ADENOSINE STIMULATION OF CELL PROLIFERATION AND MIGRATION: IMPLICATIONS FOR THE ROLE OF THE CELLULAR MICROENVIRONMENT IN TUMOUR EXPANSION

by

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This thesis is dedicated to those living with cancer, to cancer survivors, and to those who fought a courageous battle. May there someday be a cure.

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ABSTRACT

Solid tumours have a poorly formed and disorganised vasculature that leads to regions of hypoxia. In these hypoxic regions, the purine nucleoside adenosine is produced at an elevated level due to activation of 5'-nucleotidase and inhibition of adenosine kinase. The local extracellular concentration of adenosine within the solid tumour microenvionment is likely in the 3-10μM range. We and others have shown that adenosine is a potent immunosuppressant; inhibiting several anti-cancer immune responses. The objective of this thesis was to evaluate further potential tumour-promoting actions of adenosine. Specifically, I investigated the effect of adenosine on colon carcinoma cell growth and cell migration.

The effect of adenosine on tumour cell growth is unclear. Some groups have shown that adenosine induces tumour cell death while others have demonstrated that adenosine increases tumour cell growth. These contradictory results may derive from the use of nonphysiological adenosine analogues or indirect approaches through inhibition of adenosine breakdown. My first aim was to determine if authentic adenosine increased or decreased colon carcinoma cell growth. I found that adenosine consistently stimulated DNA synthesis, cell cycle progression, and cell proliferation in five human colon carcinoma cell lines at pathophysiologically relevant concentrations. The adenosine response was however, modulated by the precise conditions of the culture environment.

Adenosine also stimulated the migration of HRT-18 colon carcinoma cells at concentrations that are expected to be present in the tumour microenvironment. Checkerboard analysis revealed that adenosine increased both chemotaxis and chemokinesis of HRT-18 cells. The adenosine-mediated increase in cell migration was inhibited by antagonists to the adenosine A_{2a} and A_{2b} receptor subtypes. The A_{2a}-selective agonist CGS21680 stimulated migration of HRT-18 cells. These data suggest that adenosine likely stimulates HRT-18 cell migration through A_2 receptors.

Tumour cell growth and cell migration are integral to tumour expansion. Given that adenosine has been shown to stimulate both of these processes, as well as being immunosuppressive, interventions focused on the control of the local level of adenosine in the tumour microenvironment may be novel and effective therapeutic strategies in the pharmacotherapy of solid tumours.

ABBREVIATIONS AND SYMBOLS

AC	Adenylyl cyclase
ADA	Adenosine deaminase
Ado	Adenosine
AK	Adenosine kinase
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	bovine serum albumin
bFGF	basic fibroblast growth factor
°C	celsius
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
cDNA	complementary DNA
Ci	Curie
cpm	counts per minute
d	day
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPPIV	dipeptidyl peptidase IV
DTT	dithiothreitol
EC_{50}	concentration production half-maximal effect

ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
Fas	fibroblast associated
FasL	Fas ligand
FCS	fetal calf serum
g	gravitational force
G-protein	guanosine triphosphate binding protein
h	hour
[^{3}H]TdR	tritiated thymidine
HPLC	high performance liquid chromatography
HUVEC	human umbilical vein endothelial cells
IBD	inflammatory bowel disease
IL	interleukin
IP_3	inositol (1,4,5)-triphosphate
L	litre
M	mols/litre
MAPK	Mitogen activated kinase
min	minute
M-MLV	murine Maloney leukemia virus
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide
n	number
NCS	newborn calf serum

OvCa	ovarian cancer
PBS	phosphate buffered serum
RT-PCR	reverse transcriptase polymerase chain reaction
SAH	S-adenosylhomocysteine
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
sec	seconds
SEM	standard error of the mean
SFM	serum-free media
TCA	trichloroacetic acid
TM	tumour microenvironment
TNF-α	tumour necrois factor alpha
U	unit
VEGF	vascular endothelial growth factor

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It was my brush that touched the canvas, but many hands helped to paint the picture.

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To my family - Mum, Dad, and Aneil - thank you for your love, unwavering understanding, and for putting up with me.

"I don't have much to say about 'art'. For me, art is not linked to a specific discipline or a particular aspect of life. It has nothing to do with technical skill or prowess, but a lot to do with a spiritual dimension, the ability to 'understand' – but in a non-intellectual manner." - Charles Gagnon

PUBLICATIONS

Portions of this thesis have been previously published or are in press.

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1. INTRODUCTION

1.1 Preface

Adenosine is a purine nucleoside produced in the microenvironment of solid tumours due to the hypoxic conditions within. The primary objective of this thesis is to examine adenosine's possible tumour-promoting roles. There is extensive debate regarding adenosine's effect on tumour cell proliferation. Some groups have suggested that adenosine promotes the proliferation of tumour cells (Orrico *et al.*, 1991; Lelièvre *et al.*, 1998a, 1998b; Mujoomdar *et al.*, 2004), while others argue that adenosine induces tumour cell death (Tey *et al.*, 1992; Tanaka *et al.*, 1994). Moreover, one group has made claim that adenosine selectively inhibits tumour cell growth, while sparing normal cells and they reason that certain analogues of adenosine may be useful as chemotherapeutics (Fishman *et al.*, 2000). Given the uncertainty that surrounds adenosine's effect(s) on cell proliferation, this suggestion is seemingly premature. Clearly, a definitive answer to the question, 'What effect does adenosine have on tumour cell proliferation?', is urgently needed. To this end, this thesis will systematically examine the effect of adenosine on DNA synthesis and cell proliferation using five human colorectal cancer cell lines. The possible involvement of adenosine receptors in the cell proliferation process will also be examined. Furthermore, the potential effect of adenosine on the migration of one of the human colorectal carcinoma cell lines. The findings of this work will help to elucidate the role of adenosine in the tumour microenvironment in the processes of tumour cell growth and expansion.

1.2 Canadians and colorectal cancer

1.2.1 Current incidence and mortality

In 2004, over 19 000 new cases of colorectal cancer will be diagnosed in Canada. Colorectal cancer is the second leading cause of cancer-related death in men and women, second only to lung cancer (Canadian Cancer Society statistics, 2004). If colorectal cancer is detected and treated early, prognoses are typically favourable (Canadian Cancer Society, 2004). Unfortunately, convincing Canadians that regular screening for colon cancer, which may involve any number of tests including rectal exams, fecal blood tests, enemas, or colonoscopies, has not been met with the success of other cancers such as breast and cervical (Canadian Cancer Society, 2004). Early stage colon cancer is typically asymptomatic and therefore goes unnoticed; it is only when the tumour begins to affect the colon and/or rectal structures that symptoms become pronounced and people present to their physicians. More recently, there has been an increased recognition for the need for more biomarkers of colon cancer to assess risk, detect cancer at earlier asymptomatic stages, and predict prognosis (Srivastava *et al.*, 2001).

1.2.2 Risk factors for developing colorectal cancer

Currently, there are very few known biomarkers of risk for developing colon cancer. However, there are several conditions that may predispose individuals to colorectal cancer including colonic adenomas (Woodford-Richens *et al.*, 2000), a previous malignant tumour in the colon or rectum (Canadian Cancer Society, 2004), and

perhaps inflammatory bowel diseases (IBD), including both chronic ulcerative colitis and Crohn's disease (Rhodes and Campbell, 2002). A recent study reported that the risk of developing colon cancer for people with IBD increases by 0.5-1.0% yearly, 8-10 years after diagnosis and that the magnitude of risk is dependent on several factors including age at IBD diagnosis, extent of the disease, and duration of symptoms (Munkholm, 2003). Colorectal cancer accounts for approximately 15% of all deaths in IBD patients (Munkholm, 2003). The mechanism by which IBD is linked to colorectal cancer is unclear. It may be that an inherited predisposition to IBD may also predispose patients to IBD-associated cancer (as opposed to sporadic colorectal cancer) (Rhodes and Campbell, 2002). Alternatively, the inflammation that is associated with IBD has been suggested as a possible causal agent in the development of colon cancer (Rhodes and Campbell, 2002).

Lifestyle choices such as lack of regular physical activity and poor dietary habits may also factor into the development of colon cancer, although their involvement is controversial (Mahmud and Weir, 2001). Dietary factors that may promote colon carcinogenesis include (i) high intake of sucrose, refined carbohydrates, omega-6 fatty acids, and (ii) reduced intake of fruits and vegetables, high-fiber whole grains, carotenoids, vitamins D, E, folic acid, and the minerals calcium, zinc, and selenium (van Poppel and van-den Berg, 1997). The role of dietary fat in the development of colorectal cancer is equally debated. Dietary fats from beef, lamb, and pork are associated with increased cancer risk more often than poultry, fish, and dairy fats (Singh *et al.*, 1997).

Approximately 80% of colon cancers are sporadic and the remaining 20% are of two inherited forms: (i) familial adenomatous polyposis coli (FAP) and (ii) hereditary non-polyposis colon cancer (HNPCC) (Murphy *et al.*, 1995). In FAP cases, only a

percentage of the numerous benign adenomatous polyps progress to malignant forms, whereas in HNPCC, cancers progress rapidly to invasive forms (Jass *et al.*, 2002).

Colon carcinogenesis has a well-defined set of genetic changes that occur resulting in malignant transformation. Loss of function of the APC (adenomatous polyposis coli) gene is associated with at least 60% of sporadic colon cancers (Jass *et al.*, 2002). Germ-line mutations in the APC gene are synonymous with large bowel cancers (Srivastiva *et al.*, 2001). A series of genetic changes occur leading to the development of a malignant tumour. These include *DCC* (deleted in colon cancer) and similar to other cancers, colon cancer is associated with a loss of the p53 gene, mutations in *K-ras*, and impaired DNA repair mechanisms (King, 2000). p53 plays a critical role in cell proliferation, cell death, and DNA repair in normal cells. These processes become unregulated in cancer cells, at least in part due to the loss of p53. Mutations in *K-ras*, a GTP-binding protein, result in a loss of GTPase activity. K-ras therefore, is maintained in a GTP-bound (active) state and is capable of relaying signals to a variety of effector molecules. Loss of function of two DNA repair genes, MSH2 and MLH1 increases the probability of errors in DNA and allows for the accumulation of genetic errors. A simplified schematic of the progression of the genetic changes resulting in either a loss or gain of function of gene products and the genesis of colon cancer is illustrated in Figure 1.1.

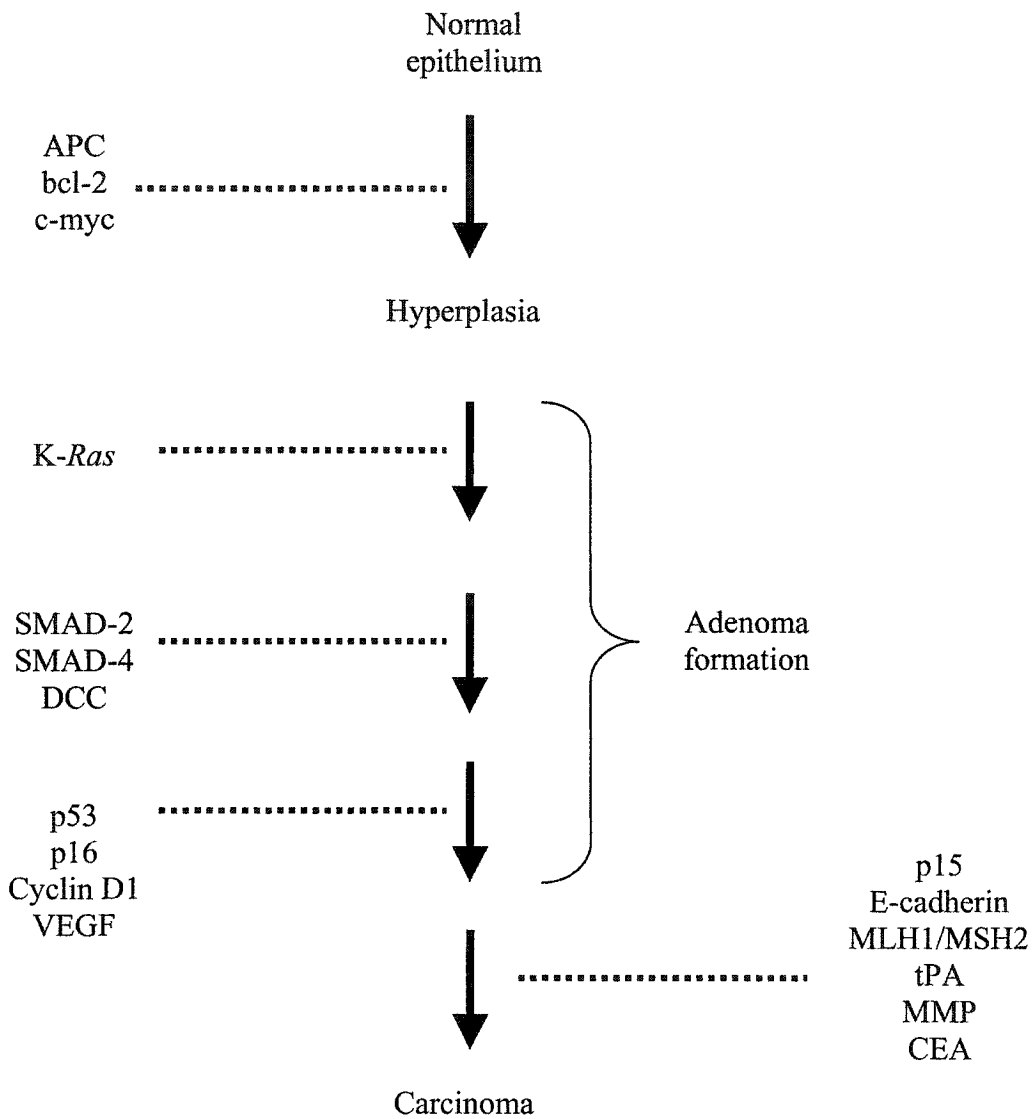

Fig. 1.1: Genetic changes during colon carcinogenesis. (Kelloff *et al.*, 2004; King, 2000)

1.2.3 Classification of colorectal tumours

Approximately 90% of all colorectal carcinomas are believed to arise from benign adenomatous polyps in the mucosal lining of the colon or rectum (Abeloff *et al.*, 2000). Over 75% of colorectal tumours occur in the colon with the remainder occurring in the rectum. The most prevalent colorectal tumour type is the adenocarcinoma which is a tumour developed from the glands in the mucosal lining of the colon wall (Murphy *et al.*, 1995). Other types of colorectal tumours such as carcinoid tumours, sarcomas, lymphomas, and small cell carcinomas occur rarely (Abeloff *et al.*, 2000). One of the most commonly used staging systems in the evaluation of colorectal tumours is the TNM system, where 'T' describes the level of penetration through the bowel wall; 'N' describes the number and location of any involved lymph nodes; and 'M' details the presence or absence of any metastases. There are also other staging systems that are used in conjunction with the TNM system such as the Duke's system (Sobin, 2003).

1.2.4 Therapeutic strategies

The primary therapy for colorectal tumours is surgery (Murphy *et al.*, 1995). Though the type of surgery performed depends on the stage and location of the tumour for both colon and rectal cancer, the ultimate goal is to remove the entire tumour whilst leaving as much healthy tissue as possible, thereby preserving function. In cases where the lymph nodes are involved, neo-adjuvant therapy (radiation and/or chemotherapy) is given before surgery to decrease the tumour size, which facilitates surgical removal.

Surgery is limited to tumours that are well-localized and tumours that have not invaded any vital regions.

Radiation therapy or radiotherapy is more commonly used on rectal cancers than on colon cancers given that many cancerous tissues in the colon are in close proximity to vital organs such as the small intestine, liver, and kidneys.

Chemotherapy for colorectal cancer typically begins 4-6 weeks following surgery. The two most common chemotherapeutic combinations used to treat non-metastatic colorectal cancers are: (i) 5-fluorouracil (5-FU) in combination with leucovorin for six months, and (ii) 5-FU in combination with leucovorin for 12 months (Abeloff *et al.,* 2000). 5-FU belongs to a class of anti-cancer drugs known as anti-metabolites, which are structurally similar to normal cellular components and act to disrupt DNA or RNA synthesis. Leucovorin, a folic acid co-enzyme, is given in conjunction with 5-FU to increase the effectiveness of the pyrimidine antagonist (Mycek *et al.*, 1997). Leucovorin acts to stabilize the binding of 5-FU to its target enzyme thymidylate synthetase, allowing for sustained inhibition, thereby resulting in a greater anti-cancer effect. Levamisole, an anthelminthic agent is sometimes administered in combination with 5-FU. Levamisole has immunomodulating properties and can increase the immune responsiveness in otherwise immunosuppressed patients, thereby engaging the anti-cancer immune response (Katzung, 2001).

1.3. Solid tumour microenvironment

1.3.1 Physiology and physiochemical features of solid tumours

The solid tumour microenvironment differs markedly from normal tissue. Most of the differences stem from the inherent anatomy of the tumour vasculature. Many of the blood vessels are irregular, tortuous, have arterio-venous shunts and blind ends. In addition, many tumour blood vessels do not have complete endothelial cell linings or basement membranes (Brown and Giaccia, 1998). Given the irregularities in the tumour blood vessels, their ability to deliver adequate oxygen and nutrients is compromised, resulting in local regions of hypoxia within the solid tumour (Fenton *et al.*, 1999).

The metabolic activities of tumour cells are different from their normal counterparts. The nutrient deprived state of the tumour microenvironment, coupled with the increased glucose consumption by tumours in response to changes in bioenergetics, results in low glucose levels (Guillino *et al.*, 1967). In 1930, Warburg first proposed that a high rate of glycolysis was indeed a hallmark of tumour tissue. This increased utilization of glucose as an energy source by means of both aerobic and anaerobic glycolysis results in the production of lactic acid as a by-product (Newell and Tannock, 1989). Tumours convert much of the pyruvate generated from glycolysis to lactic acid even in the presence of oxygen unlike cells of normal tissues, which do so only under oxygen-depleted conditions (Kouvroukoglou *et al.*, 1998). Lactic acid in part, contributes to the acidic environment of the solid tumour (Newell *et al.*, 1993).

1.3.2 Problems treating solid tumours

Cancers that grow as solid tumours, such as breast, lung, and colorectal cancers, typically lead to high mortality rates. The poor prognosis can be attributed in part to the fact that these cancers respond poorly to most conventional modes of treatment including radio-, chemo-, and immunotherapies (Kennedy *et al.*, 1980). There are numerous factors that can impact upon the effectiveness of anti-cancer therapies and the growth of cells in tumours. One factor that has been recognized to influence treatment outcome is the tumour microenvironment (Gnass and Hanahan, 1998). Solid tumours have regions of low oxygen tension, i.e. are hypoxic. Clinically, hypoxia is an obstacle in the treatment of cancer because hypoxia decreases the effectiveness of both drug and radiation therapies (Hill and Stanley, 1975; Kennedy *et al.*, 1980; Schlappack *et al.*, 1991; Robinson *et al.*, 2000). For instance, it has long been known that hypoxic conditions protect cells from the cytotoxic effects of radiation therapy. This was clearly demonstrated by Hewitt and Wilson (1959) in a study whereby ionizing radiation treatment was more efficacious at treating cells in normoxic environments compared to cells treated under hypoxic conditions. It has also been suggested that hypoxia-resistant tumour cells may develop a more clinically aggressive phenotype and have an increased potential for metastasis (Höckel *et al.*, 1996). Unfortunately, residual malignant cells that were protected from the cytotoxic effects of radiotherapy or chemotherapy by hypoxia may still be capable of proliferating, thus allowing the tumour to recur.

