REGULATION OF THE CHEMOKINE RECEPTOR CXCR4 ON COLORECTAL CARCINOMA CELLS BY THE TUMOUR MICROENVIRONMENT: THE ROLE OF ADENOSINE AND PROSTAGLANDINS

by

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Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
August 2006

Your file Votre référence
ISBN: 978-0-494-19593-2
Our file Notre référence
ISBN: 978-0-494-19593-2

DALHOUSIE UNIVERSITY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The chemokine receptor CXCR4 and its ligand, CXCL12, have been recently identified as important players in site-specific metastasis by promoting migration, invasion, and proliferation of tumour cells. CXCR4 is up-regulated in a number of cancer types, including colorectal cancer, and high CXCR4 expression is associated with poorer prognosis and increased mortality. There is evidence that CXCR4 expression may be influenced by the tumour microenvironment. The objective of this thesis was to determine how small molecules present within the tumour microenvironment may alter CXCR4 expression on colorectal carcinoma cells, and thereby alter the fate of the cancer.

In the first part of this study, it was found that adenosine, a purine nucleoside present in increased levels in tumours due to hypoxia, up-regulated CXCR4 mRNA and cell-surface protein expression on HT-29 colorectal carcinoma cells. This up-regulation was observed at concentrations of adenosine similar to those found within the tumour extracellular fluid, and resulted in increased migratory and proliferative responses of HT-29 cells to CXCL12. The combination of antagonists selective for the adenosine A_{2A} and A_{2B} receptors blocked the effect of adenosine on CXCR4 expression, implicating these two receptors in adenosine-mediated CXCR4 up-regulation.

Prostaglandins are present in elevated levels in tumours due to increased expression of cyclooxygenase 2 (COX-2) and other enzymes in the eicosanoid pathway. We found that several prostaglandins reduced cell-surface CXCR4 expression on HT-29 cells, particularly prostaglandin D_2 and its bioactive metabolite, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ($15dPGJ_2$). $15dPGJ_2$ is known to activate the peroxisome proliferator activated receptor γ (PPARγ). A range of synthetic PPARγ agonists in the thiazolidinedione class reduced CXCR4 expression, and $15dPGJ_2$-induced CXCR4 down-regulation was blocked by PPARγ antagonists, suggestive of a PPARγ-dependent effect. We also found evidence of involvement of NF-κB signaling in $15dPGJ_2$-mediated CXCR4 down-regulation.

In conclusion, small molecules present in the tumour microenvironment contribute to changes in CXCR4 expression on cancer cells, and thus may alter the ability of these cells to metastasize. Elucidation of the molecular pathways leading to changes in CXCR4 expression may lead to the development of novel strategies in the treatment of colorectal cancer.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ADA	adenosine deaminase
ADP	adenosine diphosphate
AK	adenosine kinase
Akt	protein kinase B
AK-T	activated killer T-cell
ALL	acute lymphoblastic leukaemia
alloxazine	benzo[g]pteridine-2,4[1H,3H]-dione
AMP	adenosine monophosphate
Ang	angiopoietin
ANOVA	analysis of variance
AP-1	activator protein-1
APC	adenomatous polyposis coli
ATP	adenosine triphosphate
BAB	binding assay buffer
8-Br-cAMP	8-bromo-cyclic adenosine 3'5'-cyclic monophosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAY10410	9,10-dihydro-15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
cDNA	complementary DNA
CGS21680	2-[p-(2-carboxyethyl)phenylethylamino]-5'-N-ethyl-carboxamidoadenosine
CHO	Chinese hamstery ovary

CLL	chronic lymphoblastic leukaemia
CNT	concentrative nucleoside transporter
CO_2	carbon dioxide
ConA	concanavalin A
COX	cyclooxygenase
CPA	N^6-cyclopentyl adenosine
$cPLA_2$	cytosolic PLA_2
CPM	counts per minute
CPT	8-cyclopentyltheophylline
CRTH2	chemoattractant receptor homologous molecule expressed on Th2 cells
CSC	8-(-3-chlorostyryl)caffeine
DAG	diacylglycerol
$15dPGJ_2$	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
DMEM	Dulbecco's modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DPPIV	dipeptidyl peptidase IV
DTT	dithiothreitol
EC_{50}	concentration producing half-maximal effect
EDTA	ethylenediaminetetraacetic acid

EGFR	epidermal growth factor receptor
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine
ei	NBTI-insensitive equilibrative nucleoside transporter
ENT	equilibrative nucleoside transporter
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
es	NBTI-sensitive equilibrative nucleoside transporter
FACS	fluorescence-activated cell sorting
Fas	fibroblast associated
5-FU	5-fluorouracil
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein-coupled receptor
GW9662	2-chloro-5-nitro-N-phenylbenzamide
h	hour
HIF	hypoxia-inducible factor
HIV	human immunodeficiency virus
HNSCC	head and neck squamous cell cancer
HPLC	high performance liquid chromatography
HRE	hypoxia response element
HUVEC	human umbilical vein endothelial cell
i.p.	intraperitoneal
i.v.	intravenous
IB-MECA	*N*(6)-(3-iodobenzyl)adenosine-5'-N-methyluronamide

Ig	immunogloubulin
IκB	inhibitor of κB
IKK	inhibitor of κB kinase
IL	interleukin
IP_3	inositol triphosphate
KDEL	lysine-aspartate-glutamate-leucine
LESTR	leukocyte-derived seven-transmembrane domain receptor
M	mols/litre
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
min	minute
MM	multiple myeloma
MMLV	Moloney murine leukemia virus
MMP	matrix metalloproteinase
MMTV	murine mammary tumour virus
mRNA	messenger RNA
MRS1523	3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate
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
NHL	non-Hodgkin's lymphoma
NK	nautral killer cell
NSAID	non-steroidal anti-inflammatory drug
NSCLC	non-small cell lung cancer
5'-NT	5'-nucleotidase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGA_2	prostaglandin A_2
PGD_2	prostaglandin D_2
PGDS	prostaglandin D synthase
15-PGDH	15-hydroxyprostaglandin dehydrogenase
PGE_2	prostaglandin E_2
PGES	prostaglandin E synthase
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGFS	prostaglandin F synthase
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostaglandin I_2, prostacyclin
PGIS	prostaglandin I synthase
PGJ_2	prostaglandin J_2
PI3K	phosphatidylinositol 3-kinase

PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
pVHL	von Hippel-Lindau tumour suppressor protein
RCC	renal cell cancer
RNA	ribonucleic acid
R-PIA	N^6-(L-2-phenylisopropyl)adenosine
RPTEC	renal proximal tubular epithelial cell
RT-PCR	reverse transcriptase-polymerase chain reaction
RXR	retinoid X receptor
s.c.	subcutaneous
SAH	*S*-adenosylhomocysteine
SCID	severe combined immunodeficiency disease
SCLC	small cell lung cancer
SDF-1	stromal cell-derived factor-1
SDS	sodium dodecyl sulfate
SE	standard error of the mean
siRNA	small interfering RNA
STAT	signal transducer and activator of transcription
T0070907	2-chloro-5-nitro-N-(4-pyridyl)benzamide

TGF	transforming growth factor
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TXA_2	thromboxane A_2
TXAS	thromboxane A synthase
VEGF	vascular endothelial growth factor
WHIM	warts, hypogammaglobulinaemia, immunodeficiency, and myelokathexis
Δ^{12}-PGJ_2	Δ^{12}-prostaglandin J_2

ACKNOWLEDGEMENTS

I am grateful for financial support provided for this project by the Natural Sciences and Engineering Research Council of Canada (NSERC), the Killam Trusts, and the Cancer Research Training Program.

I would like to give a heartfelt thanks to my supervisor, Jonathan, for providing a wonderful working environment and for the continual encouragement. Thanks for "embracing the opportunity" to take a chance on a pharmacist with a "passion for science".

Thanks to the Nachtigal, Sinal, and Renton labs for helping me advance my project, particularly Dr. Mark Nachtigal, Dr. Trevor Shepherd, Dr. Michelle Mujoomdar, and Tanya McCarthy for assistance with experimental design. I appreciate the guidance provided by members of my advisory committee, Dr. Jana Sawynok and Dr. Theresa Peterson. Sincere thanks to the ladies in the pharmacology office for the countless times they helped me. I would also like to acknowledge Dr. Tannis Jurgens for introducing me to research and for ongoing advice throughout my PhD.

To the members of the Blay lab, both past and present, I will look back at our time together with great fondness. Michelle, I have always looked up to you; you have been "super-helpful". Ernest, it was "sweet" that you finally came out of your shell (after a fair amount of prying). Erica, my partner in crime, it was delightful to work so closely with you. Thanks for the editing! Elena, you spoiled us! Susan, my "sister", I hope you thoroughly enjoy med school. David, I appreciated your positive outlook. Heather, you taught me so much!

I would like to acknowledge my friends in Halifax for their encouragement. Beth, I always appreciated our lunch dates on campus and the e-mail banter, and I am thankful to you and Karl for your friendship. I still can't believe that Jason had the nerve to move away on us. Princess Becky, we will be friends for life. Jonny, when are you going to start looking for a house in Ontario? To my family at West End, it was an honour to serve with you.

A special thank you to my parents, sister, and in-laws for their unwavering love and support. Shelley, thanks for all the drives – I will miss your pedestrian rants.

Last but not least, thanks to my husband Marc for standing by my side every step of the way.

And whatever you do, whether in word or deed, do it all in the name of the Lord Jesus, giving thanks to God the Father through him. Colossians 3:17

CHAPTER ONE

INTRODUCTION

Colorectal cancer is the second most common cause of cancer-related mortality in Canada, accounting for nearly nine thousand deaths annually (Canadian Cancer Society Canadian Cancer Statistics 2006). Treatment typically involves a combination of surgical and chemotherapeutic approaches (Wang et al., 2006). Although the survival rate is 90% with local disease, in patients presenting with advanced disease, in which metastasis has occurred, very few treatments are effective and the survival rate is less than 10% (American Cancer Society Cancer Facts and Figures 2006). Therefore, it is critically important to delineate biological processes that assist in the development of metastasis, such that novel therapies can be developed. In this project, I have examined the regulation of a chemokine receptor involved in tumour metastasis, CXCR4, by constituents of the tumour microenvironment, with the ultimate purpose of identifying targets for the prevention of colorectal carcinoma metastasis.

Chemokines

Chemokines are low-molecular-weight peptide ligands involved primarily in the trafficking of leukocytes (Murphy et al., 2000; Mellado et al., 2001). Nomenclature of chemokines is based on the number and spacing of conserved cysteine residues (Murphy et al., 2000). The four major groups of chemokines are the CXC, CC, C and CX3C chemokines. With the exception of members of the C subclass, which have only two cysteine residues, chemokines have four characteristic cysteine residues, leading to the

formation of two disulfide bonds (Rossi and Zlotnik, 2000). CXC chemokines have one amino acid between the first two cysteine residues, and CX3C chemokines have three amino acids between the first two cysteine residues; the first two cysteine residues are side by side in the CC family (Mellado et al., 2001). The name of each chemokine is built from the subclass (ex. CXC, CC, etc.) followed by "L" for ligand, and a number (Murphy et al., 2000).

Chemokine receptors are cell-surface seven-transmembrane G protein-coupled receptors (GPCRs; Murphy et al., 2000). Nomenclature of these receptors is based on the subclass of chemokine that the receptor recognizes, followed by "R" for receptor and a number. Chemokines bind to the extracellular domain of the chemokine receptor, which includes the N-terminus and three extracellular loops (Mellado et al., 2001). The intracellular domain consists of three loops and the C-terminus. Chemokine receptors associate with G_i proteins which, upon activation, lead to inhibition of adenylyl cyclase activity and mobilization of intracellular calcium (Mellado et al., 2001). Typical cellular consequences induced by chemokines include changes in gene expression, cell polarization, and chemotaxis.

There have been at least 18 chemokine receptors identified (Murphy, 2002). These include CXCR1-6, CCR1-10, CX_3CR1, and XCR1. The main ligands for each receptor are listed in Table 1.1. Many chemokine receptors have more than one known ligand, and many chemokines can activate more than one receptor. Thus, there is much promiscuity in chemokine/receptor signaling. Signaling through chemokine receptors primarily induces migration of cells of the immune system, leading to activation of several immune responses. The exact role depends on the expression pattern on specific

leukocyte subsets (Murphy et al., 2000). Other biological functions of chemokines are shown in Figure 1.1.

CXCL12/CXCR4

The gene for CXCR4 was cloned a decade ago, and its product was originally given the name LESTR (leukocyte-derived seven-transmembrane domain receptor) due to its abundant expression in several leukocyte populations (Loetscher et al., 1994). It was independently cloned and termed "fusin" because of its ability to act as a co-receptor for HIV fusion and entry (Feng et al., 1996). LESTR/fusin was originally considered to be an orphan receptor. However, the chemokine CXCL12, originally identified as stromal cell-derived factor 1 (SDF-1), was then shown by two independent investigators to be a ligand for LESTR/fusin, and the name CXCR4 was proposed (Bleul et al., 1996; Oberlin et al., 1996). The *CXCR4* gene contains two exons, and is constitutively expressed (Caruz et al., 1998; Wegner et al., 1998). CXCR4 protein has been detected in many cell types, including lymphocytes, monocytes, NK cells, dendritic cells, vascular smooth muscle cells, endothelial cells, cells lining the gastrointestinal tract, microglia, neurons, and astrocytes (Zhang et al., 1998; Balkwill, 2004).

CXCL12 is the only known soluble ligand for CXCR4 (Murphy et al., 2000). CXCL12 was shown to block infection by T-trophic HIV by virtue of its ability to bind CXCR4 (Bleul et al., 1996; Oberlin et al., 1996). Until recently, CXCR4 was considered to be the only receptor for CXCL12, but the orphan receptor RDC1 is now recognized as another possible receptor, and the name CXCR7 has been proposed (Balabanian et al.,

2005). CXCL12 is encoded by three exons, and is ubiquitously expressed (Shirozu et al., 1995). The *CXCL12* gene is located in chromosome 10.

Unlike mice deficient in other chemokine/receptors, mice lacking CXCL12 or CXCR4 die *in utero* (Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998; Murphy et al., 2000). CXCL12-/- mice died late in embryogenesis or shortly after birth (Nagasawa et al., 1996). Phenotypic defects included impaired B-cell progenitor formation and bone marrow myeloid cell formation, and cardiac dysfunction due to impaired ventricular septum formation. The phenotype of CXCR4-/- mice was found to be identical to that of CXCL12-/- mice (Tachibana et al., 1998; Zou et al., 1998). CXCR4 -/- mice had impaired ventricular septum formation and bone marrow haematopoiesis. Additionally, aberrant migration of cells of the cerebellum occurred during development of CXCR4-/- mice, (Zou et al., 1998), as did impaired vascularization of the gastrointestinal tract (Tachibana et al., 1998). Thus, CXCL12/CXCR4 signaling is required during the development of the haematopoietic, cardiac, vascular, and nervous system.

Signaling through the CXCL12/CXCR4 axis regulates the homing of haematopoietic progenitor cells to the bone marrow and their retention therein. These progenitor cells express high levels of CXCR4, and are attracted to CXCL12 produced by stromal cells in the bone marrow (Aiuti et al., 1997). CXCL12 also acts as a chemoattractant for haematopoietic and tissue-committed stem cells in the contexts of inflammation, tissue regeneration, and development (Kucia et al., 2005).

Although there have not been reports of disease states caused by CXCL12 or CXCR4 deficiency, warts, hypogammaglobulinaemia, immunodeficiency, and

myelokathexis (WHIM) syndrome is caused by a truncating mutation in the *CXCR4* gene which confers increased receptor activity (Hernandez et al., 2003). In this disorder, myeloid cells are retained in the bone marrow, consistent with the recognized ability of CXCL12/CXCR4 signaling to cause bone marrow retention of progenitor cells (Aiuti et al., 1997).

CXCR4 in cancer

In the past five years, several publications have demonstrated the importance of the CXCL12/CXCR4 axis in the progression of over twenty cancer types (Balkwill, 2004). Types of cancer in which the CXCL12/CXCR4 axis is involved are listed in Table 1.2, and Appendix A describes the involvement of CXCL12/CXCR4 expression and signaling in these cancers in more detail.

Müller and colleagues published a landmark study demonstrating the importance of the CXCL12/CXCR4 axis in site-specific metastasis (Müller et al., 2001). In this study, it was found that CXCR4 expression was undetectable in normal epithelial cells, but consistently up-regulated in breast cancer cells at both the mRNA and protein level. CXCR4 expression was also high on primary breast cancer cells. MDA-MB-231 human breast carcinoma cells, which expressed CXCR4, underwent morphological changes in response to CXCL12, and CXCL12 induced directional migration of these cells, indicating that CXCR4 was active. CXCL12 was highly expressed in tissues taken from human organ sites to which breast cancer cells metastasize, including lymph nodes, lung, liver, and bone marrow, but expressed at low levels in tissues that represent rare sites of metastasis, including the kidney, skin, and muscle. Interestingly, MDA-MB-231 cells

also migrated towards protein extracts of lung and liver, an effect that was inhibited with neutralizing anti-CXCR4 or anti-CXCL12 antibodies. Furthermore, a neutralizing anti-CXCR4 antibody reduced lung and lymph node metastasis after tail-vein injection or orthotopic implantation of MDA-MB-231 cells in mice, demonstrating the biological importance of this chemokine/receptor pair in the process of metastasis. The findings from this paper are depicted in Figure 1.2

Balkwill and colleagues also made seminal contributions to the CXCR4/cancer metastasis field by describing the role of CXCR4 in the migration of ovarian cancer cells (Scotton et al., 2001). CXCR4 mRNA was expressed in 4 of 6 ovarian cancer cell lines, as well as in 8 of 10 primary tumour biopsies and 19 of 20 samples from ovarian cancer ascites. CXCL12 induced functional responses in the form of intracellular calcium flux and chemotaxis of CXCR4-expressing IGROV and CAOV-3 ovarian cancer cells, demonstrating that CXCR4 was active. High levels of CXCL12 were present in ascitic fluid taken from patients with ovarian cancer, which the authors speculated would form a gradient for migration of cancer cells to the peritoneal cavity. Further investigations by this group revealed that CXCL12 stimulated the growth of IGROV cells, an effect that was blocked with a neutralizing anti-CXCR4 antibody and with the CXCR4 antagonist AMD3100, demonstrating the involvement of the CXCL12/CXCR4 signaling pathway in growth stimulation (Scotton et al., 2002).

Taichman and colleagues also published an important paper describing the significance of the CXCL12/CXCR4 axis in cancer metastasis, specifically in prostate cancer metastasis to bone (Taichman et al., 2002). They found that human osteoblasts and osteosarcoma cell lines with an osteoblast phenotype expressed CXCL12 mRNA and

secreted CXCL12 protein, and prostate cancer cell lines (DU145, PC3, LNCaP, and C4-2B) expressed CXCR4 mRNA and protein. CXCL12 induced ERK phosphorylation in PC3 cells and increased adherence of PC3 and C4-2B cells as well as MCF-7 human breast carcinoma cells to osteosarcoma cells and human bone marrow endothelial cells. CXCL12 increased the transendothelial migration of LNCaP and DU145 cells, and the invasion of PC3 and C4-2B cells in Matrigel, which was blocked with a neutralizing anti-CXCR4 antibody. Medium from osteoblast cultures also enhanced invasion of these cells. The authors speculated that prostate cancers may use the CXCL12-CXCR4 pathway in metastasis to bone, since prostate cancer cells expressing CXCR4 would migrate across endothelial barriers and basement membranes and adhere to components of the bone marrow in response to a CXCL12 gradient.

As illustrated in these studies, as well as those described in Appendix A, typical responses of cancer cells to CXCL12 include migration and invasion due to cytoskeletal rearrangement and up-regulation of matrix metalloproteinases (MMPs; Müller et al., 2001; Scotton et al., 2001; Taichman et al., 2002; Bartolomé et al., 2004; Singh et al., 2004), transendothelial migration (Taichman et al., 2002), and adhesion to components of extracellular matrix or other cell types (Taichman et al., 2002), which is mediated by integrin signaling (Cardones et al., 2003; Hartmann et al., 2005). In some cases, CXCL12 may also induce proliferation or promote survival of cancer cells (Scotton et al., 2002). Overall, cellular changes elicited by activation of CXCL12/CXCR4 signaling promote the survival and metastasis of cancer cells.

Many signaling pathways can be activated downstream of CXCL12 activation of CXCR4 in cancer cells. For example, CXCL12 has been shown to increase ERK1/2

phosphorylation (Barbero et al., 2002; Libura et al., 2002; Taichman et al., 2002; Zhou et al., 2002; Hwang et al., 2003; Castellone et al., 2004; Katayama et al., 2005; Yasumoto et al., 2006;), Akt phosphorylation (Kijima et al., 2002; Lee et al., 2004; Katayama et al., 2005), PI3K activation (Lee et al., 2004), and calcium flux (Jordan et al., 1999; Möhle et al., 1999; Scotton et al., 2001; Schrader et al., 2002; Lee et al., 2004;). For further information see Appendix A.

In many ways, the process of metastasis is similar to leukocyte and stem cell trafficking throughout the body, processes which involve the CXCL12/CXCR4 axis (Kucia et al., 2005). It appears that cancer cells, which express CXCR4, exploit this signaling pathway, leading to homing and retention in sites that are rich in CXCL12.

The CXCL12/CXCR4 axis may also promote tumour angiogenesis. Vascular endothelial growth factor (VEGF) and CXCL12 were shown to synergistically increase angiogenesis in an *in vivo* Matrigel assay and promote proliferation and migration of human umbilical vein endothelial cells (HUVECs) *in vitro* (Kryczek et al., 2005).

Since CXCL12/CXCR4 signaling has been shown to produce changes in cancer cells that are indicative of a more aggressive phenotype, it is not surprising that CXCR4 expression has been associated with disease progression, increased recurrence, and reduced survival in many cancer types, as listed in Table 1.3 and in more detail in Appendix B. It has been suggested that CXCR4 expression may be useful as an indicator of prognosis (Kim et al., 2005a) or a tumour biomarker (Cabioglu et al., 2005). Interestingly, in 13 of 14 cases of ductal carcinoma *in situ* (DCIS) of the breast, CXCR4 protein expression was detected, whereas CXCR4 levels were undetectable in adjacent normal breast epithelium (Schmid et al., 2004). Therefore, acquisition of CXCR4

expression may occur very early in malignant transformation, further supporting its potential as a biomarker. Although mutations in the *CXCR4* gene have not been reported in the context of cancer, patients with a single nucleotide polymorphism in the 3' untranslated region of the *CXCL12* gene had reduced incidence of long distance metastasis of epidermoid non-small cell lung cancer (NSCLC; Coelho et al., 2006).

CXCR4 in colorectal cancer

CXCR4 is abundantly expressed by colorectal carcinoma cells compared to other chemokine receptors (Dwinell et al., 1999; Jordan et al., 1999). The involvement of CXCR4 expression in colorectal cancer progression was first shown by Roos and colleagues (Zeelenberg et al., 2003). CT-26 mouse colon carcinoma cells were transfected with CXCL12 extended with a Lys-Asp-Glu-Leu (KDEL) sequence. The KDEL receptor functions to retain resident endoplasmic reticulum (ER) proteins, which contain a C-terminal KDEL sequence, in the ER. With this "intrakine approach", CXCL12-KDEL binds to the KDEL receptor and is retained in the ER, and CXCR4 protein which binds to CXCL12 is also retained in the ER, preventing its expression at the cell-surface (Zeelenberg et al., 2001; Zeelenberg et al., 2003). This approach was first developed as an attempt to reduce HIV infection (Chen et al., 1997). After intrasplenic injection, CXCL12-KDEL-transfected CT-26 cells, which had reduced cell-surface CXCR4 protein expression, did not form liver metastases, whereas control cells did (Zeelenberg et al., 2003). The incidence of lung metastasis was also reduced with CXCL12-KDEL-transfected cells, and survival was increased. Interestingly, unlike Zlotnik's group, who suggested that CXCR4 expression was necessary for the movement

of tumour cells to secondary sites (Müller et al., 2001), Zeelenberg and colleagues found that CXCR4 expression was not required for migration of CT-26 cells to the lungs, but rather for tumour expansion at secondary sites (Zeelenberg et al., 2003). Therefore, these authors concluded that CXCR4 is necessary for the outgrowth of colon cancer micrometastases.

Using immunohistochemistry, Ottaiano and colleagues found high CXCR4 expression (70-100% of cells stained positively) in a greater number of colorectal carcinoma tissues compared to normal tissues (Ottaiano et al., 2005). Cell-surface CXCR4 protein was expressed at high levels (40-80% of cells stained positively) on SW620, SW48, and SW480 colorectal carcinoma cells, and at moderate levels on Caco-2 and LoVo cells (approximately 20% of cells stained positively). CXCL12 enhanced the chemotaxis of SW480 cells as well as their adhesion to fibronectin and collagen type I/III, and both effects were blocked with an anti-CXCR4 neutralizing antibody. CXCL12 also induced cytoskeletal changes, proliferation, and ERK1/2 phosphorylation in SW480 cells. Similarly, Schimanski and colleagues found that SW480, SW620, and HT-29 colorectal carcinoma cells expressed CXCR4 protein, as did 96 of 96 colorectal carcinoma tissue samples (Schimanski et al., 2005). CXCL12 induced chemotaxis of SW480 and SW620 cells. Kim and colleagues found that in patients with colorectal cancer with liver metastases, CXCR4 mRNA expression was over 100-fold higher in liver metastases compared to the primary tumour (Kim et al., 2005a). Furthermore, as described in Table 1.3 and Appendix B, elevated CXCR4 expression in colorectal cancer is associated with disease progression and reduced survival (Kim et al., 2005a; Schimanski et al., 2005; Ottaiano et al., 2006).

Pre-clinical efficacy of anti-CXCR4 treatments

Several studies have demonstrated the efficacy of strategies designed to reduce CXCR4 expression or inhibit its activity in pre-clinical models of cancer, particularly cancer metastasis. A neutralizing anti-CXCR4 antibody prevented metastasis of MDA-MB-231 breast cancer cells (Müller et al., 2001) and reduced tumour growth after i.p. injection of Namalwa non-Hodgkin's lymphoma cells in mice (Bertolini et al., 2002). A neutralizing antibody against CXCR4 also inhibited the growth of subcutaneous tumours derived from pancreatic cancer cells that did not express CXCR4 by virtue of the ability of the antibody to block CXCR4 activity on tumour vasculature (Guleng et al., 2005).

CXCR4 peptide antagonists have proven effective in pre-clinical cancer models. The CXCR4 peptide antagonist 4F-benzoyl-TN14003 inhibited lung metastasis of MDA-MB-231 breast cancer cells (Tamamura et al., 2003), and 4F-benzoyl-TE14011 reduced pulmonary metastasis of B16-BL6 melanoma cells (Takenaga et al., 2004). Murakami and colleagues assessed the contribution of CXCR4 to the metastatic process by transducing B16 murine melanoma cells with CXCR4, followed by i.v. injection in syngeneic C57BL/6 mice (Murakami et al., 2002). CXCR4 expression in this context led to increased pulmonary metastasis, which was reduced with the CXCR4 peptide antagonist T22. Liang and colleagues showed that TN14003, a synthetic 14-mer peptide CXCR4 antagonist, inhibited *in vitro* invasion of MDA-MB-231 breast cancer cells and lung metastasis after tail vein injection of these cells without causing any toxicity (Liang et al., 2004).

Liang and colleagues also showed the pre-clinical efficacy of anti-CXCR4 treatments using a molecular approach (Liang et al., 2005). MDA-MB-231 breast cancer

cells transfected with siRNA oligonucleotides designed to knock down CXCR4 were injected into the tail veins of SCID mice. Mice also received twice-weekly i.v. injections of siRNA oligonucleotides to maintain CXCR4 knockdown. The control mice all developed lung tumours, whereas only one of six mice receiving CXCR4 siRNA-transfected cells and follow-up injections with CXCR4 siRNA developed tumours. Interestingly, one group of mice that was injected with untransfected cells but received twice-weekly injections of siRNA against CXCR4 also showed reduced lung colonization compared to control mice, indicating that naked delivery of CXCR4 siRNA oligonucleotides was sufficient to produce a beneficial effect, although the reduction was less than that which was seen with pre-transfected cells.

Other molecular approaches designed to reduce CXCR4 expression have yielded promising results in the context of tumour progression. NSCLC 95D cells in which CXCR4 expression was knocked down using antisense technology formed lung metastases in fewer mice after s.c. injection compared to CXCR4 positive cells (Su et al., 2005). Stable knockdown of CXCR4 expression in 4T1 murine breast carcinoma cells using short hairpin RNA reduced orthotopic tumour growth and lung metastasis (Smith et al., 2004). Similarly, MDA-MB-231 cells that had undergone stable knockdown of CXCR4 did not form tumours or lung metastases after orthotopic injection into mammary fat pads of SCID mice, whereas CXCR4-positive cells were tumourigenic (Lapteva et al., 2005). These studies show the importance of CXCR4 expression in both primary and secondary tumour growth.

Small molecule inhibitors of CXCR4 have also been tested in pre-clinical cancer models. Rubin and colleagues showed that the non-competitive CXCR4 antagonist

AMD3100 inhibited tumour growth after intracranial implantation of Daoy medulloblastoma cells and U87 glioblastoma cells (Rubin et al., 2003). There was no evidence of drug-induced toxicity. AMD3100 also inhibited peritoneal carcinomatosis and ascites formation after i.p. inoculation of NUGC4 human gastric carcinoma cells (Yasumoto et al., 2006).

Although CXCR4 antagonists have not yet been assessed in clinical trials to determine their therapeutic potential in cancer, they have been examined in small trials in the context of HIV treatment and haematopoietic progenitor cell mobilization. The CXCR4 antagonist, AMD3100, was generally well-tolerated in a Phase I clinical trials (Hendrix et al., 2000; Devine et al., 2004; Flomenberg et al., 2005), although one trial reported one patient with thrombocytopenia, two patients with premature ventricular contractions, and several patients with paresthesias (Hendrix et al., 2004). AMD3100 did not reduce viral load in HIV patients (Hendrix et al., 2004), but did effectively increase haematopoietic progenitor cell mobilization (Devine et al., 2004; Flomenberg et al., 2005; Lack et al., 2005; Liles et al., 2005).

Regulation of CXCR4 expression

Zeelenberg and colleagues found that CT-26 murine colon carcinoma cells grown *in vitro* expressed CXCR4 mRNA, but cell-surface protein levels were not detectable (Zeelenberg et al., 2003). When the same cells were freshly isolated from lung or liver metastases or from intrasplenic tumours, cell-surface expression was strongly up-regulated. This elevated expression was lost after 2-4 days in culture, indicating that it was not due to selection of a subpopulation of cells with high CXCR4 expression. The

authors concluded that CXCR4 expression was induced by the *in vivo* tumour microenvironment.

Multiple factors have already been shown to regulate CXCR4 expression on tumour cells and other cell types. One such factor is hypoxia (Schioppa et al., 2003; Staller et al., 2003). Solid tumours tend to be hypoxic due to structural abnormalities of tumour vasculature (Vaupel, 2004). Staller and colleagues were the first to demonstrate the involvement of hypoxia in the regulation of CXCR4 expression (Staller et al., 2003). Their goal was to identify genes regulated by the von Hippel-Lindau tumour suppressor protein (pVHL) in renal cell carcinoma cells, as pVHL is often inactivated in renal cell cancer (RCC) leading to constitutive activation of hypoxia-inducible factor-1 (HIF-1) target genes. In a microarray analysis, they found that CXCR4 mRNA expression was suppressed by reintroduction of functional pVHL into pVHL-deficient A498 RCC cells, an effect that was due to inactivation of HIF. CXCR4 protein was also down-regulated, resulting in reduced migration of RCC cells towards CXCL12. Hypoxia increased CXCR4 mRNA expression in HEK-293 human embryonic kidney cells and primary human proximal renal tubular epithelial cells (RPTECs), and a hypoxia response element (HRE) was identified within the CXCR4 promoter. The authors speculated that intratumoural hypoxia may lead to increased CXCR4 expression in diverse types of solid tumours, increasing metastasis to distant organs. Shioppa and colleagues found that hypoxia increased CXCR4 mRNA and cell-surface protein expression in several cell types, including monocytes, human monocyte-derived macrophages, tumour-associated macrophages, HUVECs, CAOV3 ovarian carcinoma cells, and MCF-7 breast carcinoma

cells leading to increased migration towards CXCL12 due to activation of HIF-1 (Schioppa et al., 2003).

VEGF, which is present in high levels in tumours, can also increase CXCR4 expression on cancer cells (Bachelder et al., 2002). Bachelder and colleagues found that reduction of VEGF expression in MDA-MB-231 breast carcinoma cells using antisense technology led to reduced invasive capacity, an effect that was attributed to reduced CXCR4 expression. VEGF restored CXCR4 expression. The authors concluded that an autocrine signaling pathway is present in breast cancer cells, whereby cells can produce VEGF which in turn increases CXCR4 expression.

Balkwill and colleagues showed that elevated tumour necrosis factor-α (TNF-α) levels found within tumours increased CXCR4 mRNA and cell-surface protein expression on IGROV-1 and TOV21G ovarian cancer cells, leading to enhanced migration towards CXCL12 (Kulbe et al., 2005). This increase appeared to be due to TNF-α-induced activation of NF-κB signaling. Interestingly, co-culture of ovarian cancer cells with macrophages also up-regulated CXCR4 expression, an effect that was blocked with an anti-TNF-α neutralizing antibody. Therefore, tumour-associated macrophages may contribute to increased CXCR4 expression on cancer cells by production of TNF-α. A significant correlation between TNF-α and CXCR4 expression was found in ovarian cancer biopsies. TNF-α was also shown to increase CXCR4 mRNA expression in astroglioma cells (Oh et al., 2001).

Transforming growth factor-β (TGF-β) increased cell-surface CXCR4 protein expression on BLM human melanoma cells, as did Matrigel and type I collagen

(Bartolomé et al., 2004). Therefore, interactions with TGF-β and matrix proteins within tumours may also increase CXCR4 expression.

Receptor	Agonist(s)	Main functions
CXCR1	CXCL8	Neutrophil migration; innate immunity; acute inflammation
CXCR2	CXCL1-3; CXCL5-8	Neutrophil migration; innate immunity; acute inflammation; angiogenesis
CXCR3	CXCL9-11	T cell migration; adaptive immunity; Th1 inflammation
CXCR4	CXCL12	B cell lymphopoiesis; bone marrow myelopoiesis; central nervous system and vascular development; HIV infection
CXCR5	CXCL13	B cell trafficking; lymphoid development
CXCR6	CXCL16	T cell migration
CCR1	CCL3, CCL5, CCL7, CCL8, CCL13-16, CCL23	T cell and monocyte migration; innate and adaptive immunity; inflammation
CCR2	CCL2, CCL7, CCL8, CCL13	T cell and monocyte migration; innate and adaptive immunity; Th1 inflammation
CCR3	CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL24, CCL26	Eosinophil, basophil, and T cell migration; allergic inflammation
CCR4	CCL17, CCL22	T cell and monocyte migration; allergic inflammation
CCR5	CCL3, CCL4, CCL5, CCL8, CCL14	T cell and monocyte migration; innate and adaptive immunity; HIV infection
CCR6	CCL20	Dendritic cell migration
CCR7	CCL19, CCL21	T cell and dendritic cell migration; lymphoid development; primary immune response
CCR8	CCL1, CCL4, CCL17	T cell trafficking
CCR9	CCL25	T cell homing to gut
CCR10	CCL26-28	T cell homing to skin
CX_3CR1	CX3CL1	T cell and NK cell trafficking and adhesion; innate and adaptive immunity; Th1 inflammation
XCR1	XCL1-2	T cell trafficking

Table 1.1: Chemokine Receptors. Adapted from Murphy, 2002.

Cancer	References
Acute lymphoblastic leukaemia (ALL)	Crazzolara et al., 2001
Brain cancer	Sehgal et al., 1998; Barbero et al., 2002; Rubin et al., 2003
Breast cancer	Müller et al., 2001
Chronic lymphocytic leukaemia (CLL)	Möhle et al., 1999
Colorectal cancer	Zeelenberg et al., 2003; Ottaiano et al., 2005; Schimanski et al., 2005
Endometrial cancer	Mizokami et al., 2004
Gastric cancer	Yasumoto et al., 2006
Glioblastoma	Zhou et al., 2002
Head and neck squamous cell cancer (HNSCC)	Katayama et al., 2005
Melanoma	Robledo et al., 2001; Cardones et al., 2003; Bartolomé et al., 2004
Multiple myeloma (MM)	Sanz-Rodríguez et al., 2001
Nasopharyngeal carncer	Hu et al., 2005
Neuroblastoma	Geminder et al., 2001
Non-Hodgkin's lymphoma (NHL)	Bertolini et al., 2002
Non-small cell lung cancer (NSCLC)	Su et al., 2005
Osteosarcoma	Laverdiere et al., 2005
Ovarian cancer	Scotton et al., 2001; Scotton et al., 2002
Pancreatic cancer	Koshiba et al., 2000; Marchesi et al., 2004
Prostate cancer	Taichman et al., 2002; Mochizuki et al., 2004; Singh et al., 2004
Renal cell cancer (RCC)	Schrader et al., 2002
Rhabdomyosarcoma	Libura et al., 2002
Small cell lung cancer (SCLC)	Kijima et al., 2002
Thyroid cancer	Hwang et al., 2003; Castellone et al., 2004

Table 1.2: Involvement of CXCL12/CXCR4 in many cancer types. This table lists cancer types in which the CXCL12/CXCR4 axis is involved.

