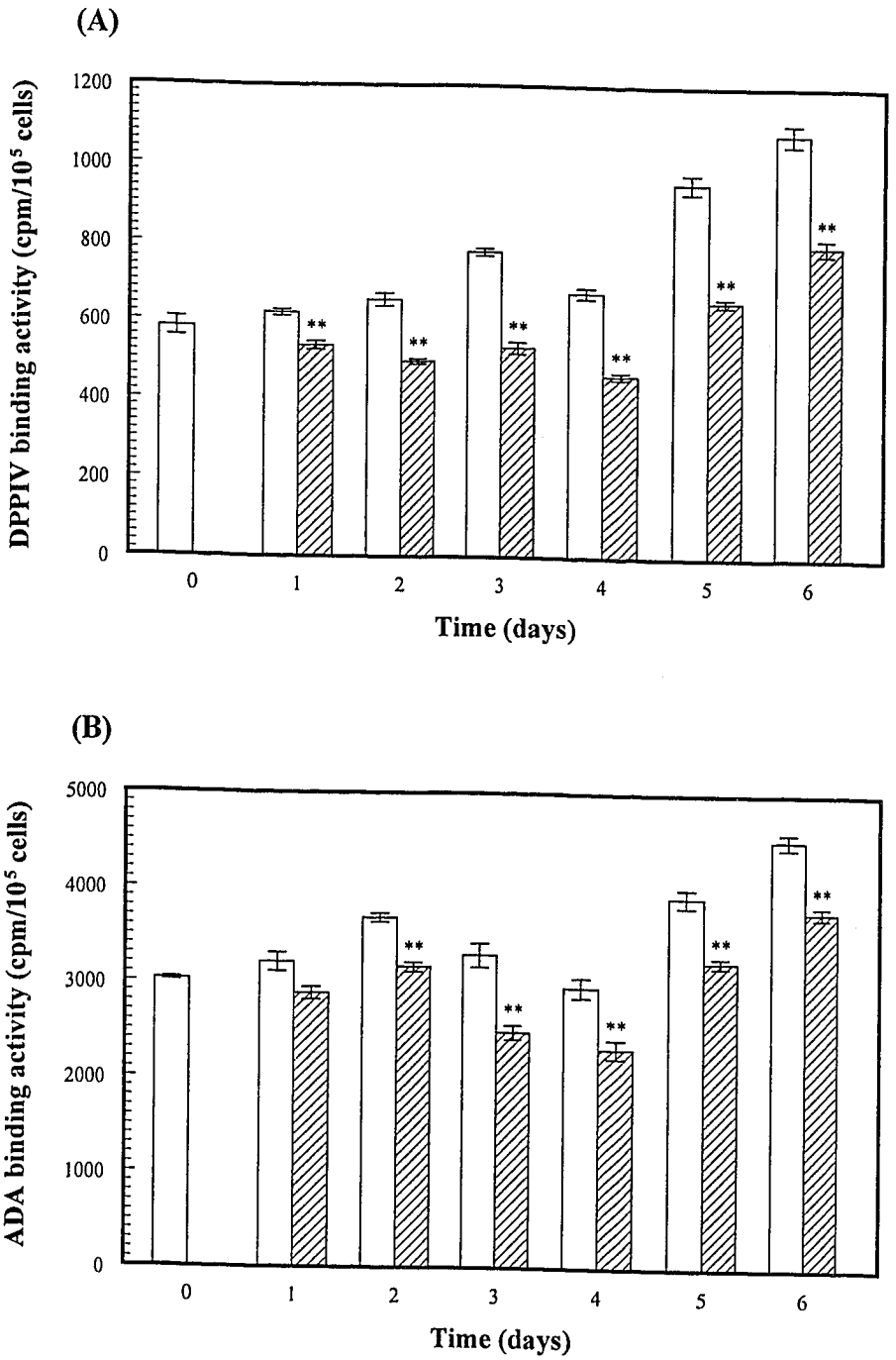


Figure 4.8 Persistent reduction of binding capacity for exogenous ADA (ecto-ADA) in response to long-term exposure to adenosine.

HT-29 cells were incubated with control media (*clear bars*) or with adenosine (50 μM, *hatched bars*) added twice daily. The cultures were then assayed for the presence of **(A)** cell-surface DPPIV protein or its **(B)** associated binding capacity for ecto-ADA. The data are mean values ± SE ($n = 4$). *, significant reduction by adenosine, $P < 0.05$. **, $P < 0.01$.

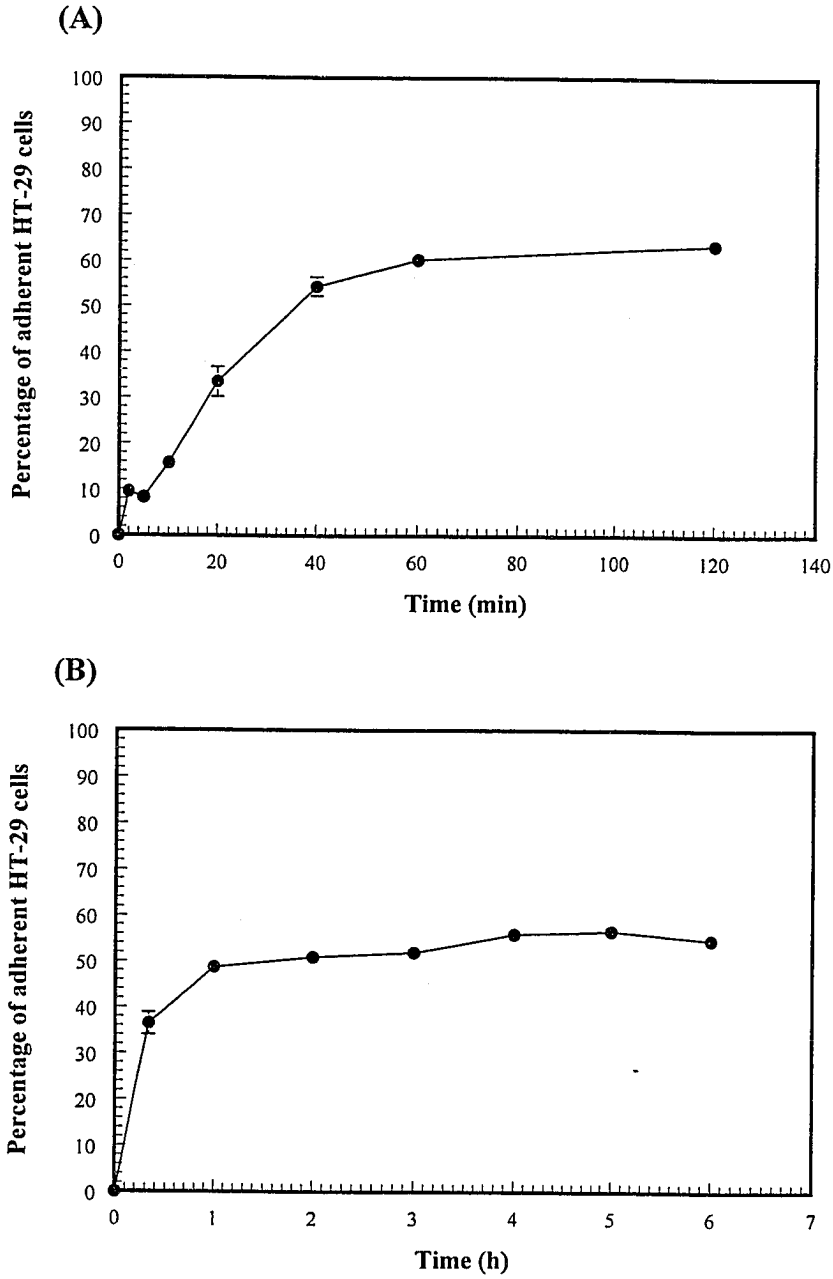


Figure 4.9 Kinetics of HT-29 cell adhesion to cellular fibronectin (cFN)-coated plates.

(A) Short-term and **(B)** long-term cell adhesion. Radiolabeled HT-29 cells were added to 4-well culture plates coated with 5 μg/ml cFN and incubated at 37°C for the times indicated. The data are mean values, $n = 4$. Most of the standard errors fall within the symbols.

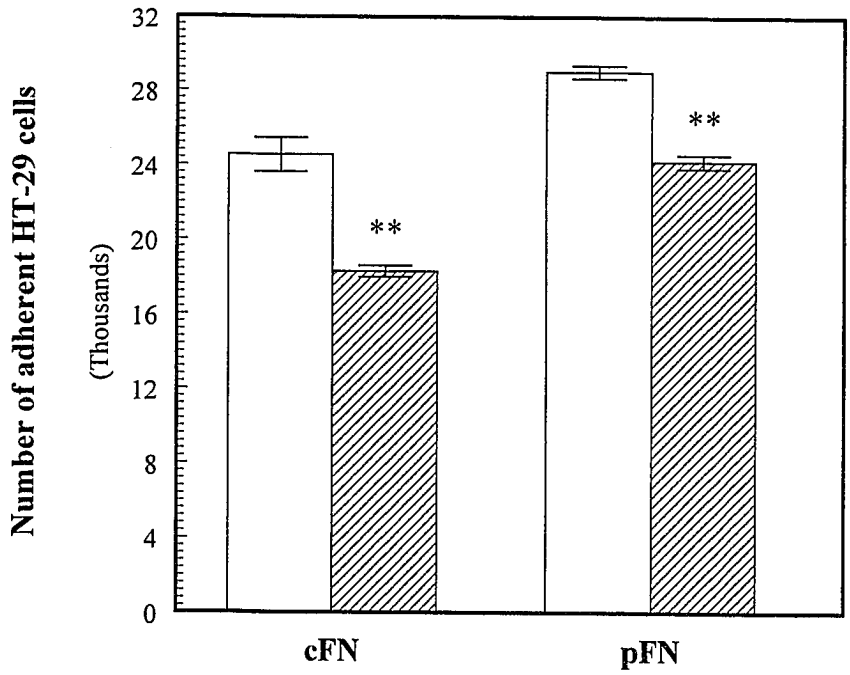


Figure 4.10 Adenosine-pretreated HT-29 cells exhibit reduced adhesion to cellular and plasma fibronectin.

HT-29 cells were treated with control vehicle (*clear bars*) or adenosine (300 μM, *hatched bars*) for 48 h, and then tested for their ability to adhere to 4-well plates coated with cellular or plasma fibronectin (5 μg/ml). The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.

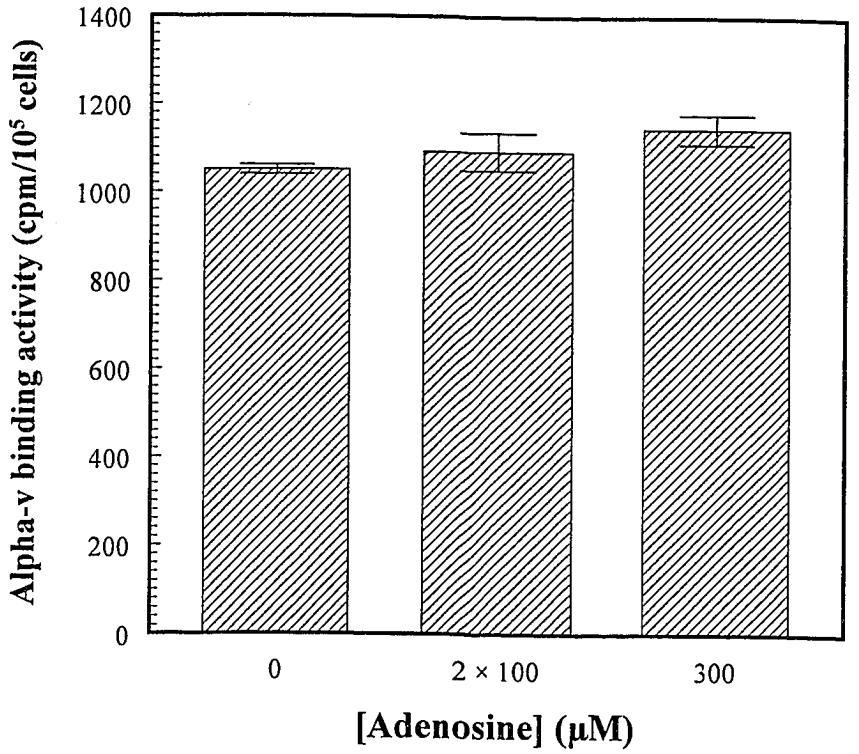


Figure 4.11 Adenosine does not regulate the expression of the α_v integrin subunit on the surface of HT-29 cells.

HT-29 cells were treated with the indicated concentrations of adenosine for 48 h. The cultures were then assayed for the presence of cell-surface α_v integrin subunit expression. The data are mean values ± SE ($n = 4$).

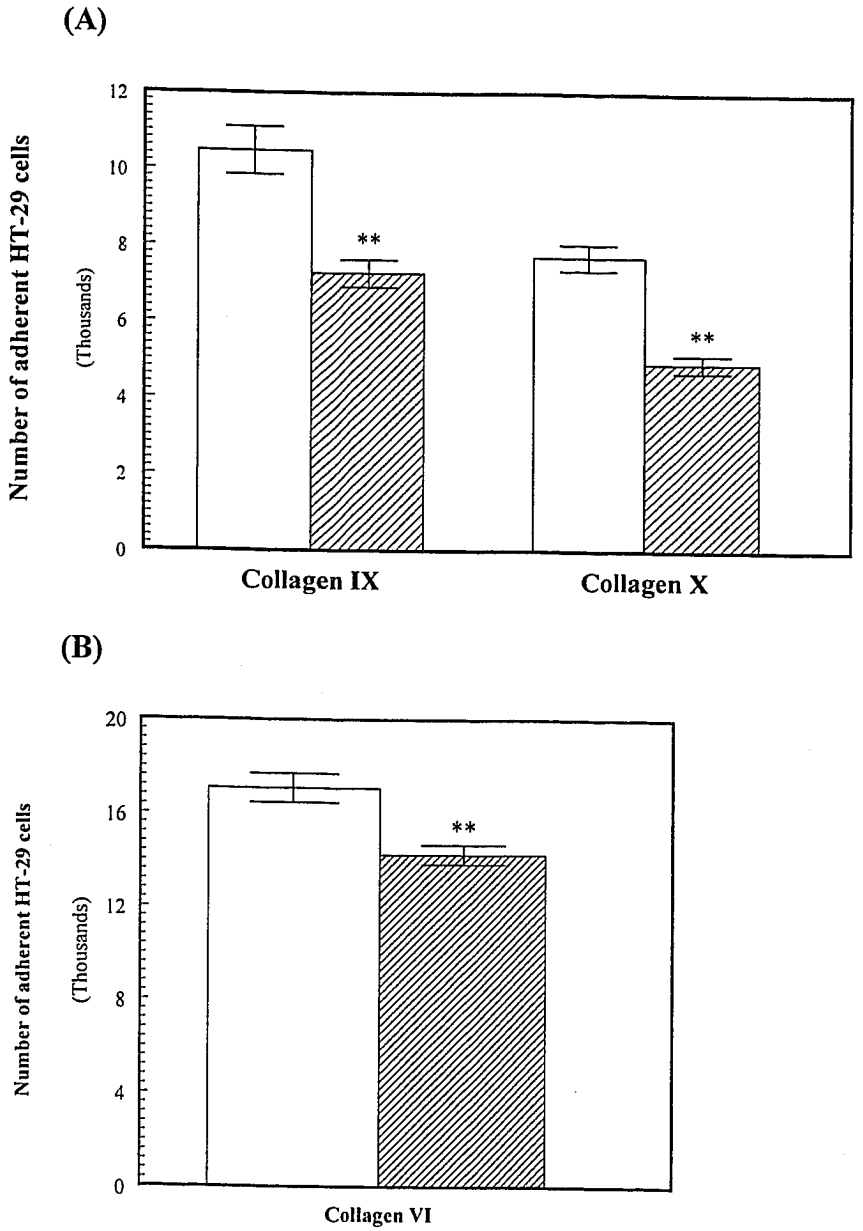


Figure 4.12 Reduced adhesion of HT-29 cells to collagen in response to adenosine. HT-29 cells were treated with control vehicle (*clear bars*) or with adenosine (100 μM daily, *hatched bars*) for 48 h, and tested for their ability to adhere to 4-well plates coated with **(A)** Sigma collagen type IX or Sigma collagen type X, or **(B)** Sigma collagen type VI (5 μg/ml). The data are mean values ± SE ($n = 6$). **, significant reduction by adenosine, $P < 0.01$.

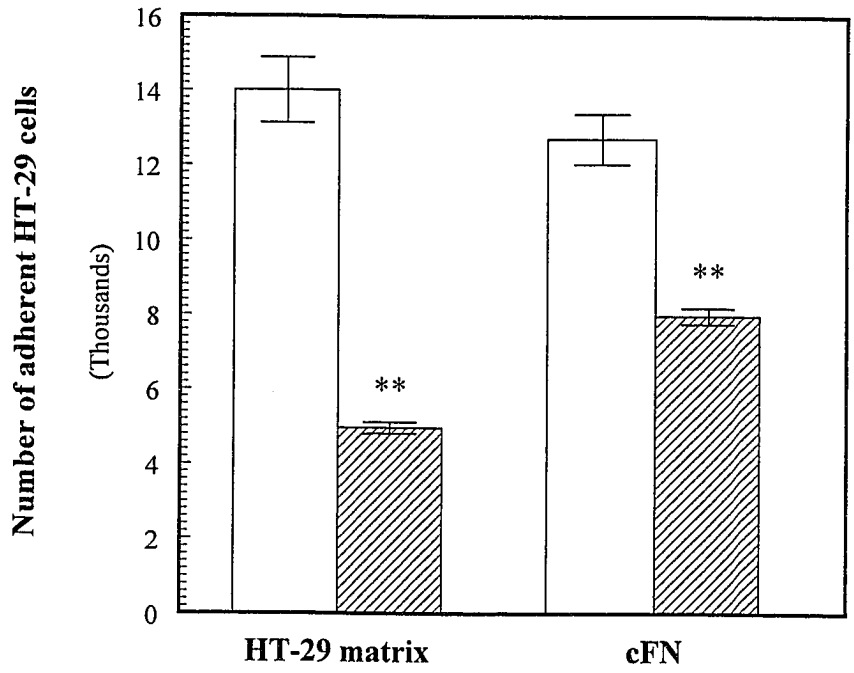


Figure 4.13 Long-term exposure to adenosine reduces adhesion of HT-29 cells to the endogenous HT-29-secreted ECM.