1.3.3 Exploiting solid tumours for treatment

The solid tumour microenvironment differs dramatically from normal tissues in its physiochemical parameters. Solid tumours are acidic, have hypoxic regions, high interstitial fluid pressure (Netti *et al.*, 1995; Pietras, 2004), and a leaky vasculature. Approaches that take advantage of these unique physiological parameters may direct cytotoxicity specifically to tumour tissue and spare normal healthy tissue. Three features of a solid tumour that may be exploited include: leaky vasculature, acidity, and hypoxia.

1.3.3.1 Leaky vasculature

Solid tumours are characterized by leaky blood vessels, in part caused by incomplete endothelial cell linings (Hashizume *et al.*, 2000). Leaky vessels may facilitate the extravasation of tumour cells into the blood stream, and subsequent metastasis (van den Brenk *et al.*, 1977). Space between the loosely connected cells lining the blood vessels may allow for the extravasation of particles up to 2μm (Hashizume *et al.*, 2000). The extent of tumour vasculature permeability has been found to correlate positively with histological grade and malignant potential (Daldrup *et al.*, 1998). The highly permeable nature of tumour blood vessels can be exploited to deliver anti-cancer agents selectively to tumour tissue.

Liposomal carriers for gene delivery or delivery of anti-cancer drugs are currently being evaluated for the treatment of solid tumours. Liposomes are stable, biocompatible, circulating drug carriers that are capable of penetrating the leaky vasculature of solid

tumours (Gabizon and Martin, 1997). Accumulation of liposomes in tumours is favoured because liposomes are able to extravasate through the leaky tumour vasculature (Harrington *et al.*, 2000). As previously mentioned, selective targeting of anti-cancer drugs to tumours is a major goal of cancer chemotherapy. Liposomes have been shown to preferentially localize to tumour tissue when injected systemically (Wells *et al.*, 2003). Current efforts are focusing on creating liposomes that can be activated to release their contents only at the intended site, thereby avoiding toxicity to non-tumour tissues (Kong *et al.*, 2000). Information about the pharmacokinetics of liposomes will help to optimize and promote tumour accumulations and thereby improve the pharmacodynamics of the encapsulated anti-cancer agent.

The delivery of monoclonal antibodies (MAbs) conjugated to toxins, radionucleotides, or anti-cancer drugs has also been explored. The efficacy of this approach is dependent on the ability of the MAbs to reach tumour cell targets (Jain and Baxter, 1988). Although leaky blood vessels in tumours will permit the extravasation of MAbs, the distribution of MAbs is heterogeneous and may not be at sufficient quantity to exert a cytotoxic effect (Jones *et al.*, 1986). Distribution is typically greatest around highly permeable blood vessels (Sands *et al.*, 1988). However, leaky blood vessels may be separated from tumour cells by stromal cells; this stromal barrier likely hinders the ability of MAbs to target tumour cells (Dvorak *et al.*, 1991). Ensuring homogeneous distribution of liposomes and biomolecules including MAbs within the solid tumour would allow for the selective targeting of tumour cells.

1.3.3.2 Tumour acidity

In 1973, Eagle demonstrated that transformed cell lines grew over a broader pH range than normal cells. The optimum growth conditions of the virally-transformed cells were more acidic than the well-defined pH optimum of 7.6-7.8 for normal murine fibroblasts (Eagle, 1973). The average pH across a variety of tumour tissues has been reported to be 0.5 units lower than that of the surrounding normal tissues and may be still lower in local regions of hypoxia within the tumour (Rotin *et al.*, 1986). In general, intracellular pH (pH_i) is similar in both tumour tissue and normal tissue (Kozin *et al.*, 2001); conversely, extracellular pH (pH_e) is higher in normal tissue (Kozin *et al.*, 2001). A low pH_e can decrease the effectiveness of anti-cancer agents (Vukovic and Tannock, 1997) and may increase the invasiveness of tumour cells (Martinez-Zaguilan *et al.*, 1996). Evidence suggests that the expression of pro-angiogenic factors is higher in tumour cells at low pH_e (Fukumura *et al.*, 2001). Furthermore, high lactate levels were found to be predictive of metastasis, tumour recurrence, and patient survival in cases of cervical cancer (Walenta *et al.*, 2000).

Although tumour cells may be resistant to acidic conditions that would typically cause cell death of normal cells, it may be possible to enhance tumour acidity to a point where the tumour cells are no longer capable of surviving the severely low pH. Experimentally in rat tumours, systemic acidosis has been achieved by increasing the level of carbon dioxide in the air that is breathed, or by providing bicarbonate in the drinking water (Guillino *et al.*, 1965). Administration of glucose can also decrease

tumour pH through its conversion to lactic acid under anaerobic conditions (Jahde and Rajewsky, 1982).

In efforts to exploit the acidic conditions of the tumour microenvironment, chemotherapeutic agents, whose activity is more effective at low pH_e, have been used. For example, transport of charged drugs whose uptake is by passive diffusion, will be facilitated at a pH_e that favours the non-ionized form of the drug (Tannock and Rotin, 1989).

Intracellular acidosis can induce apoptosis. To avoid this, tumour cells have been shown to increase the expression of pH regulators that prevent intracellular acidification (Murakami *et al.*, 2001). Four major types of pH regulators have been identified: the proton pump, the sodium-proton exchanger family, the bicarbonate transporter family, and the monocarboxylate transport family (Izumi *et al.*, 2003). There are inhibitors of all four types of pH regulators that target these intracellular pH control mechanisms. Debrowsky *et al.*, (1991) demonstrated that weak acids such as succinate and the monomethylester of succinate have the ability to induce intracellular acidification of EMT-6 and MGH-UI cells. Intracellular acidification increased with decreasing pH_e, although monomethylsuccinate was able to decrease pH_i at a neutral pH_e (Debrowsky *et al.*, 1991). The ultimate goal of these approaches is to increase the acid load and initiate cytotoxicity.

1.3.3.3 Tumour hypoxia

The vasculature of solid tumours differs dramatically from normal tissue vasculature in that it is disordered, highly irregular, leaky, and has decreased blood flow (Jain, 2002). This contrast between the solid tumour vasculature and the highly ordered normal tissue vasculature occurs when tumours undergo angiogenesis, the growth of new blood vessels. The angiogenic process in tumours is not as tightly regulated as it is in normal tissue, leading to improperly formed blood vessels. Most tumours and metastases originate as small avascular structures that only develop new blood vessels once they grow beyond a few millimetres in size (Hanahan and Folkman, 1996). Tumours arising from epithelial structures that are separated from the underlying vasculature by a basement membrane would be predicted to initiate as avascular structures, but tumours may also arise in or metastasize to well-vascularized areas. Within such vascularized tissues, tumours may co-opt existing blood vessels (Holash *et al.*, 1999).

The vasculature of solid tumours may not be capable of optimally supporting the tumour tissue, thereby impairing the uniform delivery of nutrients and oxygen to all areas of the tumour (Kato *et al.*, 2002; Sutherland *et al.*, 1986). This results in areas of the tumour that are nutrient-deprived and have low oxygen tensions, or are hypoxic.

It has been shown that hypoxia can modulate the proliferative state of tumour cells. For example, hypoxia can trigger cells to enter a quiescent state (Höckel and Vaupel, 2001) or prolong cell cycle times (Webster *et al.*, 1998), but in both of these cases the tumour cells remain viable. Importantly, most anti-cancer agents exert their

actions on cells that are rapidly proliferating. Given that the hypoxic cell fraction within a solid tumour can vary from 5-22% (Leith *et al.*, 1991), there is a substantial proportion of tumour cells that is protected from the cytotoxic effect of anti-cancer agents because they may be in a resting state. In addition, hypoxia is also capable of conferring a protective advantage to cells exposed to radiation therapy (Scott and Greco, 2004). Therefore, there exists a population of cells that remain unaffected by both radiation and chemotherapy. These residual malignant cells may be capable of proliferating and causing re-growth of the tumour. Hypoxia has also been shown to induce proliferation of endothelial cells. This increase in cell growth does not seem to be due to a direct depletion of oxygen, but rather inhibition of mitochondrial electron transfer, since an inhibitor of the mitochondrial respiratory chain mimicked hypoxia (Schäfer *et al.*, 2003).

An approach to selectively target tumour cells within hypoxic regions is to use cytotoxic agents that are only toxic in the absence of oxygen. These agents therefore would not target normal, fully oxygenated tissues. Lin *et al.*, (1972) first described the basis for the selectivity of these hypoxia-selective agents. These researchers hypothesized that hypoxic cells may have a greater capacity for reductive reactions than well-oxygenated tissues. Moreover, they believed that this unique characteristic of solid tumours could be exploited through the design and development of chemotherapeutic agents that were only cytotoxic after being reduced *in vivo* at the site of the tumour. Such 'bio-reductive' chemotherapeutic agents include the quinone alkylating agents (e.g. mitomycin C) and the nitroaromatic heterocyclic hypoxic cell sensitizers (e.g. metronidazole) (Kennedy *et al.*, 1980).

The leading compound in the class of bio-reductive drugs is tirapazamine (TPZ). TPZ has shown promising results in a number of clinical trials when used in combination with radiotherapy and/or cisplatin-based chemotherapy to treat cervical, head and neck, and non-small-cell lung cancers (Craighead *et al.*, 2000; von Pawel *et al.*, 2000). TPZ is converted intracellularly into a cytotoxic radical, that under low oxygen conditions causes DNA damage resulting in cell death (Peters *et al.*, 2001).

A second, unusual approach to exploit the hypoxic tumour has been to target anaerobic bacteria to the hypoxic regions of solid tumours. An association between bacterial infections and cancer dates back more than one hundred years. At that time, an observation was made that the prognosis of cancer patients who contracted bacterial infections was better than those that had not. Currently, several bacterial strains that grow under anaerobic conditions are being investigated for clinical use (Minton *et al.*, 1995; Theys *et al.*, 2003). One anaerobic strain, *Clostridium* spp., has been engineered to produce proteins with anti-cancer properties such as tumour necrosis factor-alpha (TNF-α) (Theys *et al.*, 2001).

Targeted gene delivery to hypoxic regions of tumours is yet another possible way to exploit tumour-associated hypoxia. In this case a therapeutic gene, perhaps encoding a cytotoxic protein, is placed under the control of a hypoxia-induced promoter. Gene expression only occur in hypoxic areas (Dachs and Tozer, 2000). Genes encoding enzymes that activate pro-drugs have been employed in gene-directed therapy. These include cytosine deaminase, which can sensitize tumour cells to the pro-drug 5-fluorocytosine, which is then converted to the cytotoxic agent 5-FU (Dachs *et al.*, 1997), and cytochrome P450 2B6, which is known to activate cyclophosphamide to its toxic

metabolite (Griffiths *et al.*, 2000). Collectively, these approaches show that targeting tumour tissue hypoxia is feasible and hold considerable promise for tumour-specific therapy.

1.4 Adenosine

Adenosine is a purine nucleoside composed of a nitrogenous base and a pentose sugar unit (ribose), that lacks any phosphate groups. Nucleotides are mono-, di-, or triphosphate esters of nucleosides (Champe and Harvey, 1994). Purines and pyrimidine nucleotides are involved in most cellular processes and their roles range from structural, metabolic, or energetic, to regulatory. They make up the backbone of RNA and DNA; adenosine triphosphate is the major source of energy for most cellular processes; and guanosine triphosphate is critical to signal transduction events (Rudolph, 1994).

1.4.1 Adenosine production and metabolism

The two major pathways of adenosine formation depicted in Figure 1.2 are (i) the enzymatic conversion of adenosine monophosphate (AMP) to adenosine via 5'-nucleotidase (5'-NT) (EC 3.1.3.5) and (ii) the hydrolysis of S-adenosylhomocysteine (SAH) by SAH hydrolase (EC 3.3.1.1) and alkaline phosphatase (AP) (EC 3.1.3.1) (Picher *et al.*, 2003). Under normoxic conditions, SAH is formed by S-adenosylmethionine (SAM) through the transfer of the methyl group (Kroll *et al.*, 1993). During ischemia or under hypoxic conditions, the major route of adenosine production is

via 5'-NT, also known as CD73, which is activated under these conditions (Synnestvedt *et al.*, 2002). Regardless of the source of adenosine, it is phosphorylated by adenosine kinase or deaminated by adenosine deaminase (Moriwaki *et al.*, 1999).

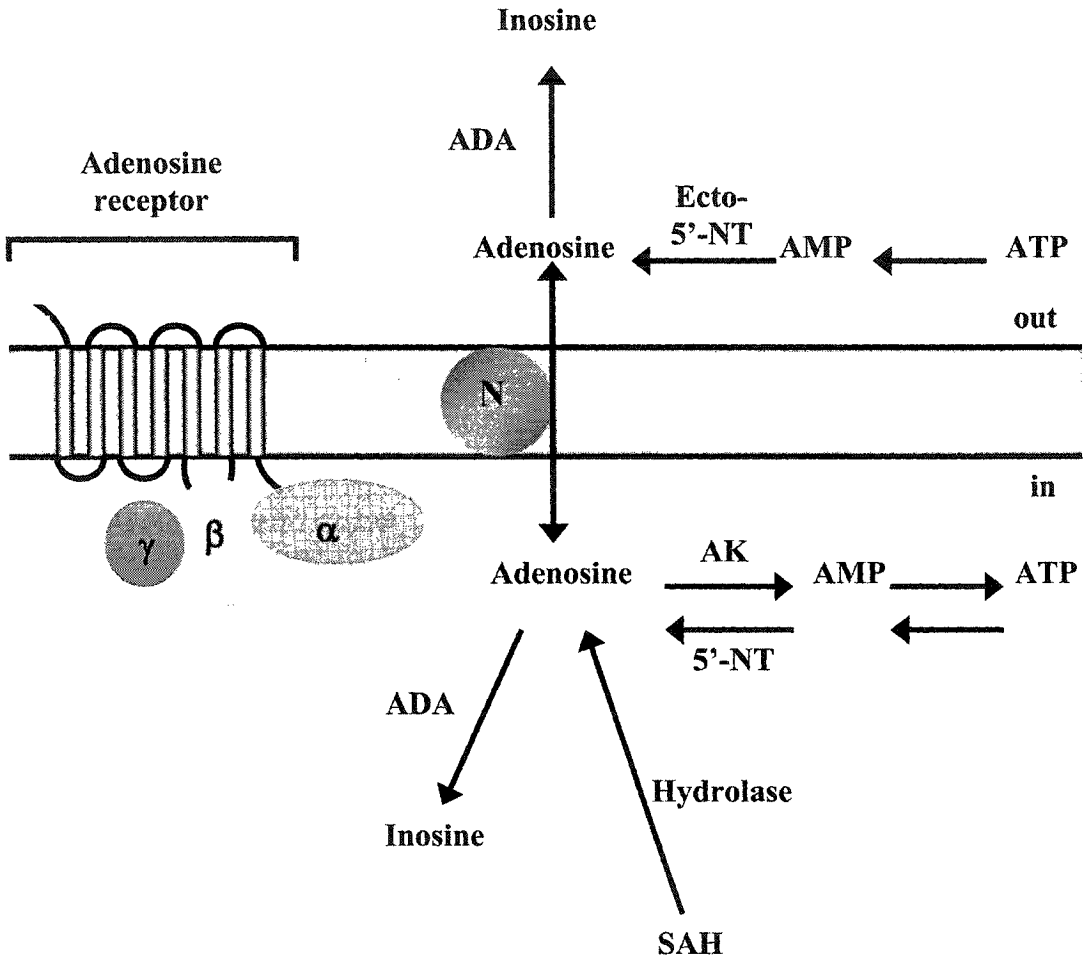

Fig. 1.2: Adenosine production and metabolism. (modified from Blackburn, 2003)

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

1.4.1.1 Adenosine deaminase

Adenosine aminohydrolase, more commonly known as adenosine deaminase (ADA) is responsible for the enzymatic conversion of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine, respectively (Cristalli *et al.*, 2001). ADA is expressed in most human tissues, but the strongest expression of this key enzyme in purine metabolism is in the lymphoid tissues such as the lymph nodes, spleen, and thymus (Moriwaki *et al.*, 1999). ADA is both a cytosolic enzyme and an externally-bound enzyme (ecto-ADA) (Franco *et al.*, 1998).

There have also been several reports of the extraenzymatic properties of ecto-ADA including stimulating the release of excitatory amino acids (Catania *et al.*, 1991) and binding to the cell-surface adenosine A_1 and A_{2b} receptor subtypes (Saura *et al.*, 1996; Herrera *et al.*, 2001).

Human ADA deficiency, leading to increased plasma adenosine and deoxyadenosine levels, has been shown to have a profound impact particularly on the immune system. Giblet (1972) linked the autosomal recessive disorder known as severe combined immunodeficiency (SCID) to ADA deficiency. Although the importance of ADA activity on immune function is clearly illustrated by the attenuation of the immune response observed in SCID patients, there are additional examples of clinical pathologies that are associated with decreased ADA activity. These include acquired immunodeficiency syndrome (AIDS) (Franco *et al.*, 1998), rheumatoid arthritis, and certain leukemias (Smyth *et al.*, 1978; Simpkins *et al.*, 1981)

ADA is known to bind to CD26, a marker of activation on T-lymphocytes. CD26 is also known as dipeptidyl peptidase IV (DPPIV). The functional role of the ADA/DPPIV complex is as yet unclear, but it has been proposed that the complex may serve to regulate local levels of adenosine at sites of CD26/DPPIV expression including the surface of T-cells and tumour cells (Dong *et al*, 1996). DPPIV has been implicated in the development of cancer, in that the down-regulation of DPPIV is thought to be a critical event in the transformation of normal melanocytes to melanoma cells (Wesley *et al.*, 1999). Over-expression of DPPIV has been linked to decreased invasiveness of melanoma cells (Pethiyagoda *et al.*, 2001) and ovarian cancer cells (Kajiyama *et al.*, 2003). Most recently, our lab has demonstrated that adenosine, at concentrations likely to be present in the tumour microenvironment, down-regulates DPPIV on human colon carcinoma cells (Tan *et al.*, 2004), strongly arguing for yet another tumour-promoting role of adenosine.

1.4.1.2 Adenosine kinase

Adenosine kinase (AK) is one of the most abundant mammalian purine nucleoside kinases (Maj *et al.*, 2000). Adenosine kinase is a key enzyme involved in the regulation of both intracellular and extracellular adenosine concentrations (Kowaluk and Jarvis, 2000). The activity of AK is inhibited at high concentrations of adenosine (Maj *et al.*, 2000) and under oxygen and glucose deprivation (Lynch *et al.*, 1998). It is believed that this inhibition, which has been shown to occur during myocardial and cerebral ischemia (Lasley *et al.*, 1995; Phillis *et al.*, 1991), may serve to potentiate the cardiac and

neural protective mechanisms of adenosine (Mullane *et al.*, 1995; Tatlisumak *et al.*, 1998).

1.4.1.3 Adenosine transporters

The regulation of intracellular and extracellular adenosine concentrations, in addition to being mediated by AK and ADA, is also further dependent on nucleoside transporters. These transporters are believed to play a major role in the regulation of physiological concentrations of nucleosides, including adenosine (Cass *et al.*, 1999). The nucleoside transport systems can be broadly classified based on their sodium dependence. Equilibrative transporters are sodium-independent and inhibited by nitrobenzylthioinosine (NBTI). They can be further classified based on their relative sensitivities to inhibition by NBTI. Equilibrative sensitive (es) are inhibited by nanomolar concentrations of NBTI whereas equilibrative insensitive (ei) transporters are resistant to concentrations of NBTI up to 1μM. These transporters accept both purine and pyrimidine nucleosides and are commonly referred to as ENT-1 and ENT-2 respectively.