Cancer	Reference
Acute lymphoblastic leukaemia (ALL)	Crazzolara et al., 2001
Breast cancer	Kato et al., 2003; Cabioglu et al., 2005
Chronic lymphocytic leukaemia (CLL)	Ishibe et al., 2002
Colorectal cancer	Kim et al., 2005a; Schimanski et al., 2005; Ottaiano et al., 2006
Gastric cancer	Yasumoto et al., 2006
Head and neck squamous cell cancer (HNSCC)	Katayama et al., 2005
Melanoma	Scala et al., 2005
Non-small cell lung cancer (NSCLC)	Su et al., 2005
Osteosarcoma	Laverdiere et al., 2005
Prostate cancer	Mochizuki et al., 2004
Renal cell cancer (RCC)	Staller et al., 2003

Table 1.3: CXCR4 expression as an indicator of prognosis and disease progression. This table lists cancer types in which CXCR4 expression is correlated with disease progression, increased recurrence, and/or reduced survival.

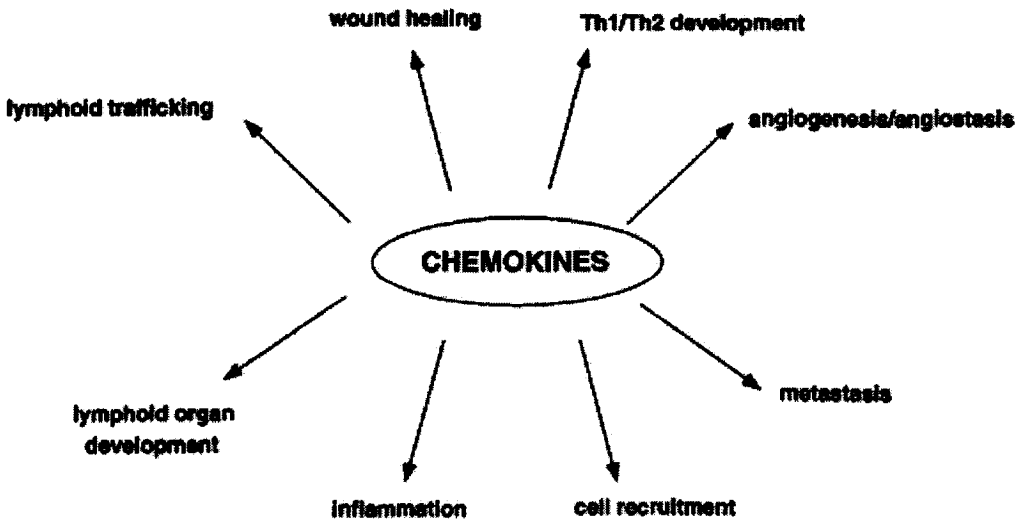


Figure 1.1: Biological functions of chemokines and chemokine receptors. Taken from Rossi and Zlotnik, 2000.

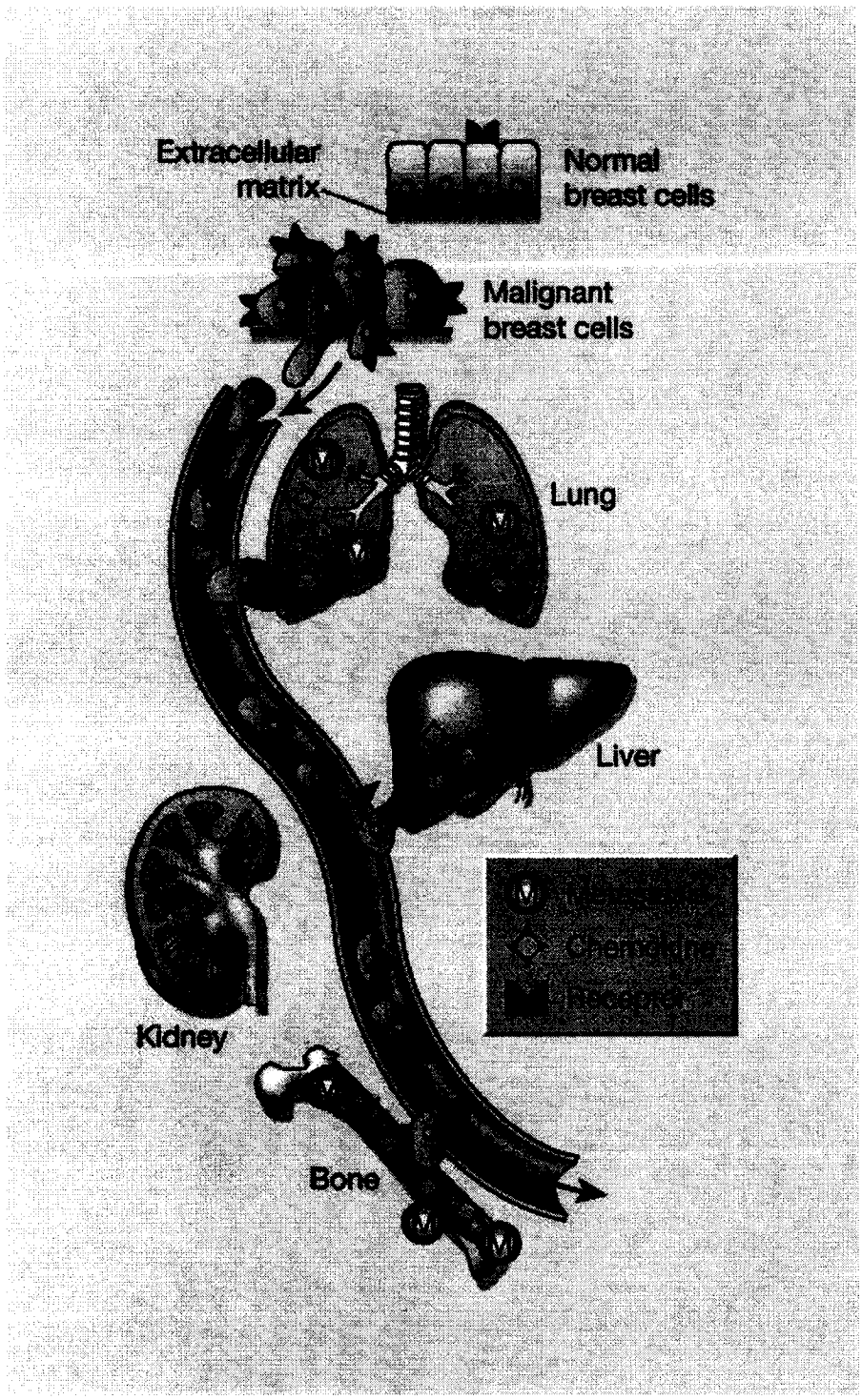


Figure 1.2: CXCL12/CXCR4 in breast cancer metastasis. The chemokine receptor CXCR4 is up-regulated during malignant transformation, leading to dissemination of cells to sites rich in the chemokine, CXCL12, and resulting in formation of metastases. Taken from Liotta, 2001.

Overall Objectives

CXCR4 expression is regulated by the tumour microenvironment (Zeelenberg et al., 2003). Multiple factors have already been shown to elevate CXCR4 expression on cancer cells, including hypoxia (Schioppa et al., 2003; Staller et al., 2003), VEGF (Bachelder et al., 2002), TNF-α (Oh et al., 2001; Kulbe et al., 2005), TGF-β (Bartolomé et al., 2004), and components of the extracellular matrix (Bartolomé et al., 2004). We sought to determine what other components of the tumour microenvironment may contribute to elevated CXCR4 expression, with a particular emphasis on small molecules.

The objectives of this thesis were:

1. To determine what factors within the tumour microenvironment contribute to elevated CXCR4 expression on colorectal carcinoma cells,
2. To identify signaling pathways involved in CXCR4 regulation, and
3. To determine the functional consequences of any changes in CXCR4 expression.

General Hypothesis

Small molecules present within the tumour microenvironment up-regulate CXCR4 expression on colorectal carcinoma cells leading to increased responsiveness to CXCL12, and that identification of signaling pathways involved in regulation of CXCR4 expression may lead to the development of new anti-cancer treatments.

CHAPTER TWO

ADENOSINE INCREASES CXCR4 EXPRESSION ON HT-29 COLORECTAL CARCINOMA CELLS

Portions of this chapter appeared in the following publication:

Richard CL, Tan EY, Blay J. (2006) Adenosine up-regulates CXCR4 and enhances the proliferative and migratory responses of human carcinoma cells to CXCL12/SDF-1α. *Int. J. Cancer*, in press.

INTRODUCTION

Adenosine production and metabolism

Adenosine is a purine nucleoside that acts as a precursor for several cellular metabolites, including AMP, ADP, ATP, and cAMP. Additionally, adenosine has a number of biological actions, as will be discussed below.

Formation of adenosine occurs at both the intracellular and extracellular level (Spychala, 2000). Intracellular production occurs through dephosphorylation of AMP by 5'-nucleotidase (5'-NT) or through hydrolysis of *S*-adenosyl-homocysteine (SAH) by SAH hydrolase (Fredholm et al., 2001). Intracellular removal of adenosine may occur through its phosphorylation to AMP by adenosine kinase (AK) or its degradation to inosine through adenosine deaminase (ADA). Extracellular adenosine production may result from the direct transport of adenosine out of cells, or from the dephosphorylation of the extracellular nucleotides ATP, ADP, or AMP to adenosine through the action of ectonucleotidases such as NTDPase1 (CD39) and ecto-5'-NT (CD73; Spychala, 2000; Fredholm et al., 2001; Linden, 2001). Extracellular adenosine removal can occur through its re-uptake into cells or its conversion to inosine through ecto-adenosine deaminase (ecto-ADA).

Under conditions of hypoxia and ischaemia, extracellular adenosine levels increase dramatically (Spychala, 2000; Fredholm et al., 2001; Linden, 2001). This may be in part due to increased cellular ATP utilization as a result of these stressors, leading to increased AMP formation and adenosine production; however, adenosine levels also increase due to changes in the activity of enzymes responsible for its metabolism. For example, under hypoxic conditions, elevated levels of extracellular adenosine during

hypoxia result from a combination of increased 5'-NT activity and decreased AK activity (Headrick and Willis, 1989; Decking et al., 1997).

Nucleoside transporters also participate in the regulation of intracellular and extracellular adenosine concentrations, and are classified as being equilibrative (ENT) or concentrative (CNT; Thorn and Jarvis, 1996; Damaraju et al., 2003). ENTs, which are members of the SLC29 family of transporters, transport nucleosides into and out of cells through the process of facilitated diffusion (Baldwin et al., 2004). This results in an equilibration of nucleoside concentrations within the intracellular and extracellular fluids. This class was originally functionally subdivided based upon sensitivity (*es*) or insensitivity (*ei*) to inhibition by nanomolar concentrations of NBTI (Thorn and Jarvis, 1996; Damaraju et al., 2003), but members are now identified by their molecular structure as ENT1-4 (Baldwin et al., 2004). The transporters differ in their substrate sensitivity and patterns of distribution, although each is found widely throughout the body.

In contrast to ENTs, CNTs transport nucleosides using active processes driven by transmembrane sodium gradients (Thorn and Jarvis, 1996). CNTs belong to the SLC28 family of transporters and are sub-classified as CNT1, CNT2, or CNT3 (Damaraju et al., 2003; Gray et al., 2004).

Signaling through adenosine receptors

There are four known adenosine receptors; these are denoted A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 2001) and each is a seven-transmembrane GPCR (Jacobson and Gao, 2006). The mechanisms of signaling through adenosine receptors are depicted in Figure

2.1, and receptor distribution is described in Table 2.1. Adenosine can also act intracellularly, independently of cell-surface adenosine receptors to inhibit cAMP formation by binding to a P-site on adenylyl cyclase (Dessauer et al., 1999).

Adenosine A_1 receptors couple to pertussis toxin-sensitive G_i proteins, which upon activation lead to reduced adenylyl cyclase activity and activation of phospholipase C, thereby inhibiting cAMP production and causing downstream accumulation of IP_3/DAG, calcium mobilization, and PKC activation (Freund et al., 1994; Iredale et al., 1994; Linden, 2001; Schulte and Fredholm, 2003). Signaling through A_1 receptors also causes activation of potassium channels through the $G_{\beta\gamma}$ subunit of the G protein and inactivation of Q-, P-, and N-type calcium channels (Fredholm et al., 2001; Jacobson and Gao, 2006).

Adenosine A_{2A} receptors couple primarily to G_s proteins, but may also bind to G_{olf} proteins in the striatum (Linden, 2001). In either case, activation of A_{2A} receptors leads to accumulation of cAMP and, in some cases, increased inositol phosphate formation (Schulte and Fredholm, 2003).

Adenosine A_{2B} receptors are considered to be low-affinity adenosine receptors (Feoktistov and Biaggioni, 1998). Activation of A_{2B} in human mast cells and HEK293 cells stimulated calcium mobilization and cAMP accumulation through coupling to $G_{q/11}$ and G_s proteins, respectively (Gao et al., 1999; Linden et al., 1999). ERK1/2 phosphorylation occurred downstream of A_{2B} activation in both HEK293 cells and canine mast cells (Gao et al., 1999), and activation of the arachidonic acid pathway has also been reported (Jacobson and Gao, 2006).

Signaling through adenosine A_3 receptors is very similar to that which occurs with A_1 receptors (Fredholm et al., 2001). Activation leads to adenylyl cyclase inhibition and IP_3/DAG accumulation in a $G_{i/o}$- and possibly G_q-dependent manner (Schulte and Fredholm, 2003).

Changes in mitogen-activated protein kinase (MAPK) signaling may occur downstream of signaling through any adenosine receptor, as depicted in Figure 2.1 (Schulte and Fredholm, 2003; Jacobson and Gao, 2006). In some contexts, adenosine receptors may also interact with other receptor systems, particularly dopamine receptors (Franco et al., 2000; Fuxe et al., 2005).

Biological actions of adenosine

Adenosine plays a significant role in tissue protection, and when produced during hypoxia or ischaemia, serves to protect tissues from further damage (Linden, 2001; Linden, 2005). The mechanisms of adenosine-mediated tissue protection include: (1) increased oxygen supply/demand ratio due to A_{2A}/A_{2B} receptor-mediated vasodilation and A_1 receptor-mediated reduction in oxygen demand; (2) ischaemic preconditioning through A_1 and A_3 receptors, whereby adenosine, released during short periods of ischaemia, reduces infarct size during subsequent periods of prolonged ischaemia; (3) anti-inflammatory responses mediated by A_{2A} receptors; and (4) promotion of angiogenesis (Linden, 2005).

A study by Ohta and Sitkovsky clearly demonstrated the tissue-protective effects of adenosine A_{2A} receptors (Ohta and Sitkovsky, 2001). They showed that genetic inactivation of the A_{2A} receptor in mice resulted in a dramatic increase in the severity of

concanavalin A (ConA)-induced liver injury, leading to the death of some animals. A_{2A}-/- mice were more susceptible to cytokine accumulation and tissue damage in a model of sepsis. Furthermore, ConA-, endotoxin-, and chemically-induced liver damage were exacerbated in wild-type mice treated with the A_{2A} receptor antagonist ZM241385.

Adenosine also plays an important role in wound healing. Topical application of A_{2A} receptor agonists improved wound healing in wild-type mice but not in A_{2A}-/- mice (Montesinos et al., 2002), and promoted wound neovascularization (Montesinos et al., 2004).

This adenosine-mediated promotion of angiogenesis occurs through several mechanisms. The A_{2A} agonist CGS21680 and the relatively non-selective adenosine receptor agonist NECA increased VEGF formation by macrophages (Leibovich et al., 2002). Similarly, NECA increased secretion of VEGF and IL-8 by human mastcells through activation of A_{2B} receptors, and ang-2 production through A_3 receptor activation (Feoktistov et al., 2003). CGS21680 also reduced human microvascular endothelial cell production of the anti-angiogenic factor thrombospondin, and CGS21680-induced vascular tube formation was blocked with an antibody against thrombospondin (Desai et al., 2005). Therefore, adenosine receptor activation promotes angiogenesis by increasing production of pro-angiogenic factors and suppressing production of anti-angiogenic factors.

Adenosine may also promote angiogenesis by stimulating endothelial cell proliferation. Sexl and colleagues showed that ADA reduced proliferation of HUVECs, whereas NECA and CGS21680 stimulated proliferation of HUVECs through activation of A_{2A} receptors (Sexl et al., 1995). This effect was not mimicked with forskolin, 8-Br-

cAMP, or cholera toxin, indicating that it did not involve G_s-mediated cAMP signaling. Furthermore, the effect was not blocked with pertussis toxin, ruling out the involvement of G_i proteins. The exact signaling pathway responsible for A_{2A}-mediated proliferation of HUVECs was not identified.

Other biological functions affected by adenosine include pain mediation, platelet aggregation, blood pressure regulation, mast cell degranulation, bronchoconstriction, vasodilation, sleep regulation, lipolysis, intraocular pressure regulation, motor functions, and cardiac rhythm (Fredholm et al., 2001; Jacobson and Gao, 2006). Activation of adenosine receptors can enhance or inhibit the proliferation of several cell types (Schulte and Fredholm, 2003).

One disease state attributed to defects in adenosine metabolism is severe combined immunodeficiency disease (SCID), in which ADA deficiency leads to depletion of T, B, and NK lymphocytes (Hershfield, 2005). Pathologic effects are mainly due to accumulation of deoxy-adenosine, which inhibits DNA synthesis and promotes release of cytochrome C, leading to apoptosis, but may also involve adenosine receptor-mediated immunosuppression through A_{2A} activation (Huang et al., 1997; Apasov et al., 2000; Hershfield, 2005).

Effects of adenosine on tumour progression

Since hypoxia leads to increased adenosine levels (Headrick and Willis, 1989; Decking et al., 1997), and solid tumours are hypoxic (Vaupel et al., 2004), Blay and colleagues hypothesized that adenosine levels would be elevated within hypoxic solid tumours (Blay et al., 1997). Human lung (A549) or colorectal (T-84, HT-29) carcinoma

cells or mouse MCA-38 colon carcinoma cells were injected s.c. in mice and, after tumours had formed, adenosine in the extracellular fluid was collected using microdialysis. Adenosine concentrations within these tumours were measured using HPLC, and the mean intratumoural concentration of this nucleoside was 0.49 μM. This value may underestimate concentrations within the hypoxic regions of tumours, since the dialysis probe would be surrounded by both hypoxic and normoxic areas of the tumour. The adenosine concentration in subcutaneous tissue of a control flank was only 30 nM, indicating that there is at least a ten-fold increase in adenosine production in tumours compared to normal tissue. When the microdialysis probe was perfused with the ADA inhibitor coformycin and the AK inhibitor 5-iodotubercidin to prevent adenosine breakdown, the mean extracellular adenosine concentration was 8.9 μM in tumours derived from HT-29 cells, a more than 100-fold increase over that found in normal tissue. In general, ecto-5'-NT activity tends to be increased in cancer, and ADA activity tends to be decreased; both of these changes may contribute to elevated adenosine levels noted within tumours (Spychala, 2000; Durak et al., 2005).

Adenosine has been shown to suppress anti-tumour immune responses in a variety of contexts. Adenosine, in combination with the ADA inhibitor coformycin, inhibited the anti-CD3-induced tumouricidal activity of activated killer T (AK-T) cells against P815 mastocytoma target cells (Hoskin et al., 1994). Adenosine also was shown to inhibit the proliferation of AK-T cells, and reduce the production of the cytolytic effector molecules granzyme B, perforin, Fas ligand, and TRAIL (Hoskin et al., 2002). Adenosine-mediated inhibition of AK-T cell proliferation was found to be a result of A_3 adenosine receptor activation. Adenosine was also shown to inhibit integrin $\alpha_4\beta_7$-mediated adhesion of AK-

T cells to murine MCA-38 colon carcinoma cells through activation of A_3 receptors on AK-T cells (MacKenzie et al., 1994; MacKenzie et al., 2002).

Previous work done in our laboratory showed that adenosine consistently increased the proliferation of several human colorectal and breast carcinoma cell lines, with an EC_{50} ranging from 3.8-30 μM (Mujoomdar et al., 2003; Mujoomdar et al., 2004). Proliferative responses were shown using both [^{3}H]thymidine incorporation assays and direct measurements of cell number. Maximal effects occurred when cells were seeded at low density (Mujoomdar et al., 2003; Mujoomdar et al., 2004) and cultured with low serum concentrations (Mujoomdar et al., 2004).

Further work in our laborabory demonstrated that adenosine reduced mRNA and cell-surface protein expression of dipeptidyl peptidase IV (DPPIV) on HT-29 human colorectal cancer cells, an effect associated with increased tumour invasion and metastasis (Tan et al., 2004). Interestingly, DPPIV can cleave and inactivate CXCL12 (Proost et al., 1998), so reduced expression would be expected to lead to increased availability of CXCL12 (Mizokami et al., 2004). Adenosine-mediated DPPIV down-regulation occurred with continuous exposure to adenosine concentrations as low as 12.5 μM, and was enhanced with the ADA inhibitor coformycin (Tan et al., 2004). The adenosine effect on DPPIV was not mediated by activation of traditional adenosine receptors, but rather occurred through activation of protein tyrosine phosphatases and subsequent reduction in phosphorylation of ERK1/2 (Tan et al., 2006).

Woodhouse and colleagues showed that adenosine and AMP stimulated chemotaxis and chemokinesis of A2058 human melanoma cells with an EC_{50} of 1.8 μM (Woodhouse et al., 1998). This effect was mimicked with the A_1-selective agonists CPA

and R-PIA, and blocked with the A_1 antagonists CPT and DPCPX. Furthermore, transfection of CHO cells with A_1 receptors allowed for a chemotactic response to adenosine, which was blocked with CPT and DPCPX, indicating that adenosine-mediated cell motility was due to activation of A_1 receptors. Similarly, our laboratory has also shown that adenosine stimulates migration of HRT-18 colorectal carcinoma cells (Mujoomdar M, Blay J, unpublished observations).

Adenosine receptor expression has been detected in several cancer types (Merighi et al., 2003). Despite the fact that we and others believe that adenosine promotes tumour progression through multiple mechanisms (Spychala, 2000), other groups hold that adenosine may inhibit tumour cell growth. For example, Barry and colleagues found that high concentrations (500 μM) of adenosine, AMP, ADP, and ATP reduced the growth of several cancer cell lines, including MCF-7, MDA-MB-231, OVCAR-3, OVCAR-5, and HT-29 cells (Barry and Lind, 2000). This effect of adenosine was enhanced with the ADA inhibitor coformycin and was blocked with the nucleoside transport inhibitor dipyridamole, indicating that the effect occurred at the intracellular level. Adenosine also inhibited the growth of 1321N1 astrocytoma cells in an intracellular fashion (Bradley and Bradley, 2001). Adenosine inhibited the growth of MDA-MB-468 and MCF-7 human breast cancer cells through initiation of apoptosis (Hashemi et al., 2005); however, this effect required concentrations of at least 500 μM. The nucleoside transport inhibitor NBTI blocked the growth inhibitory effect of adenosine, indicating that the effect occurred at the intracellular level. Millimolar doses of adenosine also reduced cell viability and induced apoptosis of GT3-TKB human gastric cancer cells (Saitoh et al., 2004). Once again, adenosine required cellular uptake to induce apoptosis.

It is interesting that in each of these studies, adenosine concentrations required to inhibit cell growth were very high, and far exceeded the low micromolar concentrations found to be present within tumours (Blay et al., 1997). Our laboratory has shown that adenosine-mediated stimulation of proliferation occurs at low micromolar concentrations of adenosine that would be present within tumour extracellular fluid (Mujoomdar et al., 2003; Mujoomdar et al., 2004).

Some investigators have suggested that A_3 adenosine receptor agonists may be useful in cancer treatment, and have argued that these agents, as well as adenosine itself, may selectively target cancer cells (Fishman et al., 2002; Lu et al., 2003; Merighi et al., 2003; Ohana et al., 2003; Fishman et al., 2004; Gessi et al., 2004). In 73 colorectal cancer patient samples, higher expression of the A_3 adenosine receptor was found on cancerous versus normal tissues (Gessi et al., 2004). This increase was seen at the protein level, but not at the mRNA level, and was also reflected on peripheral blood cells, indicating it may be useful as a diagnostic marker. Fishman and colleagues found that the A_3 receptor agonist CF101 reduced the growth of HCT-116 tumours grown subcutaneously in nude mice (Fishman et al., 2004). CF101 also reduced the formation of liver metastasis in mice inoculated in the spleen with CT-26 murine colon carcinoma cells (Ohana et al., 2003). In an HCT-116 xenograft model, CF101 had additive anti-tumour effects when combined with 5-fluorouracil (5-FU), and prevented 5-FU-induced myelotoxicity. IB-MECA, an A_3 receptor agonist, inhibited the anchorage-independent growth of MCF-7, ZR-75, T47D, and Hs578T breast cancer cells (Lu et al., 2003). However, this was not due to activation of A_3 receptors, because A_3 receptor mRNA was not detected in these cells and over-expression of A_3 made no difference in the response

to IB-MECA. Instead, IB-MECA-induced growth inhibition was mediated by reduction in estrogen receptor expression. Adenosine itself also inhibited anchorage-independent growth of MCF-7 cells, but a dose of 1 mM was required for this effect.

Although there is some controversy in the literature as to whether adenosine inhibits or promotes tumour progression, it is clear that adenosine is present at high concentrations within tumors (Blay et al., 1997), and thus may be one factor that contributes to high CXCR4 expression on tumours.

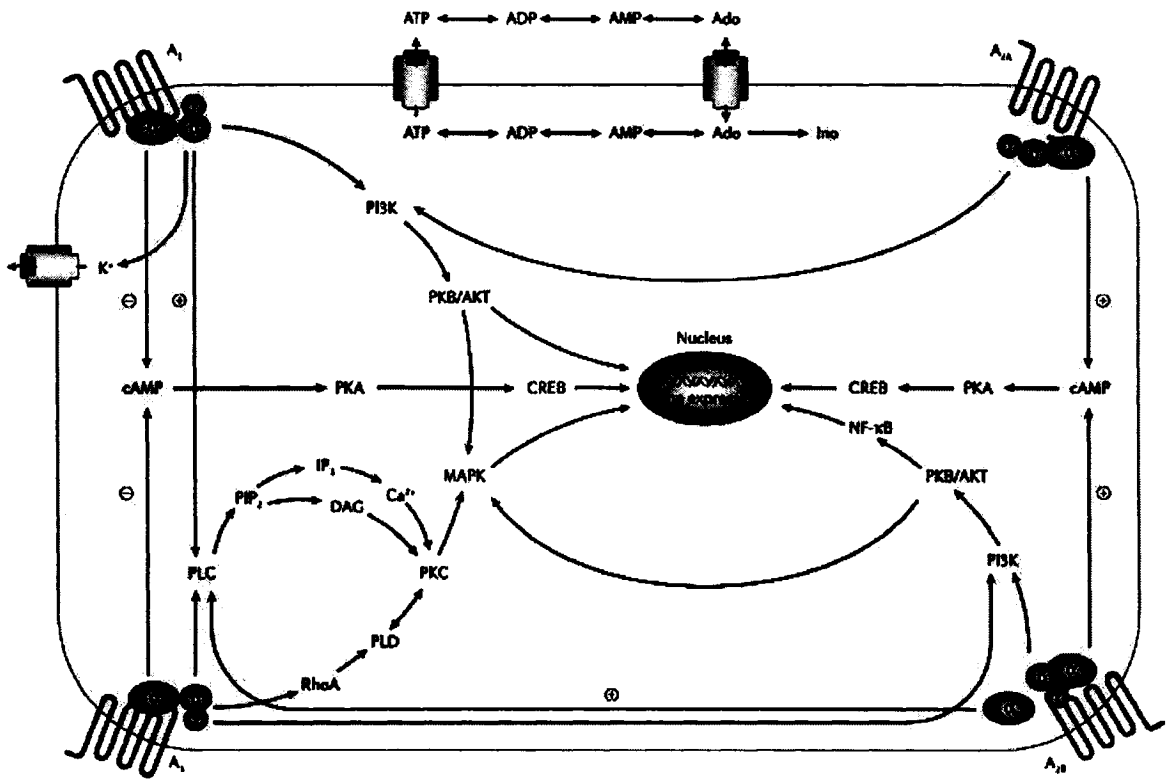


Figure 2.1: Signaling through adenosine receptors. Taken from Jacobson and Gao, 2006.

Receptor	Distribution
A_1	High: brain, dorsal horn of the spinal cord, eye, adrenal gland, atria Intermediate: skeletal muscle, liver, kidney, adipose tissue, salivary glands, esophagus, colon, testis Low: lung, pancreas
A_{2A}	High: spleen, thymus, leukocytes, blood platelets, striatopallidal GABAergic neurons, olfactory bulb Intermediate: heart, lung, blood vessels Low: other brain regions
A_{2B}	High: caecum, colon, bladder Intermediate: lung, blood vessels, eye, mast cells Low: adipose tissue, adrenal gland, brain, kidney, liver, ovary, pituitary gland
A_3	Expression has not been well-characterized; expressed in most parts of the brain, lung, adrenal gland, thyroid, spleen, liver, heart, intestine, and testis

Table 2.1: Adenosine receptor distribution. Adapted from Fredholm et al., 2001.

Objectives

Adenosine is present in high levels within tumours due to the presence of hypoxia (Blay et al., 1997), and several tumour types have been shown to express adenosine receptors (Merighi et al., 2003). Since CXCR4 expression on colorectal carcinoma cells is elevated by constituents of the tumour microenvironment (Zeelenberg et al., 2003), we sought to determine if adenosine might be one factor that contributes to high CXCR4 expression.

The objectives for the work described in this chapter were:

1. To determine whether the tumour metabolite adenosine regulates CXCR4 expression on colorectal carcinoma cells,
2. To identify the signaling pathways involved in any adenosine-mediated changes in CXCR4 expression, and
3. To determine the functional consequences of any adenosine-mediated changes in CXCR4 expression.

Hypothesis

Adenosine, which is present in high concentrations within solid tumours, elevates CXCR4 expression on colorectal carcinoma cells through activation of adenosine receptors, and this leads to increased migration and proliferation of cancer cells in response to CXCL12.

METHODS

Materials

We obtained HT-29 human colorectal carcinoma cells from the American Type Culture Collection (Manassas, VA). Media, sera, culture vessels (Nunc), TRIzol® Reagent, oligo(dT)$_{12-18}$ primer, dNTP mix, M-MLV reverse transcriptase, DTT, and 5x first strand buffer were from Invitrogen Canada (Burlington, Ontario, Canada). Adenosine, guanosine, DPCPX, CSC, alloxazine, MRS1523, NBTI, dilazep, ADA, type V collagen, custom primers, Mayer's haematoxylin solution, BSA, and sodium azide were from Sigma Chemical Co. (St. Louis, MO). EHNA was from Research Biochemicals International (Natick, MA). Inosine was from ICN Biochemicals (Irvine, CA). Coformycin was from Calbiochem (San Diego, CA). PFA was purchased from TAAB laboratories (Aldermaston, UK). Mouse anti-human monoclonal antibody against CXCR4 (clone 12G5) and mouse IgG$_{2a}$ (clone G155-178) were from BD Pharmingen (San Diego, CA). ^{125}I-labeled goat anti-mouse IgG was purchased from Perkin Elmer Life Sciences (NEN, Boston, MA). Alexa Fluor 488 goat anti-mouse IgG was from Molecular Probes (Eugene, OR). Brilliant SYBR® Green kits were from Stratagene (Cedar Creek, TX). CXCL12 was from Chemicon International (Temecula, CA). Transwell® polycarbonate inserts (8-μM pore size) were from Corning Inc. (Corning, NY). [^{3}H]Thymidine was from Amersham Biosciences Inc. (Baie d'Urfé, Quebec, Canada). Cytoseal™ 60 was from Richard-Allan Scientific (Kalamazoo, MI).

Cell Culture

HT-29 human colorectal carcinoma cells were cultured in the absence of antibiotics in DMEM containing 5% v/v newborn calf serum (NCS), and were maintained at 37°C in 80 cm^2 flasks in a humidified atmosphere of 90% air/10% CO_2. Cells were routinely passaged at sub-confluent densities by brief exposure to 0.05% trypsin/0.53 mM EDTA.

For binding assays, cells were seeded into 48-well plates in DMEM containing 10% NCS v/v at a density of 50,000 cells/well. For flow cytofluorimetry, HT-29 cells were seeded at approximately 25% of confluent density in 10-cm dishes. For real-time PCR, 35-mm plates were seeded with 500,000 cells; and for migration assays, 10^6 cells were seeded into 10-cm dishes. Cultures for proliferation assays were seeded at 25% of confluent density in 24-well plates. In all culture situations, cells were first allowed to attach for 48 h. The medium was then replaced with DMEM containing 1% NCS v/v, and after a further 48 h the cultures were treated with drugs or vehicle controls. All wells were exposed to the same final concentration of vehicle, which was either DMEM (adenosine, inosine, guanosine, 8-Br-cAMP, CXCL12, coformycin), water (EHNA, dilazep, Rp-cAMPs) or DMSO (NBTI, adenosine receptor antagonists, forskolin). The final DMSO concentration was 0.5% (v/v) in experiments with Rp-cAMPs, but did not exceed 0.05% (v/v) in other experiments. ADA inhibitors, nucleoside transport inhibitors, adenosine receptor antagonists and Rp-cAMPs were added 30 min before adenosine itself.

Radioantibody Binding Assay

An indirect radioantibody binding assay that provides quantitative measurement of proteins exposed on cultured cell monolayers (Tan et al., 2004) was used to measure cell-surface CXCR4 protein levels. Binding assays were performed after 48 h unless otherwise indicated. All steps prior to solubilization were performed at 4°C. Monolayer cultures were washed with binding assay buffer (BAB; 137 mM NaCl, 5 mM KCl, 24.8 mM Tris, 0.7 mM Na_2PO_4, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$) containing 0.2% BSA and then incubated with 125 µl BAB containing 1% BSA and 1 µg/ml of anti-CXCR4 antibody or isotype control. After a 60-min incubation, the cells were washed twice and further incubated with 125 µl BAB containing 1% BSA and 1 µCi/ml ^{125}I-labeled goat anti-mouse IgG for 60 min. The monolayers were then washed three times, and solubilized in 500 µl 0.5 M NaOH, followed by counting of radioactivity. The CXCR4-specific radioactivity was determined by subtracting the result for the corresponding isotype control, which typically ranged from 200-500 cpm. Cell counts were performed using a Coulter® Model ZM30383 particle counter (Beckman Coulter, Mississauga, Ontario, Canada), and results were corrected to cpm per 1000 cells.

Flow cytofluorimetry

Flow cytofluorimetry was also used to measure cell-surface CXCR4 protein. After a 48 h treatment with vehicle or 300 µM adenosine, HT-29 cells were released by brief exposure to trypsin/EDTA. $2x10^6$ cells were re-suspended in "FACS buffer", which consisted of PBS (140 mM NaCl, 2.7 mm KCl, 8 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$) with 2.5% BSA and 0.2% sodium azide, washed twice, and

incubated in 200 µl FACS buffer containing 4 µg anti-CXCR4 antibody or isotype control for 40 min on ice with shaking. The cells were then washed twice, and incubated in 200 µl FACS buffer containing Alexa Fluor 488 goat anti-mouse IgG (1:400 dilution) in the dark for 45 min on ice with shaking. After washing the cells three times, the cells were fixed with 1% PFA in PBS, and then analyzed with a FACScan flow cytometer (BD Immunocytometry Systems, Mountain View, CA). Forty thousand events were counted for each sample. Data were analyzed using WinMDI software.

Real-time PCR

Real-time PCR was used to quantify CXCR4 mRNA expression. Total RNA was extracted from adenosine- and vehicle-treated HT-29 cells using TRIzol® according to the manufacturer's instructions. Five micrograms of total RNA were reverse transcribed to complementary DNA (cDNA) using M-MLV reverse transcriptase and oligo$(dT)_{12\text{-}18}$ primer. Brilliant SYBR® Green was combined with cDNA, and real-time PCR amplification was performed using a Stratagene Mx3000P system (Cedar Creek, TX) with the following primer sets:

1. GAPDH: forward - 5'-catgagaagtatgacaacagcct-3';
   reverse - 5'-agtccttccacgataccaaagt-3'
2. CXCR4: forward - 5'-gcctgagtgctccagtagcc-3';
   reverse - 5'-tggagtcatagtcccctgagc-3'

Relative CXCR4 gene expression was analyzed using the manufacturer's software, standardized to GAPDH expression, and normalized to control expression at 0 h using the $2^{-\Delta\Delta cT}$ method (Livak and Schmittgen, 2001).

Cell migration assay

Migration assays were used to detect migratory responses of HT-29 cells to CXCL12. Transwell® culture inserts were coated overnight at 37°C with 3 μg/ml type V collagen and then washed with serum-free DMEM. Adenosine and vehicle-treated cells were released from culture by brief exposure to trypsin/EDTA and re-suspended in serum-free DMEM containing 0.1% BSA at $2.5x10^6$ cells/ml. One hundred microlitres of cell suspension were added to the upper chamber, and 600 μl of DMEM containing 0.1% BSA and 200 ng/ml CXCL12 or vehicle control were added to the bottom chamber. Chambers were incubated for 18 h at 37°C, and filters were fixed with ethanol and stained with Mayer's haematoxylin. Cells remaining on the upper surface of the membrane were removed using a cotton-tipped applicator, and the filter was mounted using Cytoseal 60®. Cells that had migrated to the lower surface of the membrane were visualized microscopically under 400x magnification by a blinded observer. Cells in 25 fields were counted.

Cell proliferation assay

A radiolabeled thymidine incorporation assay was used to measure changes in DNA synthesis as an indicator of proliferation in response to CXCL12. After a 48 h treatment with adenosine or vehicle, cells in 24-well plates were washed free of residual nucleoside. CXCL12 or vehicle was then added together with [^{3}H]thymidine (1 μCi/ml) and unlabeled thymidine (1 μM). The plates were incubated for 24 h to allow incorporation of [^{3}H]thymidine into newly-synthesized DNA. The plates were then placed on ice, washed twice with PBS and treated with 10% trichloroacetic acid to

precipitate DNA. The trichloroacetic acid-precipitable material was rinsed with ethanol, solubilized in 0.1M NaOH containing 1% SDS, and added to vials containing acidified scintillation fluid. Radioactivity was determined using a Beckman LS 6500 liquid scintillation counter (Beckman Coulter Canada, Mississauga, Ontario, Canada).