HT-29 cells were treated with control vehicle (*clear bars*) or adenosine (100 μM daily, *hatched bars*) for 72 h, and then tested for their ability to adhere to 4-well plates coated with the endogenous matrix secreted by confluent HT-29 cells or cFN (5 μg/ml). The data are mean values ± SE ($n = 6$). **, significant reduction by adenosine, $P < 0.01$.

DISCUSSION

The high concentrations of adenosine in the tumor microenvironment have the ability to reduce the expression of DPPIV protein on the surface of human colorectal carcinoma cells. DPPIV is a multifunctional protein that regulates the interaction of cells with both soluble and structural components of its immediate environment. This occurs through the dipeptidase activity of DPPIV which cleaves certain chemokine and neuropeptide substrates, binding of ADA to regulate extracellular levels of adenosine, and the interaction of DPPIV with the extracellular matrix. Changes in the expression of DPPIV at the surface of tumor cells would therefore be predicted to alter the behavior these cells in the tumor microenvironment. The objective of this chapter was to examine the functional consequences of the down-regulation of DPPIV on HT-29 cells by adenosine with respect to the major functions of the molecule.

DPPIV and binding of ADA

The function of DPPIV originally considered of greatest importance was its binding of ADA; thus explaining its previous identity as ADCP or ADAbp in earlier reports (Schrader and Pollara, 1978; Schrader *et al.*, 1990). This raises the possibility that a consequence of the adenosine-induced DPPIV down-regulation might (if it leads to a decline in ecto-ADA) be facilitation of the effects of adenosine itself. Indeed, we have confirmed here that adenosine-treated HT-29 cells have a reduced capacity for binding of exogenously-supplied ADA that is both quantitatively comparable to the decrease in DPPIV *and* follows the time profile of DPPIV reduction. This indicates that DPPIV is the major receptor for ADA on the surface of HT-29 cells. Alternative cell-surface binding

proteins for ADA have been reported, and intriguingly include the A_1 and A_{2B} adenosine receptors (Ciruela *et al.*, 1996; Herrera *et al.*, 2001*a*). This introduces the possibility of a mechanism for selective regulation of adenosine receptor signaling. However, it is very unlikely that ADA binding to adenosine receptors constitutes an appreciable proportion of the ADA bound to HT-29 cells. Radioantibody binding assays to detect A_{2B} receptor expression on HT-29 cells are insufficiently sensitive to detect the receptor (E.Y. Tan and J. Blay, unpublished data), even though the mRNA message is strong when compared to the A_1 receptor (M. Mujoomdar and J. Blay, unpublished data). Furthermore, Gonzalez-Gronow *et al.* (2004) found that A375 melanoma cells, which are negative for DPPIV expression, do not bind ^{125}I-labeled ADA at the cell surface. However, these same cells are known to express the ADA-binding A_1 and A_{2B} adenosine receptors at the cell surface (Merighi *et al.*, 2001). DPPIV is therefore the primary receptor for ADA on HT-29 cells and determines the binding capacity for ecto-ADA.

The baseline capacity of HT-29 cells to bind ecto-ADA remained constant over time, indicating that DPPIV protein on HT-29 cells is chronically unsaturated with ADA. This is in agreement with a number of previous reports showing the lack of saturation of DPPIV by ADA in different tissue and cell types (Schrader and Stacy, 1979; Andy and Kornfeld, 1982; Trotta, 1982; Martín *et al.*, 1995; Dong *et al.*, 1996). Importantly, we also determined that adenosine-treated HT-29 cells express reduced levels of endogenous ADA at the cell surface. The reduction of endogenous ADA with DPPIV was direct, further supporting our contention that DPPIV is the major binding protein for ADA. The dependence of cell-surface ADA expression on the levels of DPPIV in our experiments is similar to the situation in T lymphocytes, in which the number of CD26 and ADA

molecules increases in coordination following T cell activation (Martín *et al.*, 1995; Dong *et al.*, 1996). It should also be mentioned that the reduction of endogenous ADA following adenosine treatment supports our conclusion in Chapter 2 that the decline in DPPIV immunoreactivity is not due to enhanced epitope blocking by ADA.

We would predict there to be increased availability of adenosine through decreased levels of ADA at the cell surface. This has implications for the adenosine stimulation of cell growth that we have demonstrated in HT-29 and other colorectal carcinoma cell lines (Mujoomdar *et al.*, 2003). The elevated concentrations of adenosine in the proximity of tumor cells would also impair the cell-mediated anti-tumor immune response. Work from our laboratory and others have shown that adenosine can suppress the recognition/adhesion and effector phases of tumor cell destruction by cytotoxic lymphocytes (Hoskin *et al.*, 1994; MacKenzie *et al.*, 1994; Koshiba *et al.*, 1997; Hoskin *et al.*, 2002). Furthermore, the increased availability of adenosine resulting from DPPIV and ADA down-regulation would be predicted to stimulate angiogenic processes (Ethier *et al.*, 1993; Grant *et al.*, 1999; Grant *et al.*, 2001) and the motility of tumor cells (Woodhouse *et al.*, 1998). The action of adenosine that we describe here may therefore form the basis of a mechanism that *amplifies* the detrimental effects of adenosine in the context of tumorigenesis, stimulating a cascade of events that collectively contribute a selective advantage to cells configured in a solid tumor.

Previous studies have indeed identified a direct role for cell-surface DPPIV in regulating local adenosine levels and activity that occurs through its binding of ecto-ADA. Dong *et al.* (1996) found that T lymphocytes expressing ADA on the cell surface (through its association with DPPIV) were more resistant to the inhibitory effects of

adenosine on T cell proliferation and IL-2 production. The involvement of DPPIV was confirmed by this same group when they demonstrated that Jurkat cells transfected with a DPPIV construct deficient in ADA-binding were much more sensitive to the immunosuppressive effects of adenosine (Dong *et al.*, 1997). Recently, Hashikawa *et al.* (2004) reported that ecto-ADA bound to DPPIV can regulate adenosine receptor-mediated signaling (determined by the measurement of intracellular cAMP levels), and the degree to which ecto-ADA inhibits the adenosine response depends on the extent of DPPIV saturation with ADA.

Independent of adenosine metabolism, the ecto-ADA to DPPIV interaction has also been suggested to play a role in the physical adhesion of lymphocytes to colorectal carcinoma cells (Ginés *et al.*, 2002). In this mechanism, lymphocytes use their unoccupied cell-surface CD26 molecules to bind to endogenous ADA that is expressed on the surface of the carcinoma cells. Thus, the adenosine-evoked reduction of ecto-ADA on colorectal carcinoma cells might additionally inhibit immune cell attack via this adhesion mechanism.

Adenosine regulation of DPPIV dipeptidase activity

In addition to the autoregulation of adenosine levels, the reduction in DPPIV due to adenosine has the potential to impact on several processes that might facilitate invasion and metastasis. We found that the concentrations of adenosine that are present within solid tumors have the capacity to significantly down-regulate the dipeptidase activity of DPPIV. Furthermore, daily exposure to moderate levels of adenosine produced a sustained decrease in dipeptidase activity. The decline in dipeptidase activity was dose-

dependent and closely paralleled the decrease of DPPIV protein in all experiments. However, the quantitative inhibition of enzyme activity was consistently less than the extent of the protein down-regulation (i.e. suggesting a change in apparent specific activity). Boonacker *et al.* (2002) demonstrated on T lymphocytes that DPPIV activity is not always directly related to its cell-surface expression level: for example, one population of T cells can express up to six-fold more CD26 protein than a second population, while DPPIV activity is similar in both cell populations. This led to their suggestion that DPPIV protein is regulated transcriptionally while DPPIV activity might additionally be subject to post-translational regulation. This differential regulation of DPPIV protein from activity potentially represents a mechanism that keeps DPPIV activity more constant. However, the Lineweaver-Burke kinetics of DPPIV activity for control and adenosine-treated cells revealed absolutely no difference for K_m, while the V_{max} was significantly reduced for adenosine-treated cells. This excludes the possibility of differential regulation of DPPIV surface expression and dipeptidase activity. Our results are more likely explained by differences in sensitivity between the radioantibody binding assay and the colorimetric DPPIV activity assay. Riemann *et al.* (1995) also reported changes in DPPIV activities (in response to different cytokines) that were of a smaller magnitude than the measured change in protein through the use of similar assays.

In any case, the decline in net DPPIV enzyme activity would be expected to reduce the inactivation rate for certain chemokines, thereby increasing the local concentration of these peptides. The chemokine that is most susceptible to DPPIV cleavage is the CXC chemokine stromal cell-derived factor-1-alpha (SDF-1α/CXCL12; Shioda *et al.*, 1998; reviewed by Balkwill, 2003). SDF-1α is rapidly ($t_{½}$ < 1 min) and

effectively ($K_m \sim 2$ μM) truncated by DPPIV, causing a loss of its chemotactic activity (Lambeir *et al.*, 2001; Lambeir *et al.*, 2002). Consequently, a reduction in DPPIV activity would facilitate the chemotactic activities of SDF-1α and allow it to bind to its receptor CXCR4 at the cell surface (Balkwill, 2003). CXCR4 is highly expressed on many cancer cells including colon carcinoma (Dwinell *et al.*, 1999; Jordan *et al.*, 1999). Significantly, CXCR4 has been demonstrated in numerous studies as a critical regulator of the site-specific metastasis of tumor cells (Müller *et al.*, 2001; Murakami *et al.*, 2002; Taichman *et al.*, 2002). In colorectal cancer, the SDF-1α/CXCR4 signaling axis has been implicated in the outgrowth of established micrometastases, rather than in the initial movement of carcinoma cells to the metastatic site (Zeelenberg *et al.*, 2003). In either circumstance, increased adenosine concentrations in the primary tumor are predicted to down-regulate DPPIV and its associated dipeptidase activity, and remove a constraint on SDF-1α/CXCR4 regulatory action.

A previous report by Herrera *et al.* (2001*b*) found that DPPIV physically and functionally associates with CXCR4 on the surface of human peripheral blood lymphocytes. The two molecules could be coimmunoprecipitated from cellular membranes, and the treatment of lymphocytes with SDF-1α induced the formation of pseudopodia in which both DPPIV and CXCR4 colocalized. It was therefore speculated that the DPPIV/CXCR4 complex might represent a functional unit allowing DPPIV to directly mediate SDF-1α activity, at least in the case of lymphocytes. Significantly, Mizokami *et al.* (2004) recently reported the first direct link between the SDF-1α/CXCR4 pathway with the expression of DPPIV in solid tumors. They found that DPPIV-overexpressing endometrial carcinoma cells produced a decrease in soluble SDF-

1α levels compared to vector control cells, indicating that SDF-1α is constitutively degraded by cell surface DPPIV enzyme activity. Following from this work, they also found that SDF-1α stimulates the proliferation of DPPIV-negative cells, but that SDF-1α-dependent proliferation is abrogated when DPPIV is overexpressed. Thus, the imbalance between DPPIV and CXCR4, which we have shown to occur in response to adenosine, is likely to play a role in tumor progression. The function of other chemokines in the tumor microenvironment that are sensitive to DPPIV cleavage (e.g. RANTES; Khin *et al.*, 2003) remains to be determined.

Interaction of DPPIV with the extracellular matrix

Altered DPPIV levels at the surface of carcinoma cells will also affect their interaction with the extracellular matrix. We began these studies by examining the interaction of HT-29 cells with cellular fibronectin, since DPPIV binds preferentially to cell-surface fibronectin even in the presence of the soluble form of this protein (Cheng *et al.*, 1998). Another advantage of studying adhesion to cFN is that the ability of HT-29 cells to bind to this substrate appears to occur exclusively through the $\alpha_v\beta_6$ integrin (Kemperman *et al.*, 1997). There is no involvement of alternative fibronectin-binding integrins, including the more usual β_1 integrins (Stallmach *et al.*, 1994; Ebert, 1996). Since we had shown that adenosine does not reduce surface expression of the α_v subunit, we anticipated that any adenosine modulation of the interaction of HT-29 cells with cFN would occur through DPPIV. In assays of static cell adhesion to adsorbed cFN, we were able to show that the adenosine down-regulation of DPPIV was accompanied by a

reduction in binding to cFN. Furthermore, adenosine-treated HT-29 cells exhibited reduced binding to the plasma form of fibronectin.

The adenosine effect to reduce the adhesion of HT-29 cells to fibronectin through DPPIV raises the issue of the importance of binding to the ECM via integrin-*independent* mechanisms. Treatment of HT-29 cells with RGD peptide prior to the adhesion assay reduced the baseline level of cFN adhesion only moderately (~ 30%), indicating that a major component of cFN-mediated adhesion is likely taking place through DPPIV. This differs from the work of Kemperman *et al.* (1997) who showed almost complete inhibition of HT-29 cell binding to fibronectin following treatment with RGD peptide. Our results likely differ for two significant reasons: (1) Kemperman *et al.* had to use an excessively high concentration of RGD peptide (5 mM vs. 500 μM in our assays) to block adhesion to fibronectin, and (2) the protocol for their adhesion assay evaluated the binding of a very high density of cells (75,000 cells/well of a 96-well plate vs. 50,000 cells/well of a 4-well plate in our assays) that would almost certainly promote cell-to-cell interactions and possibly alter the overall mechanism of binding to fibronectin. Other groups have inhibited the binding of HT-29 cells to fibronectin through the use of α_v-blocking antibodies (von Lampe *et al.*, 1993; Stallmach *et al.*, 1994; Ebert, 1996). Interfering with the function of the α_v subunit in this way has been shown to reduce adhesion in the range of 40-80%. Although the extent of adhesion inhibition is quite variable, it does support that HT-29 cells are able to bind to fibronectin independent of integrins, which would be expected to occur through the adhesive function of DPPIV. This property is not only limited to HT-29 cells, because Fellin *et al.* (1988) reported that

hepatocytes can also bind with high affinity to fibronectin independent of RGD residues and integrins.

Reduced cellular adhesion to fibronectin, particularly cell surface-associated fibronectin, in response to adenosine in the tumor microenvironment might represent an initial step involved in the complex sequence of events leading to tumor spread. Cells with reduced levels of cell-surface DPPIV would be expected to possess less adhesive strength, thereby compromising cohesion of the tumor mass. This would facilitate detachment of cells from the primary site and permit the increased motility of the tumor cells (Danecker *et al.*, 1989). Significantly, we and others have shown that adenosine is also capable of promoting tumor cell motility (M. Mujoomdar and J. Blay, manuscript in preparation; Woodhouse *et al.*, 1998). In support of our model, highly metastatic subclones of HT-29 cells (e.g. liver metastatic HT-29LMM cells) indeed adhere poorly to fibronectin (Haier *et al.*, 1999), and they also exhibit increased tumorigenicity when inoculated into nude mice (Stallmach *et al.*, 1994).

The concept that DPPIV at the cell surface promotes/maintains cellular cohesiveness has been suggested in many studies in which the reexpression of DPPIV in carcinoma cells was found to profoundly restore normal cellular organization (Wesley *et al.*, 1999; Kajiyama *et al.*, 2002*b*; Mizokami *et al.*, 2004; Wesley *et al.*, 2005). Kajiyama *et al.* (2002*b*) speculated that DPPIV might have cadherin-like activity in ovarian carcinoma cells, thereby promoting cell-cell adhesion. Wesley *et al.* (2005) demonstrated increased levels of fibronectin proteins on the surface of DPPIV-expressing prostate carcinoma cells, which perhaps serve as a barrier against cell motility. Moreover, it is known that the overexpression of fibronectin in tumor cells can suppress the transformed

phenotype (Akamatsu *et al.*, 1996; Brenner *et al.*, 2000). Finally, other studies have found that DPPIV reintroduction into cancer cells indirectly improves cell-cell contact either by restoring the expression of E-cadherin (Kajiyama *et al.*, 2003) or CD44, the cell surface receptor for hyaluronate (Wesley *et al.*, 2004). Both E-cadherin and CD44 are critical for maintaining the intercellular adhesion of epithelial cells, while reexpression of these molecules can suppress the invasion and metastasis of carcinoma cells (Luo *et al.*, 1999; Choi *et al.*, 2000).

We found that adenosine-treated HT-29 cells also showed reduced adhesion to three different types of collagen, and that the measured reduction was comparable to the anticipated down-regulation of DPPIV. This is consistent with the results of Löster *et al.* (1995), who determined that DPPIV has binding capacity for nearly all types of collagen. Involvement of DPPIV might in fact contribute to the different patterns of adhesion of poorly and highly metastatic HT-29 subclones to different ECM substrates, even though these subclones do not have different expression of integrin subunits (Haier *et al.*, 1999). In addition, adenosine-treated HT-29 cells adhered poorly to their own endogenously-secreted ECM. The reduced adhesiveness to this physiological ECM further implicates a potential role for DPPIV in maintaining cohesion of the tumor mass. Thus, the down-regulation of DPPIV by adenosine in the tumor microenvironment likely represents one mechanism by which cell-ECM adhesion is reduced, thereby facilitating tumor cell detachment and subsequent motility and spread.

CONCLUSION

We have studied the functional consequences of the adenosine down-regulation of DPPIV with respect to the major functions and activities of the protein that occur at the surface of carcinoma cells. The results of this chapter show that the chronic down-regulation of DPPIV protein at the surface of HT-29 colorectal carcinoma cells is accompanied by reduced dipeptidyl peptidase enzyme activity, binding of ecto-ADA, and cellular interaction with the extracellular matrix. The potential impact of these alterations in cellular properties and activities are summarized in the schemes shown in Figure 4.14 and Figure 4.15. A reduction in the level of ecto-ADA at the cell surface will facilitate cellular responses to adenosine that collectively would promote tumor expansion (Figure 4.14). Reductions in the other functions of DPPIV in chemokine processing and extracellular matrix binding may further act to promote processes that will lead to the movement of carcinoma cells from their primary location (Figure 4.15). Overall, the down-regulation of DPPIV would be predicted to increase the sensitivity of cancer cells to the tumor-promoting effects of adenosine and their response to chemokines and the extracellular matrix, facilitating their expansion and metastasis.