Concentrative transport systems, conversely, are sodium dependent. As their name implies, this class of transporters is able to transport nucleosides against a concentration gradient. Concentrative transporters are divided into 6 subclasses (Table 1.1). N1, N2, and N3 transporters are more commonly referred to as CNT-2, CNT-1, and CNT-3 respectively, and catalyze inward transport or nucleosides.

Interestingly, conditions present in the tumour microenvironment may influence nucleoside transport. Recently it has been reported that chronic exposure of PC-12 pheochromocytoma cells to hypoxia down-regulated nucleoside transport, allowing for a sustained increase in the extracellular concentration of adenosine (Kobayashi *et al.*, 2000).

Table 1.1: Mammalian nucleoside transport systems

	Sodium dependent?	NBTI sensitive?	Specificity	Alternative classification
Equilibrative				
es	No	Yes (<1μM)	purines, pyrimidines	ENT-1
ei	No	Yes (>1μM)	purines, pyrimidines	ENT-2
Concentrative				
N1	Yes	No	purines, guanosine	CNT-2
N2	Yes	No	pyrimidines	CNT-1
N3	Yes	No	purines, pyrimidines	CNT-3
N4	Yes	No	pyrimidines, adenosine, guanosine	
N5	Yes	No	adenosine analogues	
N6	Yes	Yes	guanosine	

(Griffiths and Jarvis, 1996; Sinclair *et al*, 2001)

1.4.2 Adenosine production due to hypoxia

Adenosine production has been reported to be increased during ischemia and hypoxia. Hypoxia is conducive to intracellular adenine nucleotide breakdown, which results in adenosine release from the cell (Vaupel *et al.*, 1989; Vaupel *et al.*, 2001). It is the 5'-NT pathway that is thought to be the major route of adenosine production under these stress conditions (Kitakaze *et al.*, 1993). Indeed, the activity of 5'-NT is increased under hypoxic conditions in both cardiomyocytes and endothelial cells (Ledoux *et al.*, 2002).

Solid tumours are known to contain areas of hypoxia due to their poorly organized and inadequate vasculature. Accordingly, Blay *et al.* (1997) found that the extracellular fluid of murine solid tumours grown *in vivo* contained elevated levels of adenosine.

1.4.3 Adenosine receptors

The majority of adenosine's effects are mediated through cell surface receptors that bind adenosine and are more selective for adenosine than other adenine-containing nucleotides (Klotz, 2000). These receptors are 7-transmembrane G-protein coupled receptors that not only modulate adenylyl cyclase (AC) activity, but also the activity of ion channels and phospholipases (Rees *et al.*, 2003). Traditionally, adenosine receptors were termed P1 receptors and those receptors with high affinities to ATP were termed P2 receptors (Morrone *et al.*, 2003).

There are four known adenosine receptor subtypes: A_1, A_{2a}, A_{2b}, and A_3. The suggestion that multiple adenosine receptor subtypes may exist was first proposed by Van Calker and colleagues in 1979. The basis for the suggestion was the observation that adenosine could have varying effects on AC activity. Adenosine receptors are divided into two subtypes. Their characteristics are tabulated in Table 1.2. A_1 receptors, which are negatively coupled to AC and therefore inhibit cyclic adenosine monophosphate (cAMP) production and A_2 receptors are positively coupled to AC, therefore activation of A_2 receptors results in an increase in cAMP (Fredholm *et al.*, 2000). A human A_3 receptor subtype was cloned and characterized in 1993 by Salvatore and co-workers. Similar to activation of A_1 receptors, A_3 activation results in decreased AC activity. In addition to being modulated by cell-surface receptor activity, AC can be inhibited by high concentrations of adenosine (approximately 80μM) acting intracellularly at the P-site (Collis *et al.*, 1993).

The expression profile of A_1 receptors suggests strong expression of this receptor subtype in certain areas of the brain including the cortex, cerebellum, and hippocampus (Fredholm *et al.*, 2000), although its expression can be detected throughout most tissues of the body. A_1 receptors signal through $G_{1/o}$ resulting in the inhibition of AC, activation of K^+ channels, and modulation of Ca^{2+} mobilization and phospholipase C (PLC) activity (Olah and Stiles, 1995).

A_{2a} receptors are most strongly expressed in the spleen, thymus, leukocytes, platelets, striatal GABAergic neurons, and in the olfactory bulb (Fredholm *et al.*, 2000). Activation of A_{2a} through signalling via G_s and G_{olf} results in increased cAMP production

and has been associated with numerous anti-inflammatory responses (Sullivan and Linden, 1998).

The A_{2b} subtype is a low affinity receptor subtype. Therefore, a higher concentration of adenosine is required for receptor activation (Daly *et al.*, 1983). Interestingly, A_{2b} expression is highest in the caecum, bladder, and colon. Moderate expression is noted in the lung and blood vessels (Fredholm *et al.*, 2000). A_{2b} receptors couple to G_s thereby increasing cAMP, but in addition, couple to G_q resulting in Ca^{2+} mobilization (Linden *et al.*, 1998) and mitogen-activated protein kinase (MAPK) activation (Linden *et al.*, 1999). The limited availability of selective agonists and antagonists of the A_{2b} subtype remains a challenge in the pharmacological characterization of A_{2b} receptors experimentally (Merighi *et al.*, 2001).

The data regarding the expression profile of the A_3 receptor subtype are inconclusive, but suggest low-level expression in the thyroid, brain (with moderate expression in the cerebellum and hippocampus), spleen, liver, kidney, and intestine. A_3 receptors couple to G_i and G_q and result in reduced cAMP levels and increased PLC activity, respectively (Fredholm *et al.*, 2000).

Table 1.2: Characteristics of adenosine receptors

Adenosine receptor subtype	G-protein	Effect of G-protein coupling	Sites of tissue distribution
A_1	$G_{i/o}$	cAMP IP_3 Ca^{++} channels K^+ channels	High: brain, spinal cord, eye, atria, and adrenal glands Intermediate: skeletal muscle, kidney, liver, testis, and colon Low: lung and pancreas
A_{2a}	$G_{s/olf}$	cAMP IP_3	High: spleen, thymus, leukocytes, and olfactory bulb Intermediate: heart, lung, blood vessels, peripheral nerves Low: most brain regions
A_{2b}	$G_{s/q}$	cAMP IP_3	High: colon, cecum, and bladder Intermediate: blood vessels, mast cells, lung, and eye Low: brain, kidney, liver, pituitary gland, and ovary
A_3	$G_{i/q}$	cAMP IP_3 Cl^- channels K^+-ATP channels	Intermediate: cerebellum, hippocampus, and lung Low: other brain regions, adrenal gland, spleen, heart, intestine, and testis

(Fredholm *et al.*, 2001a; Linden, 2001; Rees *et al.*, 2003)

1.4.4 Adenosine receptor expression in cancers

Expression of all four of the receptor subtypes has been detected in cells of different cancers. Some cells, such as A375 melanoma cells have been reported to concurrently express all four adenosine receptor subtypes (Merighi *et al.*, 2001). Differential effects of adenosine on cancer cell growth have been reported and have partly been attributed to actions at adenosine receptors.

Adenosine A_1 receptor expression has been detected in various types of cancer including colon cancer (Lelièvre *et al.*, 1998). A_1 receptor involvement has been implicated in modulation of cell growth (Liu *et al.*, 2001; Colquhoun and Newsholme, 1997), with both stimulation and inhibition of proliferation being reported, and chemotaxis of melanoma cells (Woodhouse *et al.*, 1998). Interestingly, cisplatin (a commonly used anti-neoplastic agent) up-regulates the A_1 receptor in the rat kidney (Bhat *et al.*, 2002).

The A_{2a} receptors on T-cells have been implicated in immunosuppression under conditions where concentrations of adenosine are increased, such as in hypoxic tumours. Adenosine has been reported to impair key anti-cancer immune responses thereby conferring an advantage to the tumour; it induces apoptosis in human peripheral blood mononuclear cells through an A_{2a} receptor (Barbieri *et al.*, 1998) and to inhibits cytotoxic T-cell function (Koshiba *et al.*, 1997). In further support of the potential role of A_{2a} receptors in promoting tumour survival, activation of A_{2a} receptors has been shown to increase tumour cell and endothelial cell proliferation (Sexl *et al.*, 1995; Merighi *et al.*, 2002), VEGF up-regulation in murine macrophages (Leibovich *et al.*, 2002), and angiogenesis in a wound healing model (Montesinos *et al.*, 2004).

Insight into the properties of the A_{2b} receptor has been hindered by the lack of commercially available preparations of selective agonists and antagonists of this subtype. However, over the last several years a radioligand for the detection of the receptor protein has been made available (Ji *et al.*, 2001). A_{2b} receptors are low affinity in that high concentrations of adenosine are required for receptor activation. In other words, pathological concentrations and not physiological concentrations are needed (Fredholm *et*

al., 2001b). With respect to cancer, A_{2b} receptor expression is consistently demonstrated on microvascular cells and like the A_{2a} receptor, is believed to modulate the expression of angiogenic factors (Feoktistov *et al.*, 2002) and may therefore promote tumour angiogenesis.

Studies suggest that A_3 receptor expression is increased in tumour cells compared to normal tissues (Gessi *et al.*, 2001; Suh *et al.*, 2001), and is also increased during development (Zhao *et al.*, 2002). Differential effects on cell growth have been reported although the use of micromolar concentrations of A_3 agonists was common in these studies and such concentrations may not accurately represent A_3 receptor effects.

Given the discrepancies with respect to adenosine receptor activation and its effect on tumour cell growth, it has been suggested that the cell-type specific expression of receptor subtypes and their associated effector systems, coupled to the local adenosine concentration, dictate the effect adenosine may have (Blackburn, 2003).

1.5 Adenosine and angiogenesis

Angiogenesis refers to the formation of capillaries from pre-existing vessels (Eatock *et al.*, 2000). Physiological angiogenesis only occurs during conditions of wound healing and during the menstrual cycle. Folkman (1971) first suggested that the growth of tumours depends, in part, on their ability to induce the growth of new blood vessels. Tumour growth is known to occur exponentially up to a size of 1-2 mm^3, at which time mitosis and apoptosis are balanced and growth is halted (Folkman, 1971). Tumour expansion beyond this point requires additional blood supply. Tumour

angiogenesis is thought to depend on the production of pro-angiogenic factors by the tumour cells. There are numerous pro-angiogenic factors that have been described including basic fibroblast growth factor (b-FGF), the angiopoietins, and perhaps the best-characterized, vascular endothelial growth factor (VEGF).

It is well established that tumour tissue hypoxia induces VEGF expression (Namiki *et al.*, 1995; Takagi *et al.*, 1996; Sandner *et al.*, 1997; Tsuzuki *et al.*, 2000). One possible stepwise mechanism by which the induction of VEGF by hypoxia is believed to occur is (i) hypoxia induces expression of the transcription factor hypoxia inducible factor 1-alpha (HIF-1α), (ii) HIF-1α then binds to the hypoxia response element (HRE) in the promoter region of the VEGF gene, and (iii) transcription/translation of VEGF mRNA/protein occurs (Tsuzuki *et al.*, 2000).

Takagi and colleges (1996) found that treatment of retinal vascular cells (endothelial cells and pericytes) with A_2 receptor agonists stimulated VEGF expression in a dose-dependent fashion. Treatment of endothelial cells and pericytes with an A_{2a} receptor antagonist reduced the hypoxic stimulation of VEGF expression as did treatment with adenosine deaminase. Taken together, these results suggest that adenosine likely plays a role in the mechanism of hypoxia-induced VEGF expression through its action at adenosine receptors.

The suggestion of a pro-angiogenic role for adenosine is not surprising given the documented role of adenosine as a pro-angiogenic factor in the chick chorioallantoic membrane assay system (Dusseau *et al.*, 1986). Barcz *et al.* (2000) demonstrated that antagonism of adenosine receptors resulted in an inhibition of the angiogenic activity in human ovarian cancer cells in an *in vivo* angiogenesis assay.

1.6 Adenosine as an immunosuppressant

Adenosine is released at sites of immune cells during systemic and cellular stress (Haskó and Szabó, 1998). For example, during multiple organ failure, adenosine is released from a variety of sympathetic nerve terminals in immune organs; whereas in response to metabolic stressors such as hypoxia and ischemia, adenosine is released from the immune cells themselves (Sperlagh *et al.*, 2000). Cronstein (1994) demonstrated that concentrations of adenosine up to 100μM can be achieved in intra-organ inflammatory foci. Adenosine may exert its immunosuppressive effects by engaging cell-surface adenosine receptors. Lymphocytes have been shown to express multiple adenosine receptor subtypes (Mirabet *et al.*, 1999; Gessi *et al.*, 2001; Gessi *et al.*, 2004).

Perhaps the strongest argument for adenosine-mediated modulation of immune responses that has been documented is SCID. The basis of this syndrome is an absence of ADA activity (Resta and Thompson, 1997). The most profound effect in SCID is on the immune system, highlighting the crucial role that adenosine plays in immune responses.

1.6.1 Effects on leukocyte function and the immune/inflammatory responses

SCID has been linked to a deficiency in adenosine deaminase activity. It follows then, that levels of adenosine must be regulated to ensure proper immune function. Adenosine has profound effects on immune cell activity, involving both lymphoid on non-lymphoid cell types. For example, adenosine has been reported to inhibit eosinophil

migration (Knight *et al.*, 1997) as well as decrease the phagocytic ability of monocytes/macrophages (Eppell *et al.*, 1989). Furthermore, adenosine has been shown to decrease the production of several pro-inflammatory cytokines including monocyte-produced interleukin-12 (IL-12) and TNF-α produced by macrophages (Haskó and Szabó, 1998).

Treatment with adenosine inhibits lymphocyte activation and proliferation in a variety of assay systems (Hoskin *et al.*, 1994; Huang *et al.*, 1997). Adenosine is known to suppress interleukin-2 (IL-2) production by T-lymphocytes (Koshiba *et al.*, 1997). Given that IL-2 is a critical autocrine factor during T-cell proliferation (Waldmann, 1993), this may explain the observed decrease in lymphocyte proliferation in the presence of adenosine.

Taken together, these reports suggest that adenosine exerts an inhibitory tone on the normal immune cell function and on inflammatory processes. Adenosine may therefore, have potent and significant immunosuppressive effects in the context of the tumour microenvironment.

1.6.2 Effects on anti-cancer immune responses

In addition to modulation of systemic immune responses (Haskó *et al.*, 2002), adenosine can have profound and significant effects specifically on the body's immune response to cancer. As previously mentioned, adenosine can inhibit T-cell activation and cell proliferation thereby impairing the cytotoxic actions of T-cells. Adenosine inhibits processes involved in immune cell-mediated cytotoxicity including those involved in the

Fas ligand-induced apoptosis and the perforin/Granzyme B apoptotic pathways (Hoskin *et al.*, 2002). MacKenzie *et al.* (1997) reported that adenosine significantly inhibits the adhesion of murine activated killer T-cells to murine colon carcinoma cells. This study supports the findings by Hoskin *et al.* (1994) that adenosine can inhibit cytolysis of tumour cell targets. Blay *et al.* (1997) measured the concentration of adenosine in the extracellular fluid and found it to be within the low micromolar concentration range, concentrations that are capable of hindering anti-cancer immune responses.

1.7 Adenosine modulation of cell growth

There exists extensive controversy in the literature regarding the role of adenosine in normal and tumour cell growth. It has been reported that adenosine can inhibit or stimulate the proliferation of a variety of cell types, and that adenosine may selectively inhibit tumour cell growth (Fishman *et al.*, 1998; Ohana *et al.*, 2001).

1.7.1 Adenosine and normal cell growth

The effect of adenosine on the growth of numerous normal cell types has been investigated and the findings vary considerably, with adenosine being growth-promoting in some cases and lethal to cells in others. Given its actions as an immunosuppressant, it is not surprising that adenosine, at low micromolar concentrations in combination with coformycin, inhibited DNA synthesis in peripheral blood lymphocytes (van der Krann *et al.*, 1986). Ishii and Green (1973) reported that adenosine was also found to inhibit the

growth of 3T3 and HeLa cell cultures, although these authors did not report any quantitative data on cell growth, rather they qualitatively described tissue culture plate cell density after adenosine treatment. In contrast to the above examples, DNA synthesis and the proportion of S-phase murine mammary epithelial cells was *increased* in the presence of adenosine in the concentration range 10-100μM (Yuh and Sheffield, 1998).

A_{2b} receptor involvement has been implicated in the modulation of endothelial and smooth muscle cell growth. The growth of aortic smooth muscle cells and vascular smooth muscle cells was inhibited by adenosine and adenosine elevating agents (Dubey *et al.*, 1997; Dubey *et al.*, 2000). The effect was blocked by the A_2 antagonist KF17837, but was not mimicked by the selective A_{2a} agonist CGS21680, suggesting a possible role for the A_{2b} receptor subtype (Dubey *et al.*, 1997). Dubey and co-workers (2002) also examined the effect of adenosine on the growth of endothelial cells and unlike their previous findings on smooth muscle cells adenosine stimulated the growth of arterial endothelial cells. Interestingly, the A_{2b} receptor subtype was implicated once again in the growth-modulating response.

1.7.2 Adenosine and tumour cell growth

It has been suggested that the modulation of tumour cell growth by adenosine is receptor mediated. However, depending on the concentration of adenosine and the receptor subtype that is activated, the resulting effect on cell growth may vary. For example, Tey and colleagues (1992) found that low concentrations of adenosine (approximately 10μM) inhibited A431 human epidermoid carcinoma cell growth whereas

elevated concentrations (up to 100μM) stimulated the growth of the same cell type. These investigators implicated the A_1 receptor subtype for the inhibitory response and the A_2 receptor subtype for the stimulatory response.

Millimolar concentrations of adenosine have been reported to induce apoptosis in human leukemic cells, human melanoma cells, and human astrocytoma cells (Tanaka *et al.*, 1994; Merighi *et al.*, 2002; Abbracchio *et al.*, 2001), respectively. Given the high concentrations of adenosine used, it is plausible that adenosine actions in these studies might not only be at cell-surface receptors and adenosine may be acting intracellularly.

The suggestion has been made that adenosine acts to specifically inhibit the growth of tumour cells (Djaldetti *et al.*, 1996; Fishman *et al.*, 1998) with emphasis placed on the involvement of the A_3 receptor (Fishman *et al.*, 2000; Ohana *et al.*, 2001). It has also been suggested that the A_3 receptor should be targeted for cancer therapy (Fishman *et al.*, 2001). Indeed, Ohana and co-workers (2003) reported that the A_3 receptor agonist CF101 decreased primary colon carcinoma growth as well as liver metastases. Conversely, several reports suggest that adenosine consistently promotes the growth of human colon carcinoma cells (Lelièvre *et al.*, 1998a; Lelièvre *et al.*, 1998b) and human breast carcinoma cells (Mujoomdar *et al.*, 2004). In view of the controversy surrounding the role of adenosine, and in view of the suggestion that A_3 receptor agonists would be selective inhibitors of tumour cell growth, the precise role of adenosine in cell growth needs to be determined. It may be that the effect of adenosine depends on the cellular context.