Statistical analysis

Each figure shows a representative result from a series of experiments done on at least three independent occasions. Data were analyzed using a two-tailed Student *t*-test for unpaired data and are indicated as such if significant at the $P<0.05$ (*, #) or $P<0.01$ (**, ##) level. Where appropriate, analysis of variance (ANOVA) was performed first, followed by individual *t*-tests with Bonferroni correction using GraphPad Prism software.

RESULTS

Adenosine increases cell-surface CXCR4 protein expression

We screened several colorectal carcinoma cells lines for CXCR4 protein expression (ex. HT-29, CaCo-2, HRT-18, Colo320HSR, SW480, SW620), and found that HT-29, SW480 and SW620 cells expressed cell-surface CXCR4 protein, consistent with what has been shown in the literature (Dwinell et al., 1999; Jordan et al., 1999; Ottaiano et al., 2005; Schimanski et al., 2005). HT-29 cells expressed the highest levels of CXCR4, and have been shown in our laboratory to be responsive to adenosine (Mujoomdar et al., 2003; Tan et al., 2004). Therefore, the HT-29 human colorectal carcinoma cell line was used to study the effects of adenosine on CXCR4 expression. Cell-surface CXCR4 protein expression was primarily quantified using a radioantibody binding assay that selectively detects receptors present at the cell-surface (Tan et al., 2004). All results were normalized to cell number to take into account any changes in viability or proliferation caused by drug treatments. Maximal drug concentrations were chosen such that they did not induce more than a 20% change in cell number, and typically caused less than a 10% change in cell number.

Adenosine is present in elevated concentrations in tissues under hypoxic conditions, such as those observed within solid tumours (Blay et al., 1997). To determine if adenosine may be one factor within the tumour microenvironment that contributes to elevated CXCR4 expression, HT-29 cells were treated with a single dose of adenosine (300 μM), and cell-surface CXCR4 protein expression was measured after 0, 1, 8, 24, 48, and 72 h. As shown in Figure 2.2, adenosine produced a significant increase in CXCR4 expression that was first evident at the 24 h time point and began to decline at the 72 h

time point. The maximum up-regulation was seen after 48 h, at which time cell-surface CXCR4 protein expression was approximately 2.5 times higher on adenosine-treated cells than on control cells. Changes in the baseline level of CXCR4 that occurred over the 72 h time course were likely related to cell cycle-dependent variation in CXCR4 expression (Shibuta et al., 2002). Since the maximal adenosine effect was noted at 48 h, this time point was chosen for future analyses of adenosine-induced changes in cell-surface CXCR4 protein.

We performed flow cytofluorimetry to confirm that adenosine increases cell-surface CXCR4 protein. HT-29 cells were treated for 48 h with adenosine (300 μM), followed by trypsinization and staining using an antibody specific for CXCR4. As shown in Figure 2.3, untreated cells expressed levels of cell-surface CXCR4 protein that were barely detectable using flow cytofluorimetry. However, after treatment with adenosine, cell-surface CXCR4 protein was up-regulated on HT-29 cells such that it was detectable on a large proportion of the cells. With this method, we detected up to a 9-fold increase in cell-surface CXCR4 protein expression, suggesting that flow cytofluorimetry may be a more desirable method for measuring adenosine-mediated changes in cell-surface CXCR4 protein. However, flow cytofluorimetry requires detachment of cells by trypsinization, which may activate intracellular signaling pathways (Miller and McGee, 2002). Using radioantibody binding assays, cells are left as monolayer cultures attached to plastic, which would mimic the usual configuration in which cells are attached to a substratum. Therefore, we chose to use radioantibody binding assays for further measurements of cell-surface CXCR4 protein.

Adenosine increases CXCR4 mRNA expression

Colorectal carcinoma cells, including HT-29 cells, express CXCR4 in the cytosol as well as at the cell surface (Dwinell et al., 1999; Jordan et al., 1999; Schimanski et al., 2005). Therefore, changes in cell-surface CXCR4 protein expression could reflect, among other possibilities, an increase in trafficking of the protein to the cell surface, or an increase in transcription. To determine if adenosine increased CXCR4 expression at the level of mRNA transcription, we treated HT-29 cells with a single dose of adenosine (300 μM), and isolated RNA after 0, 24, 48, and 72 h. CXCR4 mRNA expression was quantified using real-time RT-PCR. Adenosine caused a substantial increase in CXCR4 mRNA expression after 24 h, which persisted for at least 72 h (Figure 2.4). The maximal increase in CXCR4 mRNA expression preceded maximal changes in cell-surface CXCR4 protein expression, suggesting that the effect of adenosine on cell-surface CXCR4 protein likely resulted from increased transcription.

Adenosine up-regulates CXCR4 at concentrations present in the tumour microenvironment

We examined the dose-dependency of adenosine regulation of cell-surface CXCR4 protein expression on HT-29 cells, and found that the EC_{50} was approximately 30 μM (Figure 2.5). The maximum effect was noted with 300 μM adenosine. The response diminished with 1000 μM adenosine, likely due to toxicity (data not shown).

An adenosine concentration of 30 μM exceeds that which is present within the extracellular fluid of tumours (Blay et al., 1997). However, it was found that the half-life of adenosine in monolayer HT-29 cultures was approximately 2 h (Mujoomdar et al.,

2003). Therefore, the concentration due to a single dose of adenosine would not be maintained in culture over a 48 h period. To achieve persistent levels of adenosine, we treated HT-29 cells with sequential treatments, and found that cell-surface CXCR4 expression was significantly elevated with a dose of 10 μM adenosine administered five times, or 3 μM administered twelve times over 48 h (Figure 2.6). The adenosine concentration in the extracellular fluid of solid tumours is approximately 1-10 μM (Blay et al., 1997). Therefore, by exposing HT-29 cells to lower, persistent concentrations of adenosine, we were able to show that adenosine-mediated CXCR4 up-regulation occurred at pathophysiologically-relevant concentrations, indicating that adenosine *in vivo* may contribute to elevated CXCR4 expression in tumours.

Adenosine-mediated CXCR4 up-regulation is not due to formation of inosine

Adenosine can be converted to its deamination product inosine through the action of adenosine deaminase (ADA). ADA was originally thought to be located in the cytosol, but was discovered to also be present at the cell membrane (ecto-ADA), where it can regulate extracellular adenosine concentrations through conversion to inosine (Aran et al., 1991). Previous studies in our laboratory showed that HT-29 cells express ecto-ADA (Tan EY, Blay J, unpublished observations). Therefore, cellular effects attributed to exogenously added adenosine could be due the formation of inosine. As shown in Figure 2.7, although adenosine (300 μM) produced a robust up-regulation in cell-surface CXCR4 protein on HT-29 cells, this same effect was not seen with an equimolar concentration of inosine, suggesting that the effect is due to adenosine itself rather than its deamination metabolite. The purine nucleoside guanosine (300 μM) was without

effect on CXCR4, indicating that CXCR4 up-regulation was not a non-specific purine effect, but rather was specific for adenosine (Figure 2.7).

The removal of adenosine through its conversion to inosine can be blocked by the addition of ADA inhibitors such as erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) or coformycin. HT-29 cells were pre-treated for 30 min with either EHNA or coformycin, followed by a 48 h exposure to 100 μM adenosine. In the absence of adenosine, neither ADA inhibitor affected CXCR4 protein expression (Figure 2.8). However, adenosine produced a significant up-regulation of cell-surface CXCR4 protein expression, which was further increased by pre-treatment with either EHNA or coformycin. Therefore, inhibition of the conversion of adenosine to inosine enhanced adenosine-mediated CXCR4 up-regulation, providing evidence of an adenosine-dependent effect.

To further confirm that the increase in CXCR4 was due to adenosine itself, and not inosine, we treated culture supernatants from adenosine-treated cells with ADA, which would convert any adenosine in the media to inosine. Briefly, HT-29 cells were treated with a single dose of adenosine (300 μM), and supernatants were removed after 0, 1, 2, 4, 8, 16, 24, and 48 h. The supernatants were then either untreated or treated with ADA, added to fresh HT-29 cultures, and cell-surface CXCR4 was assayed after 48 h. Supernatants taken from cultures exposed to adenosine for up to 48 h produced an up-regulation in cell-surface CXCR4 protein expression (Figure 2.9A). However, when the supernatants were treated with ADA, the CXCR4 up-regulation was substantially diminished, confirming that adenosine itself produced this effect (Figure 2.9B). Furthermore, since the ADA-treated supernatants would contain high concentrations of inosine, this ruled out the possibility that adenosine-mediated CXCR4 up-regulation was

mediated by inosine formation. This experiment also suggested that adenosine increased CXCR4 through an intracellular signaling pathway rather than through the release of an extracellular autocrine factor, since removal of adenosine from the culture supernatants was sufficient to dramatically reduce their ability to up-regulate CXCR4.

Adenosine-mediated CXCR4 up-regulation occurs through an extracellular mechanism

Adenosine typically exerts its cellular effects through its action on cell-surface adenosine receptors (Fredholm et al., 2000). Alternatively, adenosine can be transported into cells by the action of equilibrative or concentrative nucleoside transporters (Thorn and Jarvis, 1996), and act in an intracellular fashion to directly inhibit adenylyl cyclase by binding to a "P"-site (Dessauer et al., 1999). To determine if the adenosine effect on CXCR4 occurred at the extracellular level or required its uptake into the cell, we used the transport inhibitors S(4-nitrobenzyl)-6-thioinosine (NBTI) and dilazep to block adenosine uptake. Figure 2.10 shows that neither NBTI (1 μM) nor dilazep (5 μM) blocked adenosine-mediated CXCR4 up-regulation, nor did a combination of the two nucleoside transport inhibitors. Therefore, the adenosine effect on CXCR4 protein did not require transport into the cells, but rather occurred through an extracellular mechanism.

Adenosine-mediated CXCR4 up-regulation occurs through activation of adenosine receptors

There are four known adenosine receptors – A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 2001). Each receptor is a seven-transmembrane G-protein coupled receptor, and all four are expressed in HT-29 cells (Mujoomdar and Blay, unpublished observations). We used the following selective antagonists against each of the receptor subtypes to determine receptor involvement in adenosine-mediated CXCR4 up-regulation: 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, A_1), 8-(-3-chlorostyryl)caffeine (CSC, A_{2A}), benzo[g]pteridine-2,4[1H,3H]-dione (alloxazine, A_{2B}), and 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate (MRS1523, A_3). For all experiments, adenosine receptor antagonists were added 30 min before adenosine to allow time to diffuse through the culture media and bind to the receptor. The adenosine receptor antagonist concentrations that were chosen (1 μM for DPCPX, CSC, and MRS1523, 5 μM for alloxazine) are well above the known Ki values for each receptor (Ji and Jacobson, 1999; Moro et al., 2006), and did not produce any change in cell number when present in cell culture for 48 h. We chose a dose of 50 μM for adenosine, which was sufficient to induce a reproducible up-regulation of CXCR4, but low enough that it could likely be blocked by the use of adenosine receptor antagonists.

No single adenosine receptor antagonist completely blocked the effect of adenosine on CXCR4 protein (Figure 2.11). DPCPX (A_1) and MRS1523 (A_3) were both completely without effect (Figure 2.11A), whereas CSC (A_{2A}) and alloxazine (A_{2B}) both reduced the magnitude of the adenosine-mediated CXCR4 up-regulation (Figure 2.11B). Adenosine caused a 49% increase in cell-surface CXCR4 expression, which was reduced

to 31% with CSC and 19% with alloxazine. This suggested the involvement, at least in part, of adenosine A_{2A} and A_{2B} receptors.

In some circumstances, the cellular effects of adenosine can be mediated through activation of more than one adenosine receptor subtype (Zhang et al., 2004). Therefore, we used combinations of adenosine receptor antagonists to determine if the adenosine effect on CXCR4 could be mediated in this way. As shown in Figure 2.12A, blockade of all four adenosine receptors, using DPCPX, CSC, alloxazine, and MRS1523, completely abrogated adenosine-mediated CXCR4 up-regulation, indicating that this effect was indeed mediated by adenosine receptors. In fact, blockade of the A_{2A} and A_{2B} receptors alone, using CSC and alloxazine, was sufficient to completely block the adenosine effect, suggesting that adenosine signals through these two receptors to regulate CXCR4 protein expression (Figure 2.12B). The combination of DPCPX and MRS1523 antagonists had no impact on the adenosine effect (Figure 2.12C), whereas the combination of alloxazine and MRS1523 partially reduced the effect, likely due to blockade of the A_{2B} receptor with alloxazine (Figure 2.12D). Our experiments with adenosine receptor antagonists suggested that adenosine-mediated CXCR4 up-regulation occurred through its combined actions on A_{2A} and A_{2B} receptors.

Adenosine-mediated CXCR4 up-regulation does not involve cAMP/PKA signaling

Adenosine A_{2A} and A_{2B} receptors typically couple to G_s proteins, leading to activation of adenylyl cyclase and subsequent increased intracellular levels of cAMP, which in turns increases the activity of PKA (Fredholm et al., 2001). Since A_{2A}/A_{2B} receptor activation was found to be responsible for adenosine-mediated CXCR4 up-

regulation, we speculated that downstream signaling through the cAMP/PKA pathway was involved. Indeed, in several cell types, activation of this pathway has been shown to increase CXCR4 expression (Cole et al., 1999; Cristillo et al., 2002; Ödemis et al., 2002; Salcedo et al., 2003). However, we found that treatment of HT-29 cells with the stable, cell-permeable cAMP analogue 8-Br-cAMP (1 mM) did not affect cell-surface CXCR4 expression, nor did the direct adenylyl cyclase activator forskolin (50 μM, Figure 2.13A). Furthermore, the PKA inhibitor Rp-cAMPs (50 μM) did not block adenosine-mediated CXCR4 up-regulation (Figure 2.13B). These findings argue against the involvement of cAMP/PKA signaling in the adenosine effect on CXCR4 protein expression.

Adenosine-mediated CXCR4 up-regulation leads to increased migratory and proliferative responses to CXCL12

Cells expressing CXCR4 can respond to CXCL12 by migrating or proliferating (Müller et al., 2001; Scotton et al., 2001; Scotton et al., 2002; Zeelenberg et al., 2003; Oonakahara et al., 2004). In order to assess the functional consequences of adenosine-mediated CXCR4 up-regulation on HT-29 cells, we performed chemotaxis assays to assess the migratory potential of these cells, and radiolabeled thymidine incorporation assays to assess DNA synthesis and therefore proliferation.

For the migration assays, HT-29 cells were pre-treated for 48 h with adenosine (100 μM) and then seeded onto collagen-coated polycarbonate filters with 8 μm pores (Transwell® inserts). CXCL12 (200 ng/ml) was placed in the lower chamber, and cells that migrated towards CXCL12 and attached on the underside of the filter were counted. In the experiment shown in Figure 2.14, vehicle pre-treated cells did not migrate towards

CXCL12. In other experiments, there was a small migratory effect in response to CXCL12, but this did not reach statistical significance in any experiment. On the other hand, HT-29 cells pre-treated with adenosine consistently migrated towards CXCL12. As shown in Figure 2.14, the number of adenosine pre-treated cells that had migrated to the underside of the filter doubled when CXCL12 was present in the lower chamber. Therefore, adenosine-induced CXCR4 up-regulation allowed CXCL12 to produce a chemotactic response in HT-29 cells.

In the proliferation assay, HT-29 cells were again pre-treated for 48 h with adenosine (300 μM), followed by a washout to remove any remaining adenosine, since adenosine itself has been shown to stimulate proliferation of HT-29 cells (Mujoomdar et al., 2003). The cells were then treated with CXCL12, and [^{3}H]thymidine (1 μCi/ml) was added. [^{3}H]thymidine incorporation was measured as an indicator of DNA synthesis after 24 h. Vehicle pre-treated HT-29 cells only had a proliferative response to the highest concentration of CXCL12 used (200 ng/ml), whereas adenosine pre-treated cells responded in a proliferative manner even to the lowest dose of CXCL12 (50 ng/ml, Figure 2.15). Furthermore, it appeared that adenosine pre-treatment produced a leftward shift in the dose response curve to CXCL12, indicating an increased responsiveness. Therefore, both the migration and proliferation assays showed that adenosine-mediated CXCR4 up-regulation resulted in increased ability of HT-29 cells to respond to CXCL12.

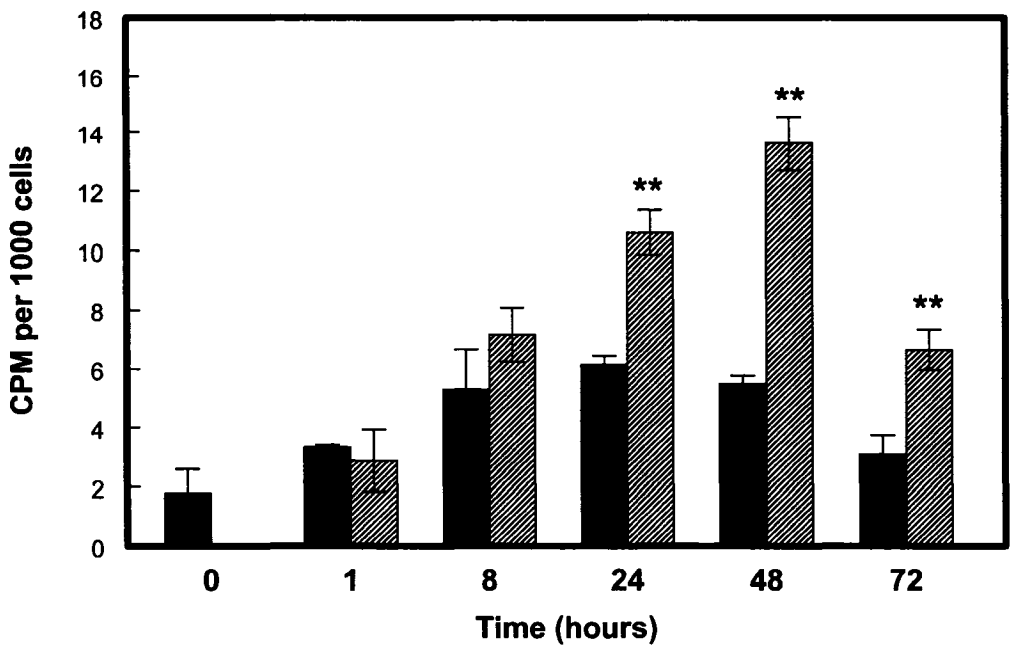


Figure 2.2: Effect of a single dose of adenosine on cell-surface CXCR4 protein expression on HT-29 cells. HT-29 cells were treated with a single dose of vehicle (solid bars) or 300 μM adenosine (hatched bars), and cell-surface CXCR4 protein was measured at the indicated time point using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant increase due to adenosine, P<0.01.

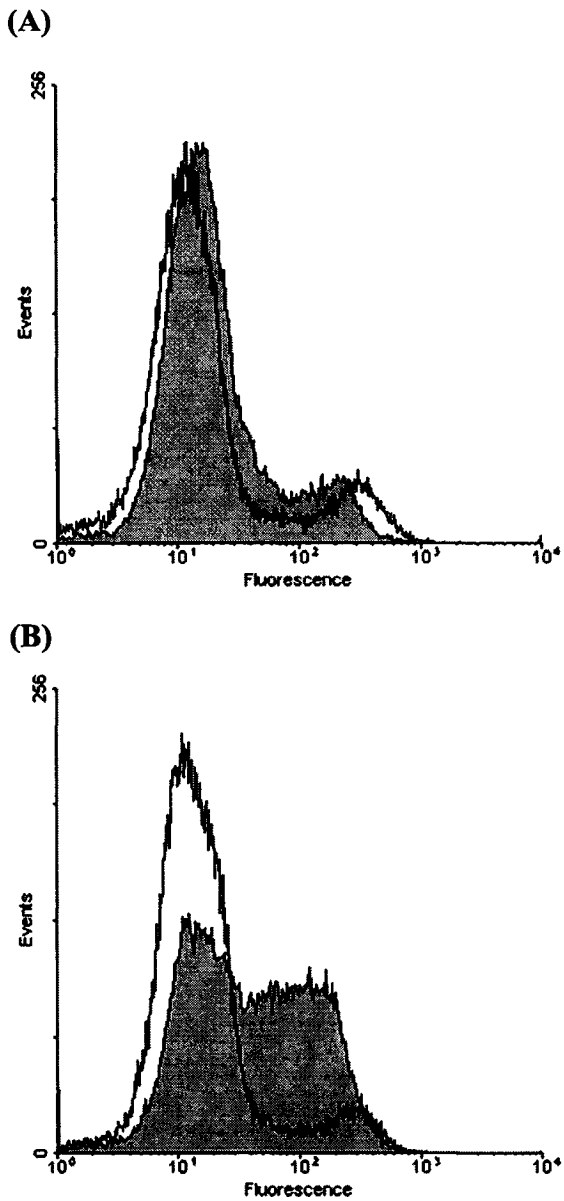


Figure 2.3: Flow cytofluorimetric detection of cell-surface CXCR4 protein expression on HT-29 cells treated with adenosine. HT-29 cells were treated with (A) vehicle or (B) 300 μM adenosine for 48 h, followed by staining with control antibody (open peaks) or anti-CXCR4 antibody (shaded peaks). Cells were analyzed by flow cytofluorimetry.

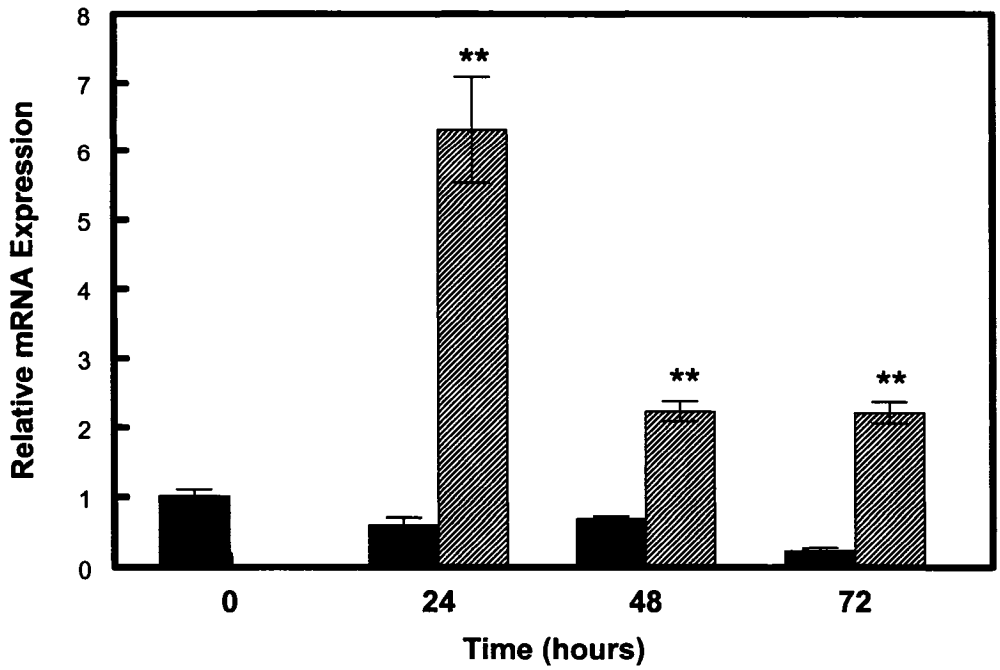


Figure 2.4: Effect of adenosine on CXCR4 mRNA expression in HT-29 cells. HT-29 cells were treated with a single dose of vehicle (solid bars) or 300 μM adenosine (hatched bars), and RNA was isolated at the indicated time points. CXCR4 mRNA was quantified using real-time PCR. The data are mean values ± SE (n=3). **, significant increase due to adenosine, P<0.01.

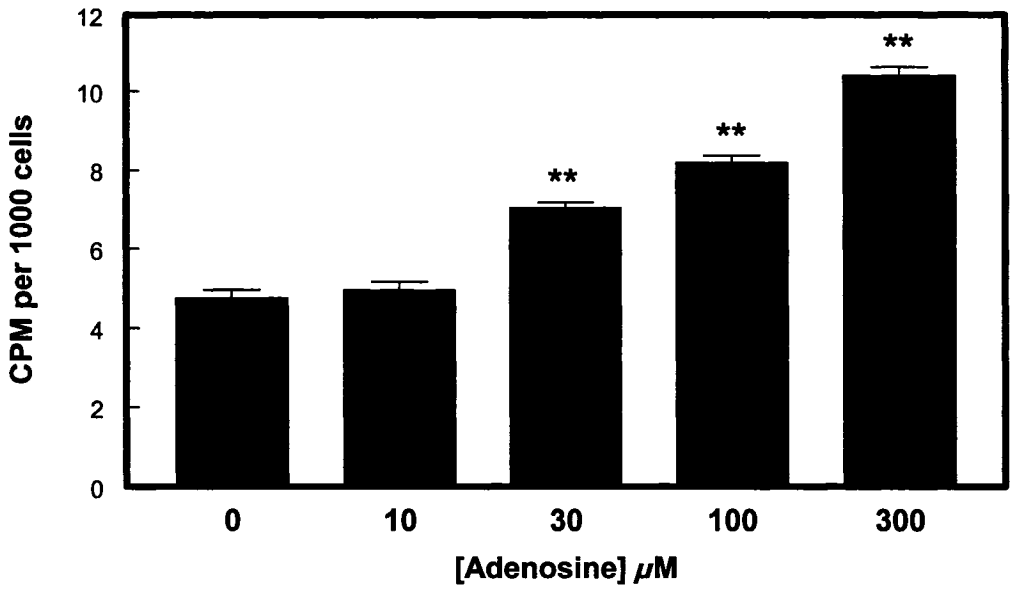
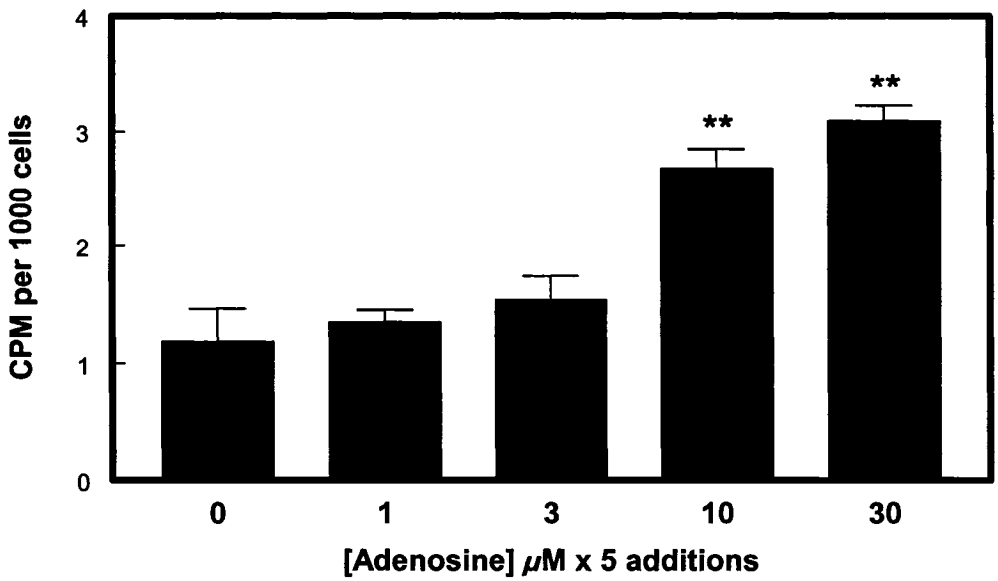


Figure 2.5: Dose-dependency of adenosine regulation of cell-surface CXCR4 protein expression on HT-29 cells. HT-29 cells were treated with a single dose of adenosine at the indicated concentrations, and cell-surface CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant increase due to adenosine, P<0.01.

(A)



(B)

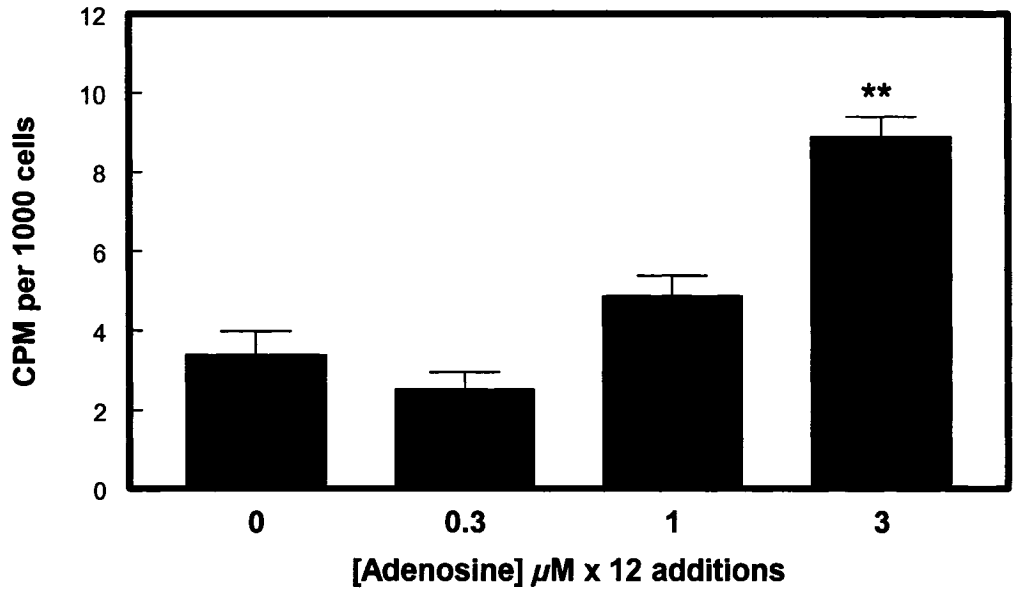


Figure 2.6: Effect of repeated additions of adenosine over a 48 h period on cell-surface CXCR4 protein expression on HT-29 cells. HT-29 cells were treated with (A) 5 sequential doses or (B) 12 sequential doses of adenosine at the indicated concentrations over a 48 h period, followed by measurement of cell-surface CXCR4 protein expression using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant increase due to adenosine, P<0.01.

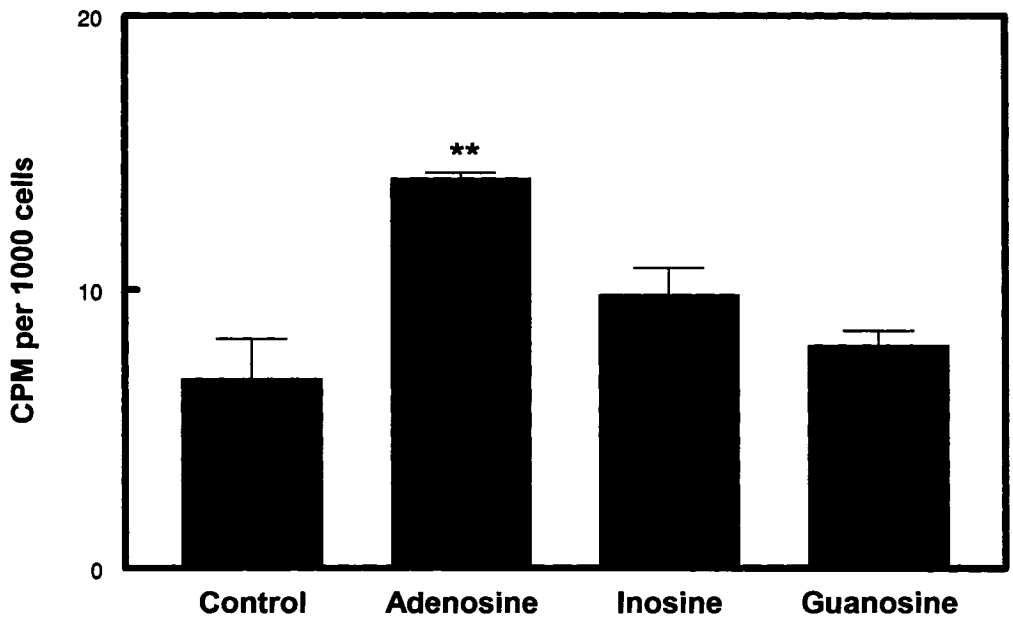
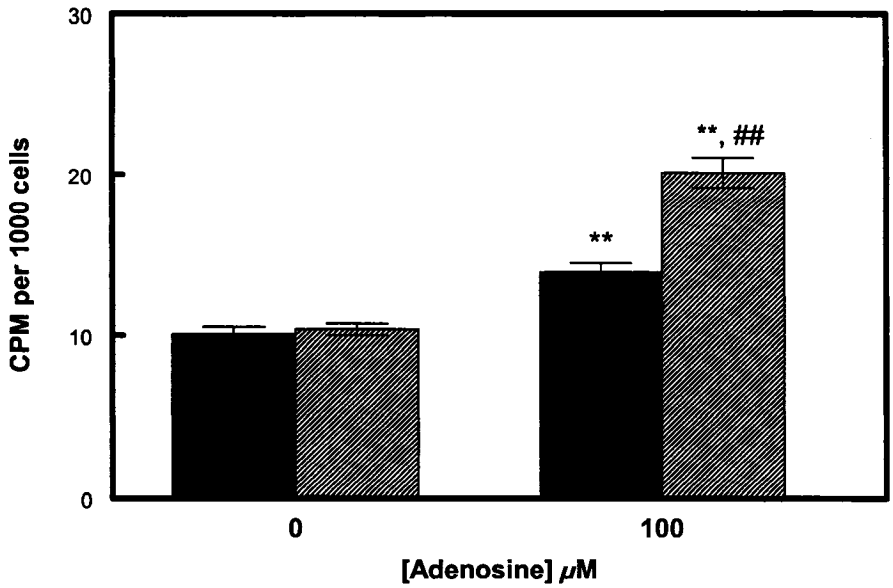


Figure 2.7: Effect of inosine and guanosine on cell-surface CXCR4 expression on HT-29 cells. HT-29 cells were treated with a single dose of vehicle, adenosine, inosine, or guanosine (300 μM each) and CXCR4 cell-surface protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant increase due to adenosine, P<0.01.

(A)



(B)

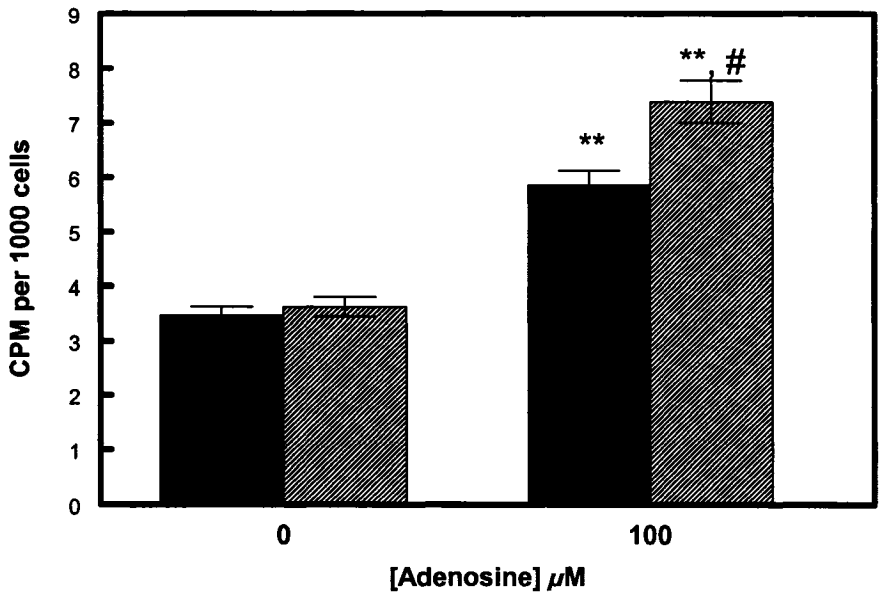


Figure 2.8: Effect of ADA inhibitors on adenosine-induced CXCR4 up-regulation on HT-29 cells. HT-29 cells were pre-treated for 30 min with vehicle (solid bars) or (A) 20 μM EHNA (B) 1 μM coformycin (hatched bars), followed by a single dose of adenosine at the indicated concentrations, and cell-surface CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant increase due to adenosine, P<0.01. ##, significant increase due to EHNA, P<0.01. #, significant increase due to coformycin, P<0.05.