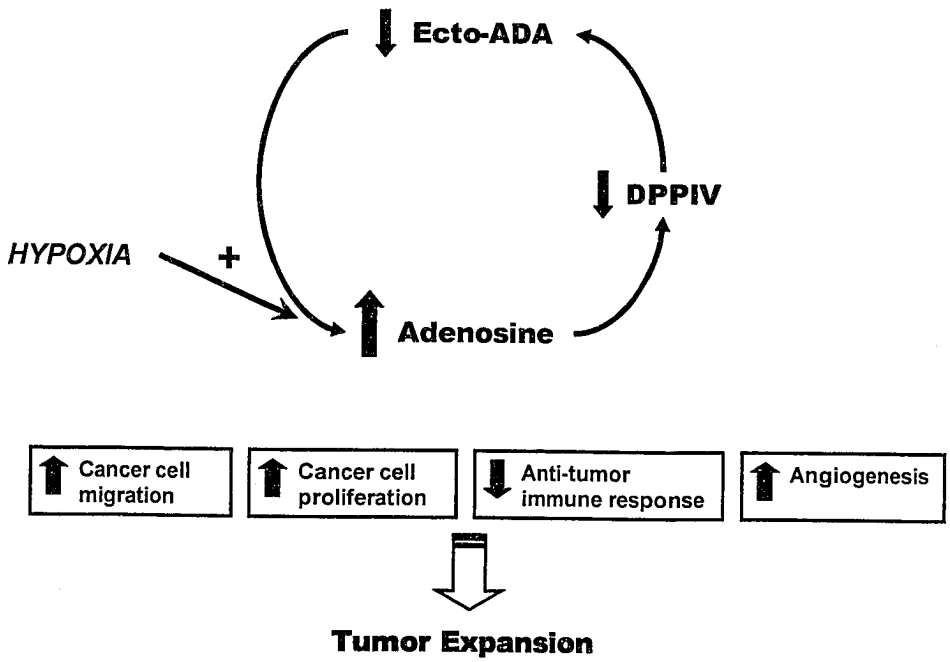


Figure 4.14 Enhancement of the tumor-promoting functions of adenosine through reduced local levels of bound ecto-ADA.

Adenosine levels in hypoxic tumors are high. Down-regulation of DPPIV leads to decreased ecto-ADA, adding to the rise in adenosine levels and therefore promoting events that facilitate tumor expansion. (From, Tan *et al.*, 2004)

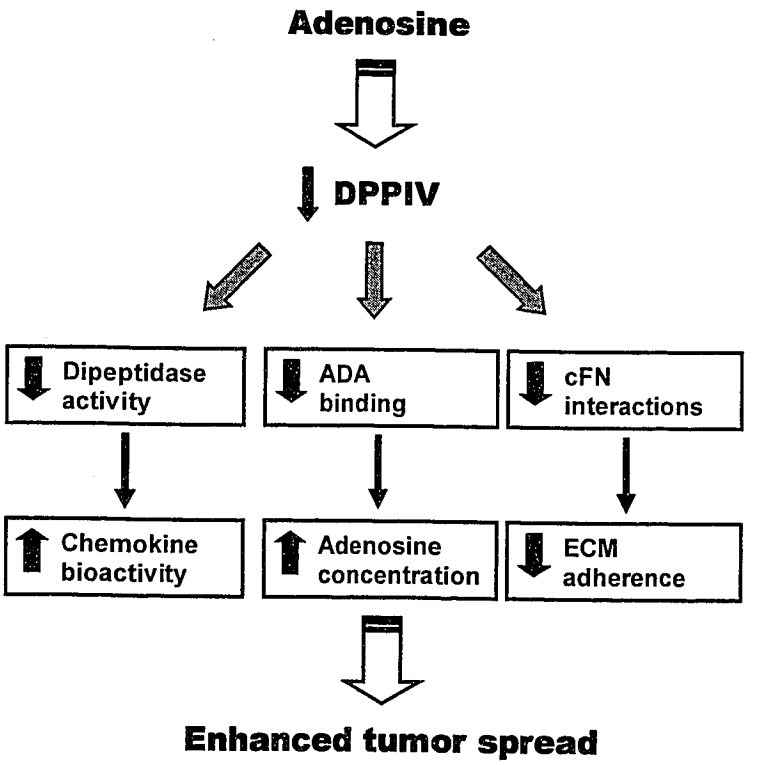


Figure 4.15 Implications of DPPIV down-regulation for other DPPIV functional activities.

Down-regulation of DPPIV also leads to decreased dipeptidase activity and reduced binding to extracellular matrix (ECM) proteins. Increased chemokine activity and reduced ECM adherence, together with the effects of adenosine, may further act to enhance the spread of tumor cells. (From, Tan *et al.*, 2004)

CHAPTER 5

CONCLUSION

Summary and conclusions

The major goal of this thesis was to determine whether the levels of adenosine present within solid tumors (due to tumor hypoxia; Blay *et al.*, 1997) would regulate the cell-surface expression of the multifunctional protein dipeptidyl peptidase IV. The effect of adenosine on DPPIV expression was assessed on human colorectal carcinoma cells. This tumor type was chosen because DPPIV expression is typically dysregulated in colorectal tumors (ten Kate *et al.*, 1985; ten Kate *et al.*, 1986), and we had previously shown that adenosine increases the growth of colorectal carcinoma cells (Mujoomdar *et al.*, 2003). We therefore hypothesized that any potential regulation of DPPIV by adenosine might perhaps represent a novel tumor-promoting role for this nucleoside.

We initially found that adenosine produced a down-regulation of DPPIV from the surface of HT-29 human colorectal carcinoma cells (Chapter 2). The effect of adenosine on DPPIV represented an authentic decrease in cell-surface protein (preceded by a reduction in DPPIV mRNA), and was not simply a reduction in DPPIV immunoreactivity that might occur from blocking by ADA or other DPPIV-binding proteins (e.g. collagen and fibronectin). In addition, the effect was specific for adenosine, and was not generalized to other nucleosides such as inosine or guanosine. Importantly, we demonstrated that the down-regulation of DPPIV could be achieved when HT-29 cells were persistently exposed to concentrations of adenosine that have been measured in the solid tumor microenvironment (~ 10 μM; Blay *et al.*, 1997). This study concluded by the determination that adenosine was acting at a cell-surface site to produce the down-

regulation of DPPIV, rather than an intracellular mechanism of action initiated by the entry of adenosine into the cell via nucleoside transporters.

In Chapter 3 of this thesis, we investigated the possibility that cell-surface adenosine receptors were responsible for triggering the adenosine regulation of DPPIV. There was absolutely no evidence that the adenosine effect occurred through adenosine receptors. This was concluded following rigorous studies using a large number of adenosine receptor antagonists, as well as selected adenosine receptor agonists. Further evidence excluding the role of conventional adenosine receptor subtypes was the lack of involvement of the cAMP-dependent protein kinase (PKA) signaling pathway, which instead stimulated an increase in DPPIV levels on HT-29 cells. We then evaluated alternative signaling pathways that have been implicated in certain aspects of adenosine action. However, we found no involvement of the PKC, PI3K, or PTK signaling pathways, or the p38 or JNK MAP kinases. Interestingly, our studies elucidated a novel cell-surface mechanism of adenosine action that required activation of a protein tyrosine phosphatase activity, leading to decreased activation of the ERK1/2 MAP kinase pathway. We believe this to be the first observation of adenosine itself causing negative regulation of the ERK1/2 signaling pathway, whereas all other reports have shown activation of ERK1/2 in response to adenosine or adenosine analogues.

Our last objective was to determine the functional consequences of the adenosine-mediated down-regulation of DPPIV on HT-29 cells (Chapter 4). The approach we took to address this issue was a direct examination of the adenosine effect on the major functions of the DPPIV molecule. The adenosine down-regulation of DPPIV was associated with a decrease in the net dipeptidase activity of DPPIV. Furthermore, the

reduction in DPPIV activity occurred in response to the levels of adenosine present in the extracellular fluid of solid tumors. We next established that reduced levels of cell-surface DPPIV following long-term adenosine treatment reduced endogenous levels of cell surface-bound ADA on HT-29 cells. Moreover, the binding capacity of the cells for exogenously administered ADA was also reduced in parallel with DPPIV. Finally, we established that the adenosine-evoked reduction of DPPIV was associated with altered adhesive properties of the cells. Adenosine pretreated HT-29 cells adhered poorly to fibronectin (cellular and plasma origin), different types of collagen, as well as the endogenous matrix secreted by colorectal carcinoma cells. Therefore, the adenosine down-regulation of DPPIV produced a persistent reduction of all of the DPPIV functional activities occurring at the surface of human colorectal carcinoma cells.

Significance and implications of findings

The ability of high concentrations of adenosine in the tumor microenvironment to down-regulate DPPIV might have substantial implications for tumor progression. We have shown that the ability of adenosine to down-regulate DPPIV from the surface of HT-29 cells is accompanied by functional alterations that should drive tumorigenesis. There is a reduction in the cellular binding capacity for ecto-ADA, which will favor the persistence of high adenosine levels. Adenosine is able to directly promote proliferation of the cancer cell population (Mujoomdar *et al.*, 2003; Mujoomdar *et al.*, 2004), in addition to many other tumor-promoting functions. The decline in cellular specific DPPIV dipeptidase activity should prolong the actions of selected chemokines and other peptides – normally substrates for DPPIV – and allow them to further promote cancer cell

proliferation and migration. Finally, adenosine causes a reduction in DPPIV-dependent adhesion of cancer cells with the extracellular matrix. Adenosine may therefore represent a soluble factor that underlies the reduced DPPIV expression in many tumors, and the association of such changes with increased invasiveness and metastasis (Dinjens *et al.*, 1990; Morrison *et al.*, 1993; Bogenrieder *et al.*, 1997; Pethiyagoda *et al.*, 2001; Kajiyama *et al.*, 2002*b*).

We have gained a preliminary understanding of the signaling pathway initiated by adenosine at the cell surface and leading to the down-regulation of DPPIV. Further understanding of this pathway by delineation of the precise PTP(s) involved should allow us to block the down-regulation of DPPIV due to adenosine and its adverse consequences. This would ultimately provide a new strategy to interfere with tumor expansion and spread. Blocking the down-regulation of DPPIV in solid tumors might also prove useful in combination with established cytotoxic or anti-angiogenic therapies. The independence of this DPPIV down-regulatory pathway from known adenosine receptors is an advantage, as the conventional signaling pathways for adenosine are involved in numerous aspects of physiologic regulation, which we would prefer to spare in any pharmacologic intervention focusing on adenosine's tumor-promoting actions.

REFERENCES

Abe, K. and Saito, H. (1998) Adenosine stimulates stellation of cultured rat cortical astrocytes. *Brain Res.* **804**, 63-71.

Aimes, R.T., Zijlstra, A., Hooper, J.D., Ogbourne, S.M., Sit, M-L., Fuchs, S., Gotley, D.C., Quigley, J.P., and Antalis, T.M. (2003) Endothelial cell serine proteases expressed during vascular morphogenesis and angiogenesis. *Thromb. Haemost.* **89**, 561-572.

Akamatsu, H., Ichihara-Tanaka, K., Ozono, K., Kamiike, W., Matsuda, H., and Sekiguchi, K. (1996) Suppression of transformed phenotypes of human fibrosarcoma cells by overexpression of recombinant fibronectin. *Cancer Res.* **56**, 4541-4546.

Akiyama, T., Ishada, J., Nakagawa, S., Ogawara, H., Watanabe, S.I., Itoh, N., Shibuya, M., and Fukami, Y. (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* **262**, 5592-5595.

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T., and Saltiel, A.R. (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.* **270**, 27489-27494.

Andy, R.J. and Kornfeld, R. (1982) The adenosine deaminase binding protein of human skin fibroblasts is located on the cell surface. *J. Biol. Chem.* **257**, 7922-7925.

Andy, R.J., Finstad, C.L., Old, L.J., Lloyd, K.O., and Kornfeld, R. (1984) The antigen identified by a mouse monoclonal antibody raised against human renal cancer cells is the adenosine deaminase binding protein. *J. Biol. Chem.* **259**, 12844-12849.

Apasov, S., Chen, J-F., Smith, P., and Sitkovsky, M. (2000) A_{2A} receptor dependent and A_{2A} receptor independent effects of extracellular adenosine on murine thymocytes in conditions of adenosine deaminase deficiency. *Blood* **95**, 3859-3867.

Aratake, Y., Umeki, K., Kiyoyama, K., Hinoura, Y., Sato, S., Ohno, A., Kuribayashi, T., Hirai, K., Nabeshima, K., and Kotani, T. (2002) Diagnostic utility of galectin-3 and CD26/DPPIV as preoperative diagnostic markers for thyroid nodules. *Diagn. Cytopathol.* **26**, 366-372.

Arslan, G. and Fredholm, B.B. (2000) Stimulatory and inhibitory effects of adenosine A_{2A} receptors on nerve growth factor-induced phosphorylation of extracellular regulated kinases 1/2 in PC12 cells. *Neurosci. Lett.* **292**, 183-186.

Baldwin, S.A., Yao, S.Y.M., Hyde, R.J., Ng, A.M.L., Foppolo, S., Barnes, K., Ritzel, M.W.L., Cass, C.E., and Young, J.D. (2005) Functional characterization of novel human and mouse equilibrative nucleoside transporters (hENT3 and mENT3) located in intracellular membranes. *J. Biol. Chem.* **280**, 15880-15887.

Balkwill, F. (2003) Chemokine biology in cancer. *Sem. Immunol.* **15**, 49-55.

Band, C.J. and Posner, B.I. (1997) Phosphatidylinositol 3′-kinase and $p70^{s6k}$ are required for insulin but not bisperoxovanadium 1,10-phenanthroline (bpV(phen)) inhibition of insulin-like growth factor binding protein gene expression. *J. Biol. Chem.* **272**, 138-145.

Band, C.J., Posner, B.I., Dumas, V., and Contreres, J-O. (1997) Early signaling events triggered by peroxovanadium [bpV(phen)] are insulin receptor kinase (IRK)-dependent: Specificity of inhibition of IRK-associated protein tyrosine phosphatase(s) by bpV(phen). *Mol. Endo.* **11**, 1899-1910.

Barat, C. and Tremblay, M.J. (2003) Treatment of human T cells with bisperoxovanadium phosphotyrosyl phosphatase inhibitors leads to activation of cyclooxygenase-2 gene. *J. Biol. Chem.* **278**, 6992-7000.

Bardelli, A., Saha, S., Sager, J.A., Romans, K.E., Xin, B., Markowitz, S.D., Lengauer, C., Velculescu, V.E., Kinzler, K.W., and Vogelstein, B. (2003) PRL-3 expression in metastatic cancers. *Clin. Cancer Res.* **9**, 5607-5615.

Baricault, L., Fransen, J.A.M., Garcia, M., Sapin, C., Codogno, P., Ginsel, L.A., and Trugnan, G. (1995) Rapid sequestration of DPP IV/CD26 and other cell surface proteins in an autophagic-like compartment in Caco-2 cells treated with forskolin. *J. Cell Sci.* **108**, 2109-2121.

Barry, C.P. and Lind, S.E. (2000) Adenosine-mediated killing of cultured epithelial cells. *Cancer Res.* **60**, 1887-1894.

Barth, R.J., Camp, B.J., Martuscello, T.A., Dain, B.J., and Memoli, V.A. (1996) The cytokine microenvironment of human colon carcinoma. Lymphocyte expression of tumor necrosis factor-α and interleukin-4 predicts improved survival. *Cancer* **78**, 1168-1178.

Bauvois, B. (1988) A collagen-binding glycoprotein on the surface of mouse fibroblasts is identified as dipeptidyl peptidase IV. *Biochem. J.* **252**, 723-731.

Bauvois, B., De Meester, I., Dumont, J., Rouillard, D., Zhao, H.X., and Bosmans, E. (1999) Constitutive expression of CD26/dipeptidylpeptidase IV on peripheral blood B lymphocytes of patients with B chronic lymphocytic leukaemia. *Br. J. Cancer* **79**, 1042-1048.

Bauvois, B., Djavaheri-Mergny, M., Rouillard, D., Dumont, J., and Wietzerbin, J. (2000) Regulation of CD26/DPPIV gene expression by interferons and retinoic acid in tumor B cells. *Oncogene* **19**, 265-272.

Bell, M.J., Kochanek, P.M., Carcillo, J.A., Mi, Z., Schiding, J.K., Wisniewski, S.R., Clark, R.S., Dixon, C.E., Marion, D.W., and Jackson, E. (1998) Interstitial adenosine,

inosine, and hypoxanthine are increased after experimental traumatic brain injury in the rat. *J. Neurotrauma* **15**, 163-170.

Beltman, J., McCormick, F., and Cook, S.J. (1996) The selective protein kinase C inhibitor, Ro-31-8220, inhibits mitogen-activated protein kinase phosphatase-1 (MKP-1) expression, induces c-Jun expression, and activates Jun N-terminal kinase. *J. Biol. Chem.* **271**, 27018-27024.

Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S., Manning, A.M., and Anderson, D.W. (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. USA.* **98**, 13681-13686.

Bennewith, K.L. and Durand, R.E. (2004) Quantifying transient hypoxia in human tumor xenografts by flow cytometry. *Cancer Res.* **64**, 6183-6189.

Benowitz, L.I., Jing, Y., Tabibiazar, R., Jo, S.A., Petrausch, B., Stuermer, C.A.O., Rosenberg, P.A., and Irwin, N. (1998) Axon outgrowth is regulated by an intracellular purine-sensitive mechanism in retinal ganglion cells. *J. Biol. Chem.* **273**, 29626-29634.

Benowitz, L.I., Goldberg, D.E., Madsen, J.R., Soni, D., and Irwin, N. (1999) Inosine stimulates extensive axon collateral growth in the rat corticospinal tract after injury. *Proc. Natl. Acad. Sci. USA* **96**, 13486-13490.

Bermpohl, F., Löster, K., Reutter, W., and Baum, O. (1998) Rat dipeptidyl peptidase IV (DPP IV) exhibits endpeptidase activity with specificity for denatured fibrillar collagens. *FEBS Lett.* **428**, 152-156.

Bieger, S., Morinville, A., and Maysinger, D. (2002) Bisperoxovanadium complex promotes dopamine exocytosis in PC12 cells. *Neurochem. Int.* **40**, 307-314.

Blay, J and Brown, K.D. (1985) Functional receptors for epidermal growth factor in an epithelial-cell line derived from rat small intestine. *Biochem. J.* **225**, 85-94.