1.8 Adenosine and cell migration

Cell motility is a critical step in invasion and metastasis of tumour cells and therefore cancer progression. Metastasis is the spread of cancer cells from the primary tumour site to one or more distant secondary sites. The metastatic process begins with the loss of cell-cell contacts within the tumour. This is followed by invasion as the cells traverse the basement membrane and migration through the underlying stroma. The tumour cells then penetrate the blood or lymphatic vessels, which serve as modes of transport through the body, and migrate through these vessels to secondary sites where the cells exit the vessel system, or extravasate. After exiting the blood/lymphatic system, the cancer cells must then migrate to the secondary site and proliferate in their new environment (King, 2000).

It is increasingly recognized that the tissue microenvironment can be a potent source of motility cues for tumour cells (Bindels *et al.*, 2001). The movement of cells within the microenvironment can be classified as 'taxis' (directed) or 'kinesis' (random), elicited by soluble (chemo) or solid (hapto) attractants. Chemotaxis, therefore, is the movement of cells along concentrations gradients of soluble factors, while haptotaxis occurs along gradients of extracellular matrix (ECM) proteins on solid substrata. Chemo- and haptokinesis refer to the random movement of cells (Quaranta, 2002).

Numerous soluble factors present in the tumour tissue microenvironment have been identified as modulators of epithelial cell migration. These include various cytokines, chemokines, and growth factors and it is likely that additional factors may serve as potential modulators. Krishnamachary *et al.* (2003) reported that over-

expression of HIF-1α stimulated the migration of HCT116 human colon carcinoma cells through Matrigel in an *in vitro* invasion assay. Exposure of endothelial cells to hypoxic conditions also stimulated their migration (Meininger *et al.*, 1988). Antagonism of adenosine receptors blocked this pro-migratory effect. Treatment with micromolar concentrations of adenosine was also chemotactic for endothelial cells. Taken together, these data suggest that adenosine present in the tumour microenvironment, released as a result of tissue hypoxia, might act as a stimulus for cell migration.

The effect of adenosine on tumour cell proliferation remains controversial. In addition, adenosine's effect of tumour cell migration is relatively unknown. Adenosine has been shown to have immunosuppressive as well as pro-angiogenic activities. Recently it was suggested that adenosine is a tumour promotor (Spychala, 2000), although adenosine has been hypothesized to be involved in the growth and spread of cancer for more than 20 years (Phillis and Wu, 1981). There have been several reports suggestions that adenosine analogues would be selective chemotherapeutic agents. The same group has participated in a phase II clinical trial of an A_3 agonist in the treatment of metastatic colorectal cancer patients (Fishman *et al.*, 2004). Clearly, the potential for adenosine to have tumour-promoting effects must be thoroughly examined in a timely manner.

1.9 Objectives

(i) To determine if adenosine increases or decreases human colon carcinoma cell growth.

(ii) To validate the use of a [*methyl*-^{3}H]-thymidine incorporation assay to measure the effect of adenosine on DNA synthesis.

(iii) To examine potential methodological variations that may account for the conflicting reports regarding the effect of adenosine on cell growth.

(iv) To examine if the adenosine response is present and, if so, whether it is altered under culture conditions that mimic the tumour microenvironment (rather than normal culture conditions).

(v) To explore the potential involvement of cell-surface adenosine receptors in the growth-promoting effect of adenosine.

(vi) To assess the effect of adenosine on colon carcinoma cell migration

1.10 Hypotheses

(i) Adenosine, at concentrations expected to be present in the microenvironment of solid tumours, will stimulate human colon carcinoma DNA synthesis and cell proliferation.

(ii) The adenosine-stimulated increase in DNA synthesis will be mediated by cell-surface adenosine receptors.

(iii) The adenosine-stimulated increase in DNA synthesis and cell proliferation will occur under conditions representative of those within the tumour microenvironment.

(iv) Concentrations of adenosine that stimulate DNA synthesis and cell proliferation will also stimulate the migration of human colon carcinoma cells.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell lines

HT-29, T84, HRT-18, Colo320*HSR*, and Caco-2 human colorectal carcinoma cell lines, A427 and A549 human lung carcinoma cell lines, MCF-7 and T47-D human breast carcinoma cell lines, and the A375 human melanoma cell line were obtained from American Type Culture Collection. The SKOV3 human ovarian carcinoma cell line, primary human ovarian cancer cells (OvCa), and primary human fibroblasts were obtained from Dr. M. Nachtigal (Dalhousie University, Halifax, NS, Canada). Primary human umbilical vein endothelial cells (HUVEC) were obtained from Dr. A. Issekutz (Dalhousie University, Halifax, NS, Canada).

Table 2.1: Site of origin and degree of differentiation of colorectal carcinoma cell lines

Colorectal carcinoma cell line	Site of origin	Degree of differentiation
HT-29	colon	well-differentiated
T84	lung metastasis	moderately differentiated
HRT-18	ileocecal	moderately differentiated
Colo320*HSR*	colon	poorly differentiated
Caco-2	colon	can spontaneously differentiate in culture

Table 2.2: Calculated doubling times for colorectal carcinoma cell lines under basal growth conditions.

Colorectal carcinoma cell line	Doubling time (h)
HT-29	36.0
T84	57.6
HRT-18	48.3
Colo320*HSR*	47.8
Caco-2	53.8

2.1.1 Cell culture medium and medium supplements

Dulbecco's modification of Eagle's medium (DMEM) containing 4.5g/L glucose and 2mM L-glutamine, and glucose-free DMEM (2mM L-glutamine) were obtained from Sigma (St. Louis, MO, USA). Heat-inactivated newborn calf serum (NCS), heat-inactivated fetal calf serum (FCS), and trypsin/EDTA were obtained from Invitrogen Canada (Burlington, ON, Canada). Endothelial cell growth supplement (ECGS) was obtained from Sigma.

2.1.2 Reagents

Adenosine, adenosine monophosphate (AMP), adenosine triphosphate (ATP), adenosine deaminase (ADA), MTT (1-4(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan), collagen IX (Sigma classification), Mayer's haematoxylin solution,

MRS1191 (3-ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate), MRS1220 (9-chloro-2-(2-furyl)-5 phenylacetylamino- 1,2,4]triazolo[1,5-c]quinazoline], and MRS1523 (3-propyl-6-ethyl-5-[(ethyl-thio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate) were obtained from Sigma.

Coformycin was obtained from Calbiochem (La Jolla, CA, USA).

DPCPX (8-cyclopentyl-1,3-dipropylxanthine), CSC (1,3,7-trimethyl-8-(3-chlorostyryl)xanthine), alloxazine (benzo[g]pteridine-2,4(1H,3H)-6-thioinosine), and 5'-iodotubercidin were obtained from Research Biochemicals Inc (Natick, MA, USA).

M-MLV reverse transcriptase, dNTPs, TRIzol® reagent, dithiothreitol (DTT), oligo-dT, 5X first strand buffer, oligonucleotide primers, DNA ladder, 10X PCR buffer (with $MgCl_2$), PCR Optimizer™ Kit, and *Taq* polymerase were obtained from Invitrogen (Burlington, ON, Canada).

[*methyl*-^{3}H]-thymidine (specific activity: 25Ci/mmol) was obtained from Amersham (Baie d'Urfé, PQ, Canada).

2.1.3 Cell culture vessels

All cell culture vessels were obtained from NUNC with the exception of Transwell® inserts that were obtained from Costar (Corning, NY, USA). These polycarbonate filter inserts had a diameter of 6.5 mm and pore size of 8μm.

2.2 Methods

2.2.1 Cell culture procedures and treatment protocols

2.2.1.1 Carcinoma cell culture

All human carcinoma cell lines were maintained in 80 cm^2 flasks at 37°C in a humidified atmosphere of 90% air/10% CO_2. All cell lines were maintained in the absence of antibiotics, in DMEM containing 2mM L-glutamine and either 10% (v/v) NCS (HT-29, T84, HRT-18, A427, A548, A375, and SKOV3), 5% NCS (Colo320*HSR*, MCF-7, and T-47D), or 10% FCS (Caco-2, HUVECs, OvCa cells, and primary human fibroblasts). DMEM containing bicarbonate together with CO_2 provided the physiological bicarbonate buffering system.

Cells were subcultured as required by trypsinization. Typically, medium was removed and 2.5ml of a buffered solution containing 0.05% trypsin/0.53mM EDTA was added to each flask. The cells were exposed to the trypsin solution until they were observed to round up from the plastic cell culture surface at which time the excess trypsin solution was removed. The flask containing residual trypsin solution was returned to the incubator until a single cell solution was obtained. Fresh medium containing serum was added to the flask and the cell suspension was divided as necessary.

2.2.1.2 Cell counting and assessment of cell viability using trypan blue

To determine cell number and to assess cell viability, a small volume (typically 50μl) of the cell suspension was diluted in 50μl of 0.4% trypan blue and further diluted with 100μl of phosphate buffered saline (PBS). A haemocytometer was then loaded with the cell suspension and typically at least 100 cells were counted. Cell number was calculated based on the cell count, the area counted, and the dilution factor used. Cell viability as measured was the number of unstained cells (alive) per total cells counted [total = stained (dead) + unstained].

2.2.1.3 MTT assay

HT-29 cells were seeded in flat bottom 96-well plates. At a culture density between 40-70% confluency, cells were treated with adenosine ranging from 1μM to 6mM or vehicle control (SFM). Forty-eight hours later, 500μg/ml of MTT was added to each well of the culture plate and incubated for 3h. Spectrometric absorbance at 492nm was measured using a plate reader. Data are expressed as mean absorbance values ± SEM.

2.2.1.4 Culture of HT-29 spheroids

Flat bottom 96-well plates were coated with a 50μl base layer of 2.5% agarose (in sterile water). Plates were left at RT under sterile conditions for up to 1h and placed at

37°C for 3h. HT-29 cells were seeded at a density of 5 x 10^4 cells onto agarose base in DMEM containing 0.5g/L glucose and 1% NCS. The culture plate was swirled and placed in incubator until spheroid formation was observed. The final volume after treatments did not exceed 200µl. Spheroid media was typically replenished every 2-3d by removing 50-100µl of conditioned media and adding 50-100µl of fresh media.

2.2.1.5 Drug treatment solutions

Adenosine, AMP, and ATP were prepared in serum-free DMEM (SFM). DPCPX, CSC, alloxazine, MRS1191, MRS1220, and MRS1523 were dissolved in dimethylsulfoxide (DMSO). MTT was prepared in saline. Coformycin and 5'-iodotubercidin were prepared in sterile water. Final DMSO concentrations under experimental conditions did not exceed 0.5%.

2.2.2 Tumour cell growth experiments

2.2.2.1 [*methyl*-^{3}H]-thymidine incorporation assay

Carcinoma cell cultures were counted and seeded in full growth medium (with serum) at the desired cell density in 24-well plates at a volume of 1ml/well. The cultures were then incubated for 48h to ensure full cell attachment. After 2d, the medium was changed to medium containing low serum (0.5 or 1% NCS) and the cultures were incubated in low-serum conditions for 2d. On d4, the cultures received the experimental treatments or vehicle control (SFM or SFM + DMSO). [*methyl*-^{3}H]-thymidine

([^{3}H]TdR) was also added on d4 unless otherwise indicated. Final concentrations of [^{3}H]TdR and thymidine were 1μCi/mL and 1μM respectively. Additions followed a randomized scheme amongst the wells to minimize variability relating to plate effects. Plates were then returned to the incubator for a further 48h.

On d6, the experimental plates were placed on ice and the medium was removed and discarded using a 19-gauge needle attached to a vacuum apparatus. The wells were then washed twice with 1X PBS (140mM NaCl, 2.7mM KCl, 8.0mM Na_2HPO_4, 1.5mM KH_2PO_4, 0.5mM $MgSO_4 \bullet 7H_2O$, and 0.9mM $CaCl_2 \bullet 2H_20$). A volume of 1ml of cold 10% trichloroacetic acid (TCA) was then added to the wells and the plates were incubated on ice for 60 min. After 1h, the TCA was removed and 1ml of 100% ethanol was added to each well. The plates were then removed from the ice, the ethanol was removed, and the plates were air-dried for approximately 1h. When the wells were dry, 0.5ml of 0.1M NaOH/1% SDS (v/v) was added to each well. The plates were placed on a rotary shaker at room temperature for 2h to solubilize the cells. The contents of the wells were then added to vials containing acidified scintillation fluid and the radioactivity of the samples were determined using a Beckman LS 5000TA liquid scintillation counter (Beckman Coulter Canada).

2.2.2.2 Measurement of cell number

Cells were counted and seeded in either 4- or 24-well plates on d0 and allowed to attach for approximately 4h. Cells were then treated with adenosine or vehicle and given repeated additions of fresh adenosine or vehicle at 2-3d intervals. Cell number

was measured at indicated time points. For counting, cells were trypsinized using 0.5ml of trypsin/EDTA, diluted in Diluton®, and subsequently counted using a Coulter Counter™ ZM30383 (Beckman Coulter). The instrument settings for all carcinoma cells were: current 100, gain 4, attenuation 32, full scale 10, polarity +, manometer 500µl, upper threshold 99.9 and lower threshold 10.

2.2.3 RT-PCR for human adenosine receptors

2.2.3.1 Isolation of RNA

Total RNA was isolated from HT-29, T84, HRT-18, Colo320*HSR*, and Caco-2 cells using the TRIZOL® reagent according to the manufacturer's instructions. Briefly, 1-5x10^6 cells were centrifuged at 300 x g for 10 min at 4°C. The resulting cell pellet was resuspended in 1ml of TRIZOL® in a 15ml centrifuge tube. The mixture was pipetted repeatedly to ensure complete cell lysis. The mixture was then incubated at room temperature (RT) for 5 min and then transferred to a 1.5ml microcentrifuge tube. Two hundred microlitres of chloroform was added to the mixture, the tubes were vigorously shaken, and then incubated for 2-3 min at RT. The aqueous phase containing the RNA was separated from the organic phase by centrifugation at 12 000 x g for 10 min at 4°C. The aqueous phase was then carefully removed and transferred to a fresh 1.5ml microcentrifuge tube. Ice-cold isopropanol was added at a volume of 500µl and the solution was mixed by inversion several times and incubated at RT for 10 min. Precipitated total RNA was pelleted by centrifugation at 12 000 x g for 10 min at 4°C. The supernatant was removed and the remaining pellet was washed with 1ml of ice-cold

75% ethanol, and centrifuged at 7 500 x g for 5 min at 4°C. The ethanol was removed and the RNA pellet was either allowed to air dry for 1h at RT or dried using a vacuum drying apparatus. The RNA pellet was then resuspended in 50µl of sterile pyrogen-free water. RNA concentration was determined using a spectrophotometer. Total RNA samples were visualized in a 1.5% agarose gel containing ethidium bromide to ensure RNA integrity and stored at –80°C until use.

2.2.3.2 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Reverse transcription of total RNA to complementary DNA (cDNA) was performed by combining 3µg of total RNA, 4µl of 5X first strand buffer, 2µl DTT, 1µl 10mM dNTPs, 1µl of oligo-dT (1mg/ml), 1µl of M-MLV-RT, and sterile water up to 12µl in a microcentrifuge tube. Samples were then placed in a 37°C water bath for 1h, followed by 10 min at 80°C. Samples were stored at -20°C until use.

PCR reactions were performed by combining 1µl of cDNA sample with 5µl 10X PCR buffer (containing $MgCl_2$), 1µl of 10mM dNTPs, 0.5µl of each primer [(A_1 forward- CATTGGGCCACACACCTACT; reverse- GATGACCTTCTCGAACTCGC), (A_{2a} forward- CTGCTCATGCTGGGTGTCTA; reverse- TTCAAAGGTTCTTGCTGCCT), (A_{2b} forward- GTCATTGCTGTCCTCTGGGT; reverse – GCTGGCTGGAAAAGAG TGAC); and (A_3 forward- TTCTCATGTGCGCCATCTAT; reverse- ATGTAAAAATC CCTTGGCCC)], 0.3µl of *Taq* polymerase, and sterile pyrogen-free water to 25µl. Conditions for PCR were optimized using PCR Optimizer™ Kit (Invitrogen). PCR was performed in an automatic theromocycler (Techne Genius) using the following

conditions: an initial denaturation step of 94°C for 2 min, followed by the cycling program of (i) 94°C for 30 sec, (ii) 59.5 for 30 sec, (iii) 72°C for 35 sec. There were 30 cycles followed by a final extension step of 72°C for 10 min. Products were then visualized on a 1.5% agarose gel containing ethidium bromide.

2.2.4 HPLC measurements of adenosine breakdown and production

For studies of adenosine breakdown or production, sub-confluent cultures cultures of carcinoma cells in 6-well plates were first changed to a serum-free basic medium (PBS) containing 1.2mM $CaCl_2$, 0.4mM $MgSO_4$, 11.1mM glucose, and 1X RPMI-1640 vitamins. Where stated, exogenous adenosine and/or inhibitors of adenosine metabolism were then added. Coformycin and 5'-iodotubercidin were added at a final concentration of 10 and 1μM respectively. Cultures were incubated at 37°C in a CO_2-free atmosphere. Rates of metabolism of exogenous adenosine were measured over 90 min following an addition of 10μM adenosine. Endogenous adenosine was measured as the accumulation over a 6h period. Culture medium (0.5ml) was collected and the adenosine was combined with 0.5% chloroacetylaldehyde for 20 min at 100°C to form the fluorescent derivative 1-N^6-ethenoadenosine. Measurement of adenosine was made by high performance liquid chromatography (HPLC) using a Waters 2690 Separations Module and 474 Fluorescent Detector, with a Nucleosil 100 C_{18} column and a mobile phase of 50mM acetate buffer (pH 4.5), 2.2 mM 1-octanesulfonic acid, and 18% acetonitrile at a flow rate of 2ml/min. The excitation and emission wavelengths used were 270 and 418nm, respectively. Adenosine standards within the appropriate expected

concentration range were prepared fresh similarly as above and were run with each sample set. Analysis was performed using Millenium© software.

2.2.5 HRT-18 cell migration assay

Transwell® inserts were coated with Sigma collagen type IX in SFM used at a final concentration of 1μg/ml. Inserts in 24-well companion plates were placed in the 37°C incubator overnight to ensure even coating. After 24h, the inserts were washed three times with SFM. SFM containing 1% bovine serum albumin (BSA) was added to the bottom chamber (wells of the 24-well plate) at a volume of 600μl and treatments were added typically in volumes less than 30μl. Cultures of HRT-18 cells (70-90% confluent) were trypsinized and DMEM containing 10% NCS was added to the flask to inactivate the trypsin. The HRT-18 cell suspension was centrifuged at 300 x g for 5 min at 4°C. The supernatant was decanted and the cells were resuspended in SFM/1% BSA and counted. A cell suspension of 2.5 x 10^6 cells/ml was prepared, transferred to a polypropylene tube, and placed on a shaker at RT for 10 min. A volume of 100μl (250 000 cells) was then added to each top chamber of the Transwell and the plate was placed in the incubator for 4h.

After 4h, the inserts were washed three times in 1X PBS and fixed in 100% ethanol for 30 sec, after which time they were placed in Mayer's haematoxylin stain for 10 min. The inserts were then washed in tap water, and placed in acid alcohol with agitation for 15 sec. The inserts were then placed in a tap water bath for 10 min. A cotton-tipped applicator wetted with 100% ethanol was used to scrape the cells from the

top side of the filter, leaving the cells that had migrated through the filter safely intact on the underside. The filter was then excised using a razor blade and thoroughly washed in xylene. It was left to air-dry for no more than 2 min and subsequently mounted on a slide and cover-slipped prior to microscopic analysis.