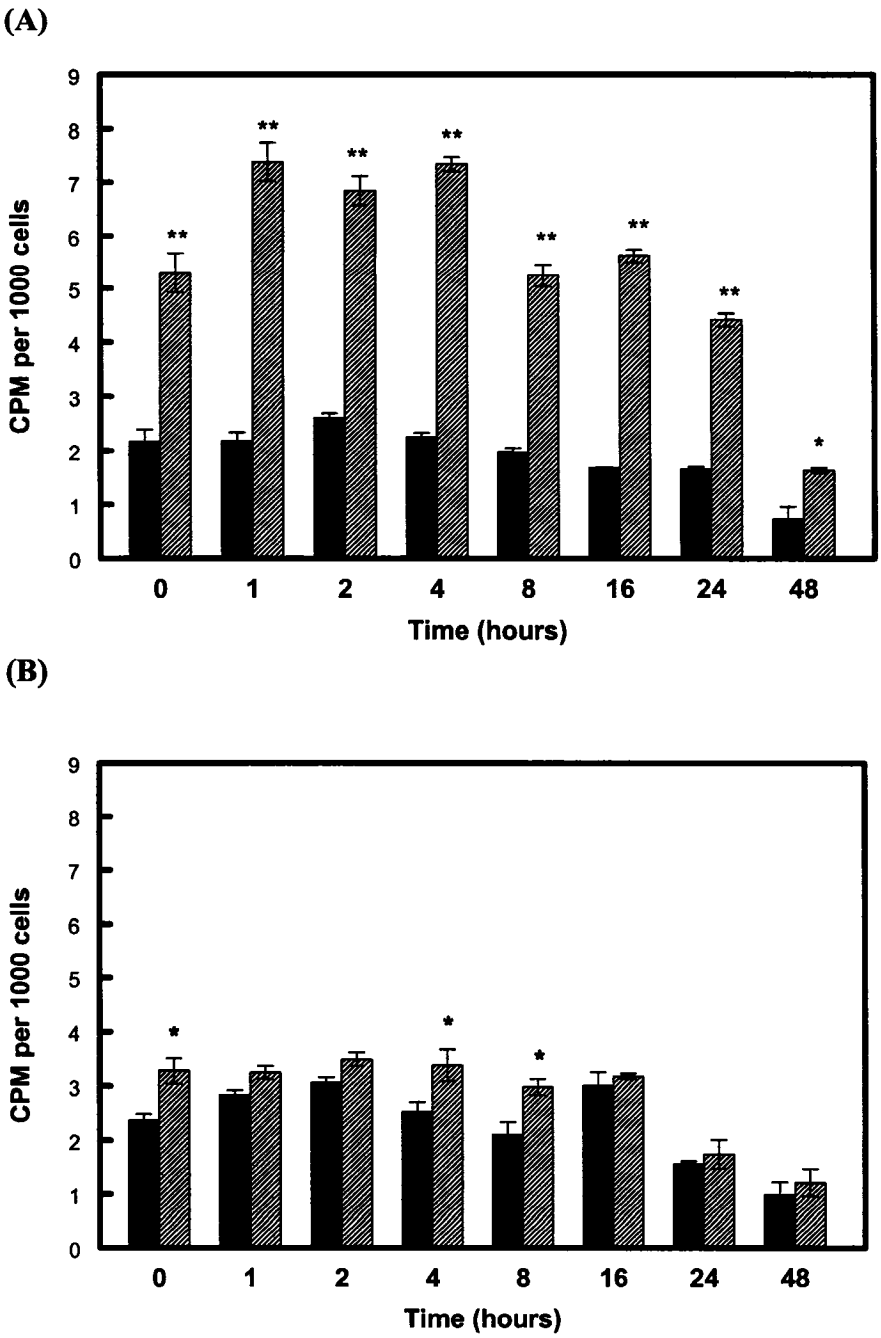


Figure 2.9: Effect of ADA treatment of supernatants taken from adenosine-treated cells on CXCR4 up-regulation. HT-29 cells were treated with a single dose of vehicle (solid bars) or 300 μM adenosine (hatched bars), and the supernatant media were removed from each well after the times indicated. The supernatant media were (A) untreated or (B) treated with ADA (1 U/ml for 5 min). These supernatants were then added to fresh HT-29 cell monolayers, and cell-surface CXCR4 protein was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=3). **, significant increase due to adenosine, $P<0.01$; *, $P<0.05$.

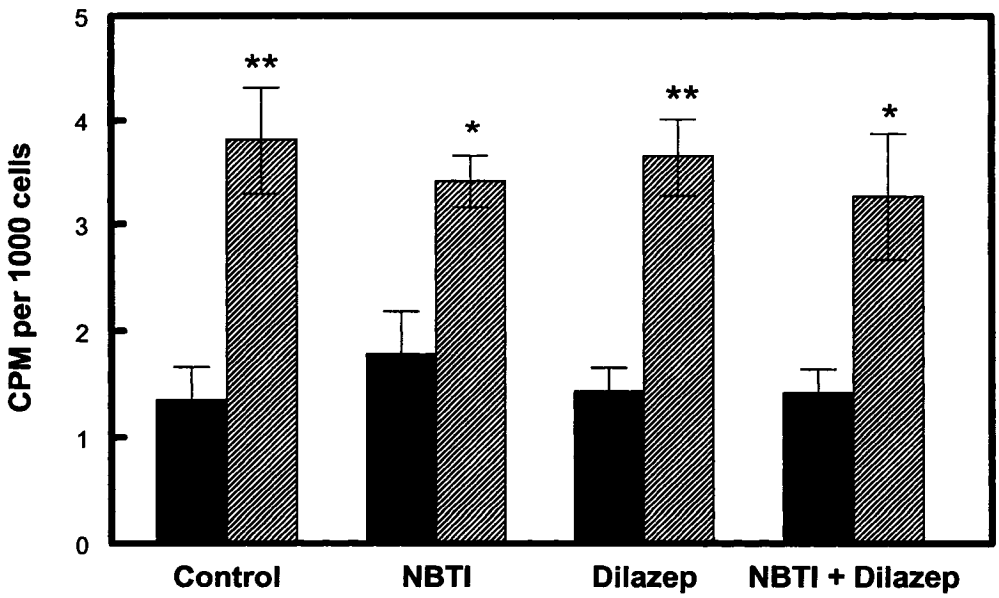
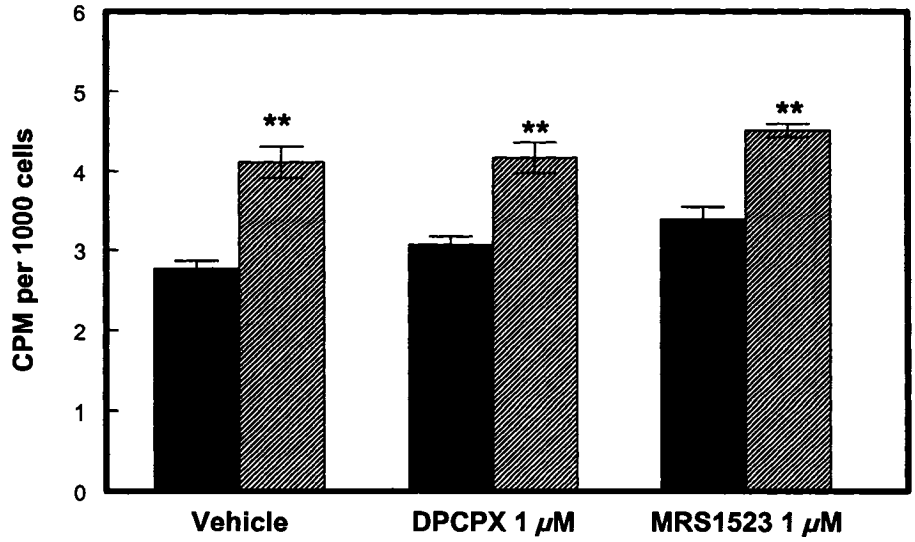


Figure 2.10: Effect of adenosine transport inhibitors on adenosine-induced CXCR4 up-regulation on HT-29 cells. HT-29 cells were pre-treated for 30 min with vehicle, NBTI (1μM), dilazep (5 μM) or a combination of NBTI/dilazep, followed by addition of vehicle (solid bars) or 300 μM adenosine (hatched bars). CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant increase due to adenosine, $P<0.01$; *, $P<0.05$.

(A)



(B)

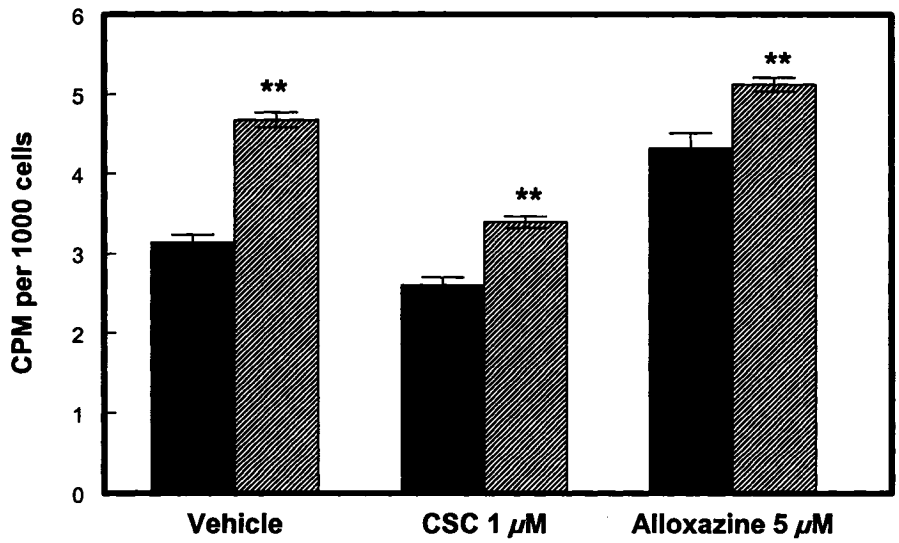


Figure 2.11: Effect of single adenosine receptor antagonists on adenosine-induced CXCR4 up-regulation on HT-29 cells. HT-29 cells were pre-treated for 30 min with vehicle or (A) 1 μM DPCPX or 1 μM MRS1523, or (B) 1 μM CSC or 5 μM alloxazine, followed by addition of vehicle (solid bars) or 50 μM adenosine (hatched bars). CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=6). **, significant increase due to adenosine, P<0.01.

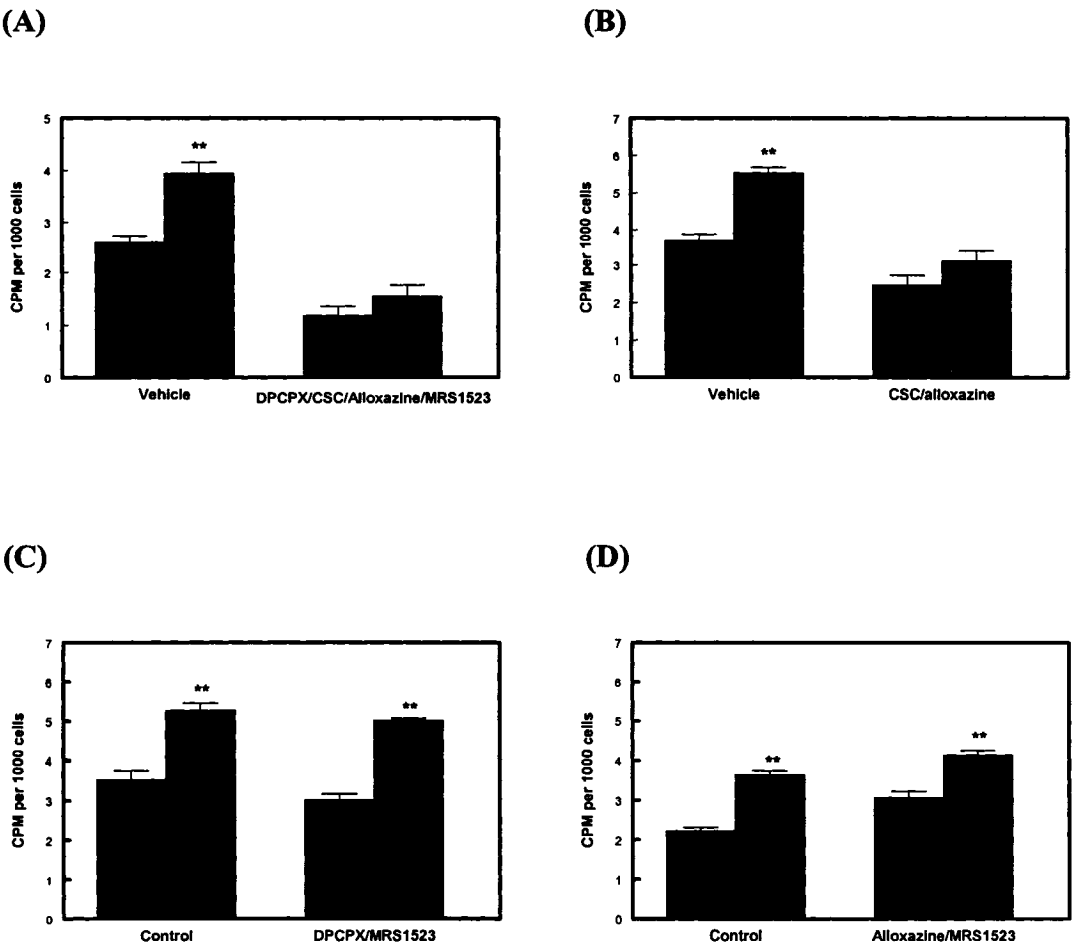


Figure 2.12: Effect of combinations of adenosine receptor antagonists on adenosine-induced CXCR4 up-regulation on HT-29 cells. HT-29 cells were pre-treated for 30 min with vehicle or (A) 1 μM DPCPX, 1 μM CSC, 5 μM alloxazine, and 1 μM MRS1523, (B) 1 μM CSC and 5 μM alloxazine, (C)1 μM DPCPX and 1 μM MRS1523, or (D) 5 μM alloxazine and 1 μM MRS1523, followed by addition of vehicle (solid bars) or 50 μM adenosine (hatched bars). CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE, (A) (n=4), (B-D) (n=6). **, significant increase due to adenosine, P<0.01.

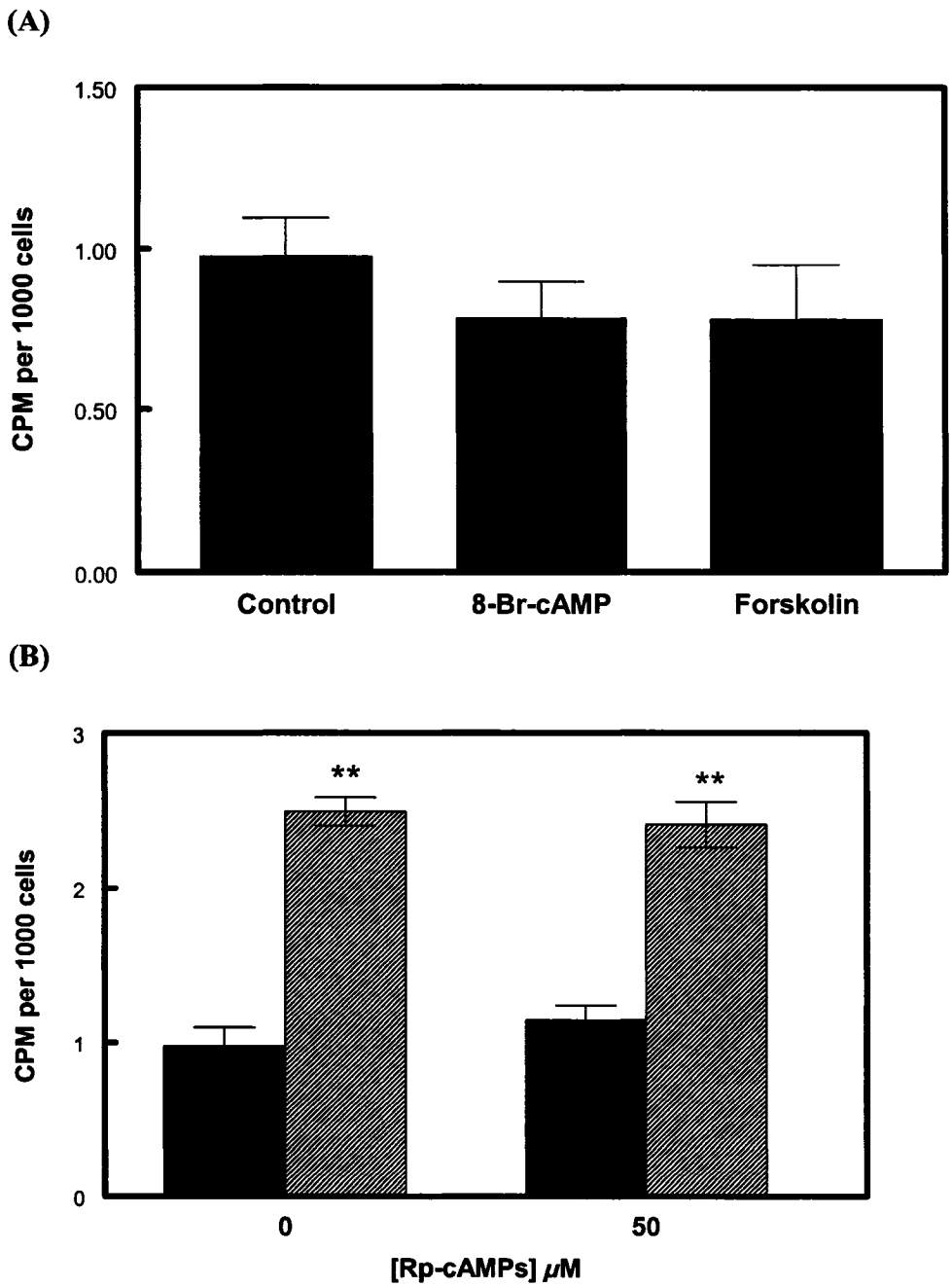


Figure 2.13: Involvement of cAMP/PKA signaling in adenosine-induced CXCR4 up-regulation on HT-29 cells. (A) HT-29 cells were treated with 1 mM 8-Br-cAMP or 50 μM forskolin, and cell-surface CXCR4 was measured 48 h later. (B) HT-29 cells were treated with Rp-cAMPs at the indicated concentrations, followed by addition of vehicle (solid bars) or 300 μM adenosine (hatched bars). Cell-surface CXCR4 was measured 48 h later. The data are mean values ± SE (n=4). **, significant increase due to adenosine, P<0.01.

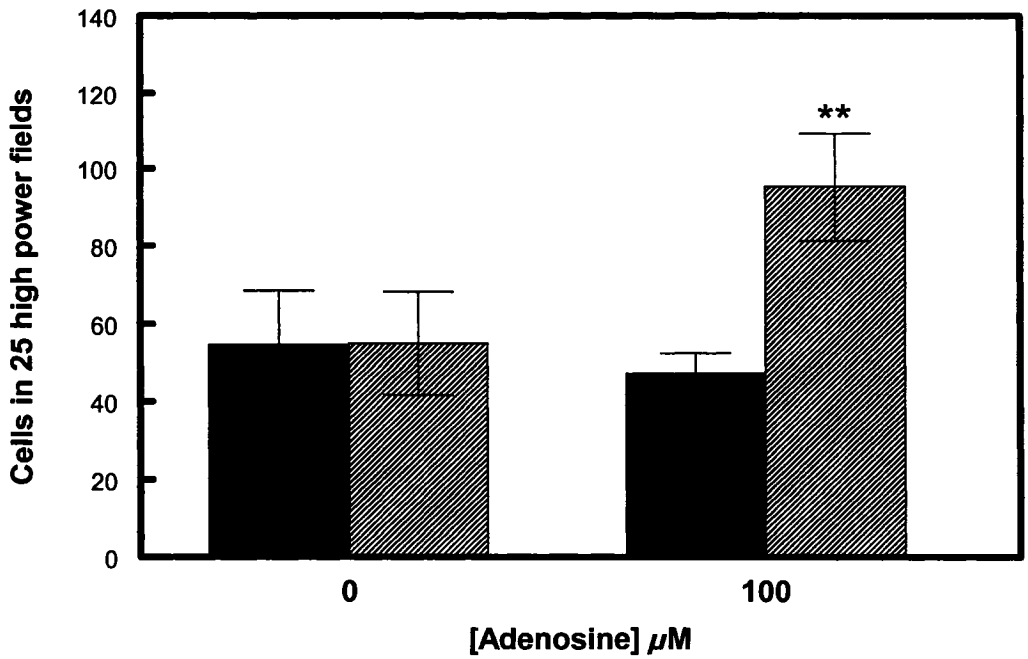


Figure 2.14: Effect of adenosine pre-treatment on migration of HT-29 cells towards CXCL12. HT-29 cells were treated for 48 h with adenosine at the indicated concentrations, and the migratory response to vehicle (solid bars) or 200 ng/ml CXCL12 (hatched bars) was then measured. The data are mean values ± SE (n=6). **, significant increase due to CXCL12, P<0.01.

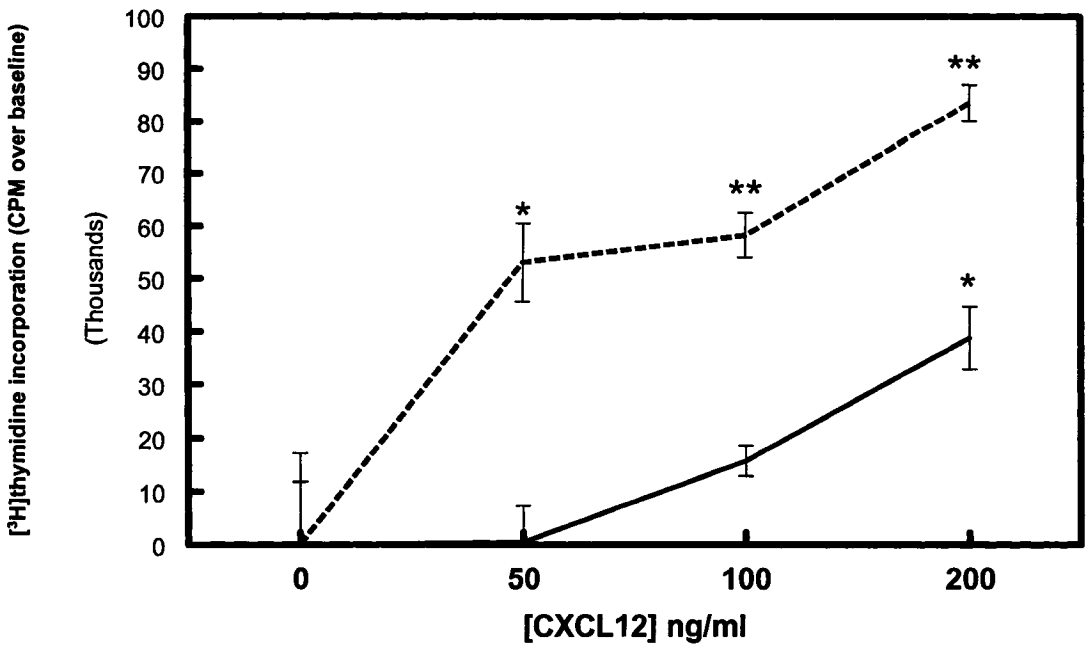


Figure 2.15: Effect of adenosine pre-treatment on proliferation of HT-29 cells in response to CXCL12. HT-29 cells were treated for 48 h with vehicle (solid line) or 300 μM adenosine (hatched line), followed by treatment with CXCL12 at the indicated concentrations. Proliferation was measured after 24 h using a [^{3}H]thymidine incorporation assay to assess DNA synthesis. The data are mean values ± SE (n=6). **, significant increase due to CXCL12, P<0.01; *, P<0.05.

DISCUSSION

The chemokine receptor CXCR4 is highly expressed on many tumour types (Zlotnik, 2004), and has been implicated in the process of site-specific metastasis (Müller et al., 2001). Specifically, cancer cells expressing CXCR4 are able to respond to the CXCR4 ligand, CXCL12, in a migratory, invasive, and/or proliferative fashion (Müller et al., 2001; Scotton et al., 2002; Taichman et al., 2002; Zeelenberg et al., 2003; Oonakahara et al., 2004; Schimanski et al., 2005). Furthermore, high CXCR4 expression has been associated with poorer prognosis and reduced survival for patients suffering from colorectal cancer (Kim et al., 2005a; Schimanski et al., 2005; Ottaiano et al., 2006). Since CXCR4 expression may be influenced by the tumour microenvironment (Zeelenberg et al., 2003), we sought to determine what specific factors within the extracellular milieu of the tumour may regulate CXCR4 expression. In this chapter, the objective was to determine whether adenosine, a purine nucleoside present in increased levels in tumours due to hypoxia (Blay et al., 1997), could contribute to elevated CXCR4 expression.

Adenosine increases CXCR4 expression

We used a radioantibody binding assay, which specifically detects receptors present at the cell-surface (Tan et al., 2004), to measure changes in CXCR4 protein expression in response to adenosine. Although others have measured cell-surface CXCR4 expression with a radioligand assay using ^{125}I-labeled CXCL12 (Salcedo et al., 1999; Peng et al., 2005), we chose to use a radioantibody binding assay instead due to the signal amplification afforded by the secondary ^{125}I-labeled IgG antibody. Furthermore,

as CXCL12 may bind to other receptors besides CXCR4, such as RDC1 (Balabanian et al., 2005), results obtained with ^{125}I-labeled CXCL12 may not be specific for CXCR4, depending on receptor expression on a given cell type. The anti-CXCR4 antibody that we used in our experiments (clone 12G5) does not bind to RDC1 (Balabanian et al., 2005).

We found that a single dose of 300 μM adenosine significantly increased cell-surface CXCR4 protein expression on HT-29 cells (Figure 2.2). An increase in cell-surface CXCR4 protein expression was also noted using flow cytofluorimetry (Figure 2.3). We focused primarily on cell-surface CXCR4 protein rather than total CXCR4 protein because the receptor must be present at the cell-surface to "sense" CXCL12 and elicit functional responses (Cole et al., 1999; la Sala et al., 2002; Brühl et al., 2003; Kulbe et al., 2005). Therefore, cell-surface protein expression provides a more accurate indicator of the functional consequences of changes in CXCR4 expression than total protein expression. However, we also permeabilized cells using paraformaldehyde (PFA) and saponin prior to radioantibody binding assays and flow-cytofluorimetry, and found that adenosine did indeed increase total CXCR4 protein as well (Richard CL, Blay J, unpublished observations).

As shown in Figure 2.2, adenosine induced a greater than 2.5-fold increase in cell-surface CXCR4 protein by 48 h. This increase persisted for at least 72 h after a single dose of adenosine. Therefore, within the context of cancer, cells would retain elevated CXCR4 protein expression after they have left the adenosine-rich tumour environment, and would likely have increased CXCL12 sensitivity for some time after emigration.

An increase in cell-surface CXCR4 protein expression was not noted until 24 h after treatment of HT-29 cells with a single dose of adenosine (Figure 2.2). Cole and colleagues found that CXCR4 surface protein levels increased for up to a maximum of 12 h following dibutyryl-cAMP treatment, which was shown to be a result of changes in CXCR4 protein trafficking (Cole et al., 1999). However, the onset of adenosine-mediated CXCR4 protein up-regulation was much slower, beginning 24 h after treatment and persisting for at least 48 h. Comparing these results, it seemed unlikely that changes in protein trafficking were involved in adenosine-mediated CXCR4 up-regulation.

To see if adenosine instead increased CXCR4 at the mRNA level, we performed real-time RT-PCR. Changes in CXCR4 mRNA expression typically correlate with changes in CXCR4 protein; however, in some cases, as was seen with cytokine-mediated changes in CXCR4 expression in neutrophils, this typical correlative relationship is not observed (Brühl et al., 2003). In our case, in accordance with changes in cell-surface protein, we found that adenosine induced a substantial increase in CXCR4 mRNA (Figure 2.4). The peak increase in mRNA expression preceded that which was noted for cell-surface CXCR4 protein. Therefore, it is likely that adenosine-mediated CXCR4 up-regulation occurs at the level of transcription.

Many effects on cancer cells that are attributed to adenosine have been demonstrated using very high doses of this nucleoside, sometimes in the millimolar range (Barry and Lind, 2000; Saitoh et al., 2004; Hashemi et al., 2005). However, even within the hypoxic environment of a tumour, extracellular adenosine concentrations are in the low micromolar range (Blay et al., 1997), although higher levels may be achieved in areas of necrosis. We sought to determine whether the effect of adenosine on CXCR4

protein expression could be achieved at relevant intratumoural concentrations. Although 30 μM adenosine was required to increase CXCR4 protein expression after a single dose (Figure 2.5), which was higher than what has been noted within tumours (1-10 μM; Blay et al., 1997), the half-life of adenosine in these cultures is approximately 2 hours (Mujoomdar et al., 2003); by the time CXCR4 protein was measured (after 48 h) most of the exogenously added adenosine would have been eliminated. In order to provide continuous exposure of HT-29 cells to pathophysiologically relevant concentrations of adenosine over a 48 h period, we repeatedly added lower doses of adenosine. Using this approach, we found that persistent exposure to low micromolar concentrations also increased cell-surface CXCR4 protein expression on HT-29 cells (Figure 2.6). Therefore, adenosine may be one factor present within the tumour microenvironment that contributes to elevated CXCR4 expression on cancer cells.

Studies designed to elicit biological effects of adenosine are often performed with adenosine analogues or synthetic receptor agonists rather than with adenosine itself. Such experiments must be interpreted with caution. Effects noted with analogues may differ from those seen with adenosine. For example, the adenosine analogue 2-chloroadenosine was cytotoxic against 1321N1 astrocytoma cells, whereas bona fide adenosine was not (Bradley and Bradley, 2001). Synthetic adenosine receptor agonists may affect cells independently of adenosine receptor activation, as was seen with A_3 receptor agonist *N*(6)-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA)-mediated breast cancer cell growth inhibition (Lu et al., 2003). Also, since adenosine effects are sometimes mediated through the activation of more than one receptor subtype, such as A_{2A}/A_{2B} receptor-mediated inhibition of IL-2-induced STAT5 phosphorylation

(Zhang et al., 2004), adenosine effects may be missed when single agonists are used. This could happen even with relatively non-selective adenosine receptor agonists, such as NECA, due to stoichiometric differences between these agonists and adenosine (Jacobson and Gao, 2006).

As a consequence of these many factors, we chose to use adenosine itself in the experiments we performed. However, adenosine can be broken down in cell culture, leading to the production of inosine through an irreversible deamination reaction catalyzed by adenosine deaminase (ADA). Therefore, cellular effects attributed to adenosine may be mediated inosine formation. Although there are no distinct cell-surface receptors for inosine, it has many biological activities, including immunomodulatory, anti-inflammatory, and neuroprotective functions, and it can activate adenosine A_3 receptors (Haskó et al., 2004).

To determine if inosine production was responsible for adenosine-mediated CXCR4 up-regulation, our first approach was to examine the effect of inosine itself on CXCR4 protein by HT-29 cells. A single dose of 300 μM inosine failed to produce a statistically significant increase in CXCR4 protein (Figure 2.7). Although in some experiments we did see a small effect, inosine did not reproducibly up-regulate cell-surface CXCR4 protein expression to as great of an extent as that which was seen with adenosine. Also, the adenosine effect was not a non-specific purine nucleoside effect, since we did not observe the same CXCR4 response with an equimolar concentration of guanosine (Figure 2.7).

To further rule out inosine involvement in CXCR4 up-regulation, we blocked the conversion of adenosine to inosine using the ADA inhibitors EHNA and coformycin,

which would reduce CXCR4 up-regulation if inosine was indeed responsible. However, we found that instead of blocking the effect of adenosine on CXCR4 protein expression, coformycin and EHNA enhanced this effect (Figure 2.8). Inhibition of ADA would increase the persistence of adenosine in the culture media; by increasing the stability of adenosine in cell culture, the ADA inhibitors coformycin and EHNA increased adenosine-mediated CXCR4 up-regulation.

A final approach to rule out inosine involvement in the adenosine effect on CXCR4 involved the treatment of culture supernatants with ADA. In previous experiments, we found that supernatants collected from cells treated with a high dose of adenosine (300 μM) elevated CXCR4 receptor expression when added to fresh cultures of HT-29 cells (Richard CL, Blay J, unpublished observations). This could be due to: (1) persistence of adenosine in culture supernatants, (2) conversion of adenosine to inosine, which may also be active, or (3) secretion of a stable autocrine factor capable of elevating CXCR4 receptor expression. To test these possibilities, we treated HT-29 cells with a single dose of 300 μM adenosine, collected supernatants at various time points over a 48 h period, treated the collected media with ADA or vehicle, and replaced the media on fresh HT-29 cultures with these supernatants. Cell-surface CXCR4 protein expression was measured 48 h later. As expected, untreated supernatants from adenosine-treated cells increased CXCR4 receptor expression on HT-29 cells (Figure 2.9A). However, after ADA treatment, supernatants from adenosine-treated cells had very little effect on CXCR4 receptor expression (Figure 2.9B). Removal of adenosine through conversion to inosine significantly reduced the effect of the supernatants on CXCR4 receptor

expression. Therefore, persistence of adenosine, rather than production of inosine or secretion of a stable autocrine factor, accounted for the activity of the supernatants.

Signaling pathway of adenosine-induced CXCR4 up-regulation

Adenosine typically exerts its effects by activation of cell-surface receptors (Fredholm et al., 2000), but can also act in an intracellular fashion to directly inhibit adenylyl cyclase by binding to a "P"-site (Dessauer et al., 1999). In fact, some effects of adenosine on tumour cell function have been mediated by an intracellular mechanism (Barry and Lind, 2000; Bradley and Bradley, 2001; Saitoh et al., 2004; Hashemi et al., 2005). Since adenosine is a hydrophilic molecule, in order to work intracellularly it must be taken into cells through nucleoside transporters (Thorn and Jarvis, 1996). There are two types of nucleoside transporters: (1) equilibrative nucleoside transporters (ENTs), which depend on facilitated diffusion and (2) concentrative nucleoside transporters (CNTs), which require active transport driven by a sodium gradient (Thorn and Jarvis, 1996; Damaraju et al., 2003; Baldwin et al., 2004). ENTs can be further classified as being either sensitive (*es*) or insensitive (*ei*) to inhibition by nanomolar concentrations of S(4-nitrobenzyl)-6-thioinosine (NBTI). Adenosine uptake into colorectal carcinoma cells is mediated by *es* and *ei* transporters (Ward and Tse, 1999). We blocked adenosine transport, using NBTI and dilazep, to assess its involvement in adenosine-mediated CXCR4 up-regulation. Both compounds block *es* transporters at nanomolar concentrations, and *ei* transporters at micromolar concentrations (Gu et al., 1996; Noji et al., 2004). We chose concentrations of NBTI and dilazep (1 μM and 5 μM, respectively) that would block both *es* and *ei* transporters. As shown in Figure 2.10, adenosine

transport inhibitors, either alone or in combination, did not block adenosine-mediated CXCR4 up-regulation. Therefore, this effect was not dependent on uptake of adenosine into cells, and did not occur through an intracellular mechanism.

Since adenosine-mediated CXCR4 up-regulation on HT-29 cells was not regulated by the uptake of adenosine into the cells, we next examined whether the effect involved activation of cell-surface adenosine receptors. Adenosine receptors are seven-transmembrane G-protein coupled receptors, classified as A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 2001). Each receptor is expressed in HT-29 cells at the mRNA level (Mujoomdar M, Blay J, unpublished observations). To elucidate the potential involvement of each of these adenosine receptor subtypes in adenosine-mediated CXCR4 up-regulation, we attempted to block the adenosine effect with selective adenosine receptor antagonists. For antagonist studies, we chose to use single concentrations of adenosine together with each antagonist. As described in the Results section, we were careful to choose an adenosine concentration that consistently up-regulated cell-surface CXCR4 protein expression but was low enough to be overcome by antagonists. Adenosine receptor antagonist concentrations were chosen to be well above the known Ki values but remained low enough to retain receptor selectivity (Ji and Jacobson, 1999; Moro et al., 2006). We are confident that this approach would effectively identify the involvement of adenosine receptors in the adenosine effect on CXCR4.

Due to the fact that blockade of all four adenosine receptors using a combination of DPCPX (A_1), CSC (A_{2A}), alloxazine (A_{2B}) and MRS1523 (A_3) abolished the adenosine effect on CXCR4 protein expression (Figure 2.12A), we concluded that adenosine-induced CXCR4 up-regulation was indeed mediated through activation of cell-surface

adenosine receptors. Interestingly, no single adenosine receptor antagonist completely blocked the adenosine effect on CXCR4 (Figure 2.11), suggesting that multiple adenosine receptors were involved. Adenosine-mediated CXCR4 up-regulation was not reduced by blockade of the A_1 receptor with DPCPX or the A_3 receptor with MRS1523 either alone or in combination (Figures 2.11A and 2.12C), ruling out the involvement of signaling through these two receptors in this effect. CSC (A_{2A}) and alloxazine (A_{2B}) each partially reduced the effect of adenosine on cell-surface CXCR4 protein expression (Figure 2.11B), and a combination of these two antagonists completely blocked the adenosine effect on CXCR4 expression (Figure 2.12B). This suggested that adenosine-induced CXCR4 up-regulation was mediated through the combined action of signaling through A_{2A} and A_{2B} receptors. The combination of alloxazine (A_{2B}) and MRS1523 (A_3) also partially reduced the adenosine effect to an extent that was similar to that observed with alloxazine alone (Figure 2.12C); this was likely due to the involvement of A_{2B} receptor signaling in adenosine-mediated CXCR4 up-regulation.

It was interesting to see that both A_{2A} and A_{2B} receptors were involved in adenosine-mediated CXCR4 up-regulation. Although these two receptors are similar in terms of their downstream signaling pathways, they differ in affinity for adenosine. Unlike the A_{2A} receptor, the A_{2B} receptor is considered to be a low-affinity adenosine receptor (Feoktistov and Biaggioni, 1998; Fredholm et al., 2001); thus, the A_{2B} receptor is activated when adenosine levels are abnormally elevated in situations such as hypoxia or necrosis. The fact that A_{2B} receptors are involved in adenosine-mediated CXCR4 up-regulation suggests that this effect may have evolved as a protective mechanism whereby cells could have increased migratory or survival capacity under stressful conditions.

Adenosine-mediated CXCR4 up-regulation would be expected to occur to a certain degree under normal conditions through activation of A_{2A}, but would likely be enhanced in critical situations by activation of A_{2B}.

Adenosine A_{2A} and A_{2B} receptors both couple positively to adenylyl cyclase, causing increased cAMP formation, PKA activation, and subsequent activation of target genes (Fredholm et al., 2001). Therefore, we considered the possibility that adenosine-mediated CXCR4 up-regulation was due to an increase in cAMP/PKA signaling. This seemed probable, as cAMP has been shown to increase CXCR4 expression in several cell types (Cole et al., 1999; Cristillo et al., 2002; Ödemis et al., 2002; Salcedo et al., 2003), although there are conflicting reports as to whether this effect occurs at the level of transcriptional regulation or trafficking of the protein to the cell surface (Cole et al., 1999; Cristillo et al., 2002). Contrary to what we expected, neither the stable cAMP analogue 8-Br-cAMP, nor the direct adenylyl cyclase activator forskolin increased CXCR4 protein levels (Figure 2.13A). In addition, the PKA inhibitor Rp-cAMPs did not block adenosine-mediated CXCR4 up-regulation (Figure 2.13B). These results argued against the involvement of cAMP/PKA signaling in adenosine-mediated CXCR4 up-regulation. However, in order to completely rule out this signaling pathway, we would need to complete a full dose-response and time course analysis for forskolin and 8-Br-cAMP and measure changes in cAMP levels in HT-29 cells induced by adenosine treatment.