Blay, J. and Poon, A.S.L. (1995) Use of cultured permanent lines of intestinal epithelial cells for the assay of okadaic acid in mussel homogenates. *Toxicon.* **33**, 739-746.

Blay, J., White, T.D., and Hoskin, D.W. (1997) The extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine. *Cancer Res.* **57**, 2602-2605.

Bogenrieder, T., Finstad, C.L., Freeman, R.H., Papandreou, C.N., Scher, H.I., Albino, A.P., Reuter, V.E., and Nanus, D.M. (1997) Expression and localization of aminopeptidase A, aminopeptidase N, and dipeptidyl peptidase IV in benign and malignant human prostate tissue. *Prostate* **33**, 225-232.

Boonacker, E.P., Wierenga, E.A., Smits, H.H., and Van Noorden, C.J.F. (2002) CD26/DPPIV signal transduction function, but not proteolytic activity, is directly related to its expression level on human Th1 and Th2 cell lines as detected with living cell cytochemistry. *J. Histochem. Cytochem.* **50**, 1169-1177.

Braun, N., Zhu, Y., Krieglstein, J., Culmsee, C., and Zimmermann, H. (1998) Upregulation of the enzyme chain hydrolyzing extracellular ATP following transient forebrain ischemia in the rat. *J. Neurosci.* **18**, 4891-4900.

Brenner, K.A., Corbett, S.A., and Schwarzbauer, J.E. (2000) Regulation of fibronectin matrix assembly by activated Ras in transformed cells. *Oncogene* **19**, 3156-3163.

Brown, J.M. (1999) The hypoxic cell: a target for selective cancer therapy – eighteenth Bruce F. Cain memorial award lecture. *Cancer Res.* **59**, 5863-5870.

Brurberg, K.G., Graff, B.A., and Rofstad, E.K. (2003) Temporal heterogeneity in oxygen tension in human melanoma xenografts. *Br. J. Cancer* **89**, 350-356.

Bühling, F., Kunz, D., Reinhold, D., Ulmer, A.J., Ernst, M., Flad, H.D., and Ansorge, S. (1994) Expression and functional role of dipeptidyl peptidase IV (CD26) on human natural killer cells. *Nat. Immun.* **13**, 270-279.

Bühling, F., Junker, U., Reinhold, D., Neubert, K., Jager, L., and Ansorge, S. (1995) Functional role of CD26 on human B lymphocytes. *Immunol. Lett.* **45**, 47-51.

Bulut, K., Meier, J.J., Ansorge, N., Felderbauer, P., Schmitz, F., Hoffmann, P., Schmidt, W.E., and Gallwitz, B. (2004) Glucagon-like peptide 2 improves intestinal wound healing through induction of epithelial cell migration in vitro – evidence for a TGF-β-mediated effect. *Regul. Pept.* **121**, 137-143.

Busso, N., Wagtmann, N., Herling, C., Chobaz-Péclat, V., Bischof-Delaloye, A., So, A., and Grouzmann, E. (2005) Circulating CD26 is negatively associated with inflammation in human and experimental arthritis. *Am. J. Pathol.* **166**, 433-442.

Camps, M., Nichols, A., and Arkinstall, S. (1999) Dual specificity phosphatases: a gene family for control of MAP kinase function. *FASEB J.* **14**, 6-16.

Canbolat, O., Durak, I., Cetin, R., Kavutcu, M., Demirci, S., and Ozturk, S. (1996) Activities of adenosine deaminase, 5′-nucleotidase, guanase, and cytidine deaminase enzymes in cancerous and non-cancerous human breast tissues. *Breast Cancer Res. Treat.* **37**, 189-193.

Ceradini, D.J., Kulkarni, A.R., Callaghan, M.J., Tepper, O.M., Bastidas, N., Kleinman, M.E., Capla, J.M., Galiano, R.D., Levine, J.P., and Gurtner, G.C. (2004) Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat. Med.* **10**, 858-864.

Champe, P.C. and Harvey, R.A. (1994) Lippencott's Illustrated Reviews. Biochemistry. 2nd Ed. Lippencott-Raven Publishers, Philadelphia, PA, USA.

Chantret, I., Barbat, A., Dussaulx, E., Brattain, M.G., and Zweibaum, A. (1988) Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: a survey of twenty cell lines. *Cancer Res.* **48**, 1936-1942.

Chen, W-T. and Wang, J.Y. (1999) Specialized surface protrusions of invasive cells, invadopodia and lamellipodia, have differential MT1-MMP, MMP-2, and TIMP-2 localization. *Ann. N.Y. Acad. Sci.* **878**, 361-371.

Chen, Y., Rathbone, M.P., and Hertz, L. (2001) Guanosine-induced increase in free cytosolic calcium concentration in mouse astrocytes in primary cultures: does it act on an A_3 adenosine receptor? *J. Neurosci. Res.* **65**, 184-189.

Chen, P., Goldberg, D.E., Kolb, B., Lanser, M., Benowitz, L.I. (2002) Inosine induces axonal rewiring and improves behavioral outcome after stroke. *Proc. Natl. Acad. Sci. USA* **99**, 9031-9036.

Cheng, H-C., Abdel-Ghany, M., Elble, R.C., and Pauli, B.C. (1998) Lung endothelial dipeptidyl peptidase IV promotes adhesion and metastasis of rat breast cancer cells via tumor cell surface-associated fibronectin. *J. Biol. Chem.* **273**, 24207-24215.

Cheng, H-C., Abdel-Ghany, M., and Pauli, B.U. (2003) A novel consensus motif in fibronectin mediates dipeptidyl peptidase IV adhesion and metastasis. *J. Biol. Chem.* **278**, 24600-24607.

Choi, S.H., Takahashi, K., Eto, H., Yoon, S.S., and Tanabe, K.K. (2000) CD44s expression in human colon carcinomas influences growth of liver metastases. *Int. J. Cancer* **85**, 523-526.

Ciccarelli, R., Di Iorio, P., Giuliani, P., D'Alimonte, I., Ballerini, P., Caciagli, F., and Rathbone, M.P. (1999) Rat cultured astrocytes release guanine-based purines in basal conditions and after hypoxia/hypoglycemia. *Glia* **25**, 93-98.

Ciccarelli, R., Di Iorio, P., D'Alimonte, I., Giuliani, P., Florio, T., Caciagli, F., Middlemiss, P.J., and Rathbone, M.P. (2000) Cultured astrocyte proliferation induced by extracellular guanosine involves endogenous adenosine and is raised by the co-presence of microglia. *Glia* **29**, 202-211.

Ciruela, F., Saura, C., Canela, E.I., Mallol, J., Lluis, C., and Franco, R. (1996) Adenosine deaminase affects ligand-induced signaling by interacting with cell surface adenosine receptors. *FEBS Lett.* **380**, 219-223.

Colquhoun, A. and Newsholme, E.A. (1997) Inhibition of human tumour cell proliferation by analogues of adenosine. *Cell Biochem. Funct.* **15**, 135-139.

Craig, C.G. and White, T.D. (1993) N-methyl-D-aspartate-evoked adenosine release from rat cortical slices: distinct purinergic sources and mechanisms of release. *J. Neurochem.* **60**, 1073-1080.

Crist, G.H., Xu, B., Berkich, D.A., and LaNoue, K.F. (2001) Effects of adenosine receptor antagonism on protein tyrosine phosphatase in rat skeletal muscle. *Int. J. Biochem. Cell Biol.* **33**, 817-830.

Cuenda, A., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P.R., and Lee, J.C. (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364**, 229-233.

Cunnick, J.M., Meng, S., Ren, Y., Desponts, C., Wang, H.G., Djeu, J.Y., and Wu, J. (2002) Regulation of mitogen-activated protein kinase signaling pathway by SHP2. *J. Biol. Chem.* **277**, 9498-9504.

Daddona, P.E. and Kelley, W.N. (1977) Human adenosine deaminase: purification and subunit structure. *J. Biol. Chem.* **252**, 110-115.

Daddona, P.E. and Kelley, W.N. (1980) Analysis of normal and mutant forms of human adenosine deaminase – a review. *Mol. Cell. Biochem.* **29**, 91-101.

Daddona, P.E., Shewach, D.S., Kelley, W.N., Argos, P., Markham, A.F., and Orkin, S.H. (1984) Human adenosine deaminase: cDNA and complete primary amino acid sequence. *J. Biol. Chem.* **259**, 12101-12106.

Damaraju, V.L., Damaraju, S., Young, J.D., Baldwin, S.A., Mackey, J., Sawyer, M.B., and Cass, C.E. (2003) Nucleoside anticancer drugs: the role of nucleoside transporters in resistance to cancer chemotherapy. *Oncogene* **22**, 7524-7536.

Danecker, G.W., Piazza, A.J., Steele, G.D., and Mercurio, A.M. (1989) Relationship between extracellular matrix interactions and degree of differentiation in human colon carcinoma cell lines. *Cancer Res.* **49**, 681-686.

Dang, N.H., Torimoto, Y., Deusch, K., Schlossman, S.F., and Morimoto, C. (1990*a*) Comitogenic effect of solid-phase immobilized anti-1F7 on human CD4 T cell activation via CD3 and CD2 pathways. *J. Immunol.* **144**, 4092-4100.

Dang, N.H., Torimoto, Y., Schlossman, S.F., and Morimoto, C. (1990*b*) Human CD4 helper T cell activation: functional involvement of two distinct collagen receptors, 1F7 and VLA integrin family. *J. Exp. Med.* **172**, 649-652.

Dang, N.H. and Morimoto, C. (2002) CD26: an expanding role in immune regulation and cancer. *Histol. Histopathol.* **17**, 1213-1226.

Darmoul, D., Lacasa, M., Baricault, L., Marguet, D., Sapin, C., Trotot, P., Barbat, A., and Trugnan, G. (1992) Dipeptidyl peptidase IV (CD26) gene expression in enterocyte-like colon cancer cell lines HT-29 and Caco-2. Cloning of the complete human coding sequence and changes of dipeptidyl peptidase IV mRNA levels during cell differentiation. *J. Biol. Chem.* **267**, 4824-4833.

Dawicki, D.D., Chatterjee, D., Wyche, J., and Rounds, S. (1997) Extracellular ATP and adenosine cause apoptosis of pulmonary artery endothelial cells. *Am. J. Physiol.* **273**, L485-L494.

De Falco, E., Porcelli, D., Torella, A.R., Straino, S., Iachininoto, M.G., Orlandi, A., Truffa, S., Biglioli, P., Napolitano, M., Capogrossi, M.C., and Pesce, M. (2004) SDF-1 involvement in endothelial phenotype and ischemia-induced recruitment of bone marrow progenitor cells. *Blood* **104**, 3472-3482.

De Meester, I., Vanham, G., Kestens, L., Vanhoof, G., Bosmans, E., Gigase, P., and Scharpé, S. (1994) Binding of adenosine deaminase to the lymphocyte surface via CD26. *Eur. J. Immunol.* **24**, 566-570.

De Meester, I., Korom, S., Van Damme, J., and Scharpé, S. (1999) CD26, let it cut or cut it down. *Immunol. Today* **20**, 367-375.

Decking, U.K., Schlieper, G., Kroll, K., and Schrader, J. (1997) Hypoxia-induced inhibition of adenosine kinase potentiates cardiac adenosine release. *Circ. Res.* **81**, 154-164.

Di Iorio, P., Kleywegt, S., Ciccarelli, R., Traversa, U., Andrew, C.M., Crocker, C.E., Werstiuk, E.S., and Rathbone, M.P. (2002) Mechanisms of apoptosis induced by purine nucleosides in astrocytes. *Glia* **38**, 179-190.

Di Iorio, P., Ballerini, P., Traversa, U., Nicoletti, F., D'Alimonte, I., Kleywegt, S., Werstiuk, E.S., Rathbone, M.P., Caciagli, F., and Ciccarelli, R. (2004) The antiapoptotic effect of guanosine is mediated by the activation of the PI 3-kinase/AKT/PKB pathway in cultured rat astrocytes. *Glia* **46**, 356-368.

Dickenson, J.M., Blank, J.L., and Hill, S.J. (1998) Human adenosine A_1 receptor and $P2Y_2$-purinoceptor-mediated activation of the mitogen-activated protein kinase cascade in transfected CHO cells. *Br. J. Pharmacol.* **124**, 1491-1499.

Dickenson, J.M., Reeder, S., Rees, B., Alexander, S., and Kendall, D. (2003) Functional expression of adenosine A_{2A} and A_3 receptors in the mouse dendritic cell line XS-106. *Eur. J. Pharmacol.* **474**, 43-51.

Dihlmann, S. and von Knebel Doeberitz, M. (2005) Wnt/β-catenin-pathway as a molecular target for future anti-cancer therapeutics. *Int. J. Cancer* **113**, 515-524.

Dinjens, W.N.M., ten kate, J., van der Linden, E.P.M., Wijnen, J.T., Khan, P.M., and Bosman, F.T. (1989*a*) Distribution of adenosine deaminase complexing protein (ADCP) in human tissues. *J. Histochem. Cytochem.* **37**, 1869-1875.

Dinjens, W.N.M., ten Kate, J., Wijnen, J.T., van der Linden, E.P.M., Beek, C.J.L., Lenders, M-H.J.H., Khan, P.M., and Bosman, F.T. (1989*b*) Distribution of adenosine deaminase-complexing protein in murine tissues. *J. Biol. Chem.* **264**, 19215-19220.

Dinjens, W.N.M., ten Kate, J., Kirch, J.A.J.M., Tanke, H.J., van der Linden, E.P.M., van den Ingh, H.F.G.M., van Steenbrugge, G.J., Khan, P.M., and Bosman, F.T. (1990) Adenosine deaminase complexing protein (ADCP) expression and metastatic potential in prostatic adenocarcinomas. *J. Pathol.* **160**, 195-201.

Dong, R-P., Kameoka, J., Hegen, M., Tanaka, T., Xu, Y., Schlossman, S.F., and Morimoto, C. (1996) Characterization of adenosine deaminase binding to human CD26 on T cells and its biologic role in immune response. *J. Immunol.* **156**, 1349-1355.

Dong, R-P., Tachibana, K., Hegen, M., Munakata, Y., Cho, D., Schlossman, S.F., and Morimoto, C. (1997) Determination of adenosine deaminase binding domain on CD26 and its immunoregulatory effect on T cell activation. *J. Immunol.* **159**, 6070-6076.

Drucker, D.J. (2001) The glucagon-like peptides. *Endocrinology* **142**, 521-527.

Dubey, R.K., Gillespie, D.G., and Jackson, E.K. (2002) A_{2B} adenosine receptors stimulate growth of porcine and rat arterial endothelial cells. *Hypertension* **39**, 530-535.

Durak, I., Cetin, R., Canbolat, O., Cetin, D., Yurtarslani, Z., and Unal, A. (1994) Adenosine deaminase, 5′-nucleotidase, guanase, and cytidine deaminase activities in gastric tissues from patients with gastric cancer. *Cancer Lett.* **84**, 199-202.

Dwinell, M.B., Eckman, L., Leopard, J.D., Varki, N.M., and Kagnoff, M.F. (1999) Chemokine receptor expression by human intestinal epithelial cells. *Gastroenterology* **117**, 359-367.

Ebert, E.C. (1996) Mechanisms of colon cancer binding to substratum and cells. *Digest. Dis. Sci.* **41**, 1551-1556.

El Yacoubi, M., Ledent, C., Parmentier, M., Costentin, J., and Vaugeois, J-M. (2000) SCH 58261 and ZM 241385 differentially prevent the motor effects of CGS 21680 in mice: evidence for a functional 'atypical' adenosine A_{2A} receptor. *Eur. J. Pharmacol.* **401**, 63-77.

English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu, S., and Cobb, M.H. (1999) New insights into the control of MAP kinase pathways. *Exp. Cell Res.* **253**, 255-270.

Ethier, M.F., Chander, V., and Dobson, J.G. (1993) Adenosine stimulates proliferation of human endothelial cells in culture. *Am. J. Physiol.* **265**, H131-H138.

Ethier, M.F. and Dobson, J.G. (1997) Adenosine stimulation of DNA synthesis in human endothelial cells. *Am. J. Physiol.* **272**, 1470-1479.

Ezeamuzie, C.I. and Philips, E. (2003) Positive coupling of atypical adenosine A_3 receptors on human eosinophils to adenylyl cyclase. *Biochem. Biophys. Res. Commun.* **300**, 712-718.

Faure, M., Voyno-Yasenetskaya, T.A., and Bourne, H.R. (1994) cAMP and beta gamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J. Biol. Chem.* **269**, 7851-7854.

Faure, R., Vincent, M., Dufour, M., Shaver, A., and Posner, B.I. (1995) Arrest at the G2/M transition of the cell cycle by protein-tyrosine phosphatase inhibition: studies on a neuronal and a glial cell line. *J. Cell. Biochem.* **58**, 389-401.

Fellin, F.M., Barsigian, C., Rich, E., and Martinez, J. (1988) Binding and cross-linking of rabbit fibronectin by rabbit hepatocytes in suspension. *J. Biol Chem.* **263**, 1791-1797.

Feoktistov, I., Goldstein, A.E., and Biaggioni, I. (1999) Role of p38 mitogen-activated protein kinase and extracellular signal-regulated kinase kinase in adenosine A_{2B} receptor-mediated interleukin-8 production in human mast cells. *Mol. Pharmacol.* **55**, 726-734.

Fischer, S., Sharma, H.S., Karliczek, G.F., and Schaper, W. (1995) Expression of vascular permeability factor/vascular endothelial growth factor in pig cerebral microvascular endothelial cells and its upregulation by adenosine. *Brain Res.* **28**, 141-148.

Fishman, P., Bar-Yehuda, S., and Vagman, L. (1998) Adenosine and other low molecular weight factors released by muscle cells inhibit tumor cell growth. *Cancer Res.* **58**, 3181-3187.

Fishman, P., Bar-Yehuda, S., Ohana, G., Pathak, S., Wasserman, L., Barer, F., and Multani, A.S. (2000) Adenosine acts as an inhibitor of lymphoma cell growth: a major role for the A3 adenosine receptor. *Eur. J. Cancer* **36**, 1452-1458.

Fishman, P., Bar-Yehuda, S., Madi, L., and Cohn, I. (2002*a*) A3 adenosine receptor as a target for cancer therapy. *Anti-Cancer Drugs* **13**, 437-443.