Blinded counts were performed at 400X magnification using an Olympus microscope (Don Mills, ON, Canada). Counts from three non-overlapping high power fields were made for each slide. Extrapolations to counting area (mm^2) were made using a predetermined scaling factor or were expressed as number of cells/filter insert.

3. RESULTS

3.1 Effect of adenosine on DNA synthesis and proliferation of human colorectal carcinoma cell and validation of the use of the [*methyl*-^{3}H]-thymidine incorporation assay system

The question of whether or not adenosine promotes the growth of tumour cells has not been answered definitively by studies in the current literature. Therefore, the first objective of this thesis was to investigate the effect of adenosine on DNA synthesis and cell proliferation in a panel of five human colorectal carcinoma cell lines grown in monolayer culture. The cell lines selected for this study differed in (i) origin (Table 2.1; Materials and Methods), (ii) growth rate (Table 2.2; Materials and Methods), and (iii) degree of differentiation (Table 2.1; Materials and Methods).

Many studies that have examined the effect of adenosine on cell growth have used the [*methyl*-^{3}H]-thymidine ([^{3}H]TdR) incorporation assay as a measurement of DNA synthesis (van der Krann *et al.*, 1986; Ethier *et al.*, 1993; Djaldetti *et al.*, 1996; Yuh and Sheffield, 1998; Fishman *et al.,* 1998; Morrone *et al.,* 2003) Using this assay system, adenosine has been reported either to increase or to decrease DNA synthesis. Could differences in experimental assay protocols contribute to this discrepancy? An accompanying objective was therefore to evaluate our [^{3}H]TdR incorporation assay experimental protocol to ensure that it was an appropriate tool for accurately measuring effects of adensine on DNA synthesis.

3.1.1 Effect of adenosine on [^{3}H]TdR incorporation in HT-29 cells

Sub-confluent (approximately 40-60%) HT-29 cell cultures were treated from four days after seeding with adenosine at a final concentration ranging from 3-300μM, and [^{3}H]TdR incorporation was determined as a measure of DNA synthesis. Figure 3.1 is a typical response curve to adenosine for HT-29 cells. HT-29 cells responded to adenosine treatment with a dose-dependent increase in DNA synthesis. Typically, this stimulatory effect by adenosine was first observed at low (< 10) micromolar concentrations. Maximal stimulation occurred at 30-100μM range and stimulation persisted even at adenosine concentrations as high as 300μM.

3.1.2 Validation of the [^{3}H]TdR incorporation assay for use in measuring the effect of adenosine on cell growth

The [^{3}H]TdR incorporation assay has been used extensively to measure effects on DNA synthesis. Despite this, a rigorous evaluation of our standard [^{3}H]TdR incorporation assay protocol was performed to confirm that it would accurately reflect the effect of adenosine on DNA synthesis and therefore cell growth. Specifically, ways in which exogenously added adenosine or specific experimental conditions could alter thymidine incorporation were examined.

It was possible that exogenously added adenosine might alter [^{3}H]TdR uptake and intracellular thymidine pools, resulting in changes in [^{3}H]TdR incorporation unrelated to a specific effect of adenosine on DNA synthesis. To address this, HT-29 cells were

treated on d4 as usual with adenosine (3-300μM) and the [^{3}H]TdR addition was delayed for 24h. By this time, the adenosine would have been completely metabolized and could therefore not effect thymidine incorporation. This excludes any possible effects of adenosine on thymidine pools. The result was again, one of increased DNA synthesis, with a similar, although not identical dose-response profile to that seen with addition at t=0 (Fig. 3.2).

We observed significant differences in the magnitude of the adenosine response when cells were incubated with sera of varying concentrations and sources (these data are presented in Results section 3.2). It was therefore possible that there were factors present in the serum that dampened or blocked the adenosine response. Therefore, when a higher concentration of serum was present in the culture media the magnitude of the adenosine response would be lower. To evaluate the extent of this potential confounding influence, a portion of newborn calf serum was dialyzed to remove low molecular weight factors. HT-29 cells were then seeded in either undialyzed 10% NCS or dialyzed 10% NCS. The cultures were downshifted to 1% NCS or dialyzed 1% NCS, respectively, and treated with adenosine (3-300μM). Both cultures responded to adenosine with a very similar increase in DNA synthesis. (Fig. 3.3).

An alternative explanation was that the serum contained a high concentration of thymidine and/or adenosine that was capable of altering the observed response. An excess of thymidine in the culture media could impact upon DNA synthesis. Low millimolar concentrations of thymidine have been reported to alter mammalian cell cycle kinetics (Bostock *et al.,* 1971). An appreciable amount of adenosine in the culture medium could have shifted the adenosine response curve rightward. Therefore, an

otherwise stimulatory concentration of adenosine in the presence of serum-derived adenosine could have become less stimulatory or even growth-inhibitory. We had measured the amount of adenosine present in newborn and fetal calf sera, as well as sera from other sources and found it to range from 1-3μM (Mujoomdar *et al.,* 2004). Note, these measurements were performed on neat serum. During the [^{3}H]TdR incorporation assay, the cells are exposed to serum containing 10% NCS for 48h and then the culture medium is changed to contain only 1% NCS for the remainder of the assay. Therefore, the cells would have been exposed to only a trace amount of serum-derived adenosine, which would not have been capable of altering the cellular response to adenosine. Measurements of thymidine were not performed, but similar to adenosine, it was unlikely that appreciable amounts of thymidine are capable of modulating the adenosine response were present in the serum for duration of the assay.

Although we believed that the concentration of thymidine present in the culture medium is not sufficiently high to interfere with DNA synthesis, we wanted to examine the effect of altering the specific activity of [^{3}H]TdR either by changing (i) the amount of radioactive thymidine, or (ii) the amount of 'cold' (unlabeled) thymidine. Moreover, could the use of higher or lower concentrations of [^{3}H]TdR be responsible for the variation in the cellular responses to adenosine?

The radioactive concentration of [^{3}H]TdR used in our standard assay protocol is 1μCi/ml and unlabeled thymidine is added to a final concentration of 1μM. Figure 3.4 depicts the result of culturing HT-29 cells (in either undialyzed or dialyzed newborn calf serum) in the presence of 1μCi/ml of [^{3}H]TdR and 10μM unlabeled thymidine. The response was an increase in DNA synthesis in response to adenosine treatment. These

data agree with our previous findings in breast carcinoma cells where we determined that increased or decreased amounts of [^{3}H]TdR and unlabelled thymidine had no effect of the adenosine response (Mujoomdar *et al.*, 2004). These data suggest that the concentration of [^{3}H]TdR that is used in the [^{3}H]TdR incorporation assay does not alter the stimulatory effect of adenosine on DNA synthesis in HT-29 cells and does not account for discrepancies in the literature regarding adenosine's effect on DNA synthesis. Moreover, these data indicate that the [^{3}H]TdR incorporation assay is a valid measure of the mitogenic response of adenosine.

3.1.3 T84, HRT-18, Colo320*HSR*, and Caco-2 cell lines

Modifications to our standard [^{3}H]TdR incorporation assay protocol did not change the response of HT-29 cells to adenosine. Adenosine consistently increased DNA synthesis in HT-29 cells. Collectively, the data together with other studies on MCF7 and T-47D human breast carcinoma cells (Mujoomdar *et al.*, 2004), strongly suggest that the [^{3}H]TdR incorporation assay accurately reflects the effect of adenosine on DNA synthesis. Therefore using the [^{3}H]TdR incorporation assay, four additional colon carcinoma cell lines were studied to determine if they too, responded to adenosine with an increase in DNA synthesis. The cells were treated in the same way as the HT-29 cells. Adenosine caused an increase in DNA synthesis in all cell lines tested (Fig. 3.5).

The EC_{50} values (effective [single dose] concentration of adenosine producing half-maximal stimulation of DNA synthesis) for all five cell lines were calculated and are show in Table 3.1. The calculated EC_{50} values ranged from 3.8-30μM. Caco-2 cells

were the only cell line to show significant variability, perhaps because of phenotypic changes that occur as these cells differentiate in culture (Pinto *et al.*, 1983). Blay and colleagues (1997), proposed that local levels of adenosine within solid tumours *in vivo* might well be in the 3-10μM range. Three of the five cell lines examined had EC_{50} values that fell within this range using only single doses of adenosine.

A single dose of adenosine was consistently able to elicit a stimulatory effect. The use of an adenosine analogue was not required to produce a sustained concentration of drug over a longer period of the assay as has been done by other groups, nor was inhibition of adenosine breakdown necessary, which has also been used to elevate adenosine concentration (Barry and Lind, 2000).

3.1.4 Effect of dosing schedule on the HT-29 'adenosine response'

Thus far, adenosine had always been added to cultures four days after seeding. Our standard assay protocol includes a two-day 'adaptation' period after the cells are seeded, to accommodate cell attachment and spreading. This adaptation period is followed by a two-day exposure to media containing a low concentration of serum. Fishman and co-workers (1998) treated cells with adenosine at the time of seeding and found that adenosine decreased tumour cell growth. To determine whether the dosing schedule modulated the adenosine response, HT-29 cells were treated with adenosine either at the same time as seeding or two days after seeding, our standard approach. Figure 3.6 demonstrates that no difference in the adenosine response was observed if the cells received adenosine treatment on d0 (at time of seeding) or d2 (2 days after seeding).

The half-life of adenosine in culture is relatively short, approximately 2h in HT-29 cell cultures (Mujoomdar *et al.,* 2003). Therefore, we wanted to examine the effect of treating HT-29 cells with smaller doses of adenosine repeatedly over a 12h period. This method of repeatedly treating cells with lower concentrations of adenosine approximated a low micromolar adenosine exposure, similar to what cells *in vivo* would see. This 'continuous' dosing approach produced a leftward shift in the adenosine dose-response curve relative to the single-addition dose-response curves indicating a decrease in the EC_{50} or an increase in potency (Fig. 3.6).

There are varying degrees of hypoxia within a solid tumour (Leith *et al.*, 1991). There are some areas in which oxygen levels are normal and therefore adenosine production would be low. Conversely, there are likely areas that produce very high levels of adenosine, for example near necrotic areas or in hypoxic foci. Therefore, the concentration of adenosine likely varies in a solid tumour, thereby exposing cells *in vivo* to a range of adenosine concentrations. These data indicate that the growth of 4 of 5 of the colon carcinoma cell lines was stimulated by a single adenosine dose as low as 10μM. Although adenosine is produced at that level *in vivo*, the continuous dosing data indicate that colon carcinoma cells also respond to much lower continuous doses of adenosine, nearly one order of magnitude lower, with an increase in DNA synthesis. Therefore, a continuous exposure of low micromolar or high nanomolar concentrations of adenosine would also be expected to increase DNA synthesis *in vivo.*

3.1.5 Effect of adenosine on HT-29 cell proliferation

To confirm that adenosine did not only trigger cells to undergo DNA synthesis, but also induced an increase in cell proliferation, cell number was measured. HT-29 cells were cultured for up to 12d and received adenosine (30μM) or vehicle (SFM) on intervening days. Cell number was measured on d2, d4, d6, d8, d10, and d12 (Fig. 3.7a). Adenosine treatment resulted in a significant increase in cell number over vehicle-treated cells. This increase was observed beginning at d8 and extended to d12 cultures. Additionally, HT-29 cells were exposed to adenosine (10-100μM) and as expected, responded with an increase in cell number (Fig. 3.7b) in a dose-dependent manner over a similar concentration range observed to stimulate DNA synthesis (see Fig. 3.1). Therefore, in addition to stimulating DNA synthesis, adenosine also increases cell proliferation. Therefore there is no evidence that adenosine simply increases DNA synthesis and prevents cells from progressing through to mitosis.

It was possible that toxicity superimposed upon a stimulation of growth would explain the lack of no net change in cell proliferation at early time points, although trypan blue exclusion tests were performed on cultures treated with adenosine up to 300μM and no evidence of toxicity was apparent (data not shown). In order to exclude the possibility that adenosine treatment at earlier time points resulted in toxicity given that no stimulatory response to adenosine was observed for d2-d6 cultures, the effect of adenosine on HT-29 cell viability was measured using the MTT assay. HT-29 cell viability in the presence of adenosine was measured. HT-29 cell viability was unaffected by concentrations of adenosine up to 300μM (Fig. 3.8) which is outside of the

concentration range that was used to study the growth-modulating effects of adenosine in this thesis. The JAM assay method, which measures DNA fragmentation and cell death (Matzinger, 1991), was also used to measure the effect of adenosine on apoptosis in HT-29 cells. Adenosine, at concentrations that stimulated DNA synthesis did not induce apoptosis (data not shown).

3.1.6 Effect of adenine nucleotides on HT-29 growth

Given that adenosine may be derived extracellularly from adenine nucleotides within the tumour microenvironment (see Introduction Figure 1.2), the effects of 30μM AMP and ATP on DNA synthesis in all five cell lines were tested. Figure 3.9 shows a representative result for HT-29 cells. Both AMP and ATP significantly increased DNA synthesis in HT-29 cells. These effects were not blocked by the 5'-NT inhibitor AMP-CP (data not shown). However, the effectiveness of this agent was not confirmed by measurements of adenosine production. It cannot be excluded therefore, that both AMP and ATP were metabolized to adenosine during the assay. Therefore, although these data do suggest that AMP and ATP may also promote DNA synthesis in colon carcinoma cells, whether or not this effect can be attributed to intact AMP and ATP remains to be determined.

3.2 Effect of cell culture environment on the adenosine response in HT-29 cells

Although the response of colon carcinoma cell lines to adenosine was consistently one of increased growth, the magnitude of the adenosine response would sometimes vary between experiments. The next objective was to indentify variables in the experimental protocol that could account for the variation observed between experiments. Influence of the cell culture environment on the adenosine response may help to explain the disparities in the literature with respect to the effect of adenosine on cell growth.

3.2.1 Effect of cell density on the adenosine response in HT-29 cells

HT-29 cells were grown to cell densities ranging from approximately 50-800 cells/mm^2 and exposed to 30μM adenosine. HT-29 cells at all densities responded to adenosine with an increase in DNA synthesis. However, the response was density-dependent, in that the most pronounced response occurred at a low cell density (Fig. 3.10). This effect was also observed when HT-29 cell cultures at different cell densities were exposed to a range of adenosine concentrations (3-100μM) (Fig. 3.11). The difference in magnitude cannot be explained simply by differences in the availability of adenosine (i.e. dense cultures would take up and metabolize adenosine more rapidly than sparse cultures). If so, the adenosine response curve for high-density cell cultures would be shifted rightward with respect to low-density cultures. This was not seen (Fig. 3.11). This influence of cell density may instead be associated with the degree of cell-cell contact and resulting cell-cell communication.

3.2.2 Effect of serum supplementation on the adenosine response in HT-29 cells

Our standard protocol for examining an adenosine-mediated increase in DNA synthesis involved a two-day exposure to low serum-containing media (1% NCS) instead of standard growth medium containing 10% NCS. The rationale behind the serum-downshift was to slow cell growth to allow for an optimal mitogenic effect. Figure 3.12 depicts the effect of 'serum-downshift' on the adenosine response expressed relative to control cultures. HT-29 cells were seeded on d0 in medium containing 10% NCS. On d2, cells received either fresh 10% NCS (open bar), or media containing 1% NCS (hatched bar). On d4, cells were treated with adenosine (30 or 100μM) and DNA synthesis was measured. All cells, irrespective of their experimental conditions responded to adenosine treatment with an increase in DNA synthesis. The most pronounced response was observed in cells whose basal growth rate was restrained by low serum-containing medium.

3.2.3 Effect of cell density and serum supplementation in combination

Since both cell density and serum were found to independently modulate the adenosine response, the effect of these culture parameters in combination was examined. Sparse cultures (25% confluent [amount of culture vessel surface covered by cells]) or dense cultures (75% confluent) were exposed to medium containing either 1% NCS (Fig. 13a) or medium containing 10% FCS (Fig. 13b) and treated with 30μM adenosine. Fetal

calf serum provides a higher level of growth support than NCS. Both the sparse and dense cultures exposed to 1% NCS responded to adenosine with increased growth, although the magnitude of the adenosine response for the sparse culture was greater than the dense culture. Conversely, cells exposed to 10% FCS did not respond to adenosine regardless of their culture density. These data suggest that serum supplementation is critical in determining whether or not cells will respond to the growth-promoting effects of adenosine.

3.3 Effect of adenosine receptor antagonists on the adenosine-mediated increase in tumour cell growth

Thus far, my findings have demonstrated that adenosine consistently increases DNA synthesis in colon carcinoma cells. In addition, adenosine stimulates HT-29 cell proliferation. The next objective was to determine how the cellular response to adenosine was mediated. It was fully expected that conventional receptor subtypes (A_1-A_3) would be involved. A logical question was therefore, would adenosine receptor antagonism attenuate the adenosine-mediated increase in DNA synthesis? All five colon carcinoma cell lines were used in preliminary experiments and HT-29 cells were used for subsequent studies.

3.3.1 Effect of single receptor antagonists on the adenosine response

To examine the involvement of adenosine receptors in the adenosine-mediated stimulation of DNA synthesis in carcinoma cell lines, a panel of receptor antagonists was used in an attempt to block the adenosine response. All of these agents had been shown to be active in other *in vitro* systems in the Blay or Hoskin laboratories (Dalhousie University, Nova Scotia, Canada), and all showed antagonist activity in some of the following experiments. Cells were pretreated with these agents at a final concentration of 20μM for 30 min, after which time adenosine was added to a final concentration of 30μM. DPCPX is a selective antagonist of A_1 receptors; CSC is selective for A_{2a} receptors; alloxazine is an A_{2b}-selective antagonist; and MRS1191, MRS1220, and MRS1523 are all A_3-selective receptor antagonists. The results, which were highly variable, are shown in Table 3.2. The adenosine response in HT-29 cells was blocked by all four receptor antagonists in certain experiments, but this inhibition was not consistent. Antagonism of the A_1 receptor subtype was the least effective at blocking the adenosine response whereas antagonism of the A_{2b} receptor subtype resulted in inhibition of the adenosine response most often. In eight of twenty-one experiments, the A_{2b} antagonist, alloxazine, blocked the adenosine response. All five of the cell lines were sensitive to A_{2b}-receptor antagonism in at least one experiment. The possible causes for this variation in spite of repeated careful experimentation, are discussed in Section 3.4.

3.3.2 Effect of multiple adenosine receptor antagonists in combination on the adenosine response

The results from the single antagonist experiments suggested that the adenosine-mediated increase in DNA synthesis may not be mediated by a sole receptor subtype. For example, antagonists for all four receptor subtypes were capable of abrogating the adenosine response in HT-29 cells. In view of this, the effect of multiple receptor antagonists in combination was investigated. HT-29 cells were pre-treated with all possible combinations of DPCPX, CSC, alloxazine, and MRS1191. Antagonists were used at a final concentration of 10μM with the exception of alloxazine, which was used at a final concentration of 50μM. Cells were then treated with 10 or 30μM adenosine. Six independent experiments, using all combinations of antagonists, failed to point to any particular set of receptors that may be involved in the adenosine response. In fact, all but one of the combinations were capable of blocking the adenosine response in at least one of the six experiments performed for each combination (Table 3.3). The combination of all four types of antagonists was only effective in 66% of experiments (Table 3.3).

Combinations of adenosine receptor antagonists were more effective at blocking the adenosine response than single receptor antagonists. Therefore, it is probable that a single receptor is not responsible for mediating the adenosine response and instead multiple receptors subtypes work in concert to elicit the stimulatory effect. Furthermore, since it was not possible to block the adenosine response with a combination of all four antagonists in all experiments, there must be a further receptor or other target of adenosine action that has the potential to play a part.