The signaling pathway downstream of A_{2A} and A_{2B} receptor activation that leads to CXCR4 up-regulation remains to be determined. In addition to cAMP/PKA signaling, activation of A_2 receptors can activate multiple other signaling pathways (Fredholm et al.,

2001). For example, both A_{2A} and A_{2B} receptors stimulate phospholipase C activity, leading to IP_3 accumulation and activation of protein kinase C pathways (Gao et al., 1999; Linden et al., 1999; Fresco et al., 2004; Jacobson and Gao, 2006). However, it is not known if this is involved in CXCR4 up-regulation. Several signaling pathways have been shown to increase CXCR4 expression, including those leading to activation of the transcription factors NF-κB (Helbig et al., 2003) and HIF-1 (Staller et al., 2003). However, adenosine activation of A_2 receptors has actually been shown to decrease NF-κB activation (Li et al., 2000; Majumdar and Aggarwal, 2003), and a link between adenosine signaling and HIF-1 remains elusive. Therefore, further clarification is needed before we are able to speculate on the signaling mechanism responsible for adenosine-mediated CXCR4 up-regulation.

Functional consequences of adenosine effect on CXCR4

In response to ligand activation, cells expressing CXCR4 can migrate towards CXCL12 (Müller et al., 2001; Scotton et al., 2001; Taichman et al., 2002; Oonakahara et al., 2004; Schimanski et al., 2005) and/or have an increased rate of proliferation (Scotton et al., 2002; Zeelenberg et al., 2003). In addition, elevated levels of CXCR4 at the cell surface have been shown to increase functional responses to CXCL12 (Cole et al., 1999; la Sala et al., 2002; Kulbe et al., 2005). Therefore, we speculated that the adenosine-mediated increase in cell-surface CXCR4 protein expression would correlate with increased migratory and proliferative responsiveness of HT-29 cells to CXCL12.

We found that untreated HT-29 cells were generally unresponsive to CXCL12, in terms of both migration and proliferation (Figures 2.14 and 2.15). This was somewhat

surprising, since HT-29 cells consistently expressed detectable levels of CXCR4 in radioantibody binding assays; however, we demonstrated with flow cytofluorimetry that the percentage of untreated cells expressing detectable CXCR4 was low (Figure 2.3A). Therefore, it is possible that there was not a sufficient number of CXCR4-expressing HT-29 cells to produce a quantifiable response to CXCL12. Consistent with this possibility, as the percentage of CXCR4-expressing HT-29 cells increased after adenosine treatment (as shown with flow cytofluorimetry, Figure 2.3B), quantifiable migratory and proliferative responses to CXCL12 were noted with adenosine-treated cells (Figures 2.14 and 2.15). Using a chemotaxis assay designed to measure migration, we showed that after adenosine pre-treatment, but not vehicle pre-treatment, there was a two-fold increase in the number of cells that migrated towards CXCL12 compared to vehicle (Figure 2.14). Similarly, using a radiolabeled thymidine incorporation assay, to assess DNA synthesis and therefore proliferation, adenosine pre-treatment produced a leftward shift in the CXCL12 dose-response curve, suggesting increased responsiveness (Figure 2.15). Therefore, as has been shown with other cell types, increased cell-surface CXCR4 protein expression induced by adenosine resulted in increased functional responses to CXCL12 (Cole et al., 1999; la Sala et al., 2002; Kulbe et al., 2005).

Relevance of adenosine effect on CXCR4

The data presented in this chapter provide sound evidence for a role of adenosine in increasing CXCR4 receptor expression on HT-29 colorectal carcinoma cells grown in monolayer culture. This increase is likely in addition to that seen with hypoxia, since monolayer cultures, especially dense cultures, tend to be hypoxic (Pettersen et al., 2005).

The question must be asked – why has this mechanism of CXCR4 regulation evolved? High concentrations of adenosine are present in tissues under stressful conditions, such as ischaemia/reperfusion, hypoxia, and necrosis (Fredholm et al., 2001). In these situations, adenosine has been shown to provide tissue protection (Linden, 2001; Ohta and Sitkovsky, 2001; Linden, 2005). Proposed mechanisms of tissue protection include an increased oxygen supply/demand ratio, ischaemic preconditioning, promotion of anti-inflammatory responses, and enhancement of angiogenesis (Linden, 2005). Perhaps an additional mechanism of tissue protection is elevation of CXCR4 expression, which may provide a survival benefit and also assist in the movement of cells away from damaged areas. Tögel and colleagues found that CXCR4 levels were increased on proximal tubular cells of the kidney after induction of acute renal failure in a murine model (Tögel et al., 2005). This may be an effect that occurs under diverse hypoxic situations, since in many cell types, hypoxia has been shown to increase CXCR4 expression through activation of the transcription factor hypoxia inducible factor 1 (HIF-1; Schioppa et al., 2003; Staller et al., 2003). It has been suggested that hypoxia-induced CXCR4 up-regulated is an adaptive protective mechanism (Tögel et al., 2005). Although hypoxia-induced CXCR4 up-regulation has been attributed to activation of HIF-1, it would be interesting to see if hypoxia-induced adenosine production would further increase CXCR4 expression, as is seen with vascular endothelial growth factor (VEGF) production (Leibovich et al., 2002). Therefore, although adenosine-induced CXCR4 up-regulation likely evolved to provide tissue protection, this mechanism appears to be exploited by tumour cells to increase survival and migratory ability.

Our laboratory has shown that adenosine at concentrations present within the extracellular fluid of solid tumours has multiple tumour promoting effects. These include enhancement of proliferation of cancer cells (Mujoomdar et al., 2003; Mujoomdar et al., 2004), modulation of cell-cell and cell-extracellular matrix interactions (MacKenzie et al., 2002; Tan et al., 2004), and interference with anti-tumour immune responses (MacKenzie et al., 1994; Hoskin et al., 2002). Interestingly, adenosine also decreases expression of dipeptidyl peptidase IV (DPPIV, CD26; Tan et al., 2004), an enzyme that cleaves and inactivates CXCL12 (Proost et al., 1998). Therefore, in addition to increasing the expression of CXCR4, adenosine may also increase the availability of its ligand, CXCL12 (Mizokami et al., 2004). Other investigators also consider adenosine to be important in tumour progression (for example, see Spychala, 2000). Particular attention has been given to the pro-angiogenic aspects of adenosine action, a phenomenon that has been mainly characterized in the context of wound healing but also applies to tumour progression. Adenosine receptor activation has been shown to increase proliferation of endothelial cells (Sexl et al., 1995), enhance production of pro-angiogenic factors such as VEGF, IL-8, and ang-2 (Grant et al., 1999; Feoktistov et al., 2002; Leibovich et al., 2002; Feoktistov et al., 2003), and decrease production of the anti-angiogenic protein thrombospondin (Desai et al., 2005).

The data presented in this chapter provide further evidence in support of a role for adenosine in tumour promotion. Our results show that adenosine-mediated CXCR4 up-regulation on HT-29 cells leads to increased migratory and proliferative responses to CXCL12. Within the context of a tumour, these responses would assist in the growth, invasion, and metastasis of cancer cells, which are considered to be hallmarks of cancer

(Hanahan and Weinberg, 2000). Therefore, functional changes induced by adenosine-mediated CXCR4 up-regulation may facilitate tumour progression.

This raises the question – would adenosine receptor antagonists be of value in the treatment of colorectal cancer? In the case of adenosine-mediated CXCR4 up-regulation, we were able to pinpoint the receptors responsible for this effect. However, this was not the case with other tumour-promoting effects of adenosine identified in our laboratory. For example, the proliferative effect of adenosine on tumour cells could not reproducibly be blocked with adenosine receptor antagonists or mimicked with adenosine receptor agonists (Mujoomdar M, Blay J, unpublished observations), nor could adenosine-mediated DPPIV down-regulation (Tan et al., 2006). Furthermore, although the adenosine effect on CXCR4 was mediated by A_2 receptors, adenosine inhibited anti-tumour immune responses through activation of A_3 receptors (MacKenzie et al., 1994; Hoskin et al., 2002). Consequently, since the tumour-promoting effects of adenosine cannot be attributed to a single receptor subtype, it would be difficult to predict which antagonists would be of benefit. The situation is further complicated by the fact that A_3 receptor *agonists* have been promoted as inhibitors of tumour growth (Ohana et al., 2003; Fishman et al., 2004), although some A_3 receptor agonists act independently of A_3 receptors (Lu et al., 2003). There are concerns of toxicity associated with adenosine receptor agonists and antagonists, as adenosine receptors are ubiquitously expressed and are involved in the physiological function of nearly every organ system, including the cardiovascular system, nervous system, renal system, and pulmonary system (Jacobson and Gao, 2006). It is also difficult to assess the efficacy of adenosine receptor agonists/antagonists in animal models, as there are species-dependent differences in

receptor selectivity, and the synthesis of specific agents has been difficult, especially for A_{2B} receptors (Kull et al., 1999; Jacobson and Gao, 2006).

Given that the development of adenosine receptor antagonists for the treatment of cancer may not be an option, other therapeutic strategies must be sought after. Since the effects of adenosine on tumour promotion are numerous, and are mediated by different receptor subtypes, an appealing approach would be to reduce local concentrations of adenosine specifically within tumours. In theory, this could be achieved by the local delivery of an adenosine-metabolizing enzyme, such as ADA, although the feasibility of this approach has not been examined. Also, it would be of great value to identify the signaling pathway(s) downstream of A_{2A} and A_{2B} receptor activation responsible for CXCR4 up-regulation, as this may reveal further targets for cancer treatment.

CONCLUSION

In this chapter, we have shown that the tumour metabolite adenosine increases CXCR4 expression at the mRNA and cell-surface protein levels on HT-29 cells. This up-regulation was mediated by activation of adenosine A_{2A} and A_{2B} receptors, and occurred at adenosine concentrations that have been shown to be present within the extracellular fluid of tumours. Therefore, adenosine may be one factor present within the tumour microenvironment that contributes to elevated CXCR4 expression. Furthermore, since adenosine-mediated CXCR4 up-regulation led to enhanced migratory and proliferative responses to CXCL12, this supports the role of adenosine in tumour promotion, specifically in the process of metastasis. Therapies designed to lower local adenosine concentrations may prove to be beneficial in the treatment of colorectal cancer. Further elucidation of the signaling mechanism involved in the adenosine effect on CXCR4 may also reveal novel targets for cancer treatment.

CHAPTER THREE

PROSTAGLANDINS DECREASE CXCR4 EXPRESSION ON HT-29 COLORECTAL CARCINOMA CELLS

Portions of this chapter appeared in the following publications:

Richard CL, Blay J. (Submitted). Thiazolidinedione drugs down-regulate CXCR4 expression on human colorectal cancer cells in a PPARγ-dependent manner.

Richard CL, Blay J. (Submitted). 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 down-regulates CXCR4 on carcinoma cells through PPARγ- and NF-κB-mediated pathways.

INTRODUCTION

Prostanoid production

Prostaglandins are lipid mediators involved in a multitude of biological processes. Prostanoids, including prostaglandins and thromboxanes, belong to a larger group of compounds referred to as eicosanoids, which are biologically active fatty acids derived from compounds with 20 carbons (Funk, 2001). Prostaglandin structure consists of a five-carbon ring and two side chains, and classifications are based on modifications to the ring and denoted by the letters A through J (Narumiya et al., 1999). Thromboxanes (such as TXA) have a structure that is related to the prostaglandins but contain a 6-carbon ring in place of a cyclopentane ring. Prostanoids may be further classified by the subscript 1, 2 or 3, indicating the number of double bonds present in the side chains. Humans have predominantly series 2 prostanoids, which are derived from arachidonic acid. The main prostanoids are prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2), prostaglandin I_2 (PGI_2 or prostacyclin), and thromboxane A_2 (TXA_2). Prostanoid structures are shown in Figure 3.1.

Prostanoids are rapidly synthesized from membrane-bound arachidonic acid in response to appropriate stimuli (Funk, 2001). Phospholipase A_2 (PLA_2) releases arachidonic from phospholipids in the membrane of the cell. Arachidonic acid is converted to PGG_2 and then PGH_2 through sequential cyclooxygenase and peroxidase reactions, which are catalyzed by cyclooxygenase (COX) enzymes. PGH_2 may then be converted to various prostaglandins or thromboxanes through the actions of specific synthases, which determine the relative abundance of the different prostanoids (Hata and Breyer, 2004; Helliwell et al., 2004). The synthases responsible for production of PGD_2,

PGE_2, $PGF_{2\alpha}$, prostacyclin, and TXA_2 are PGDS, PGES, PGFS, PGIS, and TXAS, respectively. Each synthase undergoes independent regulation of expression, and the prostaglandins produced by a cell depend upon which prostaglandin synthases it expresses. Prostanoid biosynthesis is depicted in Figure 3.1.

Two isoforms of COX exist, referred to as COX-1 and COX-2 (Smith et al., 1996; Smith et al., 2000). COX-1 is constitutively expressed, and is involved in the regulation of "housekeeping" functions such as renal water and electrolyte balance and gastric cytoprotection. Conversely, COX-2 levels can increase dramatically in response to certain stimuli, such as growth factors, cytokines, or various inflammatory mediators. As such, COX-2-derived prostaglandins are often implicated in inflammatory processes, pain, and fever.

Once formed, prostaglandins are released from cells via prostaglandin transporters, allowing them to act in an autocrine or paracrine fashion (Funk, 2001). Prostaglandins can be metabolized non-enzymatically to form a range of products both in the body and in cell culture. PGD_2 can be converted to cyclopentenone J-series prostaglandins, including prostaglandin J_2 (PGJ_2), Δ^{12}-PGJ_2, and 15-deoxy- $\Delta^{12,14}$-PGJ_2 ($15dPGJ_2$); PGE_2 can be converted to prostaglandin A_2 (PGA_2; Fitzpatrick and Wynalda, 1983; Aussel et al., 1987).

The predominant prostanoids in human colonic mucosa are PGE_2 and $PGF_{2\alpha}$, followed by PGD_2 (Boughton-Smith et al., 1983). Lower levels of prostacyclin and thromboxane are produced.

Prostanoid signaling

Prostanoids exert their biological effects through activation of seven-transmembrane GPCR prostanoid receptors (Narumiya et al., 1999; Funk, 2001). Nine prostanoid receptors have been identified: the PGD_2 receptors DP_1 and DP_2 (also called chemoattractant receptor homologous molecule expressed on Th2 cells, CRTH2); the PGE_2 receptors EP_1, EP_2, EP_3, and EP_4; the $PGF_{2\alpha}$ receptor FP; the prostacyclin receptor IP; and the thromboxane receptor TP (Hata and Breyer, 2004). Signaling pathways activated by prostaglandins are described in Table 3.1.

Multiple biological functions are elicited by activation of prostaglandin receptors, many of which have been identified by the use of knockout mice (Austin and Funk, 1999; Narumiya and FitzGerald, 2001). Known biological functions of prostaglandins include contraction and relaxation of smooth muscle, modulation of neuronal activity, fever generation, sleep induction, gastrointestinal tract mobility and secretions, renal water and ion transport, apoptosis, differentiation, and regulation of reproduction and platelet activity (Narumiya et al., 1999; Funk, 2001). In many cases, different prostanoids exert opposing effects on biological processes. For example, TXA_2 causes vasoconstriction and platelet aggregation whereas prostacyclin causes vasodilation and inhibits platelet aggregation.

Prostaglandins play a large role in inflammation and pain responses, and non-steroidal anti-inflammatory drugs (NSAIDs), which are commonly used to reduce pain and inflammation, inhibit COX and therefore block prostaglandin production (Smith et al., 2000). Gilroy and colleagues demonstrated that COX-2-derived prostaglandins may also have anti-inflammatory properties (Gilroy et al., 1999). Two hours after pleurisy

was induced in rats using carrageenin, COX-2 and PGE_2 levels increased and coincided with inflammatory responses. However, after 48 h there was a further increase in COX-2 levels which was associated with increased PGD_2 and $15dPGJ_2$ production, rather than PGE_2 production, leading to resolution of inflammation. Interestingly, the use of NSAIDs after this time actually increased inflammation due to impaired formation of anti-inflammatory PGD_2 and $15dPGJ_2$. Similarly, Schuligoi et al found that PGES expression increased 4 h after endotoxin-mediated systemic inflammation, whereas PGDS increased after 48 h (Schuligoi et al., 2005).

Prostaglandin effects on cancer cells

PGE_2 has several pro-tumour effects. For example, PGE_2 stimulated proliferation of HT-29, SW116, and HCA-7 human colon carcinoma cells (Qiao et al., 1995; Wang et al., 2005a). The effect on HCA-7 cells was blocked with Ras and MEK inhibitors, indicating that PGE_2 stimulation of cell growth occurred via activation of the Ras-MAPK cascade (Wang et al., 2005a). PGE_2 also induced chemotaxis of MDA-MB-231 human breast cancer cells, and EP_4 antagonists blocked basal migration, suggesting that PGE_2 may increase migration of breast cancer cells through activation of EP_4 (Timoshenko et al., 2003). In a skin carcinogenesis model, EP_2-/- mice had reduced tumour incidence compared to wild-type mice, whereas no difference was observed in EP_3-/- mice (Sung et al., 2005). Therefore, PGE_2 appears to enhance skin cancer development through activation of EP_2 receptors. PGE_2-mediated promotion of colon cancer growth may also be in part due to its ability to activate the epidermal growth factor receptor (EGFR; Pai et al., 2002). Treatment of RGM1 rat gastric mucosal cells and Caco-2, LoVo, and HT-29

human colon cancer cells with PGE_2 led to increased EGFR activation, and EGFR kinase inhibitors blocked PGE_2-induced proliferation and ERK2 phosphorylation.

Unlike PGE_2, its cyclopentenone metabolite PGA_2 reduced cell number and induced apoptosis and cell cycle changes in MCF-7 human breast cancer cells and HeLa human epithelial cervix carcinoma cells (Joubert et al., 2003).

In general, PGD_2 has anti-cancer effects. PGD_2 reduced the growth of SW480 and LS174T human colorectal carcinoma cells (Yoshida et al., 1998). PGD_2 also inhibited the growth of L-1210 murine leukemia cells after a 24 h treatment, but in culture media containing serum, over 80% of PGD_2 was metabolized after 24 h (Narumiya and Fukushima, 1985). The major product was Δ^{12}- PGJ_2, which was also shown to reduce growth of L-1210 cells but required a shorter exposure time than PGD_2. When the medium containing PGD_2 was replaced every 6 h, preventing accumulation of Δ^{12}- PGJ_2, PGD_2 no longer induced growth inhibition, suggesting that PGD_2-induced growth inhibition was due to formation of its metabolite, Δ^{12}- PGJ_2. J-series prostaglandins, including PGJ_2, Δ^{12}-PGJ_2, and $15dPGJ_2$, reduced proliferation and induced apoptosis of MDA-MB-231 and MCF-7 human breast cancer cells (Clay et al., 1999). $15dPGJ_2$ also inhibited growth and/or induced apoptosis of HT-29 human colorectal carcinoma cells (Kitamura et al., 1999; Shimada et al., 2002), DU145 human prostate carcinoma cells (Mueller et al., 2000), and HS-Sultan Burkitt lymphoma cells (Piva et al., 2005). Furthermore, treatment of MDA-MB-231 cells with $15dPGJ_2$ before s.c. transplantation into nude mice led to reduced tumour growth (Clay et al., 1999).

$15dPGJ_2$ is an agonist for the nuclear receptor PPARγ (Forman et al., 1995; Kliewer et al., 1995), and activation of PPARγ may account for the growth inhibitory

effects of $15dPGJ_2$. For example, $15dPGJ_2$ reduced the growth of PC-3 human prostate cancer cells through activation of PPARγ (Kim et al., 2005b). In addition to growth-inhibitory effects, $15dPGJ_2$ may also exert anti-cancer effects by reducing expression of pro-tumour proteins. $15dPGJ_2$ inhibited phorbol ester-induced VEGF and COX-2 mRNA and protein expression through transcriptional inhibition in SW620 human colorectal carcinoma cells (Grau et al., 2004).

Prostaglandin production in tumours

COX-2 expression is elevated in several tumour types, and is known to contribute to tumour progression. The evidence for this is particularly strong for colorectal tumours. Eberhart and colleagues were the first to show that COX-2, but not COX-1, mRNA was increased in colorectal cancer tissue compared to normal human mucosa (Eberhart et al., 1994). Other investigators have since confirmed COX-2 up-regulation in colorectal cancer (Kutchera et al., 1996; Dimberg et al., 1999). In patients with this disease, COX-2 protein expression increased during progression from primary disease to metastatic disease, and was positively correlated with tumour size, depth of invasion, lymph node invasion and metastasis, venous invasion, TNM stage, and recurrence (Soumaoro et al., 2004). Additionally, patients with COX-2-expressing tumours showed reduced survival, demonstrating that COX-2 expression is a prognostic indicator. In contrast, COX-1 was not predictive of disease progression or reduced survival.

Transgenic mice were generated in which the human *COX-2* gene was under the control of the murine mammary tumour virus (MMTV) promoter, such that COX-2 was over-expressed in the mammary gland, leading to increased prostaglandin synthesis (Liu

et al., 2001). Multiparous transgenic mice developed mammary hyperplasia, whereas control mice lacking COX-2 over-expression did not. Several transgenic mice also developed tumours in the mammary glands, which was a very rare event in control mice. The microvasculature in mammary glands of COX-2 transgenic mice contained vascular loops and arches, and there was evidence of abnormal vessel function (Chang et al., 2004). This abnormal architecture is consistent with tumour angiogenesis. Additionally, there was increased expression of angiogenic regulatory genes, including VEGF, ang 1 & 2, and Tie 1 & 2. PGE_2 was the prominent prostaglandin in mammary tissue from COX-2 transgenic mice, and PGE_2 induced expression of angiogenic genes in cell lines derived from tumours in COX-2 transgenic mice, suggesting that PGE_2 may be responsible for COX-2-induced angiogenesis. The authors concluded that COX-2 expression in the mammary gland was sufficient to induce tumour formation, and that this was due at least in part to increased angiogenesis (Liu et al., 2001; Chang et al., 2004).

Adding support to this conclusion, the NSAID indomethacin reduced both tumour incidence and microvessel density in MMTV-COX-2 mice (Chang et al., 2004). Furthermore, when MMTV-COX-2 mice were crossed with EP_2-/- mice to generate MMTV-COX-2-EP_2-/- mice, these animals no longer developed mammary hyperplasia, demonstrating the requirement of EP_2 receptors for COX-2-induced mammary hyperplasia (Chang et al., 2005).

NSAIDs, which inhibit both COX-1 and COX-2, and selective COX-2 inhibitors have been shown to reduce cancer progression in pre-clinical models and clinical trials (Gupta and Dubois, 2001). NSAIDs and COX-2 inhibitors reduce the relative risk of developing colorectal cancer by 40-50% (Gupta and Dubois, 2001; Marnett and DuBois,

2002; Wang et al., 2005b). The beneficial effects are generally believed to be due to inhibition of prostaglandin production, particularly PGE_2 production, but may involve COX-independent pathways as well (Hanif et al., 1996), possibly due to PPARγ activation (Wick et al., 2002).

Tumour-promoting effects of COX-2 over-expression appear to be primarily due to increased PGE_2 production. Badawi and co-workers found that COX-2 mRNA and protein expression were elevated in tumour tissue compared to adjacent normal tissue, and further increases were seen in metastatic tissues (Badawi and Badr, 2003). Increased COX-2 expression correlated with increased PGE_2 levels in primary and metastatic tumour tissues, and PGE_2 levels correlated with progression to metastatic disease. Other investigators have found that PGE_2 is the predominant prostaglandin in colorectal cancer, and PGE_2 levels are increased in colonic polyps and cancerous tissue compared to normal tissue (Rigas et al., 1993; Pugh and Thomas, 1994; Giardiello et al., 1998)

Backlund and colleagues examined expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), an enzyme involved in the inactivation of PGE_2, in colorectal carcinoma cell lines and tissue samples, and found that 15-PGDH mRNA and protein levels were consistently reduced compared to normal tissues (Backlund et al., 2005). Reduced expression of 15-PGDH in cancer tissues would contribute to elevated PGE_2 levels due to reduced catabolism.

Expression of cytosolic PLA_2 ($cPLA_2$) in tumours is also significant in cancer development, possibly due to changes in prostaglandin production. Dimberg and colleagues found that $cPLA_2$ mRNA was up-regulated in human colorectal tumours compared to normal mucosa (Dimberg et al., 1998). Mice deficient in $cPLA_2$ developed

fewer tumours than wild-type controls in a urethane-induced murine lung carcinogenesis model (Meyer et al., 2004). Tumours from $cPLA_2$-/- mice had significantly lower PGE_2 levels.

Elevated prostaglandin levels within tumours, particularly PGE_2, may result from changes in the expression of several enzymes involved in eicosanoid metabolism, including COX-2, $cPLA_2$, and 15-PGDH. We sought to determine if prostaglandins present within tumours may affect CXCR4 expression on colorectal cancer cells.

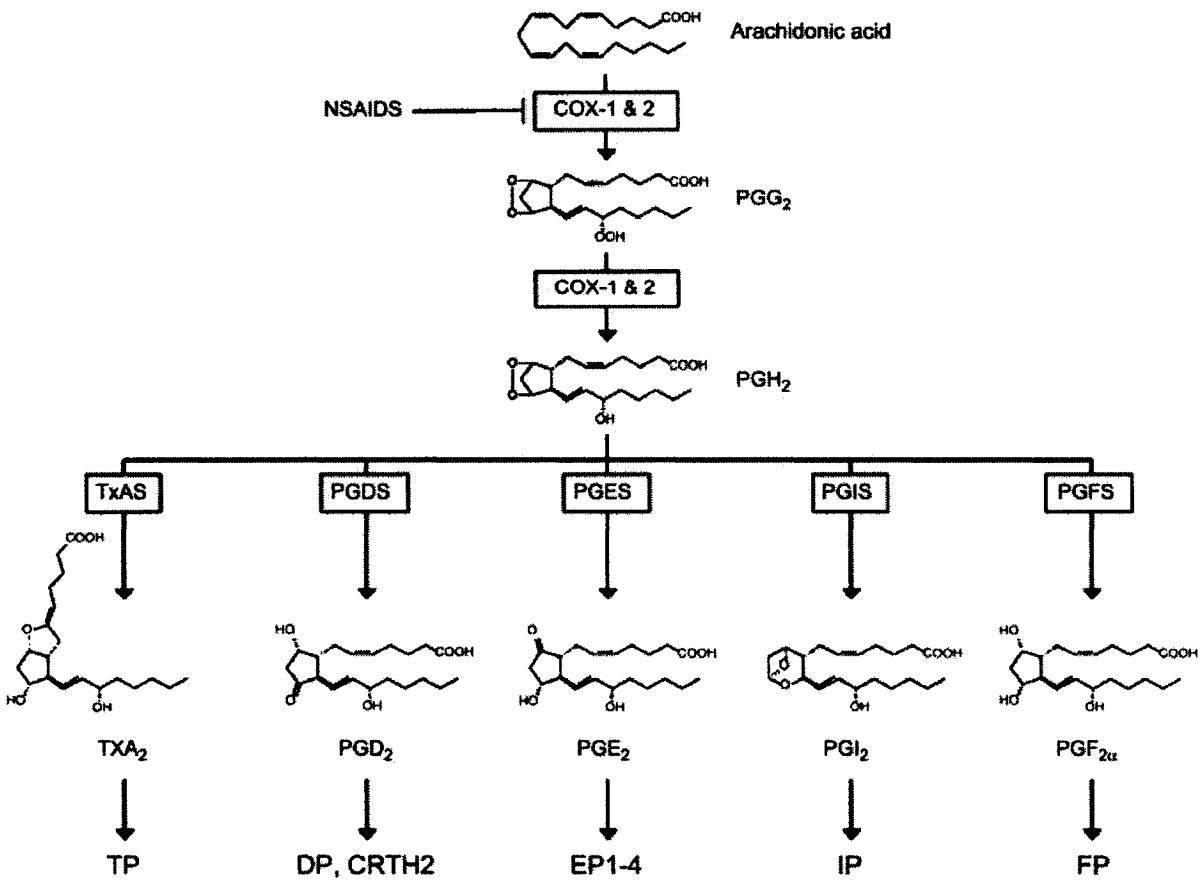


Figure 3.1: Prostanoid structure and biosynthesis. Taken from Hata and Breyer, 2004.

Receptor	G-protein coupling	Signaling
DP_1	G_s	↑ cAMP, ↑Ca^{2+}
DP_2/CRTH2	G_i	↓ cAMP, ↑Ca^{2+}, PLC, PI3K, MAPK
EP_1	Unknown	↑Ca^{2+}
EP_2	G_s	↑ cAMP, EGFR transactivation, β-catenin
EP_3	G_i, G_q, G_s	↓ cAMP, ↑ IP_3/DAG, ↑ cAMP
EP_4	G_s	↑ cAMP, PI3K, ERK1/2, β-catenin
FP	G_q	↑ IP_3/DAG, Rho, EGFR transactivation, β-catenin
IP	G_s, G_q, G_i	↑ cAMP, ↑ IP_3/DAG, ↓ cAMP
TP	G_q, G_s, G_i, G_{12}	↑ IP_3/DAG/Ca^{2+}, ↑ cAMP, ↓ cAMP

Table 3.1: Signaling through prostanoid receptors. Adapted from Hata and Breyer, 2004.

Objectives

As described in Chapter Two, we found that the tumour metabolite adenosine increased CXCR4 expression on colorectal cancer cells. In this chapter, we sought to determine if prostaglandins, which are also present in high levels within tumours, may contribute to elevated CXCR4 expression in colorectal cancer as well.

The objectives for the work described in this chapter were:

1. To determine if prostaglandins alter CXCR4 expression on colorectal cancer cells and
2. To identify signaling pathways involved in any prostaglandin-mediated changes in CXCR4 expression.

Hypothesis

Prostaglandins which are produced in increased levels in tumours increase CXCR4 expression on colorectal carcinoma cells. In particular, PGE_2, which is elevated in tumours and has pro-tumour functions, will elevate CXCR4 expression.

METHODS

Materials

HT-29 human colorectal carcinoma cells, media, sera, culture vessels, TRIzol®, Oligo(dT)$_{12-18}$ primer, dNTP mix, M-MLV reverse transcriptase, DTT, 5x first strand buffer, BSA, custom primers, mouse anti-human monoclonal antibody against CXCR4 (clone 12G5), mouse IgG$_{2a}$ (clone G155-178), ^{125}I-labeled goat anti-mouse IgG, and Brilliant SYBR® Green kits were obtained as described in Chapter Two. PGA$_2$, PGD$_2$, PGE$_2$, PGF$_{2\alpha}$, PGJ$_2$, cyclopentane, cyclopentene, and cyclopentenone were from Sigma Chemical Co. (St. Louis, MO). Rosiglitazone, ciglitazone, pioglitazone, troglitazone, MCC555, GW9662, T0070907, 15dPGJ$_2$ and CAY10410 were from Cayman Chemical (Ann Arbor, MI).

Cell Culture

HT-29 cells were cultured as described in Chapter Two. For radioantibody binding assays, cells were sub-cultured into 48-well plates in DMEM containing 10% v/v NCS at a density of 50,000 cells/well. For real-time PCR, 6-well plates were seeded at 200,000 cells/well. After allowing the cells to attach for 48 h, the medium was replaced with DMEM containing 1% NCS v/v, and drug treatments were performed after a further 48 h incubation. The vehicle for the prostaglandins, thiazolidinediones, and PPARγ antagonists was DMSO. The final DMSO concentration did not exceed 0.15% v/v. PPARγ antagonists were added 30 min before rosiglitazone or 15dPGJ$_2$.

Radioantibody binding assay

We quantified cell-surface CXCR4 protein using a radioantibody binding assay as described in Chapter Two. Radioantibody binding assays were performed 48 h after drug additions unless otherwise indicated. Briefly, plates were placed on ice, washed, and incubated with a primary antibody against CXCR4. The cells were then washed again, and incubated with a secondary ^{125}I-labeled antibody, followed by further washing and counting of radioactivity. Results are corrected to cpm per 1000 cells, and unless otherwise indicated, drug treatments did not induce greater than a 20% change in cell number.

Real-time PCR

Real-time PCR was used to quantify CXCR4 or cytokeratin 20 mRNA expression in HT-29 cells. The procedure and analysis were performed as described in Chapter Two. Briefly, RNA was isolated using TRIzol®, reverse transcribed using M-MLV reverse transcriptase, and amplified using Brilliant SYBR® Green and the following primer sets:

1. GAPDH: forward - 5'-catgagaagtatgacaacagcct-3';
   reverse - 5'-agtccttccacgataccaaagt-3'
2. CXCR4: forward - 5'-gcctgagtgctccagtagcc-3';
   reverse - 5'-tggagtcatagtcccctgagc-3'
3. Cytokeratin 20: forward – 5'-atggatttcagtcgcagaagc-3'
   reverse – 5'-ctcccatagttcaccgtgtgt-3'

Relative CXCR4 or cytokeratin 20 mRNA expression was determined using the $2^{-\Delta\Delta cT}$ method (Livak and Schmittgen, 2001), with standardization against GAPDH and normalization to 0 h control for the time course experiments and vehicle treatment for dose-response analyses.

Statistical analysis

Each figure shows a representative result from a series of experiments done on at least three independent occasions. Statistical analysis was performed using two-tailed Student *t*-test and ANOVA where appropriate.

RESULTS

Prostaglandins reduce cell-surface CXCR4 protein expression

We sought to determine if prostaglandins may affect CXCR4 protein expression on cancer cells, and thus contribute to its altered expression within tumours. We examined the effects of prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on cell-surface CXCR4 protein expression on HT-29 cells using a radioantibody binding assay. These are the predominant prostanoids in human colonic mucosa (Boughton-Smith et al., 1983). We chose not to examine the effects of the prostaglandin I_2 (PGI_2) and thromboxane A_2 (TXA_2) on CXCR4 expression, since they are found in lower levels in colonic mucosa compared to PGD_2, PGE_2, and $PGF_{2\alpha}$ (Boughton-Smith et al., 1983) and are primarily involved in the regulation of vascular and platelet function (Dogne et al., 2004; de Leval et al., 2004). Furthermore, both PGI_2 and TXA_2 are chemically unstable and are rapidly converted to inactive forms (Needleman et al., 1976; Samuelsson, 1976; Cho and Allen, 1978; Rao et al., 1980).

After a 48 h treatment, PGD_2 produced a robust, dose-dependent decrease in cell-surface CXCR4 protein on HT-29 cells (Figure 3.2). The maximum effect was noted with a dose of 30 μM, which effected an 87% reduction in cell-surface CXCR4 protein in the experiment shown.

PGE_2 also produced a dose-dependent down-regulation of CXCR4, albeit to a lesser extent than PGD_2 (Figure 3.3). A ten-fold higher dose of PGE_2 was required to produce a statistically-significant effect on CXCR4 compared to PGD_2. The maximum effect was noted with 30 μM PGE_2, which caused a 67% reduction in CXCR4 protein. $PGF_{2\alpha}$ had very little impact on CXCR4 receptor expression on HT-29 cells, and only

produced a 24% reduction at the highest dose used (Figure 3.4). Since cell-surface CXCR4 protein expression was most dramatically altered by PGD_2, we decided to focus our attention on this particular prostaglandin.

Metabolites of PGD_2 reduce CXCR4 expression

PGD_2 exerts its biological activity by activation of DP receptors, which are seven-transmembrane G-protein coupled receptors (Breyer et al., 2001). There are two subtypes of DP receptors, DP1 and DP2, but only DP1 receptors have been shown to be expressed by HT-29 cells (Hawcroft et al., 2004). However, alternatively, PGD_2 can act independently of DP receptors by its metabolism through a dehydration reaction to prostaglandin J_2 (PGJ_2), Δ^{12}-PGJ_2, and then to 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ($15dPGJ_2$; Fitzpatrick and Wynalda, 1983). This reaction occurs in cell culture media, both in the presence and absence of serum (Fitzpatrick and Wynalda, 1983; Narumiya and Fukushima, 1985; Shibata et al., 2002). Therefore, it is possible that effects noted *in vitro* with PGD_2 are actually due to the formation of J-series prostaglandins.

As shown in Figure 3.5, metabolites of PGD_2 significantly reduced cell-surface CXCR4 protein expression on HT-29 cells in a dose-dependent fashion. At a dose of 30 μM, PGJ_2 reduced CXCR4 receptor expression by 95% in the experiment shown in Figure 3.5A. Cell-surface CXCR4 protein expression was undetectable after treatment with 10 or 30 μM $15dPGJ_2$ (Figure 3.5B). $15dPGJ_2$ was a more potent and effective reducer of CXCR4 expression than its precursors, PGD_2 and PGJ_2, and could likely account for the action noted with these prostaglandins. The highest dose of $15dPGJ_2$

used in this experiment (30 μM) was toxic to HT-29 cells, causing a 65% reduction in cell number.

We next examined the time course of changes in cell-surface CXCR4 protein expression induced by PGD_2 and its metabolites, PGJ_2 and $15dPGJ_2$. All three prostaglandins (10 μM PGD_2 and PGJ_2, 3 μM $15dPGJ_2$) caused a significant down-regulation of CXCR4 by 24 h, which was maintained at 72 h (Figure 3.6). However, interestingly, at the 8 h time point only $15dPGJ_2$ reduced CXCR4 protein expression in a statistically significant manner. Although not conclusive, this again raises the possibility that PGD_2 and PGJ_2 must be converted to $15dPGJ_2$ in order to down-regulate CXCR4, and the effect noted with these two prostaglandins was due to $15dPGJ_2$ formation.