Fishman, P., Madi, L., Bar-Yehuda, S., Barer, F., Del Valle, L., and Khalili, K. (2002*b*) Evidence for involvement of Wnt signaling pathway in IB-MECA mediated suppression of melanoma cells. *Oncogene* **21**, 4060-4064.

Fishman, P., Bar-Yehuda, S., Ardon, E., Rath-Wolfson, L., Barrer, F., Ochaion, A., and Madi, L. (2003) Targeting the A_3 adenosine receptor for cancer therapy: inhibition of prostate carcinoma cell growth by A_3AR agonist. *Anticancer Res.* **23**, 2077-2083.

Fleischer, B. (1994) CD26: a surface protease involved in T-cell activation. *Immunol. Today* **15**, 180-184.

Flocke, K. and Mannherz, H.G. (1991) Isolation and characterization of 5'-nucleotidase of a human pancreatic tumor cell line. *Biochim. Biophys. Acta.* **1076**, 273-281.

Forsythe, J.A., Jiang, B-H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D., and Semenza, G.L. (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell. Biol.* **16**, 4604-4613.

Fortin, J-F., Barbeau, B., Robichaud, G.A., Paré, M-È., Lemieux, A-M., and Tremblay, M.J. (2001) Regulation of nuclear factor of activated T cells by phosphotyrosyl-specific phosphatase activity: a positive effect on HIV-1 long terminal repeat-driven transcription and a possible implication of SHP-1. *Blood* **97**, 2390-2400.

Fowler, J.C. (1993) Purine release and inhibition of synaptic transmission during hypoxia and hypoglycemia in rat hippocampal slices. *Neurosci. Lett.* **157**, 83-86.

Fredholm, B.B., Zhang, Y., van der Ploeg, I. (1996) Adenosine A_{2A} receptors mediate the inhibitory effect of adenosine on formyl-Met-Leu-Phe-stimulated respiratory burst in neutrophil leukocytes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **354**, 262-267.

Fredholm, B.B., Arslan, G., Halldner, L., Kull, B., Schulte, G., and Wasserman, W. (2000) Structure and function of adenosine receptors and their genes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **362**, 364-374.

Fredholm, B.B., Ijzerman, A.P., Jacobson, K.A., Klotz, K-N., and Linden, J. (2001) International union of pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* **53**, 527-552.

Fujiwara, H., Maeda, M., Imai, K., Fukuoka, M., Yasuda, K., Takakura, K., and Mori, T. (1992) Human luteal cells express dipeptidyl peptidase IV on the cell surface. *J. Clin. Endocrinol. Metab.* **75**, 1352-1357.

Gao, Z., Chen, T., Weber, M.J., and Linden, J. (1999) A_{2B} adenosine and $P2Y_2$ receptors stimulate mitogen-activated protein kinase in human embryonic kidney-293 cells. *J. Biol. Chem.* **274**, 5972-59080.

Garcia-Morales, P., Minami, Y., Luong, E., Klausner, R.D., and Samelson, L.E. (1990) Tyrosine phosphorylation in T cells is regulated by phosphatase activity: Studies with phenylarsine oxide. *Proc. Natl. Acad. Sci. USA* **87**, 9255-9259.

Gessi, S., Cattabriga, E., Avitabile, A., Gafa', R., Lanza, G., Cavazzini, L., Bianchi, N., Gambari, R., Feo, C., Liboni, A., Gullini, S., Leung, E., Mac-Lennan, S., and Borea, P.A. (2004) Elevated expression of A_3 adenosine receptors in human colorectal cancer is reflected in peripheral blood cells. *Clin. Cancer Res.* **10**, 5895-5901.

Ghersi, G., Chen, W-T., Lee, E.W., and Zukowska, Z. (2001) Critical role of dipeptidyl peptidase IV in neuropeptide Y-mediated endothelial cell migration in response to wounding. *Peptides* **22**, 453-458.

Ghersi, G., Dong, H., Goldstein, L.A., Yeh, Y., Hakkinen, L., Larjava, H.S., and Chen, W-T. (2002) Regulation of fibroblast migration on collagenous matrix by a cell surface peptidase complex. *J. Biol. Chem.* **277**, 29231-29241.

Giblett, E.R., Anderson, J.E., Cohen, F., Pollara, B., and Meuwissen, H.J. (1972) Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. *Lancet* **2**, 1067-1069.

Ginés, S., Mariño, M., Mallol, J., Canela, E.I., Morimoto, C., Callebaut, C., Hovanessian, A., Casadó, V., Lluis, C., and Franco, R. (2002) Regulation of epithelial and lymphocyte cell adhesion by adenosine deaminase-CD26 interaction. *Biochem. J.* **361**, 203-209.

Goh, L.B., Mack, P., and Lee, C.W. (1995) Nitrobenzylthioinosine-binding protein overexpression in human breast, liver, stomach and colorectal tumour tissues. *Anticancer Res.* **15**, 2575-2579.

Gomez, G. and Sitkovsky, M.V. (2003) Differential requirement for A2a and A3 adenosine receptors for the protective effect of inosine in vivo. *Blood* **102**, 4472-4478.

Gonzalez-Gronow, M., Shearin, T., and Pizzo, S. (1996) *Fibrinolysis* **10** (Suppl. 3), 32.

Gonzalez-Gronow, M., Hershfield, M.S., Arredondo-Vega, F.X., and Pizzo, S.V. (2004) Cell surface adenosine deaminase binds and stimulates plasminogen activation on 1-LN human prostate cancer cells. *J. Biol. Chem.* **279**, 20993-20998.

Grant, M.B., Tarnuzzer, R.W., Caballero, S., Ozeck, M.J., Davis, M.I., Spoerri, P.E., Feoktistov, I., Biaggioni, I., Shyrock, J.C., and Belardinelli, L. (1999) Adenosine receptor activation induces vascular endothelial growth factor in human retinal endothelial cells. *Circ. Res.* **85**, 699-706.

Grant, M.B., Davis, M.I., Caballero, S., Feoktistov, I., Biaggioni, I., and Belardinelli, L. (2001) Proliferation, Migration, and ERK activation in human retinal endothelial cells through A_{2B} adenosine receptor stimulation. *Invest. Ophthalmol. Vis. Sci.* **42**, 2068-2073.

Griffith, D.A. and Jarvis, S.M. (1996) Nucleoside and nucleobase transport systems of mammalian cells. *Biochim. Biophys. Acta.* **1286**, 153-181.

Griffiths, M., Beaumont, N., Yao, S.Y.M., Sundaram, M., Boumah, C.E., Davies, A., Kwong, F.Y.P., Coe, I. Cass, C.E., Young, J.D., and Baldwin, S.A. (1997*a*) Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs. *Nat. Med.* **3**, 89-93.

Griffiths, M., Yao, S.Y.M., Abidi, F., Phillips, S.E.V., Cass, C.E., Young, J.D., and Baldwin, S.A. (1997*b*) Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (*ei*) equilibrative nucleoside transporter from human placenta. *Biochem. J.* **328**, 739-743.

Grondin, G., Hooper, N.M., and LeBel, D. (1999) Specific localization of membrane dipeptidase and dipeptidyl peptidase IV in secretion granules of two different pancreatic islet cells. *J. Histochem. Cytochem.* **47**, 489-497.

Haier, J., Nasralla, M., and Nicolson, G.L. (1999) Different adhesion properties of highly and poorly metastatic HT-29 colon carcinoma cells with extracellular matrix components: role of integrin expression and cytoskeletal components. *Br. J. Cancer* **80**, 1867-1874.

Han, Z. and Wyche, J.H. (1994) Guanosine induces necrosis of cultured aortic endothelial cells. *Am. J. Pathol.* **145**, 423-427.

Han, Z., Boyle, D.L., Chang, L., Bennett, B., Karin, M., Yang, L., Manning, A.M., and Firestein, G.S. (2001) c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. *J. Clin. Invest.* **108**, 73-80.

Hanahan, D. and Folkman, J. (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353-364.

Hanski, C., Huhle, T., and Reutter, W. (1985) Involvement of plasma membrane dipeptidyl peptidase IV in fibronectin-mediated adhesion of cells on collagen. *Biol. Chem. Hoppe-Seyler* **366**, 1169-1176.

Hanski, C., Huhle, T., Gossrau, R., and Reutter, W. (1988) Direct evidence for the binding of rat liver DPP IV to collagen *in vitro*. *Exp. Cell Res.* **178**, 64-72.

Harrington, E.O., Smeglin, A., Parks, N., Newton, J., and Rounds, S. (2000) Adenosine induces endothelial apoptosis by activating protein tyrosine phosphatase: a possible role of p38α. *Am. J. Physiol. Lung Cell Mol. Physiol.* **279**, L733-L742.

Hartel, S., Gossrau, R., Hanski, C., and Reutter, W. (1988) Dipeptidyl peptidase (DPP) IV in rat organs. *Histochemistry* **89**, 151-161.

Hashikawa, T., Hooker, S.W., Maj, J.G., Knott-Craig, C.J., Takedachi, M., Murakami, S., and Thompson, L.F. (2004) Regulation of adenosine receptor engagement by ecto-adenosine deaminase. *FASEB J.* **18**, 131-133.

Haskó, G., Kuhel, D.G., Németh, Z.H., Mabley, J.G., Stachlewitz, R.F., Virág, L., Lohinai, Z., Southan, G.J., Salzman, A.L., and Szabó, C. (2000) Inosine inhibits inflammatory cytokine production by a posttranscriptional mechanism and protects against endotoxin-induced shock. *J Immunol.* **164**, 1013-1019.

Headrick, J.P. and Willis, R.J. (1989) 5′-Nucleotidase activity and adenosine formation in stimulated, hypoxic and underperfused rat heart. *Biochem. J.* **261**, 541-550.

Hegen, M., Niedobitek, G., Klein, C.E., Stein, H., and Fleischer, B. (1990) The T cell triggering molecule Tp103 is associated with dipeptidyl aminopeptidase IV activity. *J. Immunol.* **144**, 2908-2914.

Herbschleb-Voogt, E., Ten Kate, J., and Khan, P.M. (1983) Adenosine deaminase complexing protein (ADCP): a transformation sensitive protein with potentials of a cancer marker. *Anticancer Res.* **3**, 95-100.

Herrera, C., Casadó, V., Ciruela, F., Schofield, P., Mallol, J., Lluis, C., and Franco, R. (2001*a*) Adenosine A_{2B} receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. *Mol. Pharmacol.* **59**, 127-134.

Herrera, C., Morimoto, C., Blanco, J., Mallol, J., Arenzana, F., Lluis, C., and Franco, R. (2001*b*) Comodulation of CXCR4 and CD26 in human lymphocytes. *J. Biol. Chem.* **276**, 19532-19539.

Hicklin, D.J. and Ellis, L.M. (2005) Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J. Clin. Oncol.* **23**, 1011-1027.

Hirano, D., Aoki, Y., Ogasawara, H., Kodama, H., Waga, I., Sakanaka, C., Shimizu, T., and Nakamura, M. (1996) Functional coupling of adenosine A_{2a} receptor to inhibition of the mitogen-activated protein kinase cascade in Chinese hamster ovary cells. *Biochem. J.* **316**, 81-86.

Hixson, D.C., Allison, J.P., Chesner, J.E., Leger, M.J., Ridge, L.L., and Walborg, E.F. (1983) Characterization of a family of glycoproteins associated with the bile canalicular membrane of normal hepatocytes but not expressed by two transplantable rat hepatocellular carcinomas. *Cancer Res.* **43**, 3874-3884.

Hong, W. and Doyle, D. (1988) Membrane orientation of rat gp110 as studied by *in vitro* translation. *J. Biol. Chem.* **263**, 16892-16898.

Hong, W.J., Piazza, G.A., Hixson, D.C., and Doyle, D. (1989) Expression of enzymatically active rat dipeptidyl peptidase IV in Chinese hamster ovary cells after transfection. *Biochemistry* **28**, 8474-8479.

Höpfner, M., Lemmer, K., Jansen, A., Hanski, C., Rieken, E-O., Gavish, M., Mann, B., Buhr, H., Glassmeier, G., and Scherübl, H. (1998) Expression of functional P_2-purinergic receptors in primary cultures of human colorectal carcinoma cells. *Biochem. Biophys. Res. Commun.* **251**, 811-817.

Höpfner, M., Maaser, K., Barthel, B., von Lampe, B., Hanski, C., Riecken, E-O., Zeitz, M., and Scherübl, H. (2001) Growth inhibition and apoptosis induced by $P2Y_2$ receptors in human colorectal carcinoma cells: involvement of intracellular calcium and cyclic adenosine monophosphate. *Int. J. Colorectal Dis.* **16**, 154-166.

Hopsu-Havu, V.K. and Glenner, G.G. (1966) A new dipeptide naphthylamidase hydrolyzing glycyl-prolyl-beta-naphthylamide. *Histochemie* **7**, 197-201.

Hoskin, D.W., Reynolds, T., and Blay, J. (1994) Adenosine as a possible inhibitor of killer T-cell activation in the microenvironment of solid tumours. *Int. J. Cancer* **59**, 854-855.

Hoskin, D.W., Butler, J.J., Drapeau, D., Haeryfar, S.M.M., and Blay, J. (2002) Adenosine acts through an A_3 receptor to prevent the induction of murine anti-CD3-activated killer-T cells. *Int. J. Cancer* **99**, 386-395.

Houghton, A.N., Albino, A.P., Cordon-Cardo, C., Davis, L.J., and Eisinger, M. (1988) Cell surface antigens of human melanocytes and melanoma. Expression of adenosine deaminase binding protein is extinguished with melanocyte transformation. *J. Exp. Med.* **167**, 197-212.

Howell, S., Kenny, A.J., and Turner, A.J. (1992) A survey of membrane peptidases in two human colonic cell lines, Caco-2 and HT-29. *Biochem. J.* **284**, 595-601.

Huang, M., Wang, Y., Collins, M., Gu, J.J., Mitchell, B.S., and Graves, L.M. (2002) Inhibition of nucleoside transport by p38 MAPK inhibitors. *J. Biol. Chem.* **277**, 28364-28367.

Huang, M., Wang, Y., Cogut, S.B., Mitchell, B.S., and Graves, L.M. (2003) Inhibition of nucleoside transport by protein kinase inhibitors. *J. Pharmacol. Exp. Ther.* **304**, 753-760.

Hühn, J., Olek, S., Fleischer, B., and von Bonin, A. (1999) The adenosine deaminase-binding region is distinct from major anti-CD26 mAb epitopes on the human dipeptidyl peptidase IV (CD26) molecule. *Cell. Immunol.* **192**, 33-40.

Hunter, T. (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* **80**, 225-236.

Hynes, R.O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.

Idzko, M., Panther, E., Bremer, H.C., Windisch, W., Sorichter, S., Herouy, Y., Elsner, P., Mockenhaupt, M., Girolomoni, G., and Norgauer, J. (2004) Inosine stimulates chemotaxis, Ca^{2+}-transients and actin polymerization in immature human dendritic cells via a pertussis toxin-sensitive mechanism independent of adenosine receptors. *J. Cell. Physiol.* **199**, 149-156.

Imai, K., Kanzaki, H., Fujiwara, H., Maeda, M., Ueda, M., Suginami, H., and Mori, T. (1994) Expression and localization of aminopeptidase N, neutral endopeptidase, and dipeptidyl peptidase IV in the human placenta and fetal membranes. *Am. J. Obstet. Gynecol.* **170**, 1163-1168.

Ishii, K. and Green, H. (1973) Lethality of adenosine for cultured mammalian cells by interference with pyrimidine biosynthesis. *J. Cell Sci.* **13**, 429-439.

Ishii, T., Ohnuma, K., Murakami, A., Takasawa, N., Kobayashi, S., Dang, N.H., Schlossman, S.F., and Morimoto, C. (2001) CD26-mediated signaling for T cell activation occurs in lipid rafts through its association with CD45RO. *Proc. Natl. Acad. Sci. USA* **98**, 12138-12143.

Jacobson, K.A. (1998) Adenosine A_3 receptors: novel ligands and paradoxical effects. *Trends Pharmacol. Sci.* **19**, 184-191.

Jarvis, S.M., Williams, T.C., Doherty, A.J., and Griffith, D.A. (1991) Sodium-dependent nucleoside transport in epithelial cells. *In*: Role of Adenosine and Adenine Nucleotides in the Biological System. Edited by Imai, S. and Nakazawa, M. Amsterdam, Elsevier Science Publishers BV, Ch. 14, pp. 151-160.

Jiang, Y., Chen, C., Li, Z., Guo, W., Gegner, J.A., Lin, S., and Han, J. (1996) Characterization of the structure and function of a new mitogen-activated protein kinase (p38β). *J. Biol. Chem.* **271**, 17920-17926.

Jin, X., Shepherd, R.K., Duling, B.R., and Linden, J. (1997) Inosine binds to A_3 adenosine receptors and stimulates mast cell degranulation. *J. Clin. Invest.* **100**, 2849-2857.

Johnson, R.A., Yeung, S-M.H., Bushfield, M., Stubner, D., and Shoshani, I. (1991) "P"-site-mediated inhibition of adenylyl cyclase and its physiological implications. *In*: Role of Adenosine and Adenine Nucleotides in the Biological System. Edited by Imai, S. and Nakazawa, M. Amsterdam, Elsevier Science Publishers BV, Ch. 5, pp. 43-55.

Jordan, N.J., Kolios, G., Abbot, S.E., Sinai, M.A., Thompson, D.A., Petraki, K., and Westwick, J. (1999) Expression of functional CXCR4 chemokine receptors on human colonic epithelial cells. *J. Clin. Invest.* **104**, 1061-1069.

June, C.H., Fletcher, M.C., Ledbetter, J.A., Schieven, G.L., Siegel, J.N., Phillips, A.F., and Samelson, L.E. (1990) Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. *Proc. Natl. Acad. Sci. USA.* **87**, 7722-7726.

Kajiyama, H., Kikkawa, F., Maeda, O., Suzuki, T., Ino, K., and Mizutani, S. (2002*a*) Increased expression of dipeptidyl peptidase IV in human mesothelial cells by malignant ascites from ovarian carcinoma patients. *Oncology* **63**, 158-165.