3.4 Adenosine receptor expression in colon carcinoma cells

In light of the complex involvement of adenosine receptors with the adenosine growth-promoting response, the next objective of this thesis was to determine the adenosine receptor expression profile for the five human colon carcinoma cell lines.

RT-PCR was performed using oligonucleotides specific for the four human adenosine receptor subtypes. Expression of all four receptor subtypes was detected in HT-29, T84, and HRT-18 cells at variable levels (Table 3.4). The message for the A_1 receptor subtype was not expressed by Colo320*HSR* and Caco-2 cells. Interestingly, the strongest expression by all cell types was the A_{2b} receptor subtype. Inhibition of the A_{2b} receptor subtype also most consistently abrogated the adenosine response in the same five cell lines (see section 3.3.1).

I also wanted to determine if receptor expression could be modulated by cell density and serum, given that these parameters dramatically influenced the adenosine response. Changes in receptor expression in response to the cellular environment could impact upon (i) the magnitude of the adenosine response and (ii) the ability to block the adenosine response using receptor antagonists at one static time point. However, the RT-PCR results were variable despite repeated experimentation. Therefore, the results from these experiments have been appended (Appendix A).

3.5 Effect of culture format on the adenosine response

Thus far, the growth-promoting effect of adenosine on human colorectal carcinoma cells had been demonstrated in cells grown on a solid substratum in monolayer culture. The next objective of this thesis was to examine the effect of adenosine on DNA synthesis and cell proliferation in HT-29 cells grown on culture inserts and in three-dimensional tumour spheroids.

3.5.1 Effect of adenosine treatment on cells grown in Transwell® culture format

Epithelial cells *in vivo* are polarized, meaning that their plasma membrane comprises an apical surface, which faces the lumen, and a basolateral surface, which faces adjacent cells and the underlying stroma/connective tissue. The cells at both apical and basolateral sides are exposed to different environmental cues and have the ability to respond uniquely. Cells grown in Transwell® culture inserts were used to study the separate or simultaneous exposure of apical and basolateral cell surfaces to adenosine. The effect of treating one or both cell surfaces with adenosine and the resultant effect on DNA synthesis were compared.

HT-29 cells were seeded in Transwell® culture inserts and were maintained under standard culture conditions until there was evidence of a confluent monolayer. At this time cells were treated with 100μM adenosine either in the upper chamber ('apical' surface), lower chamber ('basolateral' surface), or both chambers and DNA synthesis

was measured. There was no significant difference between cultures treated apically and/or basolaterally (Fig. 3.14).

3.5.2 Effect of adenosine on HT-29 spheroid growth

Three-dimensional tumour spheroids more closely resemble tumour tissue *in vivo* than cells grown in monolayer culture (Carlsson, 1977; Carlsson and Acker, 1988). Exploitation of this culture format may provide additional information as to the effect of adenosine *in vivo*. HT-29 cells were grown until a cohesive three-dimensional cell aggregate ('spheroid') was apparent. This was defined as 'd0'. Cells were then treated with adenosine every two days (d0-d6 inclusive) at the concentrations indicated. Spheroid cell number was determined on d8. In approximately half of the experiments performed, adenosine at concentrations that were growth promoting to monolayer cell cultures resulted in a moderate increase in spheroid cell number (Fig. 3.15). The demonstration that adenosine can increase spheroid growth is significant given that spheroids more closely resemble tumour tissue *in vivo*.

It was not surprising that the magnitude of the adenosine response was lower in spheroids since adenosine is metabolized somewhat quickly ($t_{1/2} \approx 2$h for HT-29 cells) and access of tumour cells grown in spheroid format to adenosine would be much slower than for cells grown in monolayer culture, as it is for other drugs (Tannock *et al.*, 2002). It is possible in many cases, that before adenosine is able to gain substantial access to cells of the spheroid, it has undergone substantial metabolism and degradation and is no longer present at an effective concentration.

3.6 Effect of tumour microenvironment conditions on the adenosine response in HT-29 cells

The standard cell culture conditions typically used while studying processes involving cancer cells differ dramatically from the conditions to which a tumour cell is exposed *in vivo*. Two factors that are altered in the microenvironment are pH and glucose levels. The tumour microenvironment (TM) is acidic and the levels of glucose are low. It has long been known that tumour cells are quite resistant to harsh conditions such as an acidic culture environment (Eagle, 1973) and that pH, glucose, and lactate can modulate tumour cell growth (Casciari *et al.*, 1992).

Would conditions present in the TM support the adenosine response? It was necessary to verify that adenosine stimulated DNA synthesis in colon carcinoma cells cultured under TM-like culture conditions.

The extracellular pH of tumour tissue is approximately 6.8 compared with 7.4 in normal tissue (Rotin *et al.*, 1986). Therefore, HT-29 cells were exposed to culture media at pH 6.8 and 7.4 and treated with adenosine (3-300μM). Figure 3.16 highlights the ability of HT-29 cells grown in pH 6.8 culture medium to respond to adenosine with an increase in DNA synthesis, which is qualitatively very similar to that at pH 7.4.

HT-29 cells were also exposed to either culture medium containing 4.5g/L glucose (standard culture medium) or glucose-free medium. The cells were then treated with adenosine (3-300μM). Figure 3.17 highlights the ability of HT-29 cells grown in the absence of exogenous glucose to survive and respond to adenosine with an increase in

DNA synthesis. Indeed the response of cells to adenosine (expressed relative to controls) is substantially greater in conditions of glucose deprivation (Fig. 3.17).

HT-29 cells exposed to medium containing a low concentration (0.5g/L) of glucose were treated with adenosine ranging from 3-300μM and cell number was counted. There was a significant increase in HT-29 cell number with adenosine treatment (Fig. 3.18). The adenosine response curve was biphasic with maximal adenosine stimulation reached by 30μM. Although treatment of HT-29 cells with 100μM and 300μM adenosine did not decrease cell number below control values, there was a reduction in the magnitude of the response. Cell number measurements in the presence of 300μM adenosine were not significantly different from control. Adenosine at a concentration of 3μM dramatically increased cell number; this increase was not observed when HT-29 cells were cultured in media containing 4.5g/L glucose (data not shown). These findings suggest that the response of colon carcinoma cells to adenosine may be enhanced under low-glucose conditions such as those present in the TM.

3.7 Adenosine metabolism and production

The level of adenosine present in the extracellular fluid of solid tumours *in vivo* is elevated (Blay *et al.,* 1997). A contributing factor is the hypoxic environment that promotes the breakdown of adenine nucleotides to adenosine (Meghji, 1993). The next objective was to examine the relative contribution of tumour cells and non-tumour cells to adenosine production. In addition, the kinetics of adenosine metabolism was also studied.

3.7.1 The kinetics of adenosine metabolism

Many groups have opted to use adenosine analogues as replacements for native adenosine in their assay systems. This choice is often based on and justified by the well-documented finding that adenosine metabolism is rapid. While we agree that adenosine is metabolized quickly in certain cellular systems (e.g. blood – $t_{1/2}$ ~ 1sec), we did not know the kinetics of adenosine metabolism in our assay system.

The fate of a single dose of adenosine added to the culture medium was examined. Sub-confluent HT-29 cells (comparable density to that used in assays measuring DNA synthesis and cell proliferation) were treated with 10μM adenosine at t=0 and serial measurements of adenosine concentration in the medium were made every hour for six hours. The $t_{1/2}$ [time to 50% degradation (of adenosine)] was estimated to be approximately 2h (Fig. 3.19). Half-life measurement were also made for the four other cell lines used in this study and values ranged from 40min to 3h depending on the cell line and culture conditions (Mujoomdar *et al.*, 2003). These findings suggest that adenosine metabolism in our cell culture system is much slower than what has been reported for plasma and blood.

3.7.2 Adenosine production and the involvement of adenosine deaminase and adenosine kinase

The next objective was to determine the amount of adenosine produced by HT-29 cells under standard culture conditions as well as the involvement of ADA and AK in this

process. HT-29 cells were treated with either vehicle (SFM), 10μM coformycin (an inhibitor of ADA), 1μM iodotubercidin (an inhibitor of AK), or both of the enzyme inhibitors in combination; subsequently adenosine measurements were made. Endogenous adenosine production was measured as the accumulation over a 6h period. Samples were collected every hour beginning at t=0 and extending to t=6h and the rate of adenosine production was determined.

Figure 3.20 demonstrates that in the absence of enzyme inhibitors, the rate of adenosine production by HT-29 cells is low, approximately 60pmol/h. Inhibition of ADA had no effect on the endogenous production of adenosine, whereas treatment with the AK inhibitor iodotubercidin significantly increased the rate of adenosine production by HT-29 cells.

3.7.3 Production of adenosine by tumour cells exposed to hypoxia

HT-29 cells produced very little adenosine under standard culture conditions. Tumour cells *in vivo* would be exposed to hypoxic conditions. Production of adenosine in cells exposed to hypoxia according to tumour cell type would provide information about the contribution of tumour cells to the increased production of adenosine in the solid tumour microenvironment. Tumour cell lines of various site-specific cancers (colorectal, lung, breast, and ovarian) were all exposed to hypoxia (0.2% O_2) for 6h and processed for adenosine measurement. The concentration of adenosine measured in the culture medium after 6h of hypoxia was relatively low for all cell lines examined, in the tens to hundreds of nanomolar range (Fig. 3.21a).

Tumour tissue *in vivo* is comprised not only of tumour cells, but also has a stromal component as well as intervening blood vessels. In order to investigate the possible involvement of additional cell types in the production of adenosine, primary human fibroblasts and endothelial cells were exposed to hypoxia for 6h and adenosine measurements were made (Fig. 3.21b). The concentration of adenosine measured in the culture medium of fibroblasts after 6h of hypoxia was 0.4μM, which was higher than any of the tumour cell lines that were examined. Endothelial cells produced micromolar concentrations of adenosine. Interestingly, cultures of primary ovarian cancer cells produced close to 1μM adenosine, approximately a 10-fold increase over the ovarian cancer cell line (SKOV3).

3.8 Effect of adenosine on cell migration

Two studies have shown that adenosine stimulates cell migration of endothelial cells and oligodendrocytes (Meininger *et al.,* 1988; Othman *et al.*, 2003). Currently, there is only a single published report showing that adenosine stimulates tumour cell migration (Woodhouse *et al.,* 1998). Woodhouse and co-workers (1998) demonstrated that adenosine stimulated melanoma cell migration. Melanoma arises from the squamous epithelium and to date, no one has examined columnar epithelial cell cancers, from which colon carcinomas would arise. The final objective of this thesis was to examine the effect of adenosine on HRT-18 colon carcinoma cell migration.

3.8.1 Adenosine increases HRT-18 cell migration

HRT-18 cells were seeded onto Sigma collagen Type IX-coated Transwell® culture inserts. Adenosine (10-1000μM) was added to the bottom chamber. Adenosine at a concentration of 10μM increased HRT-18 cell migration (Fig. 3.22). This pro-migratory effect by adenosine persisted up to 100μM. These data suggest that pathophysiological concentrations of adenosine stimulate colon carcinoma cell migration.

To confirm that it was adenosine that was increasing HRT-18 cell migration, adenosine (10 and 100μM) or adenosine in combination with ADA (0.5U) was added to the bottom chamber. The addition of ADA to the bottom chamber inhibited the adenosine-induced increase in HRT-18 migration (Fig. 3.23). These findings confirm that it is indeed adenosine that is increasing HRT-18 cell migration.

Checkerboard analysis was performed to determine if adenosine increased chemotaxis (directed migration) or chemokinesis (random migration). Different concentrations of adenosine (1-100μM) were added to the upper chamber with the cells and to the bottom chamber as indicated. Adenosine increased both chemotaxis and chemokinesis of HRT-18 cells (Table 3.5).

3.8.2 Adenosine A_2 receptors are involved in the adenosine-induced increase in HRT-18 cell migration

To determine if adenosine receptors were involved in the adenosine-mediated increase in cell migration, HRT-18 cells were prepared as usual and then pre-treated with

adenosine receptor antagonists for 30 min. The cells were then added to the upper chamber and adenosine was added to the lower chamber. A concentration of 10μM adenosine was chosen for the antagonist study because it consistently increased HRT-18 migration. Both the A_{2a} antagonist CSC (1μM) and the A_{2b} antagonist alloxazine (5μM) blocked the adenosine-induced increase in cell migration (Fig. 3.24). CSC and alloxazine in combination, blocked the adenosine effect, but in the absence of adenosine had no effect on cell migration. The A_{2a}-selective agonist CGS21680 (0.1-30μM) caused a dose-dependent increase in HRT-18 cell migration, thereby further implicating the A_{2a} receptor subtype in the adenosine response (Fig. 3.25). Collectively these data suggest that the A_2 receptor likely mediates the adenosine-induced increase in HRT-18 cell migration. As yet, there is no truly selective A_{2b} receptor agonist available. Therefore, based on the ability of the A_{2b} antagonist to block the adenosine response, the A_{2b} receptor subtype may be involved in the adenosine-mediated increase in HRT-18 cell migrtion.

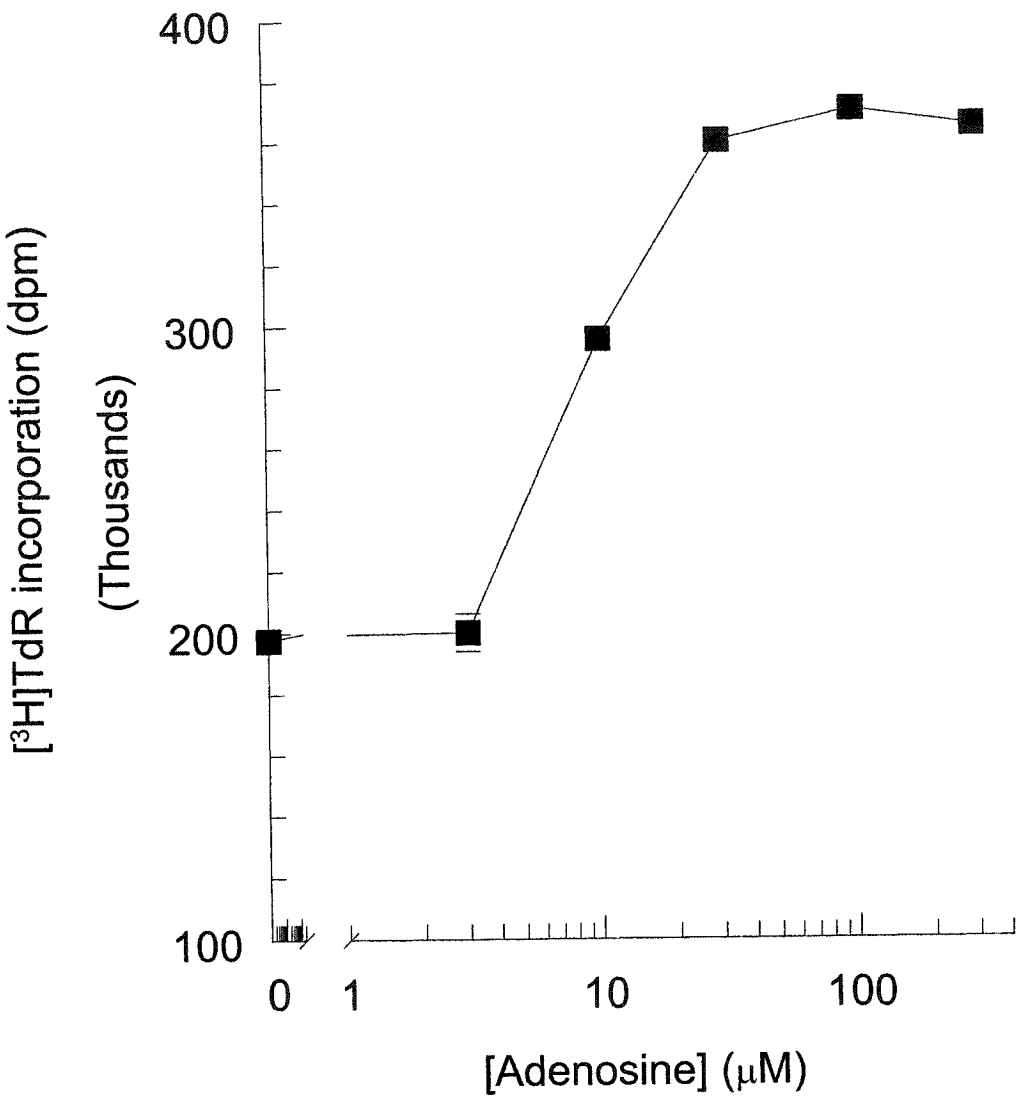

Figure 3.1: Effect of adenosine on DNA synthesis in HT-29 human colorectal carcinoma cells. Data points and error bars (may be within symbols) represent mean ± SEM for quadruplicate wells within a representative experiment. Panel is representative of six independent experiments.

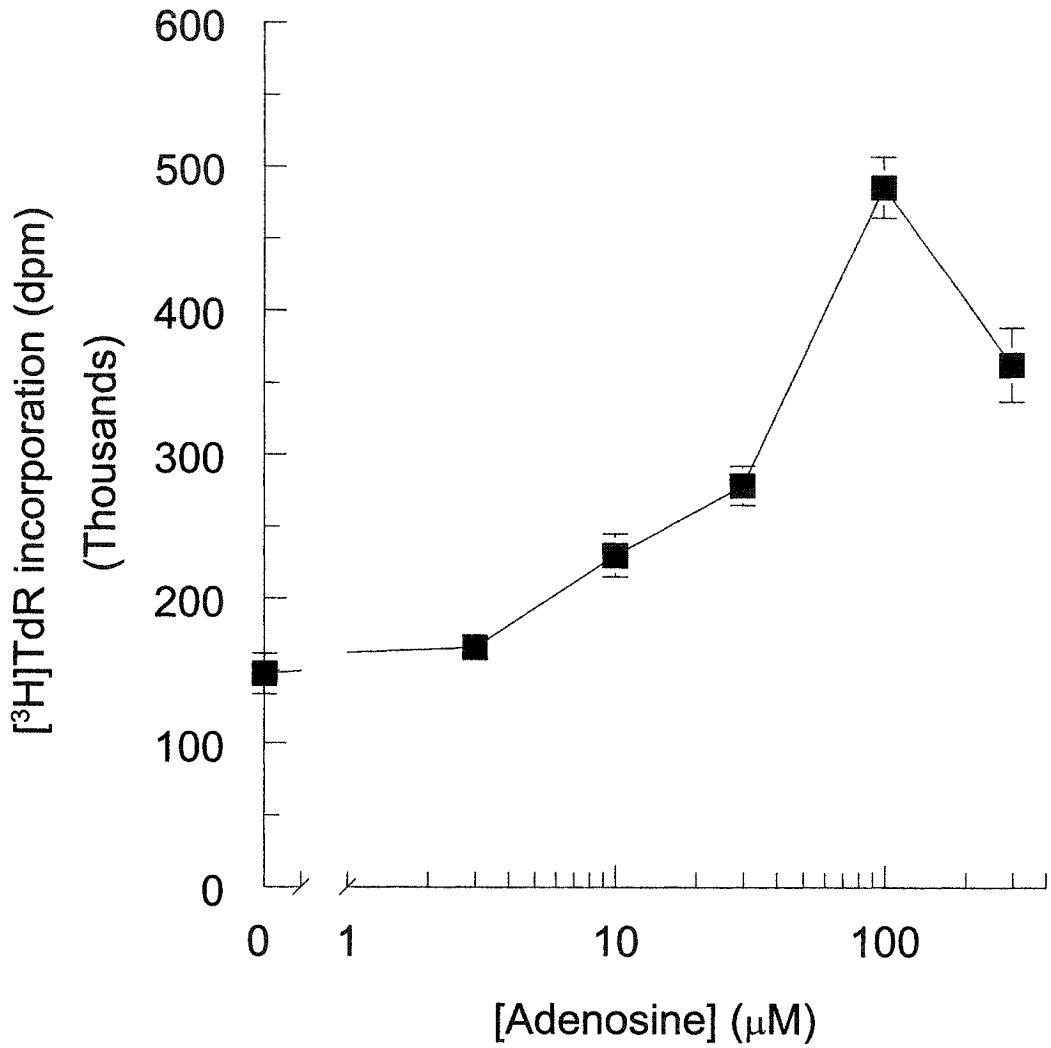

Figure 3.2: Effect of delaying [*methyl*-^{3}H]-thymidine addition on the response of HT-29 cells to adenosine. HT-29 cells were treated with a single dose of adenosine ranging from 3-300μM. Addition of [*methyl*- ^{3}H] thymidine was delayed 24h after adenosine treatment. Data points and error bars (may be within symbols) represent mean ± SEM of quadruplicate wells within a representative experiment.