We decided to further explore this marked CXCR4 down-regulation caused by $15dPGJ_2$. As described in Chapter Two of this thesis, adenosine-mediated changes in CXCR4 were due to an increase in mRNA transcription. To see if $15dPGJ_2$-mediated CXCR4 down-regulation was also apparent at the mRNA level, we treated HT-29 cells with $15dPGJ_2$ for 24 h, and then quantified CXCR4 mRNA levels using real-time RT-PCR. $15dPGJ_2$ (10 μM) caused an 88% reduction in CXCR4 mRNA expression (Figure 3.7), consistent with the reduction noted in cell-surface CXCR4 protein expression (Figure 3.5B). Thus, reduction in mRNA transcription most likely accounted for $15dPGJ_2$-induced CXCR4 down-regulation.

Synthetic PPARγ agonists reduce CXCR4 expression

The PGD_2 metabolite $15dPGJ_2$ has several biological actions, all of which occur independently of PGD_2 receptors. The most well-characterized mechanism by which

$15dPGJ_2$ affects cells is through activation of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ; Forman et al., 1995; Kliewer et al., 1995). PPARγ activation results in its heterodimerization with the retinoid X receptor (RXR), binding to peroxisome proliferator response elements (PPREs) on DNA, and subsequent activation of target gene expression (Kota et al., 2005). PPARγ is aberrantly expressed in some cancer types (DuBois et al., 1998), and in many cases its activation leads to cell death or differentiation (Brockman et al., 1998; Elstner et al., 1998; Kitamura et al., 1999).

To test whether $15dPGJ_2$-mediated CXCR4 down-regulation could be due to PPARγ activation, we attempted to reproduce the effect using other known activators of PPARγ. Rosiglitazone is a member of the thiazolidinedione class of anti-diabetic agents, and is a potent activator of PPARγ (Lehmann et al., 1995). Before examining the effect of rosiglitazone on CXCR4 expression, we first wanted to be sure that HT-29 cells were able to respond in an expected manner to PPARγ activation. We found previously using real-time RT-PCR that HT-29 cells express PPARγ (Richard CL, Blay J, unpublished observations). We looked at whether or not the known PPARγ target gene cytokeratin 20 (Gupta et al., 2001) would be regulated by rosiglitazone as expected in HT-29 cells. We treated HT-29 cells with rosiglitazone (1 μM), and quantified cytokeratin 20 mRNA isolated after 0, 2, 4, and 8 h using real-time RT-PCR. HT-29 cells expressed the PPARγ target gene cytokeratin 20, and rosiglitazone produced a significant increase in cytokeratin 20 mRNA expression (Figure 3.8). The effect was apparent after 2 h, and was further increased by 4 h and maintained at 8 h. This indicated that HT-29 cells were able to produce an expected PPARγ response.

Since PPARγ appeared to be functional in HT-29 cells, we examined the involvement of PPARγ in CXCR4 regulation. As shown in Figure 3.9, rosiglitazone produced a dose-dependent reduction in cell-surface CXCR4 protein, achieving statistical significance at a dose as low as 1 nM. Interestingly, the maximum reduction in CXCR4 by rosiglitazone was only 56%, while $15dPGJ_2$ reduced CXCR4 protein expression to the point where it was no longer detectable (Figure 3.5B). However, the fact that the potent PPARγ agonist rosiglitazone reduced CXCR4 receptor expression was in agreement with the possibility of $15dPGJ_2$ acting through a PPARγ-dependent mechanism. Furthermore, a panel of other thiazolidinedione PPARγ agonists, ciglitazone, pioglitazone, troglitazone, and MCC555, also reduced cell-surface CXCR4 protein expression on HT-29 cells (Figure 3.10), although the agents differed in potency and efficacy. The thiazolidinediones had negligible effects on cell number at the concentrations used in our experiments. The only exception was MCC555, which reduced cell number by 24% at the highest concentration (10 μM).

We performed real-time RT-PCR to see if rosiglitazone had an effect on CXCR4 mRNA. Indeed, rosiglitazone (10 nM) produced a 57% reduction in CXCR4 mRNA (Figure 3.11), similar in magnitude to the change it caused in cell-surface CXCR4 protein (Figure 3.9). As was observed with $15dPGJ_2$, rosiglitazone reduced CXCR4 expression by reducing mRNA transcription.

In an experiment designed to compare the timing of rosiglitazone-induced to $15dPGJ_2$-induced CXCR4 down-regulation, we found that both agents produced a statistically significant reduction in cell-surface CXCR4 protein expression that was evident by 8 h, and persisted until 72 h (Figure 3.12). However, it is interesting to note

that the effect of $15dPGJ_2$ was declining by the 72 h time point, whereas the rosiglitazone-induced decrease in CXCR4 was even more pronounced at the 72 h time point. This may reflect differences in the stability of these two compounds in culture. The concentration of $15dPGJ_2$ used in this experiment (1 μM) was typically sufficient to reduce CXCR4 receptor levels, although the effect noted with 3 μM was more reproducible. The change in CXCR4 receptor expression on vehicle-treated cells over the time-course likely resulted from cell cycle-dependent regulation of CXCR4 expression (Shibuta et al., 2002).

PPARγ antagonists block rosiglitazone- and $15dPGJ_2$-induced CXCR4 down-regulation

To confirm the involvement of PPARγ in regulation of CXCR4, we used two specific PPARγ antagonists, GW9662 and T0070907, to try to block the effects of rosiglitazone and $15dPGJ_2$. Each of these compounds acts as an irreversible inhibitor of PPARγ (Lee et al., 2002; Leesnitzer et al., 2002). GW9662 (1 μM) completely blocked the down-regulation of CXCR4 caused by rosiglitazone (10 nM), as did T0070907 (100 nM, Figure 3.13). Therefore, rosiglitazone-induced CXCR4 down-regulation occurred through activation of PPARγ. However, the same concentrations of GW9662 and T0070907 that blocked the rosiglitazone effect on CXCR4 failed to completely block the $15dPGJ_2$ effect (Figure 3.14). This was surprising, since $15dPGJ_2$ is known to activate PPARγ, and in general it seemed to influence CXCR4 expression in a manner similar to that seen with the potent PPARγ agonist rosiglitazone. One significant difference between activation of PPARγ by $15dPGJ_2$ and rosiglitazone is that $15dPGJ_2$ binds

covalently to PPARγ (Soares et al., 2005), whereas rosiglitazone binds non-covalently. Therefore, it seemed feasible that a higher dose of a PPARγ antagonist would be required to block the effect of $15dPGJ_2$ compared to rosiglitazone. Indeed, when we used a tenfold higher dose of each PPARγ antagonist (10 μM GW9662 and 1 μM T0070907), we were able to block $15dPGJ_2$-induced CXCR4 down-regulation of cell-surface CXCR4 protein on HT-29 cells (Figure 3.15). Therefore, $15dPGJ_2$ did indeed work in a PPARγ-dependent manner to reduce CXCR4 receptor expression.

$15dPGJ_2$-induced CXCR4 down-regulation may involve signaling through NF-κB

In addition to its ability to activate the nuclear receptor PPARγ, another cellular mechanism by which $15dPGJ_2$ can elicit its effects is by inhibition of NF-κB signaling (Straus et al., 2000). An analogue of $15dPGJ_2$, 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (CAY10410), does not inhibit NF-κB signaling but can still activate PPARγ (Lindstrom and Bennett, 2005; Shiraki et al., 2005). We found that CAY10410 reduced CXCR4 protein expression on HT-29 cells by greater than 90% at a dose of 30 μM, but the dose-response curve was shifted to the right compared to that of $15dPGJ_2$ (Figure 3.16). The highest dose of CAY10410 (30 μM) caused a 15% reduction in cell number, whereas an equimolar dose of $15dPGJ_2$ resulted in a 50% reduction in cell number.

As $15dPGJ_2$-mediated NF-κB inhibition occurs by covalent modification of proteins by its cyclopentenone structure (Straus et al., 2000; Lindstrom and Bennett, 2005), we examined whether cyclopentenone itself would decrease CXCR4 expression. Indeed, a sharp drop in cell-surface CXCR4 protein expression occurred with 300 or

1000 μM cyclopentenone (Figure 3.17A). Control compounds cyclopentane and cyclopentene did not reduce CXCR4 receptor expression (Figure 3.17B and C). Cyclopentenone caused a 33% reduction in cell number at the highest dose (1000 μM), whereas cyclopentane and cyclopentene had negligible effects on cell number. Since cyclopentenone alone was sufficient to reduce CXCR4 protein, it is likely that NF-κB inhibition results in reduced CXCR4 levels.

Prostaglandin A_2 (PGA_2), a metabolite of PGE_2, is another prostaglandin containing a cyclopentenone structure, allowing it to inhibit NF-κB signaling (Rossi et al., 2000). However it does not contain the α,β-unsaturated ketone moiety necessary to activate PPARγ signaling (Shiraki et al., 2005). PGA_2 reduced cell-surface CXCR4 protein expression (Figure 3.18), an effect that could be caused by inhibition of NF-κB signaling, but not by PPARγ activation. The highest dose of PGA_2 (30 μM) caused a 34% reduction in cell number. Interestingly, the magnitude of the PGA_2 effect on CXCR4 and the shape of its dose response curve resembled that which was seen with PGE_2 (Figure 3.3). Therefore, it is possible that PGE_2 exerted its effect on CXCR4 expression through its conversion to PGA_2, a reaction which has been shown to occur in cell culture (Aussel et al., 1987; Ishihara et al., 1991).

Although these experiments are not conclusive, the fact that CAY10410 was less potent than $15dPGJ_2$, and that cyclopentenone and PGA_2 both decreased CXCR4 receptor expression support the concept of regulation of CXCR4 through NF-κB signaling. The most likely explanation for $15dPGJ_2$-induced CXCR4 down-regulation is a combined effect on PPARγ and NF-κB signaling.

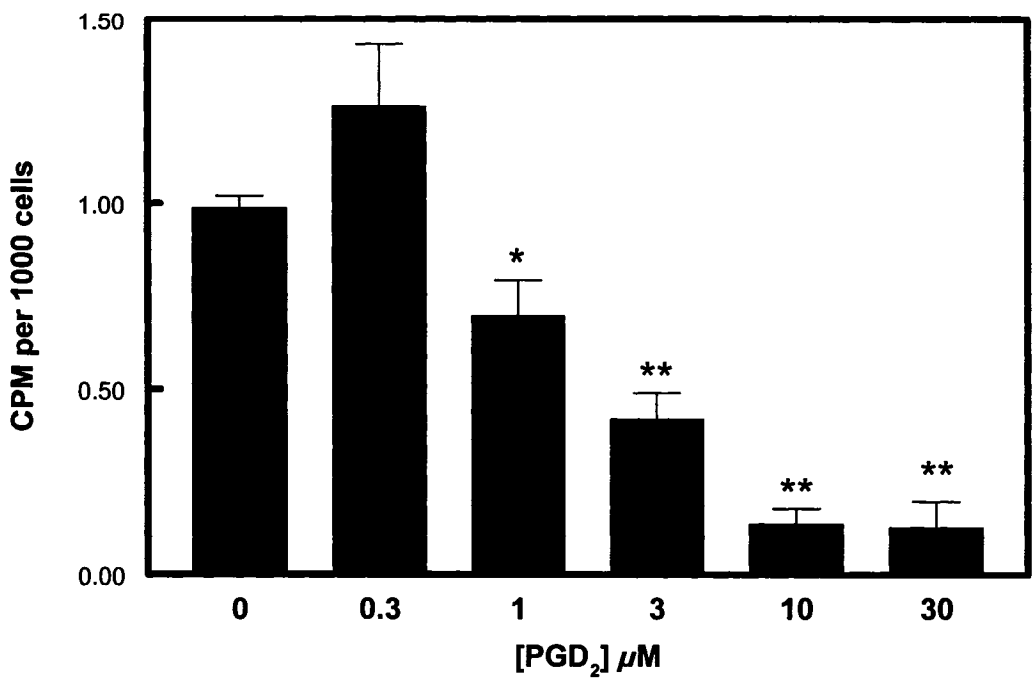


Figure 3.2: Effect of PGD_2 on cell-surface CXCR4 protein expression on HT-29 cells. HT-29 cells were treated with PGD_2 at the indicated concentrations, and cell-surface CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant decrease due to PGD_2, $P<0.01$; *, $P<0.05$.

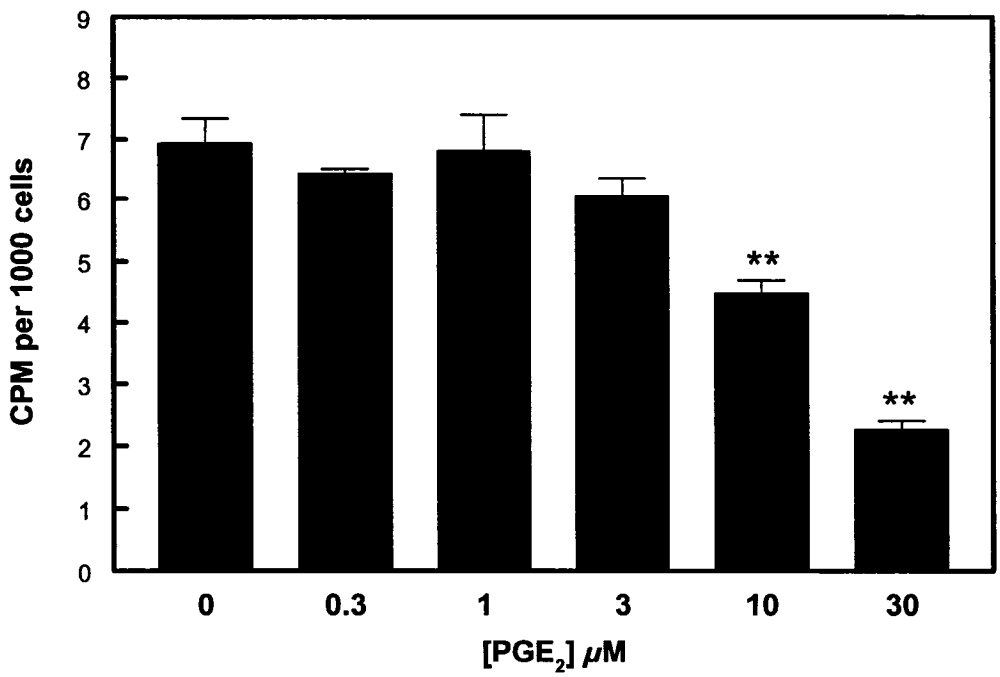


Figure 3.3: Effect of PGE_2 on cell-surface CXCR4 protein expression on HT-29 cells. HT-29 cells were treated with PGE_2 at the indicated concentrations, and cell-surface CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant decrease due to PGE_2, P<0.01.

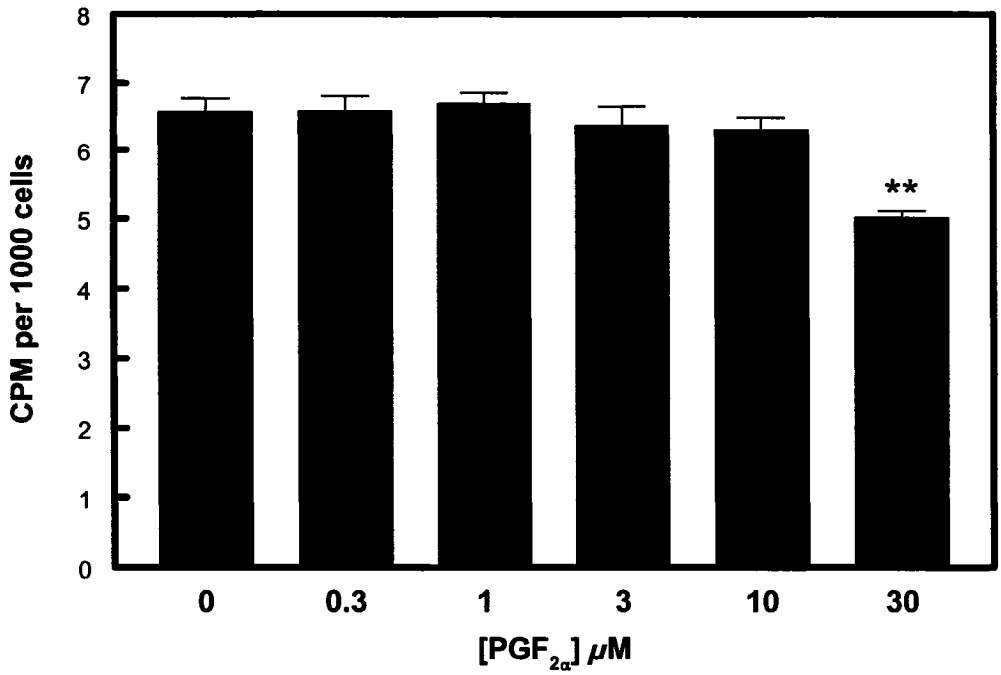


Figure 3.4: Effect of $PGF_{2\alpha}$ on cell-surface CXCR4 protein expression on HT-29 cells. HT-29 cells were treated with $PGF_{2\alpha}$ at the indicated concentrations, and cell-surface CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant decrease due to $PGF_{2\alpha}$, P<0.01.

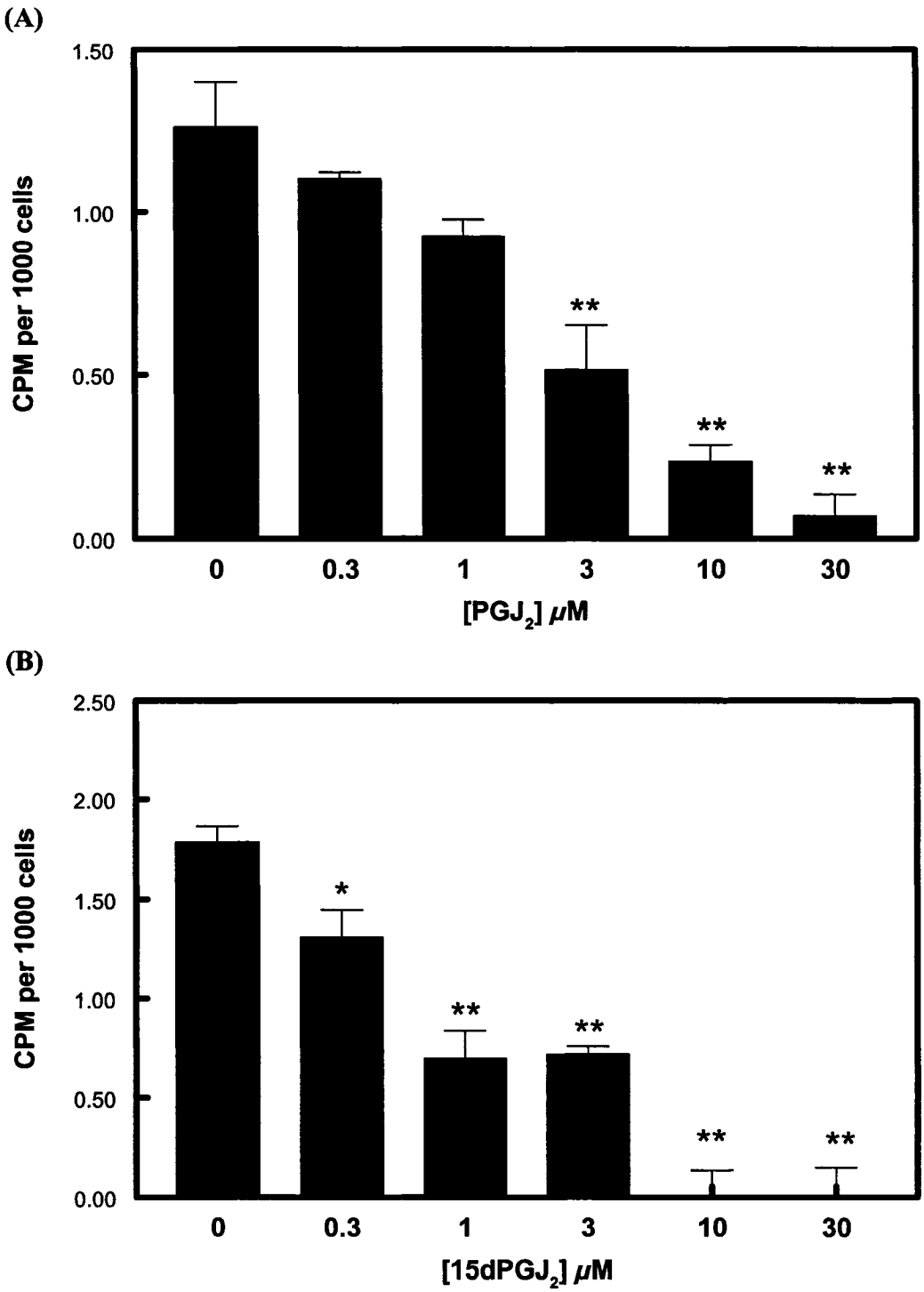


Figure 3.5: Effect of metabolites of PGD_2 on cell-surface CXCR4 protein expression on HT-29 cells. HT-29 cells were treated with (A) PGJ_2 or (B) $15dPGJ_2$ at the indicated concentrations, and cell-surface CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant decrease due to prostaglandin, $P<0.01$; *, $P<0.05$.

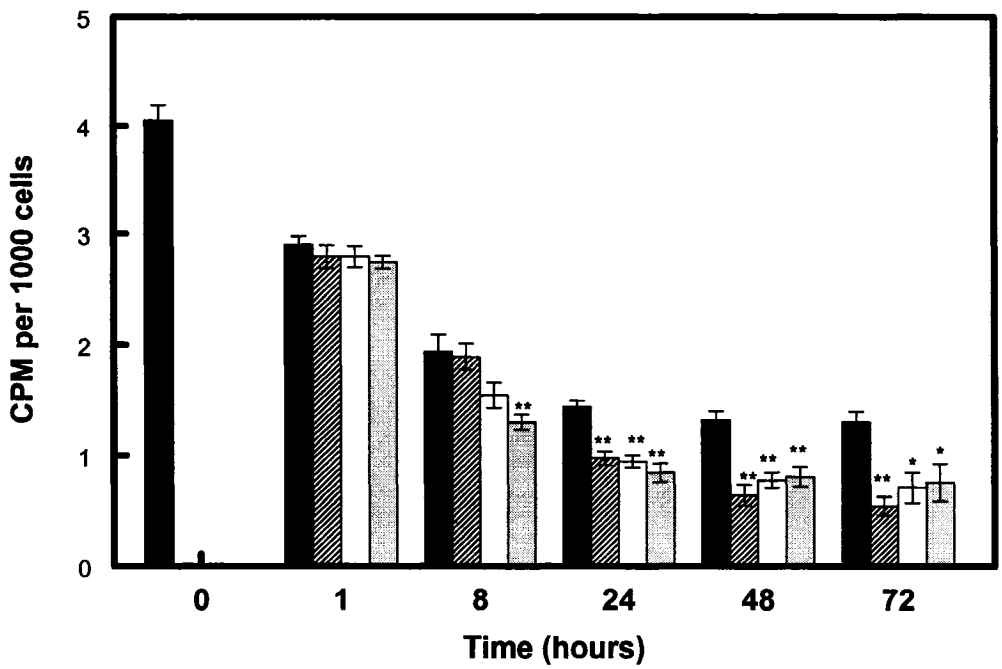


Figure 3.6: Time-dependency of changes in cell-surface CXCR4 protein expression on HT-29 cells by PGD_2 and its metabolites. HT-29 cells were treated with vehicle (solid bars), 10 μM PGD_2 (hatched bars), 10 μM PGJ_2 (open bars), or 3 μM $15dPGJ_2$ (shaded bars), and cell-surface CXCR4 protein expression was measured at the indicated time points using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant decrease due to prostaglandin, $P<0.01$; *, $P<0.05$.

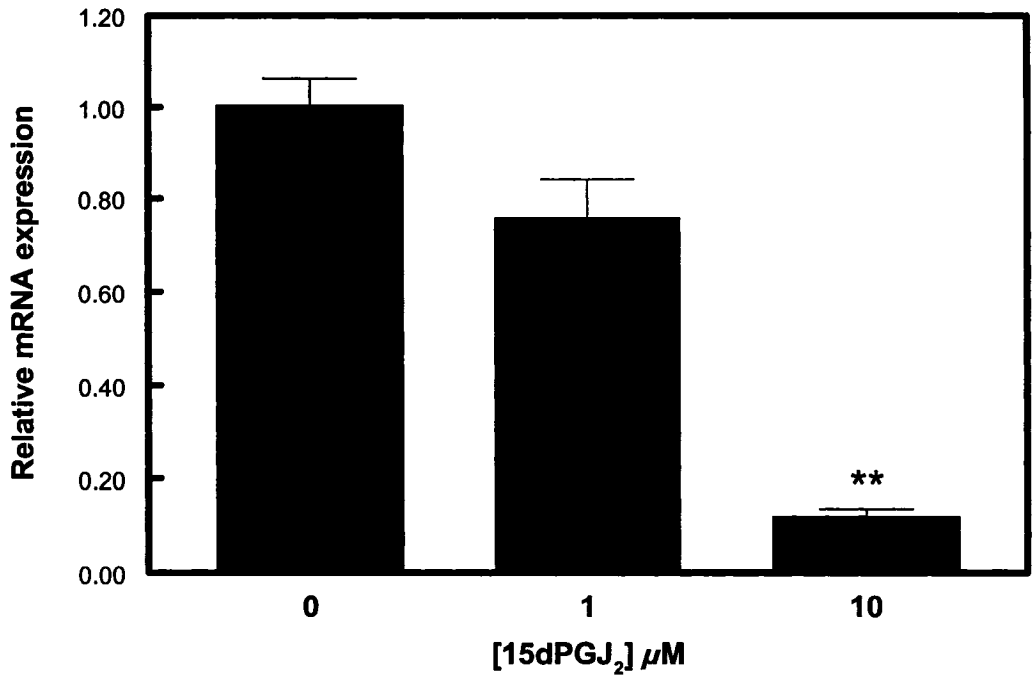


Figure 3.7: Effect of $15dPGJ_2$ on CXCR4 mRNA expression in HT-29 cells. HT-29 cells were treated with $15dPGJ_2$ at the indicated concentrations, and RNA was isolated after 24 h. CXCR4 mRNA expression was quantified using real-time PCR. The data are mean values ± SE (n=3). **, significant decrease due to $15dPGJ_2$, P<0.01.

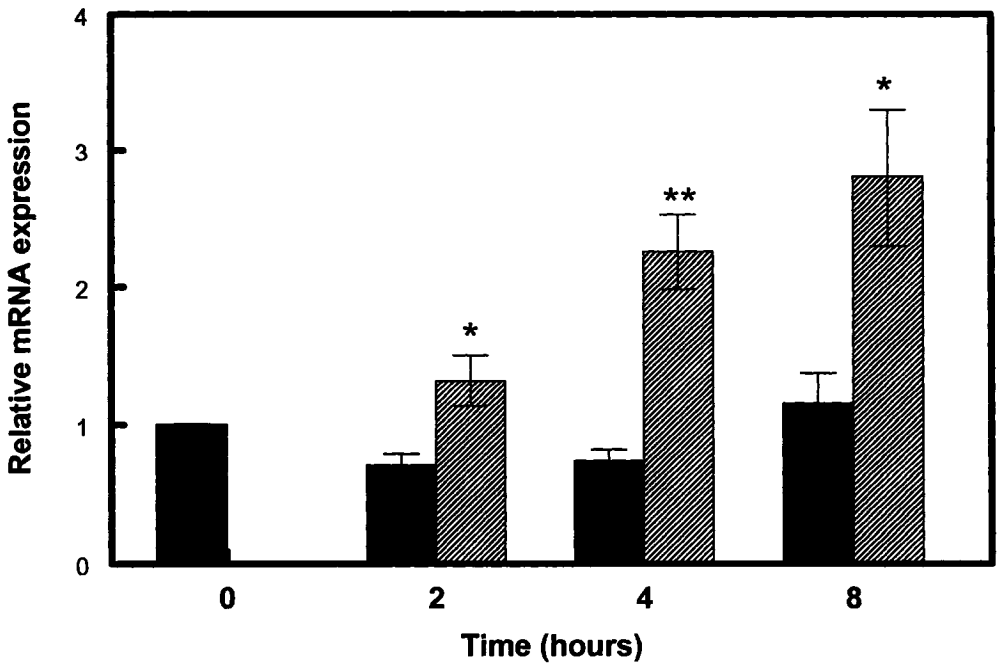


Figure 3.8: Effect of rosiglitazone on cytokeratin 20 mRNA expression in HT-29 cells. HT-29 cells were treated with vehicle (solid bars) or 1 μM rosiglitazone (hatched bars), and RNA was isolated at the indicated time points. Cytokeratin 20 mRNA expression was quantified using real-time PCR. The data are mean values ± SE (n=3). **, significant increase due to rosiglitazone, $P<0.01$; *, $P<0.05$.

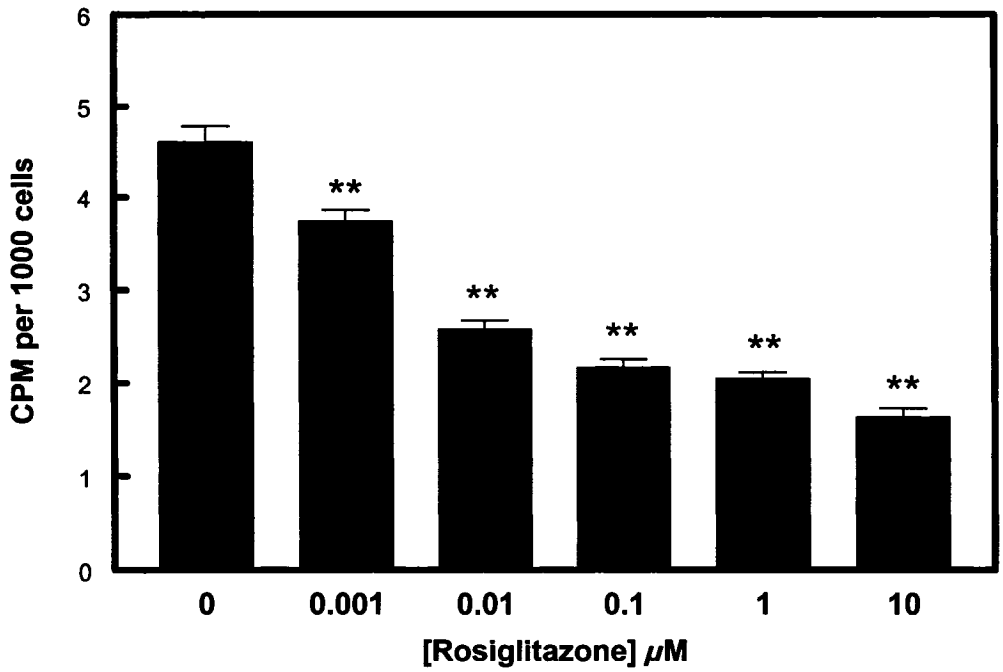


Figure 3.9: Effect of rosiglitazone on cell-surface CXCR4 protein expression on HT-29 cells. HT-29 cells were treated with rosiglitazone at the indicated concentrations, and cell-surface CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant decrease due to rosiglitazone, P<0.01.

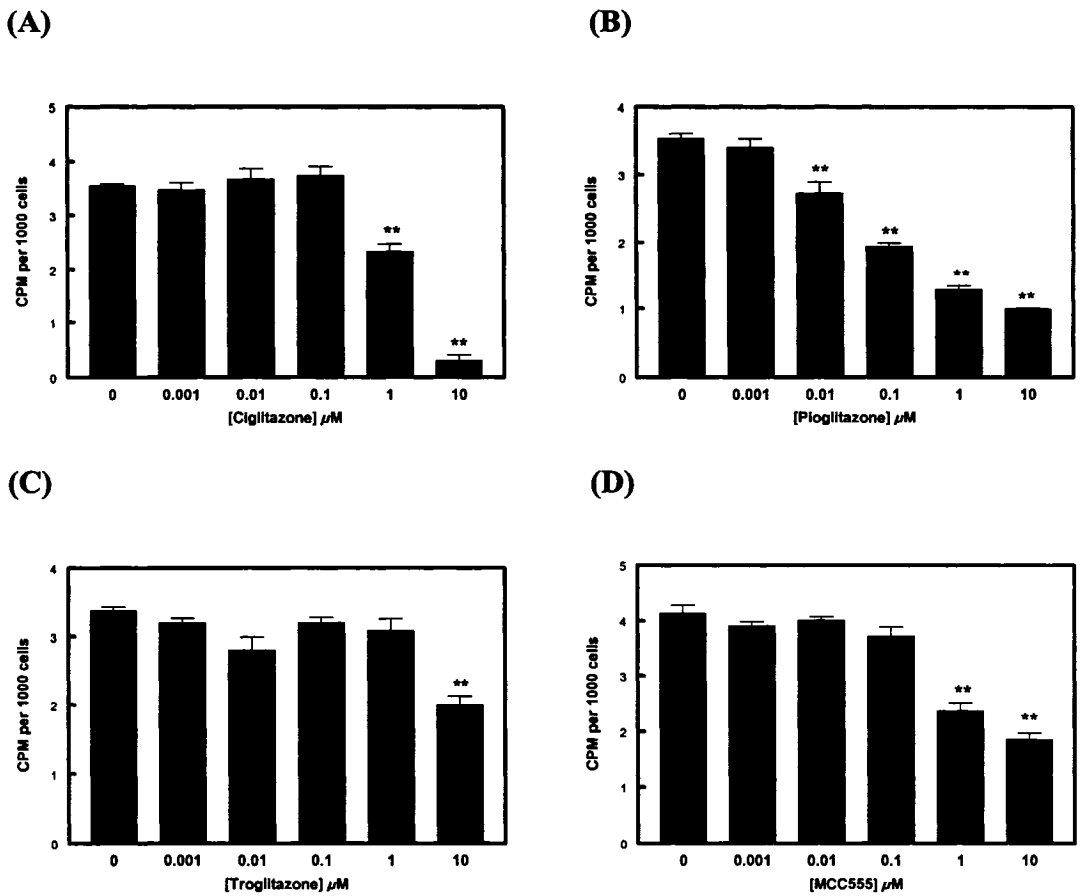


Figure 3.10: Effect of thiazolidinediones on cell-surface CXCR4 protein expression on HT-29 cells. HT-29 cells were treated with (A) ciglitazone, (B) pioglitazone, (C) troglitazone, or (D) MCC555 at the indicated concentrations, and cell-surface CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant decrease due to thiazolidinedione, P<0.01.

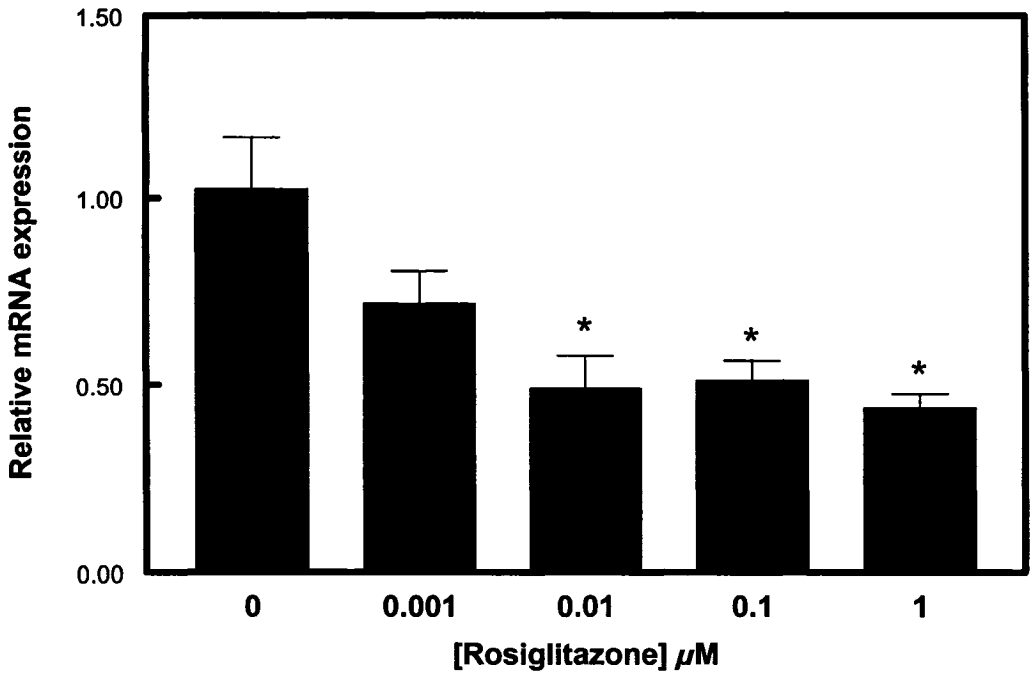


Figure 3.11: Effect of rosiglitazone on CXCR4 mRNA expression in HT-29 cells. HT-29 cells were treated with rosiglitazone at the indicated concentrations, and RNA was isolated after 24 h. CXCR4 mRNA expression was quantified using real-time PCR. The data are mean values ± SE (n=3). *, significant decrease due to rosiglitazone, P<0.05.