Kajiyama, H., Kikkawa, F., Suzuki, T., Shibata, K., Ino, K., and Mizuani, S. (2002*b*) Prolonged survival and decreased invasive activity attributable to dipeptidyl peptidase IV overexpression in ovarian carcinoma. *Cancer Res.* **62**, 2753-2757.

Kajiyama, H., Kikkawa, F., Khin, E., Shibata, K., Ino, K., and Mizutani, S. (2003) Dipeptidyl peptidase IV overexpression induces up-regulation of E-cadherin and tissue inhibitors of matrix metalloproteinases, resulting in decreased invasive potential in ovarian carcinoma cells. *Cancer Res.* **63**, 2278-2283.

Kameoka, J., Tanaka, T., Nojima, Y., Schlossman, S.F., and Morimoto, C. (1993) Direct association of adenosine deaminase with a T cell activation antigen, CD26. *Science* **261**, 466-469.

Kanai, T., Watanabe, M., Hayashi, A., Nakazawa, A., Yajima, T., Okzawa, A., Yamazaki, M., Ishii, H., and Hibi, T. (2000) Regulatory effect of interleukin-4 and interleukin-13 on colon cancer cell adhesion. *Br. J. Cancer* **82**, 1717-1723.

Kato, H., Semba, S., Miskad, U.A., Seo, Y., Kasuga, M., and Yokozaki, H. (2004) High expression of *PRL-3* promotes cancer cell motility and liver metastasis in human colorectal cancer: a predictive molecular marker or metachronous liver and lung metastasis. *Clin. Cancer Res.* **10**, 7318-7328.

Kehlen, A., Göhring, B., Langner, J., and Riemann, D. (1998) Regulation of the expression of aminopeptidase A, aminopeptidase N/CD13 and dipeptidylpeptidase IV/CD26 in renal carcinoma cells and renal tubular epithelial cells by cytokines and cAMP-increasing mediators. *Clin. Exp. Immunol.* **111**, 435-441.

Kemperman, H., Wijnands, Y.M., and Roos, E. (1997) αV integrins on HT-29 colon carcinoma cells: adhesion to fibronectin is mediated solely by small amounts of αVβ6, and αVβ5 is codistributed with actin fibers. *Exp. Cell Res.* **234**, 156-164.

Khin, E.E., Kikkawa, F., Ino, K., Kajiyama, H., Suzuki, T., Shibata, K., Tamakoshi, K., Nagasaka, T., and Mizutani, S. (2003) Dipeptdiyl peptidase IV expression in endometrial endometrioid adenocarcinoma and its inverse correlation with tumor grade. *Am. J. Obstet. Gynecol.* **188**, 670-676.

Khoo, H.E., Ho, C.L., Chhatwal, V.J., Chan, S.T., Ngoi, S.S., and Moochhala, S.M. (1996) Differential expression of adenosine A_1 receptors in colorectal cancer and related mucosa. *Cancer Lett.* **106**, 17-21.

Kikkawa, F., Kajiyama, H., Ino, K., Shibata, K., and Mizutani, S. (2003) Increased adhesion potency of ovarian carcinoma cells to mesothelial cells by overexpression of dipeptidyl peptidase IV. *Int. J. Cancer* **105**, 779-783.

Klinger, M., Freissmuth, M., and Nanoff, C. (2002) Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. *Cell. Signal.* **14**, 99-108.

Klotz, K-N. (2000) Adenosine receptors and their ligands. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **362**, 382-391.

Kobayashi, E., Nakano, H., Morimoto, M., and Tamaoki, T. (1989) Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of phospholipid/Ca2+ dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135**, 397-402.

Kobayashi, S., Zimmermann, H., and Millhorn, D.E. (2000) Chronic hypoxia enhances adenosine release in rat PC12 cells by altering adenosine metabolism and membrane transport. *J. Neurochem.* **74**, 621-632.

Koshiba, M., Kojima, H., Huang, S., Apasov, S., and Sitkovsky, M.V. (1997) Memory of extracellular adenosine A_{2A} purinergic receptor-mediated signaling in murine T cells. *J. Biol. Chem.* **272**, 25881-25889.

Kotani, T., Aratake, Y., Ogata, Y., Umeki, K., Araki, Y., Hirai, A., Kuma, K., and Ohtaki, S. (1991) Expression of dipeptidyl aminopeptidase IV activity in thyroid carcinoma. *Cancer Lett.* **57**, 203-208.

Kreisel, W., Volk, B.A., Buchsel, R., and Reutter, W. (1980) Different half-lives of the carbohydrate and protein moieties of a 110,000-dalton glycoprotein isolated from plasma membranes of rat liver. *Proc. Natl. Acad. Sci. USA* **77**, 1828-1831.

Krejsa, C.M., Nadler, S.G., Esselstyn, J.M., Kavanagh, T.J., Ledbetter, J.A., and Schieven, G.L. (1997) Role of oxidative stress in the action of vanadium phosphotyrosine phosphatase inhibitors. Redox independent activation of NF-κB. *J. Biol. Chem.* **272**, 11541-11549.

Laderoute, K.R., Mendonca, H.L., Calaoagan, J.M., Knapp, A.M., Giaccia, A.J., and Stork, P.J.S. (1999) Mitogen-activated protein kinase phosphatase-1 (MKP-1) expression is induced by low oxygen conditions found in solid tumor microenvironments. *J. Biol. Chem.* **274**, 12890-12897.

Lai, H.L., Yang, T.H., Messing, R.O., Ching, Y.H., Lin, S.C., and Chern, Y. (1997) Protein kinase C inhibits adenylyl cyclase type VI activity during desensitization of the A2a-adenosine receptor-mediated cAMP response. *J. Biol. Chem.* **272**, 4970-4977.

Lambeir, A-M., Diaz Pereira, J.F., Chacon, P., Vermeulen, G., Heremans, K., Devreese, B., Van Beeumen, J., De Meester, I., and Scharpé, S. (1997) A prediction of DPP IV/CD26 domain structure from a physico-chemical investigation of dipeptidyl peptidase IV (CD26) from human seminal plasma. *Biochim. Biophys. Acta.* **1340**, 215-226.

Lambeir, A-M., Proost, P., Durinx, C., Bal, G., Senten, K., Augustyns, K., Scharpé, S., Van Damme, J., and De Meester, I. (2001) Kinetic investigation of chemokine truncation by CD26/dipeptidyl peptidase IV reveals a striking selectivity within the chemokine family. *J. Biol. Chem.* **276**, 29839-29845.

Lambeir, A-M., Proost, P., Scharpé, S., and De Meester, I. (2002) A kinetic study of glucagon-like peptide-1 and glucagon-like peptide-2 truncation by dipeptidyl peptidase IV, *in vitro*. *Biochem. Pharmacol.* **64**, 1753-1756.

Latini, S., Bordoni, F., Pedata, F., and Corradetti, R. (1999) Extracellular adenosine concentrations during *in vitro* ischaemia in rat hippocampal slices. *Br. J. Pharmacol.* **127**, 729-739.

Lavoie, C., Chevet, E., Roy, L., Tonks, N.K., Fazel, A., Posner, B.I., Paiement, J., and Bergeron, J.J.M. (2000) Tyrosine phosphorylation of p97 regulates transitional endoplasmic reticulum assembly *in vitro*. *Proc. Natl. Acad. Sci. USA* **97**, 13637-13642.

Leaney, J.L. and Tinker, A. (2000) The role of members of the pertussis toxin-sensitive family of G proteins in coupling receptors to the activation of the G protein-gated inwardly rectifying potassium channel. *Proc. Natl. Acad. Sci. USA* **97**, 5651-5656.

Legrand, A.B., Narayanan, T.K., Ryan, U.S., Aronstam, R.S., and Catravas, J.D. (1990) Effects of adenosine and analogs on adenylate cyclase activity in cultured bovine aortic endothelial cells. *Biochem. Pharmacol.* **40**, 1103-1109.

Leith, J.T., Padfield, G., Faulkner, L., and Michelson, S. (1991) Hypoxic fractions in xenografted human colon tumors. *Cancer Res.* **51**, 5139-5143.

Lelièvre, V., Muller, J-M., and Falcón, J. (1998*a*) Adenosine modulates cell proliferation in human colonic adenocarcinoma. I. Possible involvement of adenosine A_1 receptor subtypes in HT29 cells. *Eur. J. Pharmacol.* **341**, 289-297.

Lelièvre, V., Muller, J-M., and Falcón, J. (1998*b*) Adenosine modulates cell proliferation in human colonic carcinoma. II. Differential behavior of HT29, DLD-1, Caco-2 and SW403 cell lines. *Eur. J. Pharmacol.* **341**, 299-308.

Lelièvre, V., Caigneaux, E., Muller, J-M., and Falcón, J. (2000) Extracellular adenosine deprivation induces epithelial differentiation of HT29 cells: evidence for a concomitant adenosine A_1/A_2 receptor balance regulation. *Eur. J. Pharmacol.* **391**, 21-29.

Liu, Y., Cox, S.R., Morita, T., and Kourembanas, S. (1995) Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5′ enhancer. *Circ. Res.* **77**, 638-643.

Liu, Q., and Hofmann, P.A. (2002) Antiadrenergic effects of adenosine A_1 receptor-mediated protein phosphatase 2a activation in the heart. *Am. J. Physiol. Heart Circ. Physiol.* **283**, H1314-H1321.

Liu, Q., and Hoffmann, P.A. (2003) Modulation of protein phosphatase 2a by adenosine A_1 receptors in cardiomyocytes: role for p38 MAPK. *Am. J. Physiol. Heart Circ. Physiol.* **285**, H97-H103.

Lloyd, H.G. and Fredholm, B.B. (1995) Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. *Neurochem. Int.* **26**, 387-395.

Londos, C., Cooper, D.M.F., and Wolff, J. (1980) Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. USA* **77**, 2551-2554.

Löster, K., Zeilinger, K., Schuppan, D., and Reutter, W. (1995) The cysteine-rich region of dipeptidyl peptidase IV (CD26) is the collagen-binding site. *Biochem. Biophys. Res. Commun.* **217**, 341-348.

Lu, J., Pierron, A., and Ravid, K. (2003) An adenosine analogue, IB-MECA, down-regulates estrogen receptor α and suppresses human breast cancer cell proliferation. *Cancer Res.* **63**, 6413-6423.

Luo, J., Lubaroff, D.M., and Hendrix, M.J.C. (1999) Suppression of prostate cancer invasive potential and matrix metalloproteinase activity by E-cadherin transfection. *Cancer Res.* **59**, 3552-3556.

MacKenzie, W.M., Hoskin, D.W., and Blay, J. (1994) Adenosine inhibits the adhesion of anti-CD3-activated killer lymphocytes to adenocarcinoma cells through an A_3 receptor. *Cancer Res.* **54**, 3521-3526.

MacKenzie, W.M., Hoskin, D.W., and Blay, J. (2002) Adenosine suppresses $\alpha_4\beta_7$ integrin-mediated adhesion of T lymphocytes to colon adenocarcinoma cells. *Exp. Cell Res.* **276**, 90-100.

Madi, L., Bar-Yehuda, S., Barer, F., Ardon, E., Ochaion, A., and Fishman, P. (2003) A3 adenosine receptor activation in melanoma cells. Association between receptor fate and tumor growth inhibition. *J. Biol. Chem.* **278**, 42121-42130.

Madi, L., Ochaion, A., Rath-Wolfson, L., Bar-Yehuda, S., Erlanger, A., Ohana, G., Harish, A., Merimski, O., Barer, F., and Fishman, P. (2004) The A_3 adenosine receptor is highly expressed in tumor versus normal cells: potential target for tumor growth inhibition. *Clin. Cancer Res.* **10**, 4472-4479.

Marguet, D., Bernard, A-M., Vivier, I., Darmoul, D., Naquet, P., and Pierres, M. (1992) cDNA cloning for mouse thymocyte-activating molecule. A multifunctional ecto-dipeptidyl peptidase IV (CD26) included in a subgroup of serine proteases. *J. Biol. Chem.* **267**, 2200-2208.

Marguet, D., Baggio, L., Kobayashi, T., Bernard, A-M., Pierres, M., Nielsen, P.F., Ribel, U., Watanabe, T., Drucker, D.J., and Wagtmann, N. (2000) Enhanced insulin secretion and improved glucose tolerance in mice lacking CD26. *Proc. Natl. Acad. Sci. USA* **97**, 6874-6879.

Martín, M., Huguet, J., Centelles, J.J., and Franco, R. (1995) Expression of ecto-adenosine deaminase and CD26 in human T cells triggered by the TCR-CD3 complex: possible role of adenosine deaminase as costimulatory molecule. *J. Immunol.* **155**, 4630-4643.

Marton, A., Pacher, P., Murthy, K.G., Nemeth, Z.H., Haskó, G., and Szabó, C. (2001) Anti-inflammatory effects of inosine in human monocytes, neutrophils and epithelial cells *in vitro*. *Int. J. Mol. Med.* **8**, 617-621.

Matzinger, P. (1991) The JAM test. A simple assay for DNA fragmentation and cell death. *J. Immunol. Methods* **145**, 185-192.

Merighi, S., Varani, K., Gessi, S., Cattabriga, E., Iannotta, V., Ulouglu, C., Leung, E., and Borea, P.A. (2001) Pharmacological and biochemical characterization of adenosine receptors in the human malignant melanoma A375 cell line. *Br. J. Pharmacol.* **134**, 1215-1226.

Merighi, S., Mirandola, P., Milani, D., Varani, K., Gessi, S., Klotz, K-N., Leung, E., Baraldi, P.G., and Borea, P.A. (2002) Adenosine receptors as mediators of both cell proliferation and cell death of cultured human melanoma cells. *J. Invest. Dermatol.* **119**, 923-933.

Merighi, S., Benini, A., Mirandola, P., Gessi, S., Varani, K., Leung, E., Maclennan, S., and Borea, P.A. (2005) A_3 adenosine receptor activation inhibits cell proliferation via phosphatidylinositol 3-kinase (PI3K)/Akt-dependent inhibition of the extracellular signal-regulated kinase 1/2 phosphorylation in A375 human melanoma cells. *J. Biol. Chem.* **280**, 19516-19526.

Middlemiss, P.J., Gysbers, J.W., and Rathbone, M.P. (1995) Extracellular guanosine and guanosine-5′-triphosphate increase NGF synthesis and release from cultured mouse neopallial astrocytes. *Brain Res.* **677**, 152-156.

Mizokami, Y., Kajiyama, H., Shibata, K., Ino, K., Kikkawa, F., and Mizutani, S. (2004) Stromal cell-derived factor-1α-induced cell proliferation and its possible regulation by CD26/dipeptidyl peptidase IV in endometrial carcinoma. *Int. J. Cancer* **110**, 652-659.

Morimoto, C. and Schlossman, S. (1998) The structure and function of CD26 in the T-cell immune response. *Immunol. Rev.* **61**, 55-70.

Morrison, M.E., Vijayasaradhi, S., Engelstein, D., Albino, A.P., and Houghton, A.N. (1993) A marker for neoplastic progression of human melanocytes is a cell surface ectopeptidase. *J. Exp. Med.* **177**, 1135-1143.

Muda, M., Boschert, U., Dickinson, R., Martinou, J-C., Martinou, I., Camps, M., Schlegel, W., and Arkinstall, S. (1996) MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase. *J. Biol. Chem.* **271**, 4319-4326.

Mujoomdar, M., Hoskin, D., and Blay, J. (2003) Adenosine stimulation of the proliferation of colorectal carcinoma cell lines. Roles of cell density and adenosine metabolism. *Biochem. Pharmacol.* **66**, 1737-1747.

Mujoomdar, M., Bennett, A., Hoskin, D., and Blay, J. (2004) Adenosine stimulation of proliferation of breast carcinoma cell lines: evaluation of the [^{3}H]thymidine assay system and modulatory effects of the cellular microenvironment in vitro. *J. Cell. Physiol.* **201**, 429-438.

Müller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M.E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S.N., Barrera, J.L., Mohar, A., Verástegui, E., and Zlotnik, A. (2001) Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410**, 50-56.

Mun, E.C., Tally, K.J., and Matthews, J.B. (1998) Characterization and regulation of adenosine transport in T84 intestinal epithelial cells. *Am. J. Physiol.* **274**, G261-G269.

Munoz, J.J., Tarrega, C., Blanco-Aparicio, C., and Pulido, R. (2003) Differential interaction of the tyrosine phosphatases PTP-SL, STEP and HePTP with the mitogen-activated protein kinases ERK1/2 and p38α is determined by a kinase specificity sequence and influenced by reducing agents. *Biochem. J.* **372**, 193-201.

Murakami, T., Maki, W., Cardones, A.R., Fang, H., Kyi, A.T., Nestle, F.O., and Hwang, S.T. (2002) Expression of CXC chemokine receptor-4 enhances the pulmonary metastatic potential of murine B16 melanoma cells. *Cancer Res.* **62**, 7328-7334.

Murphy, K., Gerzanich, V., Zhou, H., Ivanova, S., Dong, Y., Hoffman, G., West, G.A., Winn, H.R., and Simard, J.M. (2003) Adenosine-A2a receptor down-regulates cerebral smooth muscle L-type Ca^{2+} channel activity via protein tyrosine phosphatase, not cAMP-dependent protein kinase. *Mol. Pharmacol.* **64**, 640-649.

Nakanishi, S., Catt, K.J., and Balla, T. (1995) A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids. *Proc. Natl. Acad. Sci. USA.* **92**, 5317-5321.

Narayan, P., Mentzer, R.M., and Lasley, R.D. (2000) Phosphatase inhibitor cantharidin blocks adenosine A_1 receptor anti-adrenergic effect in rat cardiac myocytes. *Am. J. Physiol. Heart Circ. Physiol.* **278**, H1-H7.

Narravula, S., Lennon, P.F., Mueller, B.U., and Colgan, S.P. (2000) Regulation of endothelial CD73 by adenosine: paracrine pathway for enhanced endothelial barrier function. *J. Immunol.* **165**, 5262-5268.

Nemoto, E., Sugawara, S., Takada, H., Shoji, S., and Horiuch, H. (1999) Increase of CD26/dipeptidyl peptidase IV expression on human gingival fibroblasts upon stimulation with cytokines and bacterial components. *Infect. Immun.* **67**, 6225-6233.

Neumann, J., Vahlensieck, U., Boknik, P., Linck, B., Luss, H., Muller, F.U., Matherne, G.P., and Schmitz, W. (1999) Functional studies in atrium overexpressing A_1-adenosine receptors. *Br. J. Pharmacol.* **128**, 1623-1629.