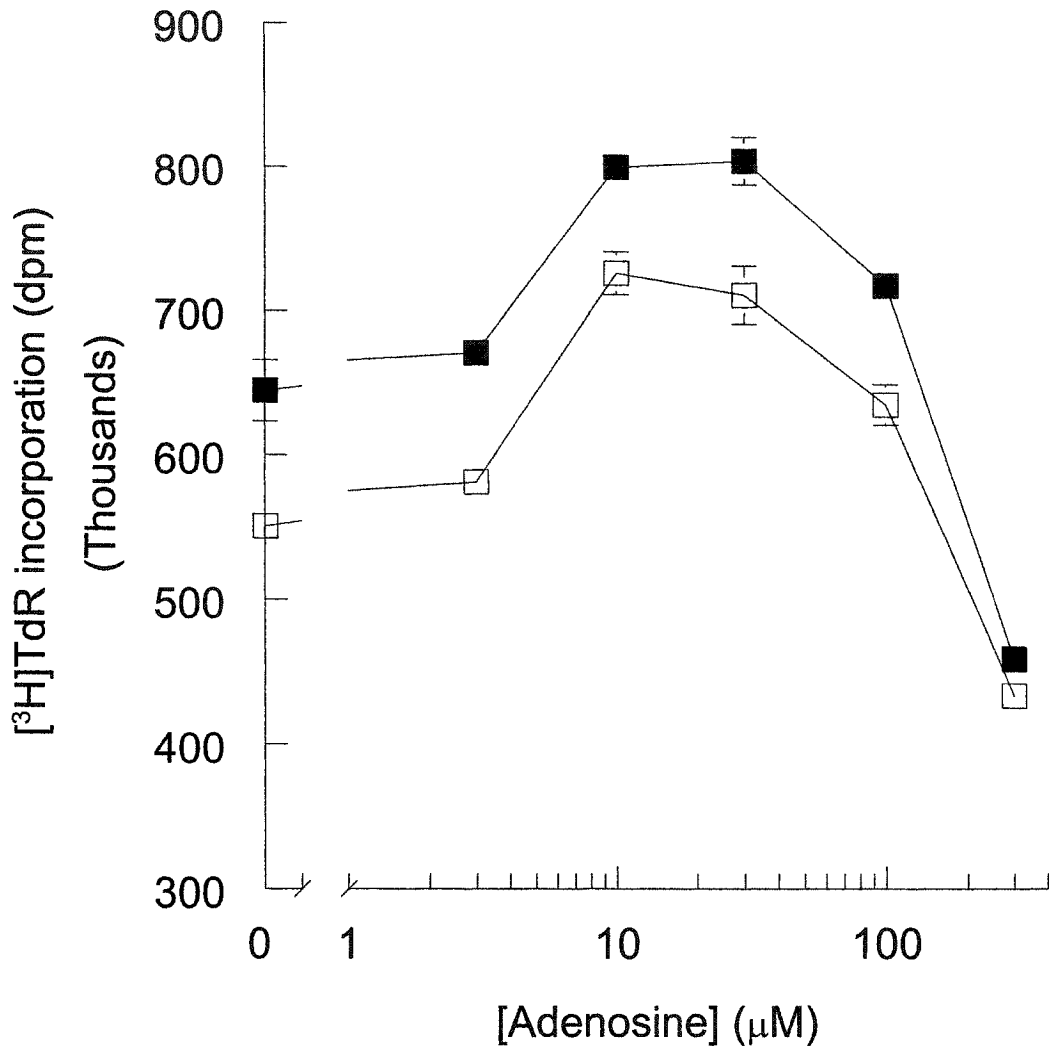

Figure 3.3: Effect of dialyzed serum on the response of HT-29 cells to adenosine. Cells cultured in undialyzed 1% NCS (□) or dialyzed 1% NCS (■) and treated with adenosine ranging from 3-300μM. Data points and error bars (may be within symbols) represent mean ± SEM of quadruplicate wells within a representative experiment.

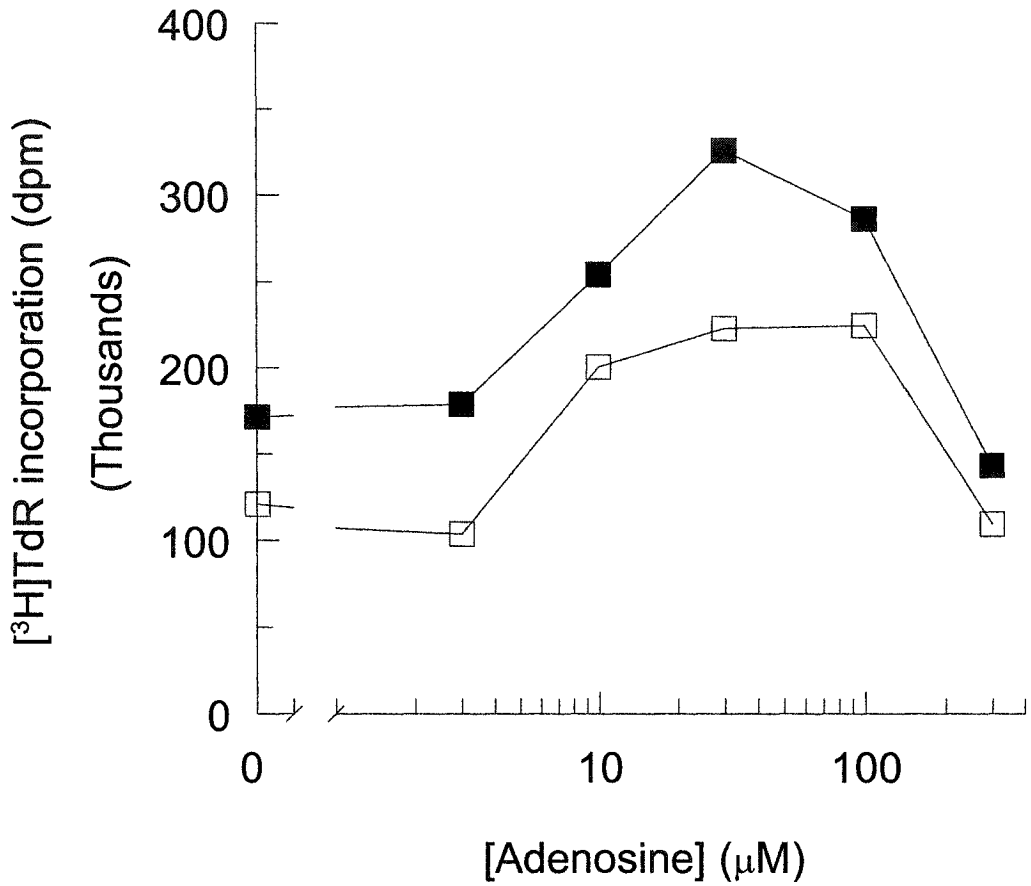

Figure 3.4: Effect of increased thymidine concentration on the adenosine response of HT-29 cells cultured in normal or dialyzed newborn calf serum. Cells cultured in undialyzed 10% NCS (□) or dialyzed 10 % NCS (■) were exposed to 10μM thymidine and treated with adenosine (3-300μM). Data points and error bars (may be within symbols) represent mean ± SEM of quadruplicate wells within a representative experiment.

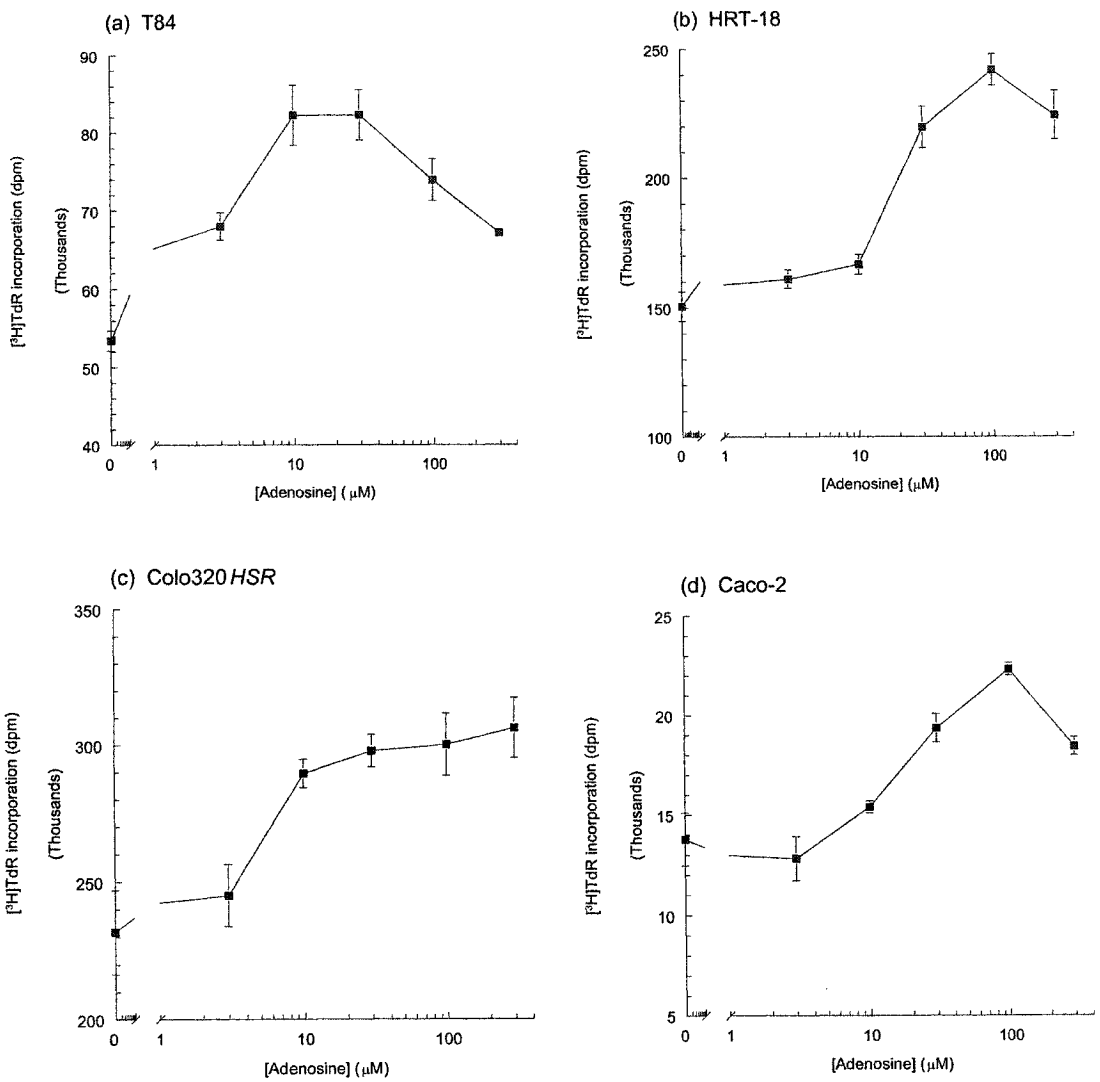

Figure 3.5 (a-d): Effect of adenosine on DNA synthesis in human colorectal carcinoma cell lines. Data points and error bars (may be within symbols) represent mean ± SEM for quadruplicate wells within a representative experiment. Each panel is representative of four independent experiments.

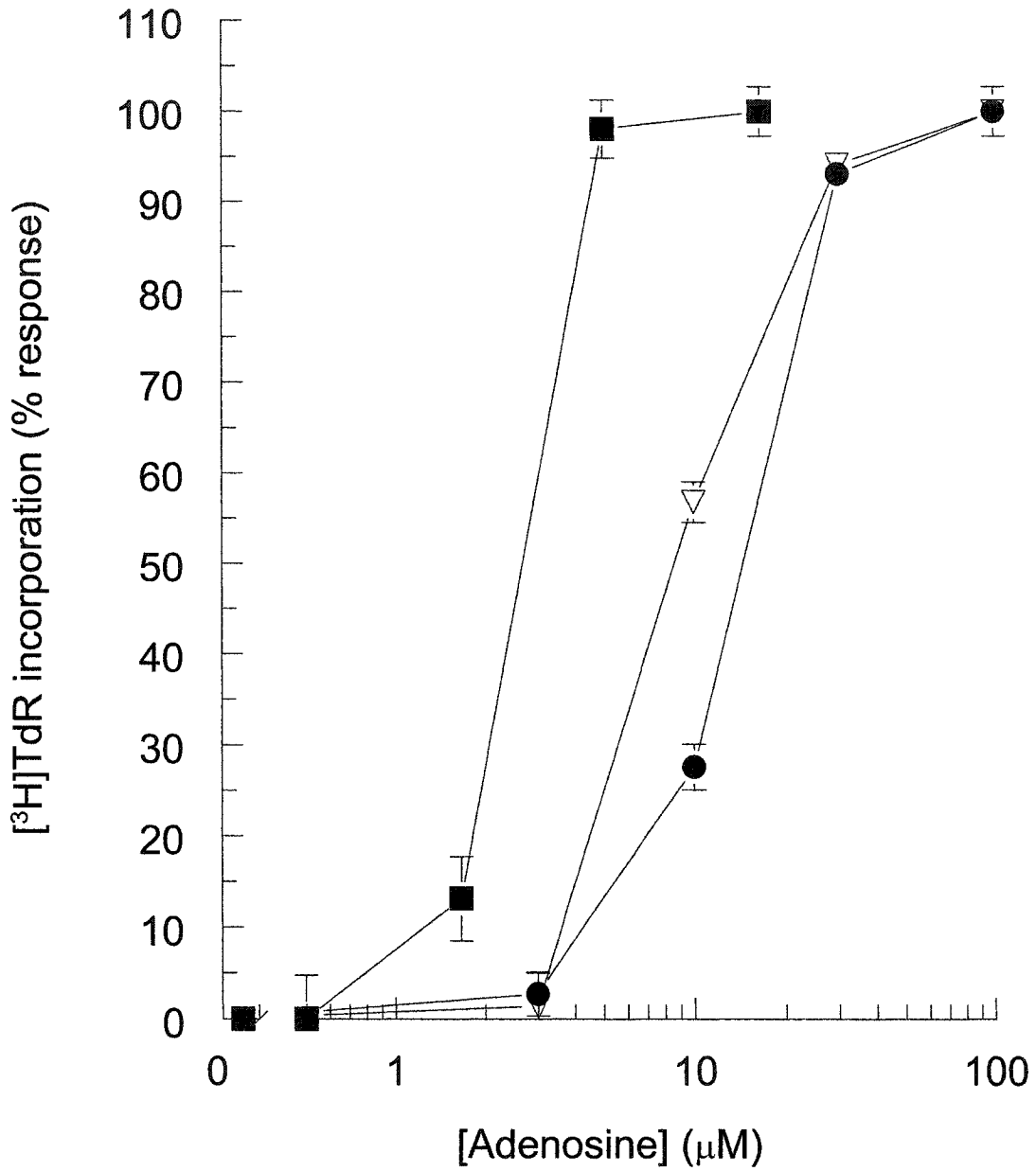

Figure 3.6: Effect of timing and schedule of *in vitro* dosing with adenosine on DNA synthesis in HT-29 cells. Adenosine was given as a single dose at time of plating (●), 2 days after plating (▽), or divided between six fractional doses given at 2h intervals 2d after plating (■). Data points and error bars (may be within symbols) represent mean ± SEM for quadruplicate wells within a representative experiment.

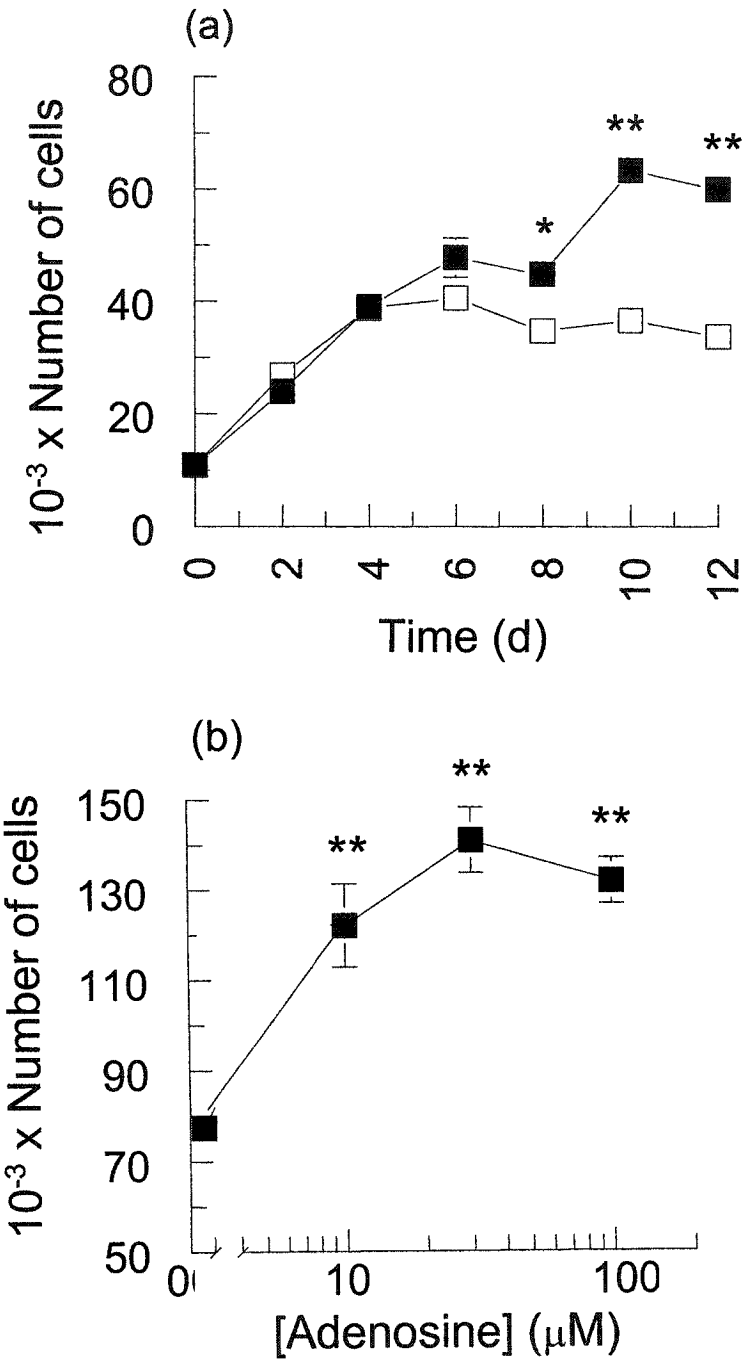

Figure 3.7: Effect of adenosine on the proliferation of HT-29 cells. (a) Time course. Cells were cultured without (□) or with (■) 30μM adenosine. (b) Concentration dependence. Cells were treated with adenosine every 2-3d at the concentrations indicated and counted after 10d. Data points and error bars (may be within symbols) represent mean ± SEM of quadruplicate wells within a representative experiment. Significant increase over control: $*P < 0.05$; $**P < 0.01$.