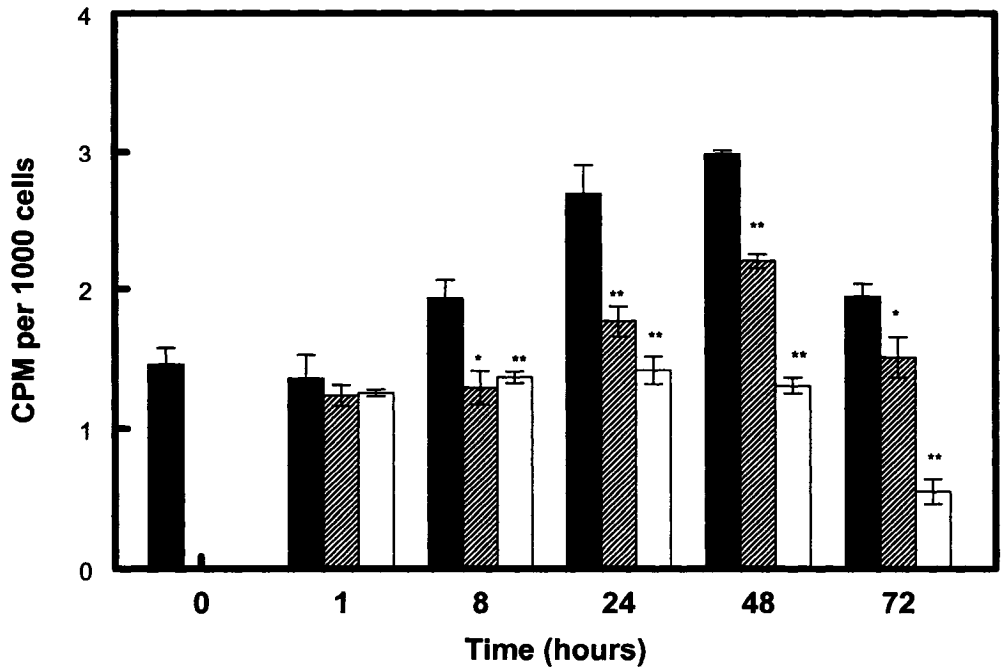


Figure 3.12: Time-dependency of rosiglitazone-induced CXCR4 down-regulation on HT-29 cells. HT-29 cells were treated with vehicle (solid bars), 1 μM $15dPGJ_2$ (hatched bars), or 10 nM rosiglitazone (open bars), and cell-surface CXCR4 protein expression was measured at the indicated time points using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant decrease due to $15dPGJ_2$ or rosiglitazone, $P<0.01$; *, $P<0.05$.

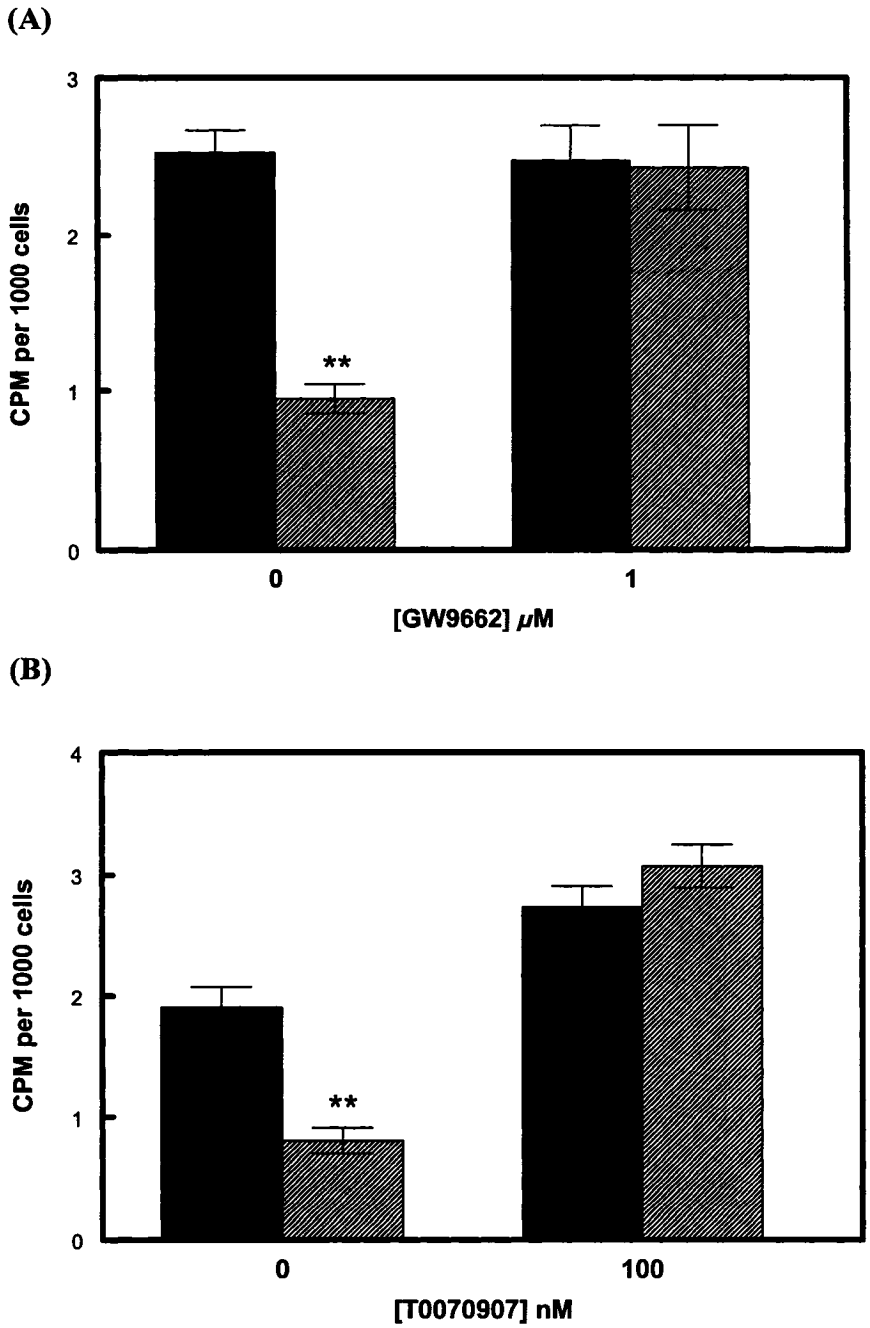


Figure 3.13: Effect of PPARγ antagonists on rosiglitazone-induced CXCR4 down-regulation on HT-29 cells. HT-29 cells were pre-treated for 30 min with (A) GW9662 or (B) T0070907 at the indicated concentrations, followed by addition of vehicle (solid bars) or 10 nM rosiglitazone (hatched bars). CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant change due to rosiglitazone, P<0.01; *, P<0.05.

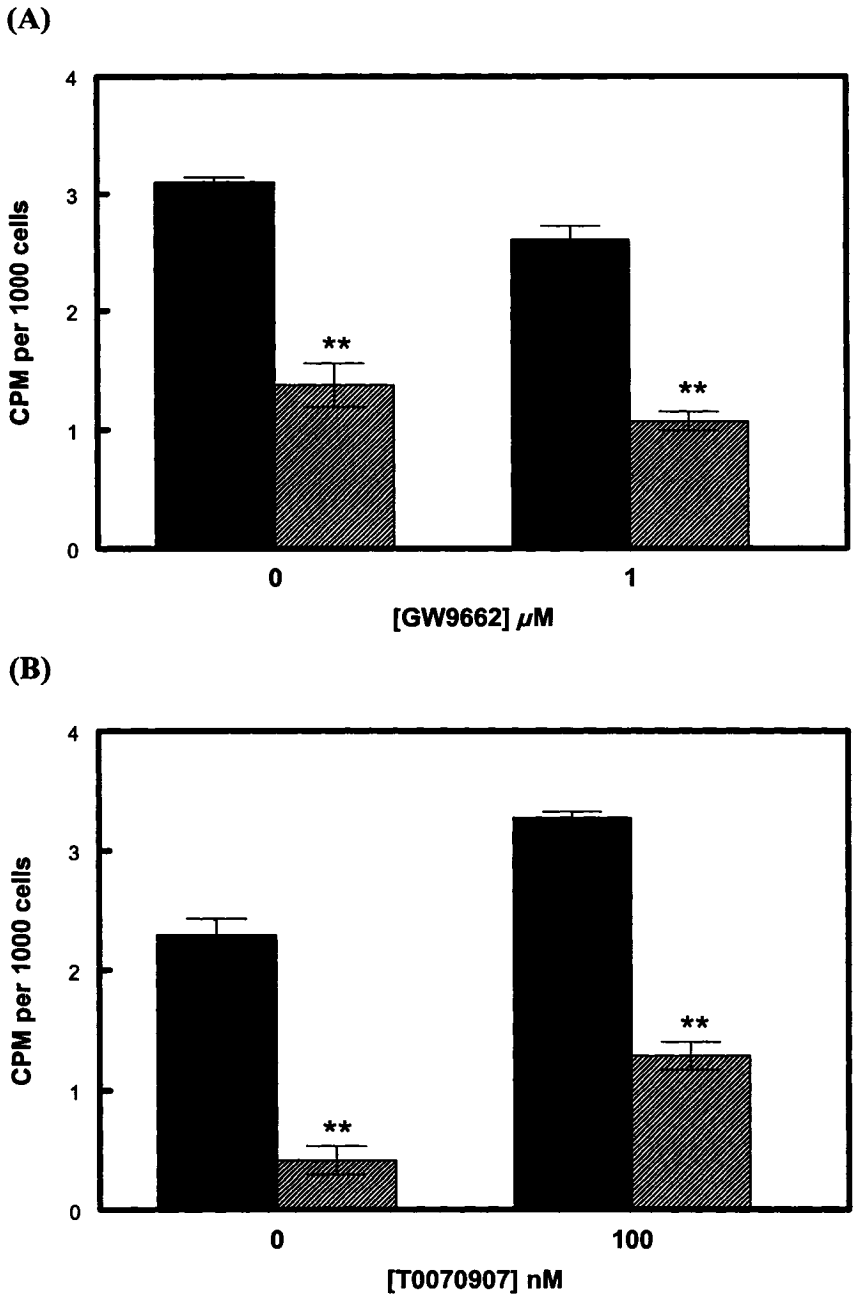
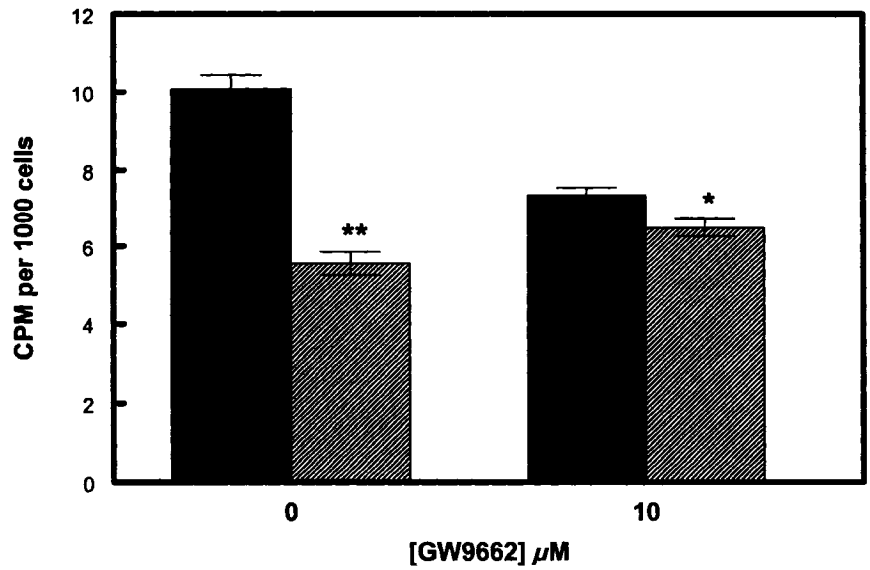


Figure 3.14: Effect of PPARγ antagonists on 15dPGJ$_2$-induced CXCR4 down-regulation on HT-29 cells. HT-29 cells were pre-treated for 30 min with (A) GW9662 or (B) T0070907 at the indicated concentrations, followed by addition of vehicle (solid bars) or 3 μM 15dPGJ$_2$ (hatched bars). CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant decrease due to 15dPGJ$_2$, P<0.01; *, P<0.05.

(A)



(B)

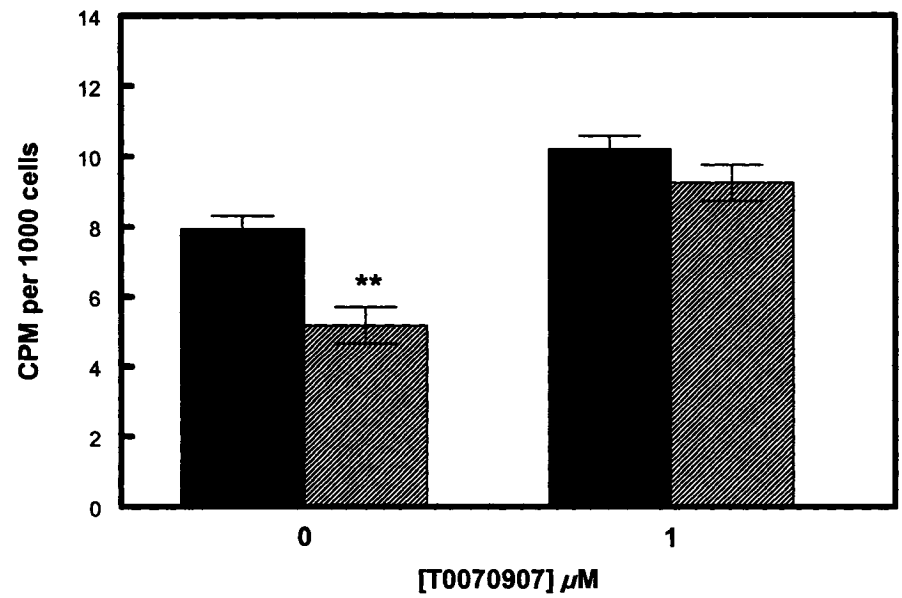


Figure 3.15: Effect of high doses of PPARγ antagonists on 15dPGJ$_2$-induced CXCR4 down-regulation on HT-29 cells. HT-29 cells were pre-treated for 30 min with (A) GW9662 or (B) T0070907 at the indicated concentrations, followed by addition of vehicle (solid bars) or 3 μM 15dPGJ$_2$ (hatched bars). CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant decrease due to 15dPGJ$_2$, P<0.01; *, P<0.05.

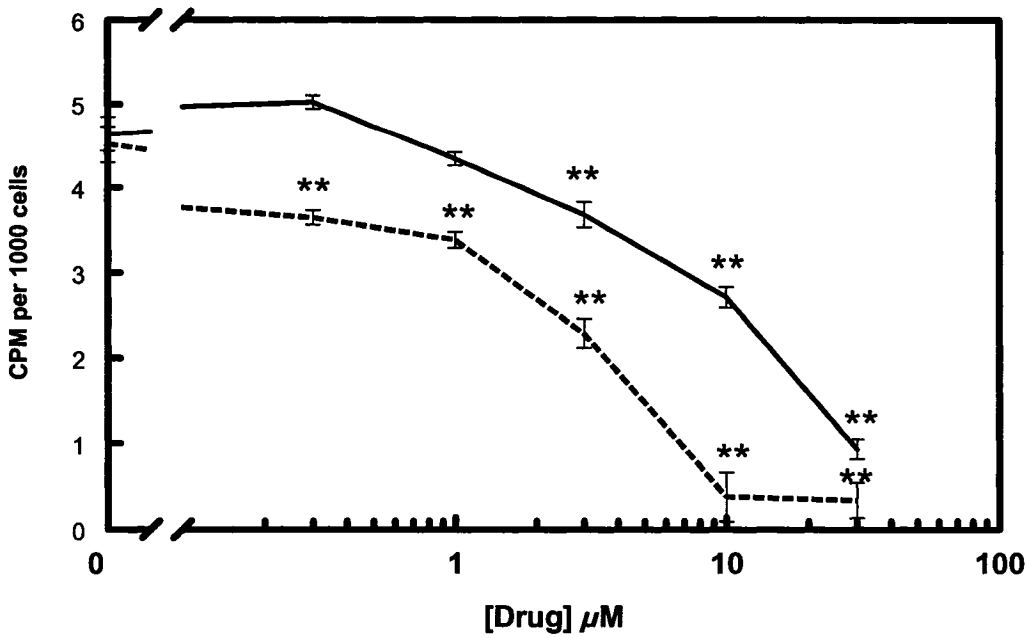


Figure 3.16: Effect of CAY10410, a 15dPGJ$_2$ analogue, on cell-surface CXCR4 expression on HT-29 cells. HT-29 cells were treated with CAY10410 (solid line) or 15dPGJ$_2$ (hatched line) at the indicated concentrations, and cell-surface CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant decrease due to CAY10410 or 15dPGJ$_2$, P<0.01.

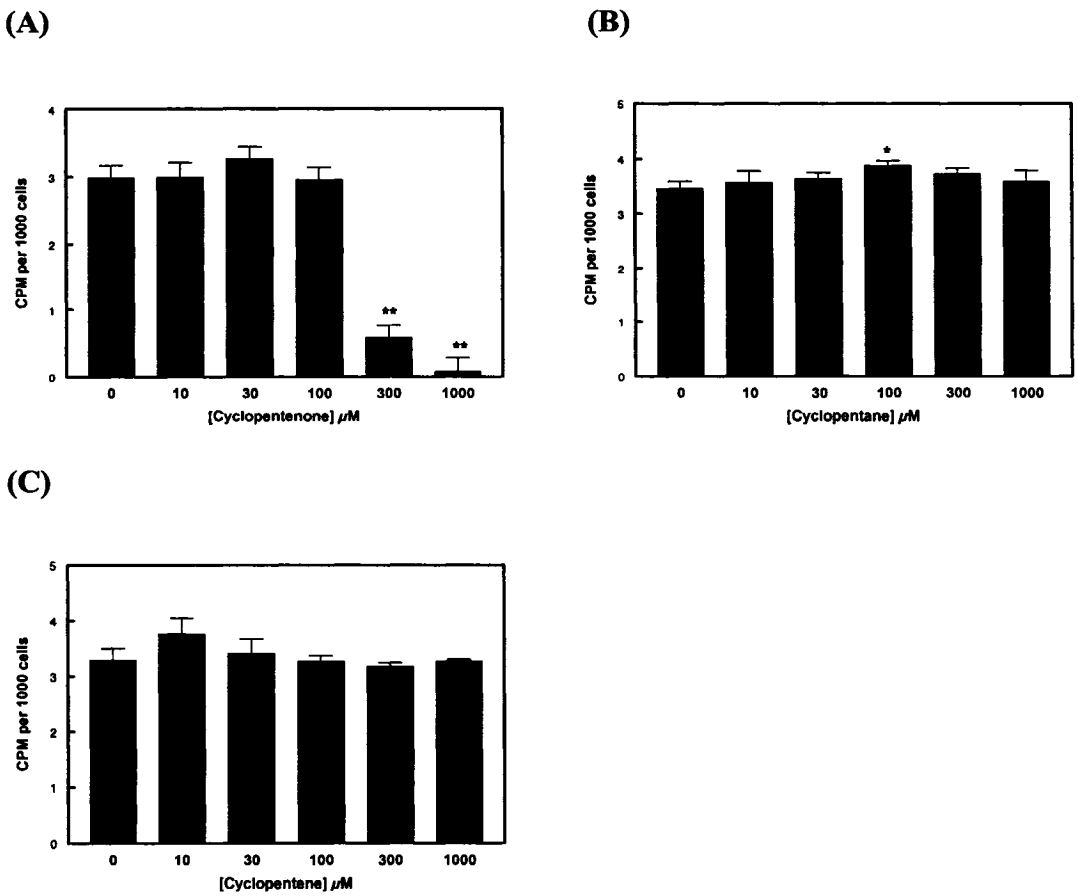


Figure 3.17: Effect of cyclopentenone and related compounds on cell-surface CXCR4 expression on HT-29 cells. HT-29 cells were treated with (A) cyclopentenone, (B) cyclopentane, or (C) cyclopentene at the indicated concentrations, and cell-surface CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant decrease due to cyclopentenone, P<0.01; *, significant increase due to cyclopentane, P<0.05.

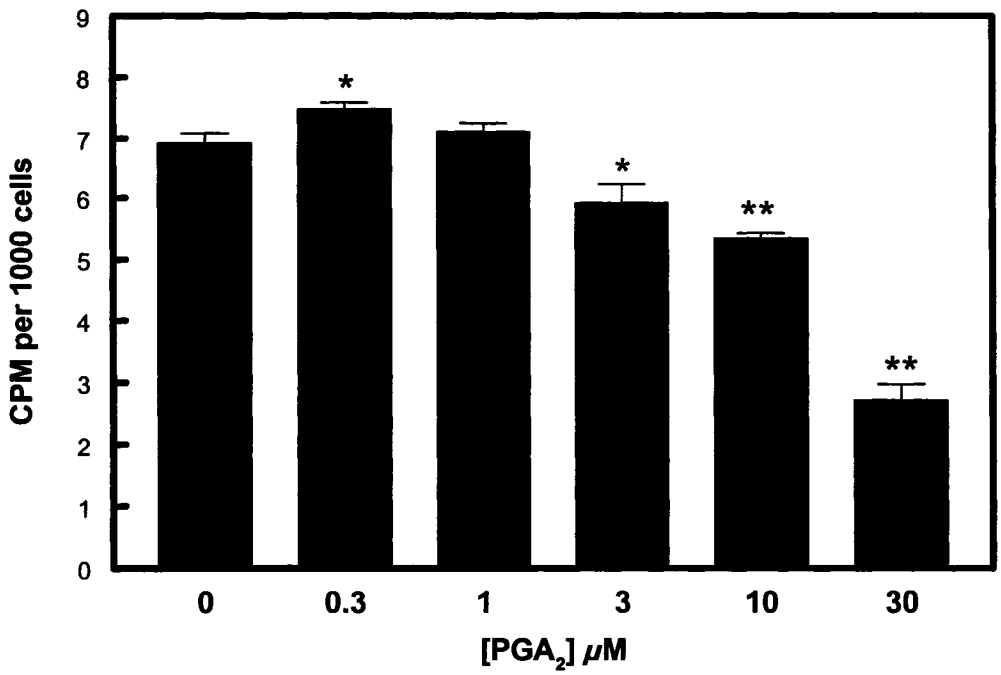


Figure 3.18: Effect of PGA_2 on cell-surface CXCR4 expression on HT-29 cells. HT-29 cells were treated with PGA_2 at the indicated concentrations, and cell-surface CXCR4 protein expression was measured after 48 h using a radioantibody binding assay. The data are mean values ± SE (n=4). **, significant change due to PGA_2, $P<0.01$; *, $P<0.05$.

DISCUSSION

Prostaglandin production is catalyzed by the sequential enzymatic actions of cytoplasmic phospholipase A_2 ($cPLA_2$), cyclooxygenase 1 (COX-1) or cyclooxygenase 2 (COX-2), and specific isomerases, also referred to as prostaglandin synthases (Smith et al., 2000). Prostaglandins are then transported out of cells via prostaglandin transporters, and can exert cellular effects in an autocrine or paracrine fashion (Funk, 2001). Within tumours, prostaglandin levels may be increased (Rigas et al., 1993; Pugh and Thomas, 1994; Yang et al., 1998; Badawi and Badr, 2003) due to elevated expression of enzymes involved in prostaglandin production, including COX-2 (Eberhart et al., 1994; Kutchera et al., 1996; Badawi and Badr, 2003; Ermert et al., 2003;), $cPLA_2$ (Soydan et al., 1996; Dimberg et al., 1998; Österstrom et al., 2002), and specific prostaglandin synthases (Ermert et al., 2003). The objective for this chapter was to determine whether prostaglandins present within the tumour microenvironment may contribute to elevated CXCR4 expression.

Prostaglandins decrease CXCR4 expression

As described in Chapter Two of this thesis, we found that adenosine, a purine nucleoside present in elevated concentrations within tumours due to hypoxia (Blay et al., 1997), up-regulated CXCR4 expression on HT-29 cells. We hypothesized that prostaglandins, which are also present in high levels within tumours (Rigas et al., 1993; Pugh and Thomas, 1994; Yang et al., 1998; Badawi and Badr, 2003), would increase CXCR4 expression in a similar manner. However, surprisingly, we found instead that all three of the primary prostaglandins tested decreased cell-surface CXCR4 protein

expression (Figures 3.2, 3.3, 3.4). Prostaglandin D_2 (PGD_2) was the most effective at lowering CXCR4 receptor expression, with a minimum effective concentration of 1 μM and maximal inhibition of 87% with a dose of 30 μM (Figure 3.2). PGD_2 produced a similar down-regulation of CXCR4 protein expression in the T47D human breast carcinoma cell line (Richard CL, Blay J, unpublished observations), suggesting that this effect may be common to other cancer types as well.

Prostaglandin E_2 (PGE_2) also down-regulated CXCR4 by up to 67%, although a dose of 10 μM was required to produce a statistically-significant down-regulation (Figure 3.3). We were particularly surprised to note any down-regulation of CXCR4 with PGE_2, as this prostaglandin has been shown to elevate CXCR4 expression in microvascular endothelial cells (Salcedo et al., 2003) and has been associated with pro-tumour effects, including stimulation of angiogenesis (Chang et al., 2004), enhancement of cancer cell proliferation (Qiao et al., 1995; Wang et al., 2005a), and stimulation of cancer cell migration (Timoshenko et al., 2003). In fact, many of the anti-cancer effects noted with non-steroidal anti-inflammatory drugs (NSAIDs) have been attributed to inhibition of PGE_2 production (Marnett and DuBois, 2002; Wang et al., 2005b). Interestingly, we were not able to reproduce the effect of PGE_2 on CXCR4 protein in the T47D breast carcinoma cell line (Richard CL, Blay J, unpublished observations), raising the possibility that PGE_2-induced CXCR4 down-regulation is not actually a common event. There is evidence in the literature that HT-29 cells do not respond as expected to PGE_2 in terms of proliferation, calcium mobilization, or changes in metabolic activity (Cassano et al., 2000; Nylund et al., 2003). Also, HT-29 cells express the PGE_2 receptor EP_4, but not

EP_{1-3} (Colucci et al., 2005). Therefore, perhaps the HT-29 cell line is not a representative model for measuring PGE_2-induced changes in CXCR4 expression.

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) had very little effect on cell-surface CXCR4 protein expression on HT-29 cells (Figure 3.4). At the highest dose tested (30 μM), there was a small decrease of 24%. Interestingly, as was the case with PGE_2, the same effect was not noted in the T47D cell line (Richard CL, Blay J, unpublished observations). In fact, $PGF_{2\alpha}$ increased cell-surface CXCR4 protein expression on T47D cells.

Since we saw the most robust effect on CXCR4 receptor expression with PGD_2, and its effect was seen in both HT-29 and T47D cells, we decided to focus our attention on this particular prostaglandin. PGD_2 can act by binding to cell-surface seven-transmembrane G-protein coupled receptors referred to as DP_1 and DP_2 (Hata and Breyer, 2004), or alternatively through its metabolism to J-series prostaglandins, which occurs non-enzymatically in cell culture (Fitzpatrick and Wynalda, 1983; Narumiya and Fukushima, 1985; Shibata et al., 2002). We found that the J-series metabolites of PGD_2, prostaglandin J_2 (PGJ_2) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ($15dPGJ_2$), both drastically reduced CXCR4 protein expression on HT-29 cells (Figure 3.5). In fact, a dose of 10 or 30 μM of $15dPGJ_2$ reduced cell-surface CXCR4 protein expression to the point where it was no longer detectable (Figure 3.5B). Similarly, using real-time RT-PCR, an 88% reduction in CXCR4 mRNA was observed with 10 μM $15dPGJ_2$, suggesting that this effect occurred at the level of transcription (Figure 3.7). This differs from$15dPGJ_2$-mediated down regulation of other proteins, including cyclin D1 and estrogen receptor α, which has been shown to occur through protein degradation rather than through changes in transcription (Qin et al., 2003).

The conversion of PGD_2 to PGJ_2 and then to the end-product $15dPGJ_2$ would be expected to occur in our HT-29 cultures, as it occurs non-enzymatically both in the presence and absence of serum (Fitzpatrick and Wynalda, 1983; Narumiya and Fukushima, 1985; Shibata et al., 2002). We looked at the timing of CXCR4 down-regulation induced by each of these prostaglandins to see if PGD_2 might require conversion to $15dPGJ_2$ in order to be active. Indeed, down-regulation of CXCR4 was noted first with $15dPGJ_2$ (Figure 3.6), consistent with the need for PGD_2 to be converted to $15dPGJ_2$ to exert its effects. We were able to detect a statistically-significant down-regulation of CXCR4 8 h after $15dPGJ_2$ treatment, but not until 24 h after PGD_2 treatment. Interestingly, Forman and colleagues found a similar lag time between the effects of these two prostaglandins in reporter gene assays, and this was attributed to the requirement of metabolic activation of PGD_2 (Forman et al., 1995). Also, Kim and colleagues found that 5 h after addition of PGD_2 to prostate cancer cell cultures, just over 1% was converted to $15dPGJ_2$, and the rate of $15dPGJ_2$ production had not leveled off at this point (Kim et al., 2005b). Narumiya and colleagues found that 12 h after incubation of PGD_2 in media, 50% was metabolized, mainly to J-series metabolites, and over 80% was metabolized after 24 h (Narumiya and Fukushima, 1985). Therefore, the kinetics of conversion of PGD_2 to $15dPGJ_2$ are consistent with PGD_2-induced CXCR4 down-regulation being mediated through metabolism to $15dPGJ_2$. To confirm this, we would need to measure the rate and extent of conversion of PGD_2 to $15dPGJ_2$ in cultures of HT-29 cells, which can only be done reliably using mass spectrometry (Powell, 2003). HT-29 cells only express one subtype of PGD_2 receptors, DP1 (Hawcroft et al., 2004). To rule out the involvement of DP receptors in the PGD_2 effect on CXCR4, we could try to

either mimic the effect of PGD_2 using a DP1 receptor agonist such as BW 245C, or block its effect using a DP1 receptor antagonist such as BW A868C (Breyer et al., 2001).

A number of anti-tumour effects have been attributed to $15dPGJ_2$. These include promotion of apoptosis (Clay et al., 1999; Sato et al., 2000; Clay et al., 2002; Shimada et al., 2002; Piva et al., 2005), cell-cycle or growth arrest (Kitamura et al., 1999; Sato et al., 2000), differentiation (Mueller et al., 1998; Kitamura et al., 1999), and inhibition of VEGF production (Grau et al., 2004). Since we have shown that $15dPGJ_2$ also reduces expression of CXCR4, a key protein in cancer metastasis, $15dPGJ_2$ may play an important role in inhibition of tumour progression. Therefore, we decided to focus our attention on this particular prostaglandin.

Role of PPARγ signaling

$15dPGJ_2$ is an endogenous ligand for the nuclear receptor nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ; Forman et al., 1995; Kliewer et al., 1995; Kliewer et al., 1997). Upon activation, PPARγ heterodimerizes with the retinoid X receptor (RXR) and binds to peroxisome proliferator response elements (PPRE) on DNA, activating target gene expression (Kota et al., 2005). Although PPARγ has been primarily characterized for its role in glucose and lipid metabolism, it is also involved in tumourigenesis, although its exact role has yet to be elucidated (Koeffler, 2003; Grommes et al., 2004). PPARγ is expressed in various tumour types, including colorectal cancer (Sarraf et al., 1998; Feilchenfeldt et al., 2004; Matthiessen et al., 2005), and PPARγ agonists have been shown to both inhibit the growth and induce apoptosis of cancer cells grown in culture (Brockman et al., 1998; Elstner et al., 1998; Sarraf et al.,

1998; Yang and Frucht, 2001; Shimada et al., 2002; Crowe and Chandraratna, 2004; Yoshizumi et al., 2004). Such agents also inhibit the growth of tumours in xenograft models (Elstner et al., 1998; Sarraf et al., 1998; Yoshizumi et al., 2004). Interestingly, in a murine model of rectal cancer, a PPARγ agonist was also shown to inhibit the metastasis of colorectal carcinoma cells to lung and lymph nodes (Yoshizumi et al., 2004).

We hypothesized that 15dPGJ$_2$-induced CXCR4 down-regulation was mediated by activation of PPARγ, which is expressed by HT-29 cells (Richard CL, Blay J, unpublished observations). This hypothesis was tested using thiazolidinedione drugs such as rosiglitazone, which are potent and selective activators of PPARγ (Lehmann et al., 1995). Rosiglitazone increased expression of the PPARγ target gene cytokeratin 20 (Gupta et al., 2001; Figure 3.8), indicating that HT-29 cells expressed functional PPARγ. As was seen with 15dPGJ$_2$, rosiglitazone reduced CXCR4 mRNA (Figure 3.11) and cell-surface protein expression (Figure 3.9) on HT-29 cells, supporting our hypothesis that 15dPGJ$_2$-induced CXCR4 down-regulation involves signaling through PPARγ. Furthermore, four other thiazolidinedione PPARγ agonists also reduced cell-surface CXCR4 protein expression (Figure 3.10). Interestingly, the order of potency of the thiazolidinedione compounds in reducing CXCR4 receptor expression (rosiglitazone > pioglitazone > ciglitazone = MCC555 > troglitazone) corresponded with their known potencies as PPARγ agonists (Lehmann et al., 1995; Reginato et al., 1998; Camp et al., 2000). Troglitazone acts as a partial agonist at PPARγ receptors in some cell lines, as does MCC555 (Reginato et al., 1998; Camp et al., 2000) whereas the other

thiazolidinediones are full agonists (Camp et al., 2000). These data imply that PPARγ activation produces CXCR4 down-regulation in HT-29 cells.

There are, however, reports of thiazolidinedione-induced cellular effects that occur independently of PPARγ. For example, ciglitazone and troglitazone inhibited the growth of both PPARγ+/+ and PPARγ-/- embryonic stem cells *in vitro* and *in vitro* (Palakurthi et al., 2001), and troglitazone inhibited the growth of oral squamous cell carcinoma cells that did not express PPARγ protein (Nakashiro et al., 2003). Therefore, although the potency correlation in our study was suggestive of PPARγ involvement, we performed experiments with antagonists to confirm the involvement of PPARγ. The two PPARγ antagonists GW9662 and T0070907 both completely blocked the effects of rosiglitazone on CXCR4 protein expression (Figure 3.13), confirming that rosiglitazone-induced CXCR4 down-regulation did indeed occur through PPARγ activation.

Although these data show conclusive evidence that PPARγ activation does indeed lead to CXCR4 down-regulation, the question still remains – is activation of PPARγ responsible for the 15dPGJ$_2$ effect on CXCR4 expression? We found several differences between 15dPGJ$_2$- and rosiglitazone-induced CXCR4 down-regulation. For example, while 15dPGJ$_2$ reduced cell-surface CXCR4 protein expression to the point where it was no longer detectable after a 48 h treatment (Figure 3.5B), the maximum reduction due to rosiglitazone at this time point was only 56% (Figure 3.9). A similar difference in magnitude was noted at the mRNA level (Figures 3.7 and 3.11). Also, although the onset of 15dPGJ$_2$-induced CXCR4 down-regulation was similar to that seen with rosiglitazone, the effect persisted longer with rosiglitazone than with 15dPGJ$_2$, and rosiglitazone produced a greater effect at the 72 h time point than after 48 h (Figure 3.12). The most

marked difference between the effect of $15dPGJ_2$ and rosiglitazone on CXCR4 receptor expression was seen in the PPARγ antagonist experiments. We were able to block both $15dPGJ_2$- and rosiglitazone-induced CXCR4 down-regulation with the PPARγ antagonists GW9662 and T0070907 (Figure 3.13 and 3.15). However, a 10-fold higher antagonist concentration was required to block the $15dPGJ_2$ effect compared to the rosiglitazone effect.

How can these differences in the $15dPGJ_2$ and rosiglitazone effects be explained? It is unlikely that $15dPGJ_2$-induced CXCR4 down-regulation is completely independent of PPARγ, since we were able to block the $15dPGJ_2$ effect with two separate PPARγ antagonists. A possible explanation is that discrepancies between CXCR4 down-regulation caused by $15dPGJ_2$ and rosiglitazone are caused by differences in the mechanism of ligand binding. $15dPGJ_2$ binds covalently to PPARγ (Soares et al., 2005), whereas rosiglitazone binds non-covalently. This may result in differences in receptor conformational changes induced by each agent. The discrepancies observed in the time course may be caused by differences in chemical stability in culture, or alternatively, since $15dPGJ_2$ covalently modifies PPARγ, it would likely be destroyed during turnover of PPARγ protein.

Our data show that CXCR4 protein expression is reduced through PPARγ activation, likely accounting at least in part for $15dPGJ_2$-induced CXCR4 down-regulation. However, the exact mechanism of PPARγ-mediated transcriptional repression of CXCR4 has yet to be determined. Although activation of PPARγ typically results in transactivation of target genes, transrepression has also been noted (Grommes et al., 2004). For example, activated PPARγ can antagonize the effects of some

transcription factors, including AP-1, STAT, and NF-κB, and thereby inhibit transcription of a number of genes, including those encoding inducible nitric oxide synthase, gelatinase B, and scavenger receptor A through inhibited dissociation of corepressor complexes from the promoter region of target genes (Ricote et al., 1998; Pascual et al., 2005). The involvement of specific transcription factors in $15dPGJ_2$-induced CXCR4 down-regulation could be examined using chromatin immunoprecipitation assays or electrophoretic mobility shift assays.

Role of NF-κB signaling

Another possible explanation for the differences between $15dPGJ_2$- and rosiglitazone-induced CXCR4 down-regulation is that $15dPGJ_2$ may be acting through more than one signaling pathway. In addition to its ability to activate the nuclear receptor PPARγ, $15dPGJ_2$ can also elicit its effects by inhibition of NF-κB signaling. NF-κB is a transcription factor that is normally retained in the cytoplasm through its interaction with IκB (Ghosh et al., 1998). Upon receipt of appropriate stimuli, IκB kinase (IKK) phosphorylates IκB, leading to its ubiquitination and subsequent degradation. NF-κB is then able to enter the nucleus and activate transcription of target genes. $15dPGJ_2$ has been shown to inhibit several steps in the NF-κB signaling pathway through covalent modification of key proteins (Straus et al., 2000). It has been shown to inhibit the actions of IKK (Rossi et al., 2000), and also to bind directly to NF-κB subunits, blocking their ability to bind to DNA (Cernuda-Morollón et al., 2001). Interestingly, NF-κB activation has also been shown to increase CXCR4 expression (Helbig et al., 2003), which makes it

rational to hypothesize that 15dPGJ$_2$-induced CXCR4 down-regulation could be due at least in part to inhibition of NF-κB signaling.