Nishihara, H., Ishikawa, S., Shinkai, K., and Akedo, H. (1973) Multiple forms of human adenosine deaminase. II. Isolation and properties of a conversion factor from human lung. *Biochim. Biophys. Acta.* **301**: 429-442.

Ohana, G., Bar-Yehuda, S., Arich, A., Madi, L., Dreznick, Z., Rath-Wolfson, L., Silberman, D., Slosman, G., and Fishman, P. (2003) Inhibition of primary colon carcinoma growth and liver metastasis by the A3 adenosine receptor agonist CF101. *Br. J. Cancer* **89**, 1552-1558.

Olah, M.E. and Roudabush, F.L. (2000) Down-regulation of vascular endothelial growth factor expression after A_{2A} adenosine receptor activation in PC12 pheochromocytoma cells. *J. Pharmacol. Exp. Ther.* **293**, 779-787.

Oravecz, T., Pall, M., Roderiquez, G., Gorrell, M.D., Ditto, M., Nguyen, N.Y., Boykins, R., Unsworth, E., and Norcross, M.A. (1997) Regulation of the receptor specificity and function of the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) by dipeptidyl peptidase IV (CD26)-mediated cleavage. *J. Exp. Med.* **186**, 1865-1872.

Pala, L., Mannucci, E., Pezzatini, A., Ciani, S., Sardi, J., Raimondi, L., Ognibene, A., Cappadona, A., Vannelli, B.G., and Rotella, C.M. (2003) Dipeptidyl peptidase-IV expression and activity in human glomerular endothelial cells. *Biochem. Biophys. Res. Commun.* **310**, 28-31.

Parsons, M., Young, L., Lee, J.E., Jacobson, K.A., and Liang, B.T. (2000) Distinct cardioprotective effects of adenosine mediated by differential coupling of receptor subtypes to phospholipases C and D. *FASEB J.* **14**, 1423-1431.

Pathak, M.K. and Yi, T. (2001) Sodium stibogluconate is a potent inhibitor of protein tyrosine phosphatases and augments cytokine responses in hemopoietic cell lines. *J. Immunol.* **167**, 3391-3397.

Pennycooke, M., Chaudary, N., Shuralyova, I., Zhang, Y., and Coe, I.R. (2001) Differential expression of human nucleoside transporters in normal and tumor tissue. *Biochem. Biophys. Res. Commun.* **280**, 951-959.

Pethiyagoda, C.L., Welch, D.R., Fleming, T.P. (2001) Dipeptidyl peptidase IV (DPPIV) inhibits cellular invasion of melanoma cells. *Clin. Exp. Metastasis* **18**, 391-400.

Pettiford, S.M. and Herbst, R. (2000) The MAP-kinase ERK2 is a specific substrate of the protein tyrosine phosphatase HePTP. *Oncogene* **19**, 858-869.

Piazza, G.A., Callanan, H.M., Mowery, J., and Hixon, D.C. (1989) Evidence for a role of dipeptidyl peptidase IV in fibronectin-mediated interactions of hepatocytes with extracellular matrix. *Biochem. J.* **262**, 327-334.

Pierce, K.D., Furlong, T.J., Selbie, L.A., and Shine, J. (1992) Molecular cloning and expression of an adenosine A_{2b} receptor from human brain. *Biochem. Biophys. Res. Commun.* **187**, 86-93.

Plate, K.H., Breier, G., Weich, H.A., and Risau, W. (1992) Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. *Nature* **359**, 845-848.

Posner, B.I., Faure, R., Burgess, J.W., Bevan, A.P., Lachance, D., Zhang-Sun, G., Fantus, I.G., Ng, J.B., Hall, D.A., Lum, B.S., and Shaver, A. (1994) Peroxovanadium compounds. A new class of potent phosphotyrosine phosphatase inhibitors which are insulin mimetics. *J. Biol. Chem.* **269**, 4596-4604.

Proost, P., De Meester, I., Schols, D., Struyf, S., Lambeir, A-M., Wuyts, A., Opdenakker, G., De Clercq, E., Scharpé, S., and Van Damme, J. (1998) Amino-terminal truncation of chemokines by CD26/dipeptidyl-peptidase IV. *J. Biol. Chem.* **273**, 7222-7227.

Proost, P., Menten, P., Struyf, S., Schutyser, E., De Meester, I., and Van Damme, J. (2000) Cleavage by CD26/dipeptidyl peptidase IV converts the chemokine LD78β into a most efficient monocyte attractant and CCR1 agonist. *Blood* **96**, 1674-1680.

Pulido, R., Zuniga, A., and Ullrich, A. (1998) PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif. *EMBO J.* **17**, 7337-7350.

Rao, B.R., Slotman, B.J., Geldof, A.A., and Dinjens, W.N. (1990) Correlation between tumor histology, steroid receptor status, and adenosine deaminase complexing protein immunoreactivity in ovarian cancer. *Int. J. Gynecol. Pathol.* **9**, 47-54.

Rego, A.C., Santos, M.S., and Oliveira, C.R. (1997) Adenosine triphosphate degradation products after oxidative stress and metabolic dysfunction in cultured retinal cells. *J. Neurochem.* **69**, 1228-1235.

Reimer, M.K., Holst, J.J., and Ahrén, B. (2002) Long-term inhibition of dipeptidyl peptidase IV improves glucose tolerance and preserves islet function in mice. *Eur. J. Endocrinol.* **146**, 717-727.

Reinhold, D., Vetter, R.W., Mnich, K., Bühling, F., Lendeckel, U., Born, I., Faust, J., Neubert, K., Gollnick, H., and Ansorge, S. (1998) Dipeptidyl peptidase IV (DP IV, CD26) is involved in regulation of DNA synthesis in human keratinocytes. *FEBS Lett.* **428**, 100-104.

Riemann, D., Kehlen, A., and Langner, J. (1995) Stimulation of the expression and the enzyme activity of aminopeptidase N/CD13 and dipeptidyl peptidase IV/CD26 on human renal cell carcinoma cells and renal tubular epithelial cells by T cell-derived cytokines, such as IL-4 and IL-13. *Clin. Exp. Immunol.* **100**, 277-283.

Riemann, D., Hansen, G.H., Niels-Christiansen, L-L., Thorsen, E., Immerdal, L., Santos, A.N., Kehlen, A., Langner, J., and Danielsen, E.M. (2001) Caveolae/lipid rafts in fibroblast-like synoviocytes: ectopeptidase-rich membrane microdomains. *Biochem. J.* **354**, 47-55.

Rogel, A., Bromberg, Y., Sperling, O., and Zoref-Shani, E. (2005) Phospholipase C is involved in the adenosine-activated signal transduction pathway conferring protection against iodoacetic acid-induced injury in primary rat neuronal cultures. *Neurosci. Lett.* **373**, 218-221.

Rothermel, J.D., Jastorff, B., and Botelho, L.H. (1984) Inhibition of glucagon-induced glycogenolysis in isolated rat hepatocytes by the Rp diastereomer of adenosine cyclic 3′,5′-phosphorothioate. *J. Biol. Chem.* **259**, 8151-8155.

Rounds, S., Yee, W.L., Dawicki, D.D., Harrington, E., Parks, N., and Cutaia, M.V. (1998) Mechanism of extracellular ATP- and adenosine-induced apoptosis of cultured pulmonary artery endothelial cells. *Am. J. Physiol.* **275**, L379-L388.

Roy, J., Audette, M., and Tremblay, M.J. (2001) Intercellular adhesion molecule-1 (*ICAM-1*) gene expression in human T cells is regulated by phosphotyrosyl phosphatase activity. Involvement of NF-κB, Ets, and palindromic interferon-γ-responsive element-binding sites. *J. Biol. Chem.* **276**, 14553-14561.

Saitoh, M., Nagai, K., Nakagawa, K., Yamamura, T., Yamamoto, S., and Nishizaki, T. (2004) Adenosine induces apoptosis in the human gastric cancer cells via an intrinsic pathway relevant to activation of AMP-activated protein kinase. *Biochem. Pharmacol.* **67**, 2005-2011.

Salvatore, C.A., Jacobson, M.A., Taylor, H.E., Linden, J., and Johnson, R.G. (1993) Molecular cloning and characterization of the human A_3 adenosine receptor. *Proc. Natl. Acad. Sci. USA* **90**, 10365-10369.

Sarret, P., Gendron, L., Kilian, P., Nguyen, H.M., Gallo-Payet, N., Payet, M.D., and Beaudet, A. (2002) Pharmacology and functional properties of NTS2 neurotensin receptors in cerebellar granule cells. *J. Biol. Chem.* **277**, 36233-36243.

Sato, Y., Fujiwara, H., Higuchi, T., Yoshioka, S., Tatsumi, K., Maeda, M., and Fujii, S. (2002) Involvement of dipeptidyl peptidase IV in extravillous trophoblast invasion and differentiation. *J. Clin. Endocrinol. Metab.* **87**, 4287-4296.

Saura, C.A., Mallol, J., Canela, E.I., Lluis, C., and Franco, R. (1998) Adenosine deaminase and A_1 receptors internalize together following agonist-induced receptor desensitization. *J. Biol. Chem.* **273**, 17610-17617.

Schmid, A.C., Byrne, R.D., Vilar, R., Woscholski, R. (2004) Bisperoxovanadium compounds are potent PTEN inhibitors. *FEBS Lett.* **566**, 35-38.

Schrader, W.P., Stacy, A.R., and Pollara, B. (1976) Purification of human erythrocyte adenosine deaminase by affinity column chromatography. *J. Biol. Chem.* **251**, 4026-4032.

Schrader, W.P. and Pollara, B. (1978) Localization of an adenosine deaminase-binding protein in human kidney. *J. Lab. Clin. Med.* **92**, 656-662.

Schrader, W.P. and Stacy, A.R. (1979) Immunoassay of the adenosine deaminase complexing proteins of human tissues and body fluids. *J. Biol. Chem.* **254**, 11958-11963.

Schrader, W.P. and Bryer, P.J. (1982) Characterization of an insoluble adenosine deaminase complexing protein from human kidney. *Arch. Biochem. Biophys.* **215**, 107-115.

Schrader, W.P., Harder, C.M., and Schrader, D.K. (1983) Adenosine deaminase complexing protein of the rabbit. *Comp. Biochem. Physiol.* **75**, 119-126.

Schrader, W.P., Harder, C.M., Schrader, D.K., and West, C.A. (1984) Metabolism of different molecular forms of adenosine deaminase intravenously infused into the rabbit. *Arch. Biochem. Biophys.* **230**, 158-167.

Schrader, W.P. and West, C.A. (1985) Adenosine deaminase complexing proteins are localized in exocrine glands of the rabbit. *J. Histochem. Cytochem.* **33**, 508-514.

Schrader, W.P., Miczek, A.D., West, C.A., and Samsonoff, W.A. (1988) Evidence for receptor-mediated uptake of adenosine deaminase in rabbit kidney. *J. Histochem. Cytochem.* **36**, 1481-1487.

Schrader, W.P., West, C.A., Miczek, A.D., and Norton, E.K. (1990) Characterization of the adenosine deaminase-adenosine deaminase complexing protein binding reaction. *J. Biol. Chem.* **265**, 19312-19318.

Schulte, G. and Fredholm, B.B. (2000) Human adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors expressed in Chinese human ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. *Mol. Pharmacol.* **58**, 477-482.

Schulte, G. and Fredholm, B.B. (2002) Signaling pathway from the human adenosine A_3 receptor expressed in Chinese hamster ovary cells to the extracellular signal-regulated kinase 1/2. *Mol. Pharmacol.* **62**, 1137-1146.

Schulte, G. and Fredholm, B.B. (2003*a*) The G_S-coupled adenosine A_{2B} receptor recruits divergent pathways to regulate ERK1/2 and p38. *Exp. Cell Res.* **290**, 168-176.

Schulte, G., and Fredholm, B.B. (2003*b*) Signalling from adenosine receptors to mitogen-activated protein kinases. *Cell. Signal.* **15**, 813-827.

Schrier, S.M., van Tilburg, E.W., van der Meulen, H., Ijzerman, A.P., Mulder, G.J., and Nagelkerke, J.F. (2001) Extracellular adenosine-induced apoptosis in mouse neuroblastoma cells. Studies on involvement of adenosine receptors and adenosine uptake. *Biochem. Pharmacol.* **61**, 417-425.

Scrivens, P.J., Alaoui-Jamali, M.A., Giannini, G., Wang, T., Loignon, M., Batist, G., and Sandor, V.A. (2003) Cdc25A-inhibitory properties and antineoplastic activity of bisperoxovanadium analogues. *Mol. Cancer Ther.* **2**, 1053-1059.

Seargeant, L.E. and Stinson, R.A. (1979) Inhibition of human alkaline phosphatases by vanadate. *Biochem. J.* **181**, 247-250.

Seidel, M.G., Klinger, M., Freissmuth, M., and Höller, C. (1999) Activation of mitogen-activated protein kinase by the A_{2A}-adenosine receptor via a rap1-dependent and via a $p21^{ras}$-dependent pathway. *J. Biol. Chem.* **274**, 25833-25841.

Senesi, S., Freer, G., Gasperini, M., Batoni, G., and Campa, M. (1988) Complete release of adenosine deaminase from mouse lymphocytes stabilized by low-pH acetate. *Biochim. Biophys. Acta.* **968**, 59-68.

Sexl, V., Mancusi, G., Baumgartner-Parzer, S., Schutz, W., and Freissmuth, M. (1995) Stimulation of human umbilical vein endothelial cell proliferation by A_2-adenosine and β_2-adrenoceptors. *Br. J. Pharmacol.* **114**, 1577-1586.

Sexl, V., Mancus, G., Höller, C., Gloria-Maercker, E., Schütz, W., and Freissmuth, M. (1997) Stimulation of the mitogen-activated protein kinase via the A_{2A}-adenosine receptor in primary human endothelial cells. *J. Biol. Chem.* **272**, 5792-5799.

Shearer, T.W. and Crosson, C.E. (2002) Adenosine A_1 receptor modulation of MMP-2 secretion by trabecular meshwork cells. *Invest. Ophthalmol. Vis. Sci.* **43**, 3016-3020.

Shi, Z.Q., Lu, W., and Feng, G.S. (1998) The Shp-2 tyrosine phosphatase has opposite effects in mediating the activation of extracellular signal-regulated and c-Jun NH_2-terminal mitogen-activated protein kinases. *J. Biol. Chem.* **273**, 4904-4908.

Shioda, T., Kato, H., Ohnishi, Y., Tashiro, K., Ikegawa, M., Nakayama, E.E., Hu, H., Kato, A., Sakai, Y., Liu, H., Honjo, T., Nomoto, A., Iwamoto, A., Morimoto, C., and Nagai, Y. (1998) Anti-HIV-1 and chemotactic activities of human stromal cell-derived factor 1α (SDF-1α) and SDF-1β are abolished by CD26/dipeptidyl peptidase IV-mediated cleavage. *Proc. Natl. Acad. Sci. USA* **95**, 6331-6336.

Singer-Sam, J., Robinson, M.O., Bellvè, A.R., Simon, M.I., and Riggs, A.P. (1990) Measurement by quantitative PCR of changes in HRPT, PGK-1, PGK-2, APRT, Mtase and Zyf gene transcripts during mouse spermatogenesis. *Nucleic Acids Res.* **18**, 1255-1259.

Spychala, J., Datta, N.S., Takabayashi, K., Datta, M., Fox, I.H., Gribbin, T., and Mitchell, B.S. (1996) Cloning of human adenosine kinase cDNA: sequence similarity to microbial ribokinases and fructokinases. *Proc. Natl. Acad. Sci. USA* **93**, 1232-1237.

Spychala, J., Mitchell, B.S., and Barankiewicz, J. (1997) Adenosine metabolism during phorbol myristate acetate-mediated induction of HL-60 cell differentiation. Changes in expression patter of adenosine kinase, adenosine deaminase, and 5′-nucleotidase. *J. Immunol.* **158**, 4947-4952.

Spychala, J. (2000) Tumor-promoting functions of adenosine. *Pharmacol. Therapeutics* **87**, 161-173.

Spychala, J. and Kitajewski, J. (2004) Wnt and β-catenin signaling target the expression of ecto-5′-nucleotidase and increase extracellular adenosine generation. *Exp. Cell Res.* **296**, 99-108.

Spychala, J., Lazarowski, E., Ostapkowicz, A., Ayscue, L.H., Jin, A., and Mitchell, B.S. (2004) Role of estrogen receptor in the regulation of ecto-5′-nucleotidase and adenosine in breast cancer. *Clin. Cancer Res.* **10**, 708-717.

Stallmach, A., von Lampe, B., Orzechowski, H-D., Matthes, H., and Riecken, E-O. (1994) Increased fibronectin-receptor expression in colon carcinoma-derived HT 29 cells decreases tumorigenicity in nude mice. *Gastroenterology* **106**, 19-27.

Stecca, B.A., Nardo, B., Chieco, P., Mazziotti, A., Bolondi, L., and Cavallari, A. (1997) Aberrant dipeptidyl peptidase IV (DPPIV/CD26) expression in human hepatocellular carcinoma. *J. Hepatol.* **27**, 337-345.

Struyf, S., De Meester, I., Scharpé, S., Lenaerts, J-P., Menten, P., Wang, J.M., Proost, P., and Van Damme, J. (1998) Natural truncation of RANTES abolishes signaling through the CC chemokine receptors CCR1 and CCR3, impairs its chemotactic potency and generates a CC chemokine inhibitor. *Eur. J. Immunol.* **28**, 1262-1271.

Struyf, S., Proost, P., Schols, D., De Clercq, E., Opdenakker, G., Lenaerts, J-P., Detheux, M., Parmentier, M., De Meester, I., Scharpé, S., and Van Damme, J. (1999) CD26/dipeptidyl-peptidase IV down-regulates the eosinophil chemotactic potency, but not the anti-HIV activity of human eotaxin by affecting its interaction with the CC chemokine receptor 3. *J. Immunol.* **162**, 4903-4909.

Synnestvedt, K., Furuta, G.T., Comerford, K.M., Louis, N., Karhausen, J., Eltzschig, H.K., Hansen, K.R., Thompson, L.F., and Colgan, S.P. (2002) Ecto-5′-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. *J. Clin. Invest.* **110**, 993-1002.