NF-κB inhibition by 15dPGJ$_2$ is dependent on the cyclopentenone ring in the structure of 15dPGJ$_2$ (Straus et al., 2000; Lindstrom and Bennett, 2005). An analogue of 15dPGJ$_2$, 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (CAY10410) does not have the required cyclopentenone structure, and is thus no longer able to inhibit the NF-κB pathway (Lindstrom and Bennett, 2005). CAY10410 does, however, retain its ability to activate PPARγ, as this part of its activity relies on the α,β-unsaturated ketone moiety (Shiraki et al., 2005). We found that CAY10410 reduced CXCR4 expression on HT-29 cells by greater than 90% at a dose of 30 μM (Figure 3.16). This is consistent with PPARγ-dependent regulation of CXCR4, as was noted with rosiglitazone. However, although it appeared that both agents produced nearly the same maximal response, the CAY10410 curve was shifted to the right compared to 15dPGJ$_2$, indicating reduced potency. There was approximately a three-fold difference in the EC$_{50}$ values between the two agents; the EC$_{50}$ was approximately 3 μM for 15dPGJ$_2$ in this experiment, and 10 μM for CAY10410. This difference in potency could possibly be explained by 15dPGJ$_2$ exerting its effects through a combination of PPARγ activation and NF-κB inhibition.

Further evidence for down-regulation of CXCR4 through NF-κB inhibition was provided by experiments with cyclopentenone itself and with the cyclopentenone prostaglandin A$_2$ (PGA$_2$), both of which can inhibit NF-κB but cannot activate PPARγ (Rossi et al., 2000; Straus et al., 2000; Lindstrom and Bennett, 2005). Both compounds reduced cell-surface CXCR4 protein (Figures 3.17A and 3.18). PGA$_2$ can be formed in cell culture from PGE$_2$ (Aussel et al., 1987; Ishihara et al., 1991), and the dose response

curves for these two prostaglandins were nearly identical (Figure 3.3 and 3.18). Therefore, PGE_2-induced CXCR4 down-regulation may be accounted for by production of the cyclopentenone prostaglandin PGA_2, just as PGD_2-induced CXCR4 down-regulation may be due to $15dPGJ_2$ formation. A dose of 300 μM was required for cyclopentenone to down-regulate CXCR4 (Figure 3.17A), whereas $15dPGJ_2$-induced CXCR4 down-regulation occurred with low micromolar concentrations. However, this was not surprising, since cyclopentenone concentrations approximately 100-fold higher than $15dPGJ_2$ are required to achieve similar levels of inhibition of NF-κB activity (Straus et al., 2000). Therefore, it is certainly possible that inhibition of NF-κB contributes to $15dPGJ_2$-induced CXCR4 down-regulation.

Our results indicate that $15dPGJ_2$ likely occurs through a combined effect on PPARγ and NF-κB signaling. Interestingly, although $15dPGJ_2$ can inhibit NF-κB signaling through direct interaction with the p50 subunit (Cernuda-Morollón et al., 2001) or in an upstream fashion by binding to IKK (Rossi et al., 2000), in some cases, inhibition of NF-κB activity by $15dPGJ_2$ can occur in a PPARγ-dependent manner through inhibition of the dissociation of repressor complexes (Ricote et al., 1998; Straus et al., 2000; Pascual et al., 2005). This may be the case in $15dPGJ_2$-induced CXCR4 down-regulation. In fact, all of the effects on CXCR4 expression attributed to PPARγ activation could be mediated by PPARγ-dependent inhibition of NF-κB. This possibility is particularly appealing, since NF-κB activation has been shown to increase CXCR4 transcription (Helbig et al., 2003). A model in which $15dPGJ_2$-mediated CXCR4 down-regulation is induced by a combination of PPARγ-dependent and PPARγ-independent NF-κB inhibition would explain many of our results. For example, the fact that

rosiglitazone did not down-regulate CXCR4 to the same extent as was seen with $15dPGJ_2$ could be explained by the fact that rosiglitazone would only cause PPARγ-dependent NF-κB inhibition, and not PPARγ-independent NF-κB inhibition. The same would be expected to occur with CAY10410. Cyclopentenone and PGA_2 on the other hand would only cause PPARγ-independent NF-κB inhibition, which may explain in part why these two agents were less potent than $15dPGJ_2$. Further experimentation is required to validate this model of $15dPGJ_2$-induced CXCR4 down-regulation on HT-29 cells.

Relevance of prostaglandin effect on CXCR4

We have shown in this chapter that two primary prostaglandins, PGD_2 and PGE_2, reduce CXCR4 protein expression on the HT-29 human colorectal carcinoma cell line, likely due to formation of their respective cyclopentenone metabolites $15dPGJ_2$ and PGA_2. These cyclopentenone prostaglandins are able to inhibit signaling through NF-κB (Rossi et al., 2000), which likely accounts at least in part for their ability to down-regulate CXCR4 as NF-κB activation increases CXCR4 expression (Helbig et al., 2003). $15dPGJ_2$-induced CXCR4 down-regulation also involved PPARγ activation, possibly due to PPARγ-dependent NF-κB activation.

Why would a mechanism of $15dPGJ_2$-induced CXCR4 down-regulation have evolved? This is likely an extension of the anti-inflammatory effects of $15dPGJ_2$, which are seen in many disease states, including inflammatory bowel disease, Alzheimer disease, and arthritis (Scher and Pillinger, 2005). It is believed that late in the inflammation process the prostaglandin profile shifts from a PGE_2-rich state to a PGD_2-rich state (and therefore $15dPGJ_2$-rich), leading to the resolution of inflammation

(Schuligoi et al., 2005). As the CXCL12-CXCR4 axis is important in trafficking of cells to areas of inflammation (Murdoch, 2000; Balkwill, 2004), reduced CXCR4 expression may be an additional mechanism by which $15dPGJ_2$ participates in the resolution of inflammation.

We had originally hypothesized that prostaglandins present within the tumour microenvironment might contribute to elevated CXCR4 expression on cancer cells. However, we found that prostaglandins produced a decrease in CXCR4 receptor expression rather than an increase. This raises the question – why are CXCR4 levels still elevated on tumours? There are several possibilities:

1. *The final level of expression of CXCR4 may depend on the balance of a number of factors that can either increase or decrease CXCR4 levels.*

In Chapter Two of this thesis, we provided evidence that the tumour metabolite adenosine increases CXCR4 expression on cancer cells. Other factors present within the tumour microenvironment can also increase CXCR4 expression on cancer cells, including VEGF (Bachelder et al., 2002), TGF-β (Bartolomé et al., 2004) and TNF-α (Kulbe et al., 2005). The presence of hypoxia increases CXCR4 expression as well (Schioppa et al., 2003; Staller et al., 2003). Therefore, although prostaglandins reduce CXCR4 expression, this may be overcome by the up-regulation induced by other components of the tumour microenvironment.

2. *Prostaglandins which lower CXCR4 expression are not present in sufficient concentrations to achieve this effect*

The single prostaglandin doses we used to induce changes in CXCR4 expression were in the low micromolar range. However, it is unlikely that concentrations this high are achieved *in vivo* (Bell-Parikh et al., 2003). It is very challenging to measure exact levels of prostaglandins within tissues, because tissue disruption causes phospholipase activation, which may generate erroneously high prostaglandin levels (Bennett, 1986). Our laboratory previously measured adenosine levels in solid tumours using microdialysis (Blay et al., 1997); a similar study would be useful to assess prostaglandin levels within tumours. Also, it would be beneficial to measure the half-life of prostaglandins added to cultures of HT-29 cells, as was done previously for adenosine (Mujoomdar et al., 2003).

Measurement of $15dPGJ_2$ levels is complicated by the fact that it is a highly reactive molecule, so much of it is bound to proteins and therefore can not be accurately measured (Powell, 2003). There is significant controversy in the literature as to whether or not pharmacologically relevant concentrations of $15dPGJ_2$ are achieved *in vivo* at all (Nosjean and Boutin, 2002; Bell-Parikh et al., 2003; Powell, 2003). Bell-Parikh and colleagues used liquid chromatography-mass spectrometry-mass spectrometry to measure $15dPGJ_2$ in cultures of 3T3-LI preadipocytes, and found extracellular concentrations of $15dPGJ_2$ in the picomolar range and intracellular levels of approximately 1 nM (Bell-Parikh et al., 2003). The concentration of $15dPGJ_2$ needed to activate PPARγ was 4.5 μM, which led the authors to the conclusion that $15dPGJ_2$ is not an endogenous ligand for PPARγ. However, in a follow-up study by Soares and colleagues, when $15dPGJ_2$ was

incubated with recombinant PPARγ, $15dPGJ_2$ bound covalently to PPARγ, reducing the amount of $15dPGJ_2$ that could be detected using mass spectrometry (Soares et al., 2005). Therefore, since much of the $15dPGJ_2$ within cells is likely bound to PPARγ or other proteins and therefore not measurable, it has yet to be concluded if pharmacologically relevant concentrations of $15dPGJ_2$ occur *in vivo*.

3. $15dPGJ_2$ may not be a predominant prostaglandin within tumours.

Prostaglandin levels are elevated in tumours due to increased expression of COX-2 (Eberhart et al., 1994; Kutchera et al., 1996; Badawi and Badr, 2003; Ermert et al., 2003) and $cPLA_2$ (Soydan et al., 1996; Dimberg et al., 1998; Österstrom et al., 2002). However, levels of the individual prostaglandins are not uniform. The exact prostaglandin profile depends on the specific prostaglandin synthases that are expressed, as well as mechanisms within the cells that can remove the prostaglandins. Several investigators have shown that PGE_2 levels are elevated within tumours compared to normal tissues (Rigas et al., 1993; Pugh and Thomas, 1994; Giardiello et al., 1998; Yang et al., 1998; Badawi and Badr, 2003) due to increased expression of PGE synthase (Yoshimoto et al., 2005) or reduced expression of an enzyme responsible for its removal, 15-hydroxyprostaglandin dehydrogenase (Backlund et al., 2005). Although we showed that both PGE_2 and $15dPGJ_2$ reduced CXCR4 receptor expression, this occurred to a greater extent and at lower concentrations with $15dPGJ_2$. However, unlike PGE_2 which is present in elevated concentration in tumours, $15dPGJ_2$ levels may actually be low in tumours compared to normal tissue. Badawi and colleagues found that levels of $15dPGJ_2$ decreased during breast cancer progression, with the lowest levels being detected in

metastatic disease, and decreased $15dPGJ_2$ expression correlated with progression to metastatic disease (Badawi and Badr, 2003). Consistent with this, in patients with familial adenomatous polyposis, PGD_2 was the least abundant prostaglandin in biopsy samples (Giardiello et al., 1998), and in tumour tissues taken from patients with colorectal cancer, patients with hepatic metastasis had significantly lower tumour PGD_2 levels than those without hepatic metastasis (Yoshida et al., 1998). In cerebrospinal fluid taken from patients with brain cancer, PGDS protein levels were reduced compared to patients without disease (Saso et al., 1998). The limiting step in $15dPGJ_2$ production is PGD_2 production through PGDS. Also, $15dPGJ_2$ produced within tumours may be sequestered by glutathione *S*-transferases or conjugated to glutathione, and then pumped out of cells via multidrug resistance proteins 1 and 3, reducing its concentration and preventing it from activating PPARγ (Paumi et al., 2003; Paumi et al., 2004). Overall, it seems that the predominant prostaglandin within tumours is PGE_2, and $15dPGJ_2$ may not be present in high levels at all.

The fact that prostaglandins reduced the expression of CXCR4, a chemokine receptor involved in tumour progression, also raises another question – why do NSAIDs and COX-2 inhibitors, which inhibit prostaglandin production, reduce the development and progression of cancer, especially colorectal cancer (Brown and DuBois, 2005)? As mentioned previously, PGE_2 has tumour-promoting functions, so regardless of what effect it has on CXCR4 expression, reducing the production of this particular prostaglandin will likely provide a benefit in the context of cancer. However, we can speculate that the use of PGE synthase inhibitors may be better than the use of NSAIDs

or COX-2 inhibitors, as these would reduce production of the tumour-promoting prostaglandin PGE_2 and theoretically could increase the synthesis of PGD_2 and its J-series metabolites by increasing the availability of the rate-limiting prostaglandin PGH_2 for PGDS. Another interesting point is that several NSAIDs can act independently of COX inhibition, and can activate PPARγ (Lehmann et al., 1997; Jaradat et al., 2001). In line with this, our laboratory has shown that NSAIDs decrease CXCR4 expression on HT-29 cells, an effect which may be partly PPARγ-dependent (Chiu D, Richard CL, Blay J, manuscript in preparation).

As CXCR4 expression is an important determinant of cancer metastasis (Müller et al., 2001; Zeelenberg et al., 2003), agents that reduce CXCR4 expression on cancer cells could prove to be useful in the prevention of metastasis. By studying the mechanisms of $15dPGJ_2$-induced CXCR4 down-regulation, we have revealed two signalling pathways by which CXCR4 levels may be reduced: activation of PPARγ and inhibition of NF-κB signalling. Agents that act on these signalling pathways may be useful in the treatment of colorectal cancer, particularly in combination with other agents in the metastatic setting.

The activity of one PPARγ agonist has been evaluated in an animal model of colorectal cancer metastasis (Yoshizumi et al., 2004). In this study, the parent compound, thiazolidinedione itself, reduced the metastasis of HT-29 cells implanted in the rectum of mice. However, in a Phase II study of patients with advanced metastatic colorectal cancer that had not responded to chemotherapy, troglitazone failed to produce an objective tumour response (Kulke et al., 2002). It is unclear whether or not this same lack of benefit would be noted with the more potent and selective PPARγ agonist, rosiglitazone, although a greater reduction in CXCR4 expression would be anticipated

based on the results of our *in vitro* study. The use of thiazolidinedione drugs in combination with chemotherapy is supported by a Phase II study in which all of six patients receiving low-dose chemotherapy with pioglitazone and a COX-2 inhibitor for the treatment of angiosarcoma experienced clinical benefit (Vogt et al., 2003). Interestingly, in pre-clinical studies, rosiglitazone had myeloprotective effects when given in combination with or following 5-fluorouracil treatment (Djazayeri et al., 2005; Djazayeri et al., 2006). Due caution must be taken in the case of colorectal cancer, particularly in patients with *APC* mutations, as pre-clinical studies in which *APC*-deficient mice were treated with thiazolidinediones have yielded opposing results, with some investigators finding an increase in polyp formation (Lefebvre et al., 1998; Saez et al., 1998; Pino et al., 2004), and others finding decreased polyp formation (Niho et al., 2003a; Niho et al., 2003b).

NF-κB regulation is often impaired in cancer, leading to constitutive activation and dysregulation of genes involved in cancer progression (Dolcet et al., 2005; Yu et al., 2005). Also, resistance to chemotherapy may be due in part to activation of NF-κB. Therefore, NF-κB inhibitors are being developed to be used in combination with chemotherapeutic agents (Nakanishi and Toi, 2005; Yu et al., 2005). It would be interesting to see if these agents also reduce metastasis by virtue of their potential ability to reduce CXCR4 expression.

CONCLUSION

In this chapter, we have shown that prostaglandins reduce cell-surface CXCR4 expression on HT-29 cells. Therefore, there are small molecules present within the tumour microenvironment that have the potential to increase CXCR4 expression, such as adenosine, and those with the ability to decrease CXCR4 expression, as was seen with prostaglandins. The relative quantities of these molecules may play a part in determining overall expression. The prostaglandin effect was particularly pronounced with PGD_2, and appeared to be mediated by its J-series metabolite, $15dPGJ_2$. $15dPGJ_2$-induced CXCR4 down-regulation occurred at both the mRNA and cell-surface protein level, and likely occurred through both PPARγ and NF-κB signaling. Therefore, agents that alter PPARγ or NF-κB signaling may have therapeutic potential in combination with established cancer treatments through their ability to reduce CXCR4 expression.

CHAPTER FOUR

CONCLUSION

The chemokine receptor CXCR4 has recently been recognized as an important player in metastasis by directing the dissemination of cancer cells and their growth at secondary sites (Müller et al., 2001; Scotton et al., 2001; Scotton et al., 2002; Taichman et al., 2002; Zeelenberg et al., 2003). CXCR4 expression is elevated in colorectal cancer, and correlates with disease progression, increased recurrence, and reduced survival (Kim et al., 2005a; Schimanski et al., 2005; Ottaiano et al., 2006). Elevated CXCR4 expression may be due in part to constituents of the tumour microenvironment (Zeelenberg et al., 2003). The objective of this thesis was to identify factors within the tumour microenvironment that contribute to elevated CXCR4 expression on colorectal carcinoma cells.

The physiology of solid tumours differs from that of normal tissues. Many differences arise from abnormal tumour vasculature. Tumour vessels are elongated and uneven in diameter, and have excessive branching and chaotic organization (Raghunand et al., 2003; Vaupel, 2004). Disruptions in endothelial lining and basement membranes contribute to increased vascular permeability (Vaupel, 2004). Abnormal vasculature leads to areas of hypoxia/anoxia, high interstitial pressure, glucose deprivation, and increased lactate production within tumours (Raghunand et al., 2003; Vaupel, 2004). The tumour microenvironment is also acidic due to increased glycolysis, reduced removal of lactic acid, and increased activation of the Na^+/H^+ exchanger NHE1 in tumour cells (Raghunand et al., 2003; Vaupel, 2004; Cardone et al., 2005). Several of these changes in tumour physiology can contribute to metastasis independent of tumour cell genetic

abnormalities, as depicted in Figure 4.1 (Raghunand et al., 2003; Cardone et al., 2005). In addition to metabolic changes, populations of fibroblasts, endothelial cells, and cells of the immune systems within the tumour microenvironment can contribute to tumour progression through secretion of growth factors, cytokines, proteases, and pro-angiogenic factors (Kopfstein and Christofori, 2006). A number of small molecules are present in increased levels within the tumour microenvironment. These include the purine nucleoside adenosine, which is present in high levels due to tumour hypoxia (Blay et al., 1997), and prostaglandins which result from increased expression of enzymes involved in the eicosanoid pathway (Rigas et al., 1993; Eberhart et al., 1994; Pugh and Thomas, 1994; Kutchera et al., 1996; Dimberg et al., 1998; Dimberg et al., 1999; Österstrom et al., 2002).

In Chapter Two of this thesis, I investigated the regulation of CXCR4 protein expression on HT-29 human colorectal carcinoma cells by adenosine. We found that adenosine increased cell-surface CXCR4 protein expression at pathophysiologically relevant concentrations. This increase was also seen at the mRNA level, suggesting that it occurred through increased transcription. Inosine formation was not responsible for the adenosine effect. Using adenosine receptor antagonists, we found that adenosine-mediated CXCR4 up-regulation occurred through activation of adenosine A_{2A}/A_{2B} receptors. We did not identify the downstream signaling pathway involved; however, we ruled out signaling through a cAMP/PKA pathway. Adenosine-induced CXCR4 up-regulation resulted in increased proliferative and migratory responses to CXCL12.

I assessed how prostaglandins affected CXCR4 expression on HT-29 cells in Chapter Three of this thesis. $PGF_{2\alpha}$ had very little effect on cell-surface CXCR4 protein

expression, whereas PGE_2 and PGD_2 both decreased CXCR4 expression. This was most pronounced with PGD_2, and was likely due to formation of cyclopentenone J-series metabolites, particularly $15dPGJ_2$, which also reduced CXCR4 mRNA expression. CXCR4 expression was decreased with a panel of thiazolidinedione PPARγ agonists, and $15dPGJ_2$-induced CXCR4 down-regulation was blocked with PPARγ antagonists. Furthermore, an analogue of $15dPGJ_2$ that retains its ability to activate PPARγ, CAY10410, decreased CXCR4 receptor expression. Therefore, we concluded that the $15dPGJ_2$ effect on CXCR4 protein expression involved signaling through PPARγ. However, we found that other cyclopentenone compounds reduced CXCR4 receptor expression, suggesting that inhibition of NF-κB signaling is also involved. We speculated that $15dPGJ_2$-induced CXCR4 down-regulation likely involves PPARγ-dependent and -independent NF-κB inhibition.

In summary, we found that there are factors within the tumour microenvironment that both increase and decrease CXCR4 expression on colorectal carcinoma cells. The relative abundance of each of these factors may determine overall CXCR4 expression. Regulation of CXCR4 expression by adenosine and prostaglandins represent novel mechanisms by which the tumour microenvironment may alter disease progression. Effective cancer therapies may include strategies to reduce CXCR4 expression, such as PPARγ agonists, NF-κB inhibitors, or treatments that lower tumour adenosine concentrations, in combination with established treatments such as chemotherapy. Also, further elucidation of the signaling pathways involved in adenosine- and prostaglandin-mediated changes in CXCR4 expression may reveal further targets.

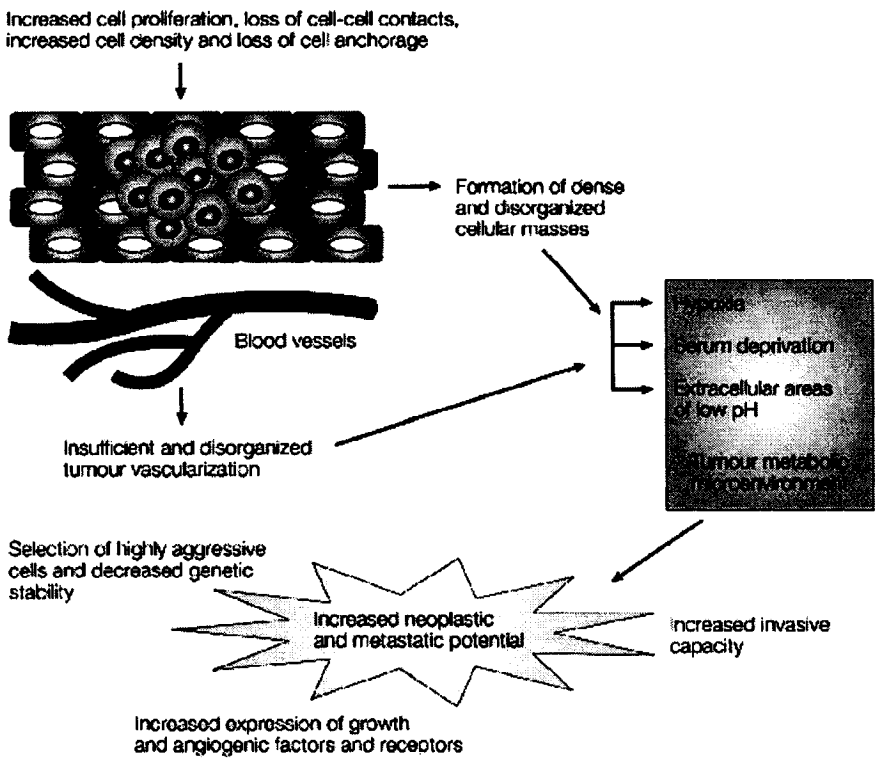


Figure 4.1: The tumour microenvironment contributes to metastasis. Taken from Cardone et al., 2005.

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Appendix A – Involvement of CXCL12/CXCR4 in many cancer types

Cancer	Comments	Reference
Acute lymphoblastic leukaemia (ALL)	Lymphoblasts taken from patients with ALL expressed higher levels of CXCR4 than lymphocytes taken from healthy donors. The proportion of cells that migrated through an endothelial layer towards CXCL12 was higher with cells expressing higher levels of CXCR4 protein.	Crazzolara et al., 2001
Brain cancer	Eleven of nineteen human glioblastoma tissue samples expressed CXCR4 mRNA, as did three different glioblastoma cell lines. Cell lines derived from other brain tumours expressed CXCR4 as well, including neuroblastoma, neuroectoderm, medulloblastoma, and astrocytoma.	Sehgal et al., 1998
	CXCR4 mRNA was expressed in several human brain tumours. CXCL12 increased proliferation and activated ERK1/2 phosphorylation of rat type I cortical astrocytes and A172 human glioblastoma cells.	Barbero et al., 2002
	Human brain tumours were found to express CXCR4 protein, including medulloblastoma, anaplastic astrocytoma, and glioblastoma. CXCL12 induced migration and increased proliferation and survival of Daoy medulloblastoma cells and U87 glioblastoma cells, both of which expressed CXCR4. Each of these effects was blocked with the non-competitive CXCR4 antagonist AMD3100.	Rubin et al., 2003
Breast cancer	Breast cancer cell lines expressed high CXCR4 mRNA and protein levels compared to normal cells. CXCL12 induced migration, invasion, and morphological changes in MDA-MB-231 human breast carcinoma cells, which expressed CXCR4. CXCL12 was highly expressed in tissues to which breast cancer cells metastasize. A neutralizing anti-CXCR4 antibody significantly reduced lung and lymph node metastasis after tail-vein injection or orthotopic implantation of MDA-MB-231 cells in SCID mice.	Müller et al., 2001
Chronic lymphocytic leukaemia (CLL)	Compared to normal B cells, malignant CLL cells expressed three- to four-fold higher cell-surface protein levels. CXCL12 induced calcium flux in CLL cells, and enhanced transendothelial migration.	Möhle et al., 1999
Colorectal cancer	After intrasplenic injection, CXCL12-KDEL transfected CT-26 cells did not form liver metastases, whereas control cells did. Lung metastases formation was also reduced with CXCL12-KDEL transfected cells compared to normal cells after tail vein injection. Mice injected with CXCL12-KDEL-transfected cells had increased survival.	Zeelenberg et al., 2003

Cancer	Comments	Reference
Colorectal cancer (cont'd)	CXCR4 was over-expressed in colorectal carcinoma tissues compared to normal tissues. Cell-surface CXCR4 protein was expressed at high levels in SW620, SW48, and SW480 cells, and at moderate levels in Caco-2 and LoVo cells, as shown by flow cytofluorimetry. CXCL12 enhanced the chemotaxis of SW480 cells as well as their adhesion to fibronectin and collagen type I/III, and both effects were blocked with anti-CXCR4 neutralizing antibodies. CXCL12 also induced cytoskeletal changes, proliferation, and ERK1/2 phosphorylation in SW480 cells.	Ottaiano et al., 2005
	SW480, SW620, and HT-29 cells expressed CXCR4 mRNA and protein, and 96/96 colorectal carcinoma tissue samples expressed CXCR4. CXCL12 induced chemotaxis of SW480 and SW620 cells	Schimanski et al., 2005
Endometrial cancer	Endometrial adenocarcinoma tissues and human cell lines expressed CXCR4 protein. CXCL12 induced proliferation of endometrial carcinoma cells.	Mizokami et al., 2004
Gastric cancer	Three of seven human gastric carcinoma cell lines expressed high levels of CXCR4 mRNA. NUGC4 human gastric carcinoma cells, which also expressed cell-surface CXCR4 protein, showed chemotactic and proliferative responses to CXCL12 that were blocked with a neutralizing anti-CXCR4 antibody. CXCL12 enhanced phosphorylation of Akt and ERK in NUGC4 cells. Sixty-seven percent of primary gastric tumours examined expressed CXCR4 protein.	Yasumoto et al., 2006
Glioblastoma	Thirteen of sixteen human glioma cell lines expressed CXCR4 mRNA, as did six of six glioblastoma tissue samples. CXCL12 induced ERK phosphorylation in LN427 and LN827 glioma cell lines, and promoted migration and survival of LN827 cells.	Zhou et al., 2002
Head and neck squamous cell cancer (HNSCC)	CXCR4 mRNA and cell-surface protein expression was detected in three of six HNSCC cell lines, and CXCL12 induced migration and proliferation of CXCR4-positive HNSCC cells. CXCR4 protein was on NHSCC tissues as well.	Katayama et al., 2005
Melanoma	CXCR4 cell-surface protein was expressed on BLM, MeWo, and A375 human melanoma cell lines, as well as on cells isolated from melanoma surgical specimens. CXCL12 enhanced the adhesion of MeWo cells to fibronectin, and increased phosphorylation of p38 MAPK.	Robledo et al., 2001
	CXCL12 enhanced the binding of B16 murine melanoma cells to endothelial cells, an effect mediated by β_1 integrin.	Cardones et al., 2003

Cancer	Comments	Reference
Melanoma (cont'd)	CXCL12 promoted the invasion of BLM human melanoma cells across basement membranes. This effect was due to CXCL12-induced cytoskeletal rearrangement caused by activation of the Rho GTPases RhoA and Rac1 and subsequent up-regulation of membrane-type 1 MMP.	Bartolomé et al., 2004
Multiple myeloma (MM)	MM cells isolated from bone marrow and MM cell lines NCI-H929 and RPMI 8226 expressed cell-surface CXCR4 protein, and cells derived from most patient samples migrated towards CXCL12. CXCL12 enhanced adhesion of NCI-H929 cells to fibronectin.	Sanz-Rodríguez et al., 2001
Nasopharyngeal cancer	Most primary human nasopharyngeal carcinoma biopsy samples and metastatic lymph nodes stained positively for CXCR4 protein. Several nasopharyngeal carcinoma cell lines also expressed CXCR4 at the mRNA level. Reduction of CXCR4 expression in 5-8F nasopharyngeal carcinoma cells using antisense technology inhibited lung metastasis formation after i.v. injection of these cells.	Hu et al., 2005
Neuroblastoma	Eight neuroblastoma cell lines expressed cell-surface CXCR4 protein expression, and SH-SY5Y human neuroblastoma cells expressing stable levels of CXCR4 migrated towards CXCL12 and adhered to bone marrow stromal cells in a CXCL12-dependent manner.	Geminder et al., 2001
Non-Hodgkin's lymphoma (NHL)	Most NHL primary samples and cell lines expressed high levels of CXCR4 mRNA and cell-surface protein. An antibody against CXCR4 reduced the transendothelial migration and proliferation of Namalwa NHL cells.	Bertolini et al., 2002
Non-small cell lung cancer (NSCLC)	CXCR4 mRNA was up-regulated in NSCLC tissues compared to normal tissues, and levels were higher in tissue samples taken from patients with metastasis than from those without metastasis. Over-expression of CXCR4 in 95C NSCLC cells led to enhanced migratory, invasive, and adhesive responses to CXLC12, all of which were inhibited with a neutralizing anti-CXCR4 antibody.	Su et al., 2005
Osteosarcoma	CXCR4 mRNA was expressed in 63% of human osteosarcoma samples, and two of three osteosarcoma cell lines.	Laverdiere et al., 2005
Ovarian cancer	CXCR4 mRNA was expressed in ovarian cancer cell lines, as well as in biopsies from primary tumours and ovarian cancer ascites. CXCL12 induced intracellular calcium flux and chemotaxis of CXCR4-expressing IGROV and CAOV-3 ovarian cancer cells. High levels of CXCL12 were present in ascitic fluid taken from patients with ovarian cancer.	Scotton et al., 2001
	CXCL12 stimulated the growth of IGROV ovarian cancer cells, an effect that was blocked with a neutralizing anti-CXCR4 antibody and with the CXCR4 antagonist AMD3100.	Scotton et al., 2002

Cancer	Comments	Reference
Pancreatic cancer	70% of human pancreatic cancer tissues stained positively for CXCR4 expression, and five human pancreatic cancer cell lines expressed CXCR4 mRNA. CXCL12 induced chemotaxis of AsPC-1 human pancreatic cells, an effect that was reduced with the CXCR4 antagonist T22.	Koshiba et al., 2000
	Six of eleven human pancreatic cancer cell lines tested expressed CXCR4 mRNA and cell-surface protein. High levels of CXCR4 mRNA were detected in tumour cells isolated from surgical samples. Normal pancreatic cells expressed lower amounts of CXCR4. Hs766T, AsPC1, A818r, and CFPAC pancreatic cancer cells, all of which expressed CXCR4, migrated towards CXCL12. CXCL12 increased the adhesion of Hs766T cells to HUVECs, and stimulated proliferation and promoted survival of Hs766T and AsPC1 cells.	Marchesi et al., 2004
Prostate cancer	Osteoblasts and osteosarcoma cell lines expressed CXCL12 mRNA and secreted CXCL12 protein. Prostate cancer cell lines (DU145, PC3, LNCaP, and C4-2B) expressed CXCR4 mRNA and protein. Treatment of PC3 cells with CXCL12 induced ERK phosphorylation. Treatment of PC3 and C4-2B human prostate cancer cells as well as MCF-7 human breast carcinoma cells with CXCL12 increased their adherence to osteosarcoma cells and bone marrow endothelial cells. CXCL12 increased the transendothelial migration of LNCaP and DU145 cells, and the invasion of PC3 and C4-2B cells in Matrigel, which was blocked with a neutralizing anti-CXCR4 antibody.	Taichman et al., 2002
	LNCaP, PC3, and DU145 human prostate cancer cell lines expressed CXCR4 mRNA and protein, and approximately half of prostate cancer tissues stained positively for CXCR4. CXCL12 induced migration of PC3 and DU145 cells in a CXCR4-dependent manner, but had no effect on proliferation.	Mochizuki et al., 2004
	LNCaP and PC3 human prostate cancer cells expressed CXCR4 mRNA and cell-surface protein at higher levels than normal prostate epithelial cells. LNCaP and PC3 cells migrated towards CXCL12, an effect that was reduced with a neutralizing anti-CXCR4 antibody. Similar results were noted in invasion assays, which was likely due to CXCL12-induced increase in the mRNA and active protein expression of several MMPs in LNCaP and PC3 cells.	Singh et al., 2004
Renal cell cancer (RCC)	One of four human RCC cell lines (A-498 cells) expressed CXCR4 mRNA, and CXCR4 mRNA was up-regulated in RCC tumour samples compared to normal tissue. A-498 cells express cell-surface CXCR4 protein expression, and treatment with CXCL12 induced calcium flux.	Schrader et al., 2002

Cancer	Comments	Reference
Rhabdomyo-sarcoma	Several rhabdomyosarcoma cell lines expressed cell-surface CXCR4 protein. CXCL12 did not induce proliferation of rhabdomyosarcoma cells, but did induce phosphorylation of ERK1/2. CXCL12 also increased cell motility, caused cytoskeletal rearrangement, induced chemotaxis, increased adhesion to fibronectin and laminin, and stimulated secretion of MMP-2.	Libura et al., 2002
Small cell lung cancer (SCLC)	CXCR4 mRNA and cell-surface protein were detected in ten SCLC cell lines, and CXCL12 induced the proliferation of NCI-H69 SCLC cells. CXCL12 increased adherence and motility of NCI-H446 SCLC cells, and induced morphological changes such as filopodia formation.	Kijima et al., 2002
Thyroid cancer	Anaplastic thyroid cancer cell lines (FRO, NPA, and ARO) expressed CXCR4 mRNA, but only one cell line (ARO) expressed cell-surface CXCR4 protein and migrated towards CXCL12.	Hwang et al., 2003
	Human thyroid carcinoma cell lines expressed CXCR4 protein, and CXCR4 was up-regulated in primary papillary thyroid carcinomas compared to normal thyroid tissue. CXCL12 activated ERK1/2 and Akt, increased proliferation, inhibited apoptosis, and increased migration and invasion of FB2 human thyroid cancer cells.	Castellone et al., 2004

Appendix B – CXCR4 expression as an indicator of prognosis and disease progression

Cancer	Comments	Reference
Acute lymphoblastic leukaemia (ALL)	In childhood ALL, high CXCR4 expression on lymphoblasts was associated with increased infiltration in the liver or spleen.	Crazzolara et al., 2001
Breast cancer	In invasive ductal carcinomas, high CXCR4 expression was associated with more extensive lymph node metastasis, but not with not haematogenous metastasis or survival.	Kato et al., 2003
	CXCR4 co-expression with HER2-neu was an indicator of more extensive lymph node involvement in breast cancer.	Cabioglu et al., 2005
Chronic lymphocytic leukaemia (CLL)	High CXCR4 expression on B cells was associated with reduced survival in patients with familial CLL.	Ishibe et al., 2002
Colorectal cancer	In patients with colorectal cancer with liver metastasis, higher CXCR4 expression was found on liver metastases compared to the primary tumour. In patients with stage I/II disease, high CXCR4 mRNA expression in tumour samples was associated with increased disease recurrence. In patients with stage IV disease, patients with high CXCR4 had decreased overall survival. CXCR4 expression was found to be a predictor of overall survival.	Kim et al., 2005a
	High CXCR4 expression was associated with increased lymph node involvement and distant metastasis, as well as with reduced three-year survival.	Schimanski et al., 2005
	A significant association was found between CXCR4 expression and lymph node involvement. CXCR4 expression was associated with reduced disease-free survival, although the association was statistically significant using univariate analysis but not multivariate analysis. The combination of high CXCR4 and high VEGF expression was strongly predictive of reduced disease-free survival.	Ottaiano et al., 2006
Gastric cancer	Primary tumours that expressed CXCR4 protein correlated with peritoneal carcinomatosis.	Yasumoto et al., 2006
Head and neck squamous cell cancer (HNSCC)	High CXCR4 expression was associated with increased occurrence of distant metastases and reduced survival.	Katayama et al., 2005
Melanoma	CXCR4 expression was associated with reduced disease-free survival and overall survival.	Scala et al., 2005

Cancer	Comments	Reference
Non-small cell lung cancer (NSCLC)	High CXCR4 protein expression correlated with the presence of metastatic disease.	Su et al., 2005
Osteosarcoma	CXCR4 mRNA expression was inversely correlated with overall survival and event-free survival, and was positively correlated with the occurrence of metastasis.	Laverdiere et al., 2005
Prostate cancer	CXCR4 expression was a positive predictor of bone metastasis, particularly in patients with elevated PSA levels.	Mochizuki et al., 2004
Renal cell cancer (RCC)	High CXCR4 expression was associated with poor tumour-specific survival, independent of tumour stage and differentiation grade.	Staller et al., 2003