Taichman, R.S., Cooper, C., Keller, E.T., Pienta, K.J., Taichman, N.S., and McCauley, L.K. (2002) Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res.* **62**, 1832-1837.

Takasuga, S., Katada, T., Ui, M., and Hazek, O. (1999) Enhancement by adenosine of insulin-induced activation of phosphoinositide 3-kinase and protein kinase B in rat adipocytes. *J. Biol. Chem.* **274**, 19545-19550.

Tan, E.Y., Mujoomdar, M., and Blay, J. (2004) Adenosine down-regulates the surface expression of dipeptidyl peptidase IV on HT-29 human colorectal carcinoma cells. Implications for cancer cell behavior. *Am. J. Pathol.* **165**, 319-330.

Tanaka, T., Camerini, D., Seed, B., Torimoto, Y., Dang, N.H., Kameoka, J., Dahlberg, H.N., Schlossman, S.F., and Morimoto, C. (1992) Cloning and functional expression of the T cell activation antigen CD26. *J. Immunol.* **149**, 481-486.

Tanaka, T., Kameoka, J., Yaron, A., Schlossman, S.F., and Morimoto, C. (1993) The costimulatory activity of the CD26 antigen requires dipeptidyl peptidase IV enzymatic activity. *Proc. Natl. Acad. Sci. USA* **90**, 4586-4590.

Tanaka, T., Umeki, K., Yamamoto, I., Sakamoto, F., Noguchi, S., and Ohtaki, S. (1995) CD26 (dipeptidyl peptidase IV/DPP IV) as a novel molecular marker for differentiated thyroid carcinoma. *Int. J. Cancer* **64**, 326-331.

ten Kate, J., Wijnen, J.T., van der Goes, R.G.M., Quadt, R., Griffioen, G., Bosman, F.T., and Khan, P.M. (1984) Quantitative changes in adenosine deaminase isozymes in human colorectal adenocarcinomas. *Cancer Res.* **44**, 4688-4692.

ten Kate, J., Wijnen, J.T., Boldewijn, J, Khan, P.M., and Bosman, F.T. (1985) Immunohistochemical localization of adenosine deaminase complexing protein in intestinal mucosa and in colorectal adenocarcinoma as a marker for tumour cell heterogeneity. *Histochem. J.* **17**, 23-31.

ten Kate, J., van den Ingh, H.F.G.M., Khan, P.M., and Bosman, F.T. (1986) Adenosine deaminase complexing protein (ADCP) immunoreactivity in colorectal adenocarcinoma. *Int. J. Cancer* **37**, 479-485.

Tey, H.B., Khoo, H.E., and Tan, C.H. (1992) Adenosine modulates cell growth in human epidermoid carcinoma (A431) cells. *Biochem. Biophys. Res. Commun.* **187**, 1486-1492.

Tey, H.B., Tan, C.H., and Khoo, H.E. (1994) Modulation of DNA synthesis via adenosine receptors in human epidermoid carcinoma (A431) cells. *Biofactors* **4**, 161-165.

Thulesen, J., Hartmann, B., Hare, K.J., Kissow, H., Orskov, C., Holst, J.J., and Poulsen, S.S. (2004) Glucagon-like peptide 2 (GLP-2) accelerates the growth of colonic neoplasms in mice. *Gut* **53**, 1145-1150.

Tilley, S.L., Wagoner, V.A., Salvatore, C.A., Jacobson, M.A., and Koller, B.H. (2000) Adenosine and inosine increase cutaneous vasopermeability by activating A_3 receptors on mast cells. *J. Clin. Invest.* **105**, 361-367.

Tonks, N.K. and Neel, B.G. (2001) Combinatorial control of the specificity of protein tyrosine phosphatases. *Curr. Opin. Cell Biol.* **13**, 182-195.

Torimoto, Y., Dang, N.H., Vivier, E., Tanaka, T., Schlossman, S.F., and Morimoto, C. (1991) Coassociation of CD26 (dipeptidyl peptidase IV) with CD45 on the surface of human T lymphocytes. *J. Immunol.* **147**, 2514-2517.

Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D., and Kirilovsky, J. (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* **266**, 15771-15781.

Trincavelli, M.L., Tuscano, D., Marroni, M., Klotz, K-N., Lucacchini, A., and Martini, C. (2002) Involvement of mitogen activated protein kinase cascade in agonist-mediated human A_3 adenosine receptor regulation. *Biochim. Biophys. Acta* **1591**, 55-62.

Trotta, P.P. and Balis, M.E. (1978) Characterization of adenosine deaminase from normal colon and colon tumors. Evidence for tumor-specific variants. *Biochemistry* **17**, 270-277.

Trotta, P.P. (1982) Identification of a membrane adenosine deaminase binding protein from human placenta. *Biochemistry* **21**, 4014-4023.

Van Calker, D., Müller, M., and Hamprecht, B. (1978) Adenosine inhibits the accumulation of cyclic AMP in cultured brain cells. *Nature* **276**, 839-841.

Van Calker, D., Müller, M., and Hamprecht, B. (1979) Adenosine regulates, via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.* **33**, 999-1005.

Van Damme, J., Struyf, S., and Opdenakker, G. (2004) Chemokine-protease interactions in cancer. *Sem. Cancer Biol.* **14**, 201-208.

Van Den Oord, J.J. (1998) Expression of CD26/dipeptidyl-peptidase IV in benign and malignant pigment-cell lesions of the skin. *Br. J. Dermatol.* **138**, 615-621.

Van der Weyden, M.B. and Kelley, W.N. (1976) Human adenosine deaminase: Distribution and properties. *J. Biol. Chem.* **251**, 5448-5456.

Vaupel, P., Kallinowski, F., and Okunieff, P. (1989) Blood flow, oxygen and nutrient supply, and metabolic microenvironment in solid tumors: a review. *Cancer Res.* **49**, 6449-6465.

Veikkola, T. and Alitalo, K. (1999) VEGFs, receptors and angiogenesis. *Semin. Cancer Biol.* **9**, 211-220.

Visser, F., Vickers, M.F., Ng, A.M.L., Baldwin, S.A., Young, J.D., and Cass, C.E. (2002) Mutation of residue 33 of human equilibrative nucleoside transporters 1 and 2 alters sensitivity to inhibition of transport by dilazep and dipyridamole. *J. Biol. Chem.* **277**, 395-401.

Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* **269**, 5241-5248.

von Lampe, B., Stallmach, A., and Riecken, E.O. (1993) Altered glycosylation of integrin adhesion molecules in colorectal cancer cells and decreased adhesion to the extracellular matrix. *Gut* **34**, 829-836.

Walborg, E.F., Tsuchida, S., Weeden, D.S., Thomas, M.W., Barrick, A., McEntire, K.D., Allison, J.P., and Hixson, D.C. (1985) Identification of dipeptidyl peptidase IV as a protein shared by the plasma membrane of hepatocytes and liver biomatrix. *Exp. Cell Res.* **158**, 509-518.

Wallman-Johansson, A. and Fredholm, B.B. (1994) Release of adenosine and other purines from hippocampal slices stimulated electrically by hypoxia/hypoglycemia. Effect of choromethiazole. *Life Sci.* **55**, 721-728.

Wang, H. and Gilles-Baillien, M. (1992) Alkaline phosphatase and ATPases in brush-border membranes of rat jejunum: distinct effects of divalent cations and of some inhibitors. *Arch. Int. Physiol. Biochim. Biophys.* **100**, 289-294.

Wang, T., Sodhi, J., Mentzer, R.M., and Van Wylen, D.G. (1994) Changes in interstitial adenosine during hypoxia: relationship to oxygen supply:demand imbalance, and effects of adenosine deaminase. *Cardiovasc. Res.* **28**, 1320-1325.

Ward, J.L. and Tse, C.M. (1999) Nucleoside transport in human colonic epithelial cell lines: evidence for two Na^+-independent transport systems in T84 and Caco-2 cells. *Biochim. Biophys. Acta* **1419**, 15-22.

Ward, J.L., Sherali, A., Mo, Z-P., and Tse, C-M. (2000) Kinetic and pharmacological properties of cloned human equilibrative nucleoside transporters ENT1 and ENT2, stably expressed in nucleoside transporter-deficient PK15 cells. *J. Biol. Chem.* **275**, 8375-8381.

Weisz, O.A., Machamer, C.E., and Hubbard, A.L. (1992) Rat liver dipeptidylpeptidase IV contains competing apical and basolateral targeting information. *J. Biol. Chem.* **267**, 22282-22288.

Wesley, U.V., Albino, A.P., Iwari, S., and Houghton, A.N. (1999) A role for dipeptidyl peptidase IV in suppressing the malignant phenotype of melanocytic cells. *J. Exp. Med.* **190**, 311-322.

Wesley, U.V., Tiwari, S., and Houghton, A.N. (2004) Role for dipeptidyl peptidase IV in tumor suppression of human non small cell lung carcinoma cells. *Int. J. Cancer* **109**, 855-866.

Wesley, U.V., McGroarty, M., and Homoyouni, A. (2005) Dipeptidyl peptidase inhibits malignant phenotype of prostate cancer cells by blocking basic fibroblast growth factor signaling pathway. *Cancer Res.* **65**, 1325-1334.

Williams, B.A., Manzer, A., Blay, J., and Hoskin, D.W. (1997) Adenosine acts through a novel extracellular receptor to inhibit granule exocytosis by natural killer cells. *Biochem. Biophys. Res. Commun.* **231**, 264-269.

Woodhouse, E.C., Amanatullah, D.F., Schetz, J.A., Liotta, L.A., Stracke, M.L., and Clair, T. (1998) Adenosine receptor mediates motility in human melanoma cells. *Biochem. Biophys. Res. Commun.* **246**, 888-894.

Wolber, C. and Fozard, J.R. (2005) The receptor mechanism mediating the contractile response to adenosine on lung parenchymal strips from actively sensitized, allergen-

challenged Brown Norway rats. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **371**, 158-168.

Wu, Z-L., O'Kane, T.M., Scott, R.W., Savage, M.J., and Bozyczko-Coyne, D. (2002) Protein tyrosine phosphatases are up-regulated and participate in cell death induced by polyglutamine expansion. *J. Biol. Chem.* **277**, 44208-44213.

Wu, X., Zeng, H., Zhang, X., Zhao, Y., Sha, H., Ge, X., Zhang, M., Gao, X., and Xu, Q. (2004) Phosphatase of regenerating liver-3 promotes motility and metastasis of mouse melanoma cells. *Am. J. Pathol.* **164**, 2039-2054.

Yamabe, T., Takakura, K., Sugie, K., Kitaoka, Y., Takeda, S., Okubo, Y., Teshigawara, K., Yodoi, J., and Hori, T. (1997) Induction of the 2B9 antigen/dipeptidyl peptidase IV/CD26 on human natural killer cells by IL-2, IL-12 or IL-15. *Immunology* **91**, 151-158.

Yamaguchi, J., Kusano, K.F., Masuo, O., Kawamoto, A., Silver, M., Murasawa, S., Bosch-Marce, M., Masuda, H., Losordo, D.W., Isner, J.M., and Asahara, T. (2003) Stromal cell-derived factor-1 effects ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. *Circulation* **107**, 1322-1328.

Yamashita, K., Tachibana, Y., Matsuda, Y., Katunuma, N., Kochibe, N., and Kobata, A. (1988) Comparative studies of the sugar chains of aminopeptidase N and dipeptidylpeptidase IV purified from rat kidney brush-border membrane. *Biochemistry* **27**, 5565-5573.

Yan, S., Marguet, D., Dobers, J., Reutter, W., and Fan, H. (2003) Deficiency of CD26 results in a change of cytokine and immunoglobulin secretion after stimulation by pokeweed mitogen. *Eur. J. Immunol.* **33**, 1519-1527.

Yao, S.Y.M., Ng, A.M.L., Muzyka, W.R., Griffiths, M., Cass, C.E., Baldwin, S.A., and Young, J.D. (1997) Molecular cloning and functional characterization of nitrobenzylthioinosine (NBMPR)-sensitive (*es*) and NBMPR-insensitive (*ei*) equilibrative nucleoside transporter proteins ($rENT_1$ and $rENT_2$) from rat tissues. *J. Biol. Chem.* **271**, 28423-28430.

Zeelenberg, I.S., Ruuls-Van Stalle, L., and Roos, E. (2003) The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma metastases. *Cancer Res.* **63**, 3833-3839.

Zhang, H., Conrad, D.M., Butler, J.J., Zhao, C., Blay, J., and Hoskin, D.W. (2004) Adenosine acts through A2 receptors to inhibit IL-2-induced tyrosine phosphorylation of STAT5 in T lymphocytes: role of cyclic adenosine 3′,5′-monophosphate and phosphatases. *J. Immunol.* **173**, 932-944.

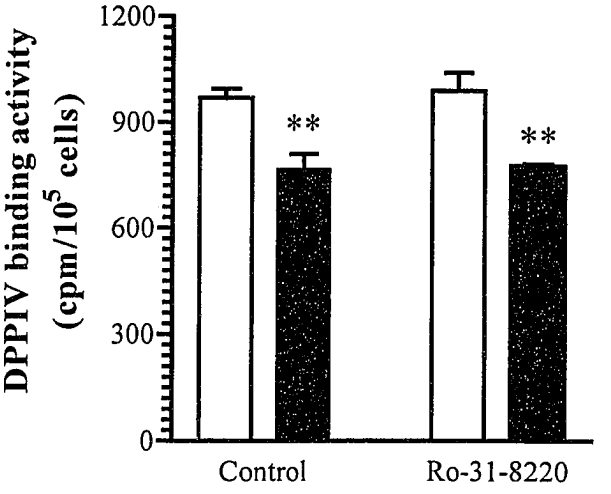
Zhong, H., Shlykov, S.G., Molina, J.G., Sanborn, B.M., Jacobson, M.A., Tilley, S.L., and Blackburn, M.R. (2003) Activation of murine lung mast cells by the adenosine A_3 receptor. *J. Immunol.* **170**, 338-345.

Zhou, Q-Y., Li, C., Olah, M.E., Johnson, R.A., Stiles, G.L., and Civelli, O. (1992) Molecular cloning and characterization of an adenosine receptor: the A_3 adenosine receptor. *Proc. Natl. Acad. Sci. USA* **89**, 7432-7436.

Zhou, B., Wang, Z-X., Zhao, Y., Brautigan, D.L., and Zhang, Z-Y. (2002) The specificity of extracellular signal-regulated kinase 2 dephosphorylation by protein phosphatases. *J. Biol. Chem.* **277**, 31818-31825.

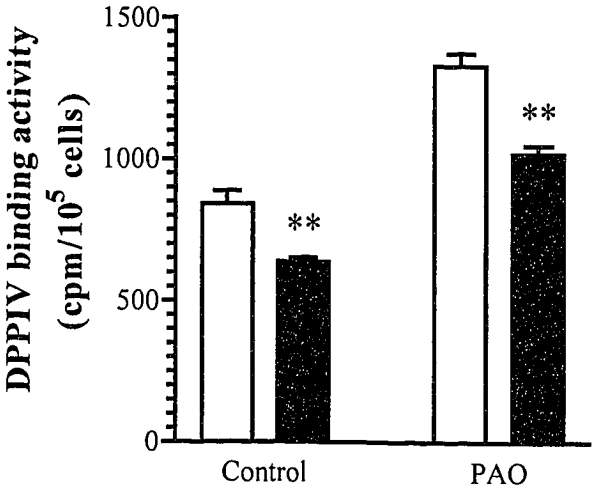
Zhu, L., Tamvakopoulos, C., Xie, D., Dragovic, J., Shen, X., Fenyk-Melody, J.E., Schmidt, K., Bagchi, A., Griffin, P.R., Thornberry, N.A., and Roy, R.S. (2003) The role of dipeptidyl peptidase IV in the cleavage of glucagon family peptides. *In vivo* metabolism of pituitary adenylate cyclase-activating polypeptide-(1-38). *J. Biol. Chem.* **278**, 22418-22423.

Zukowska-Grojec, Z., Karwatowska-Prokopczuk, E., Rose, W., Rone, J., Movafagh, S., Ji, H., Yeh, Y., Chen, W-T., Kleinman, H.K., Grouzmann, E., and Grant, D.S. (1998) Neuropeptide Y: a novel angiogenic factor from the sympathetic nerves and endothelium. *Circ. Res.* **83**, 187-195.



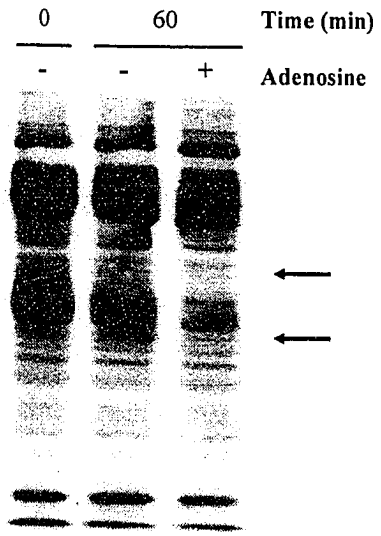
Appendix A The PKC inhibitor Ro-31-8220 does not block the adenosine-mediated down-regulation of DPPIV.

HT-29 cells were pretreated for 30 min with Ro-31-8220 (5 μM). The cells were then treated with control vehicle (*clear bars*) or 30 μM adenosine with 2.5 μM coformycin (*filled bars*). Forty-eight h later surface expression of DPPIV was measured. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.



Appendix B The PTP inhibitor phenylarsine oxide does not block the adenosine-mediated down-regulation of DPPIV.

HT-29 cells were pretreated for 30 min with phenylarsine oxide (PAO, 1 μM). The cells were then treated with control vehicle (*clear bars*) or 30 μM adenosine with 2.5 μM coformycin (*filled bars*). Forty-eight h later surface expression of DPPIV was measured. The data are mean values ± SE ($n = 4$). **, significant reduction by adenosine, $P < 0.01$.



Appendix C Adenosine reduction of 40-50 kDa range of tyrosine phosphorylated proteins in HT-29 cells.

HT-29 cells were stimulated with adenosine (300 μM) or control vehicle and then harvested at the indicated time points. Cell lysates (20 μg) were subjected to Western blot analysis for total tyrosine phosphorylated proteins. The region of proteins indicated between the arrows demonstrates the greatest changes in tyrosine phosphorylation.