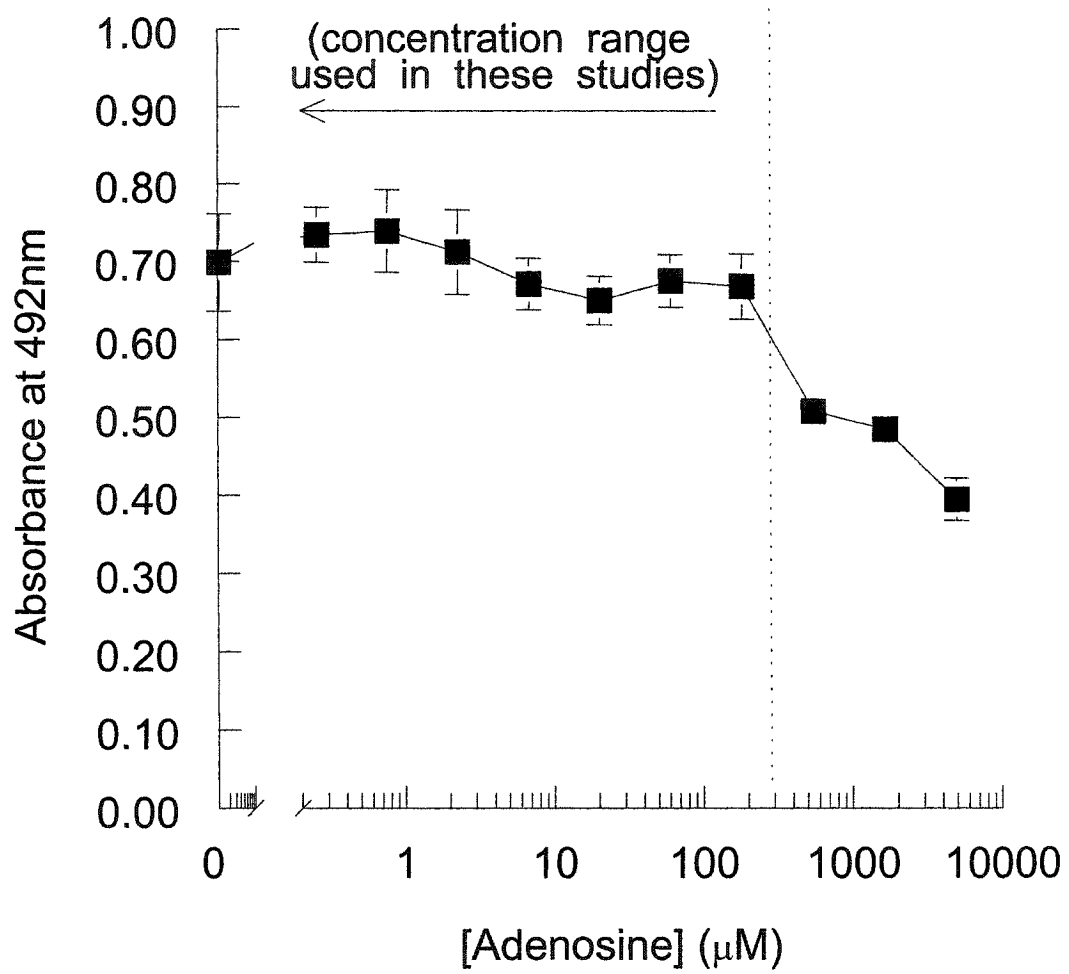

Figure 3.8: Effect of adenosine on the viability of HT-29 cells. HT-29 cells were exposed to adenosine. Viability was assessed using an MTT assay. Data points and error bars (may be within symbols) represent mean ± SEM of eight wells within a representative experiment.

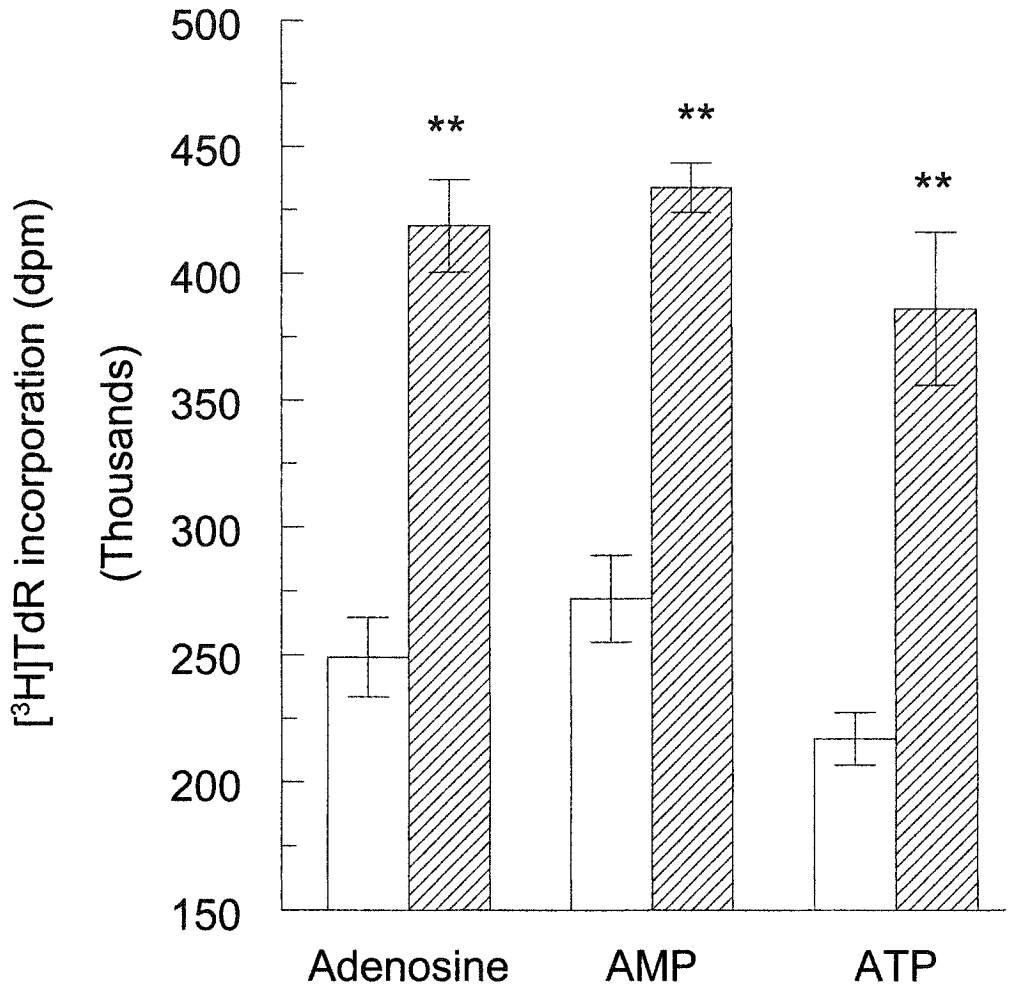

Figure 3.9: Effect of adenosine and adenine nucleotides on DNA synthesis in HT-29 cells. Cells were cultured without (open bar) or with (hatched bar) 30μM adenosine, AMP, or ATP, in separate experiments. Data and error bars represent mean ± SEM for quadruplicate wells within a representative experiment. Significant increase over control: $^{**}P < 0.01$.

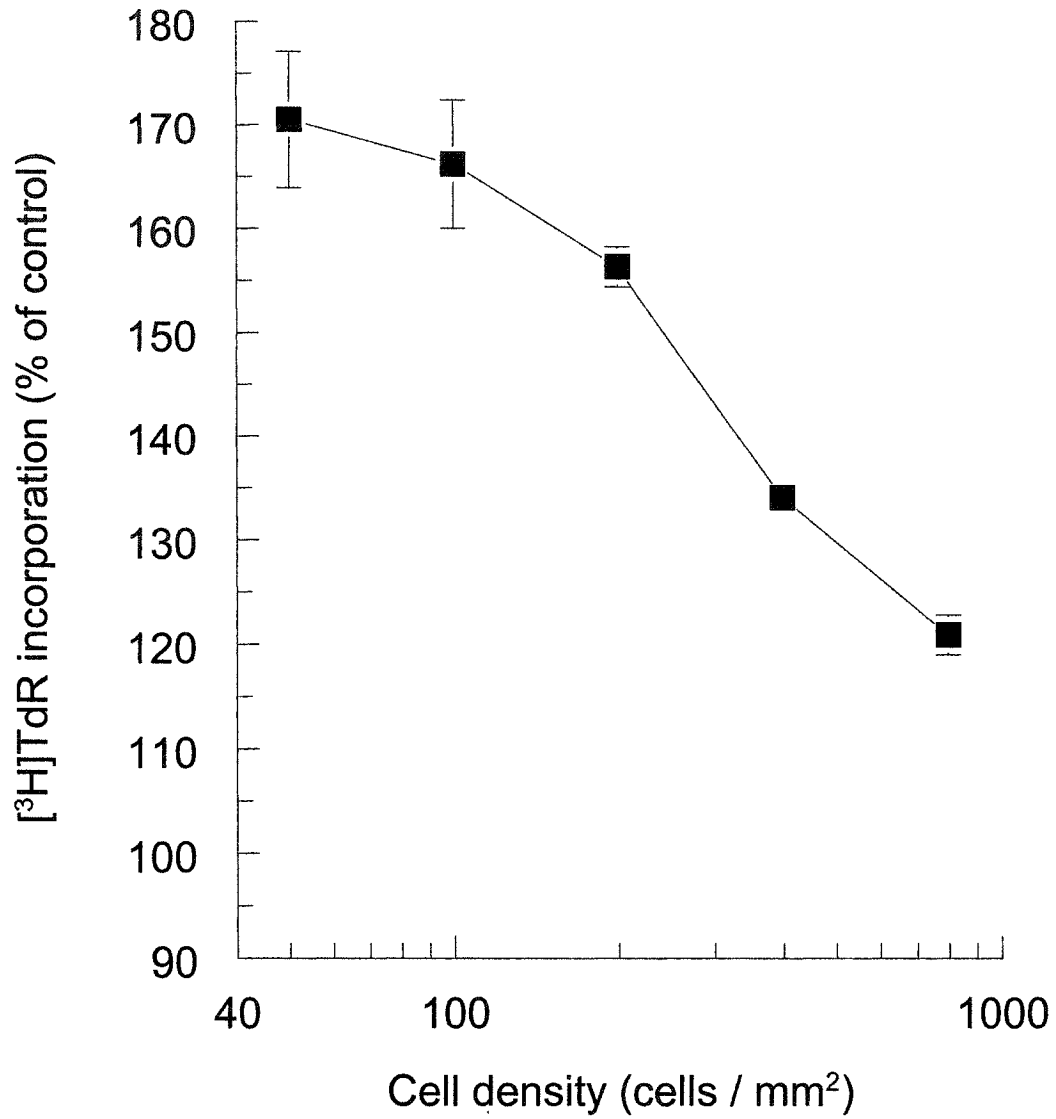

Figure 3.10: Density-dependence of the adenosine response in HT-29 cells. Data points and error bars (may be within symbols) represent mean ± SEM of quadruplicate wells within a representative experiment.

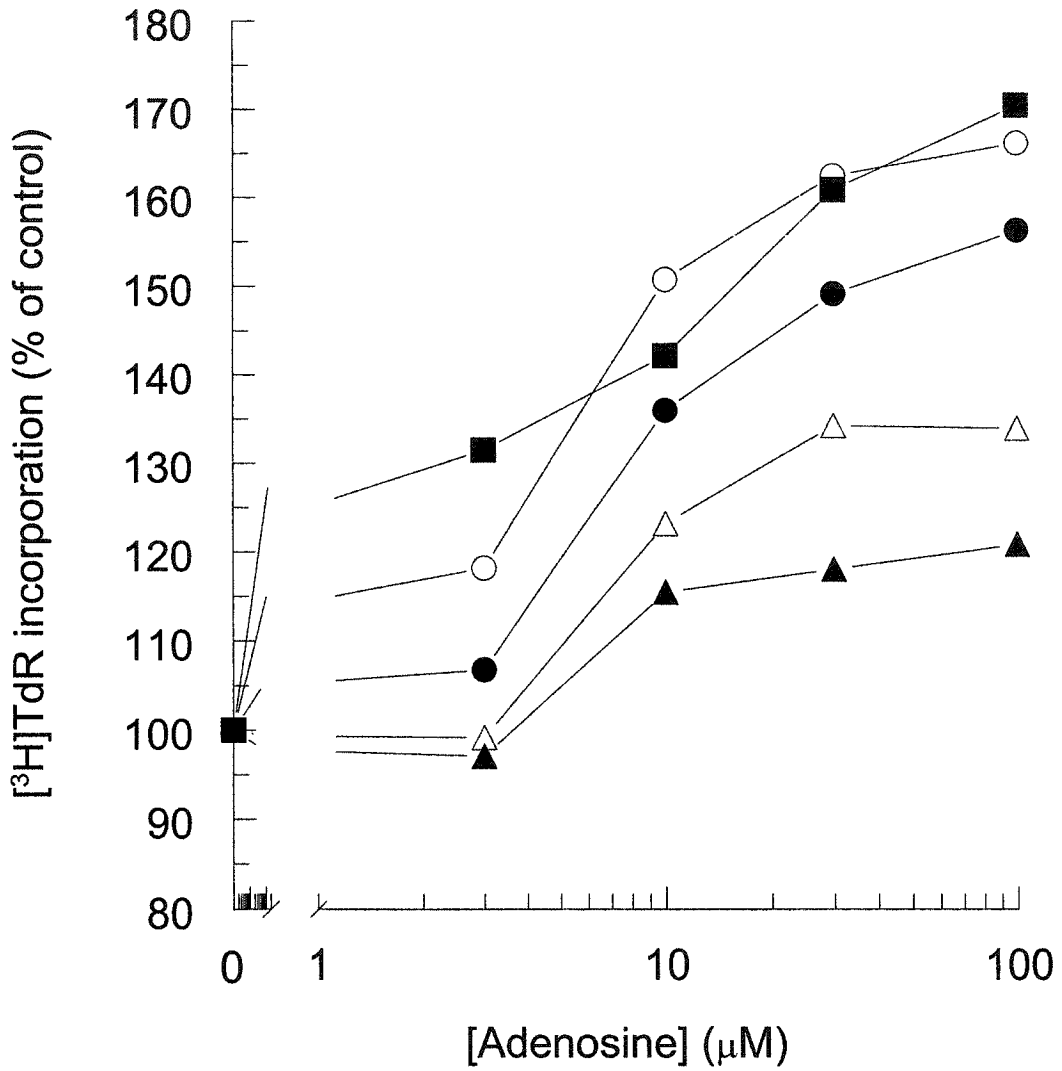

Figure 3.11: Concentration dependence of cell density on the magnitude of the adenosine response in HT-29 cells. HT-29 cells cultures at various densities (x 10^3 cells/mm^2): (■) 50; (○) 100; (●) 200; (△) 400; and (▲) 800. Data points and error bars (may be within symbols) represent mean ± SEM of quadruplicate wells within a representative experiment.

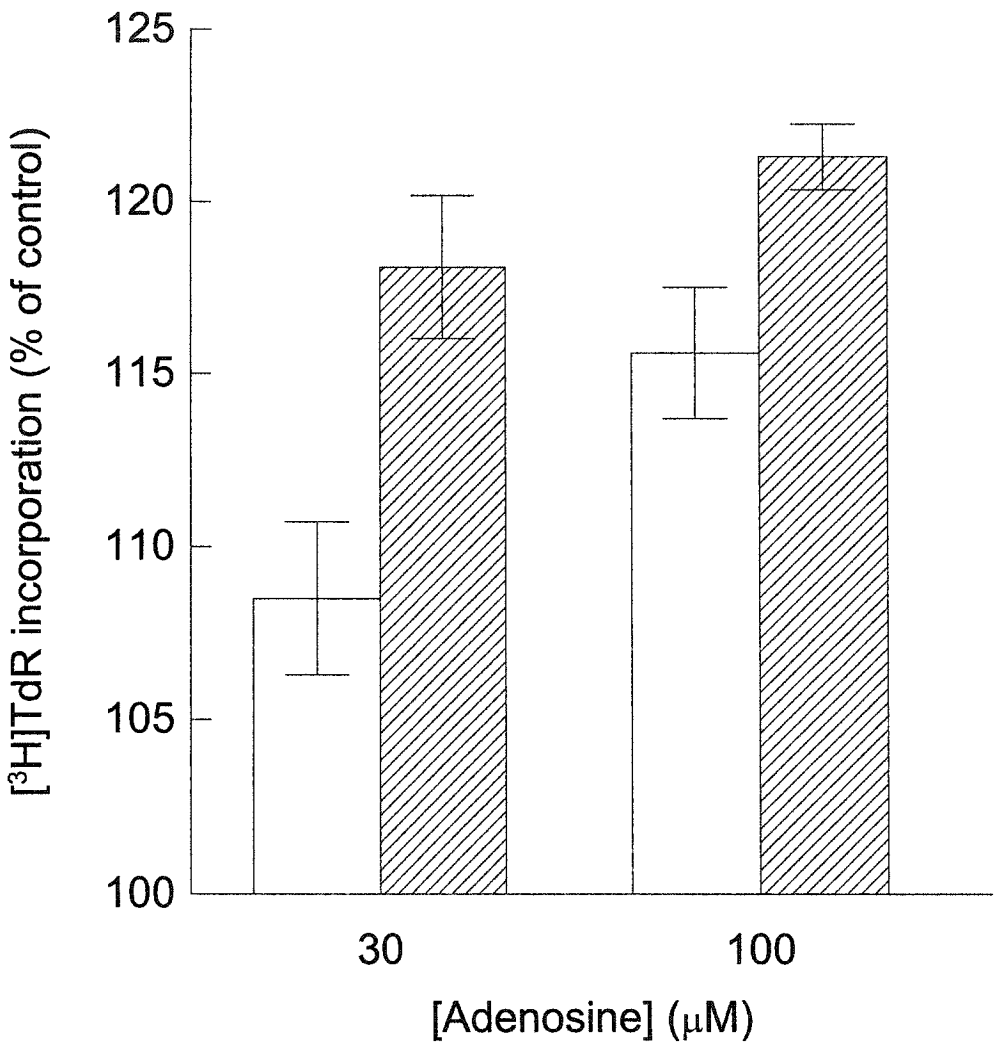

Figure 3.12: Effect of serum concentration on the response of HT-29 cells to adenosine. HT-29 cells exposed to either 10% NCS (open bars) or 1% NCS (hatched bars) and treated with adenosine (30 and 100μM). Data and error bars represent mean ± SEM of quadruplicate wells within a representative experiment and are expressed as percent increase over control (SFM treated cells).

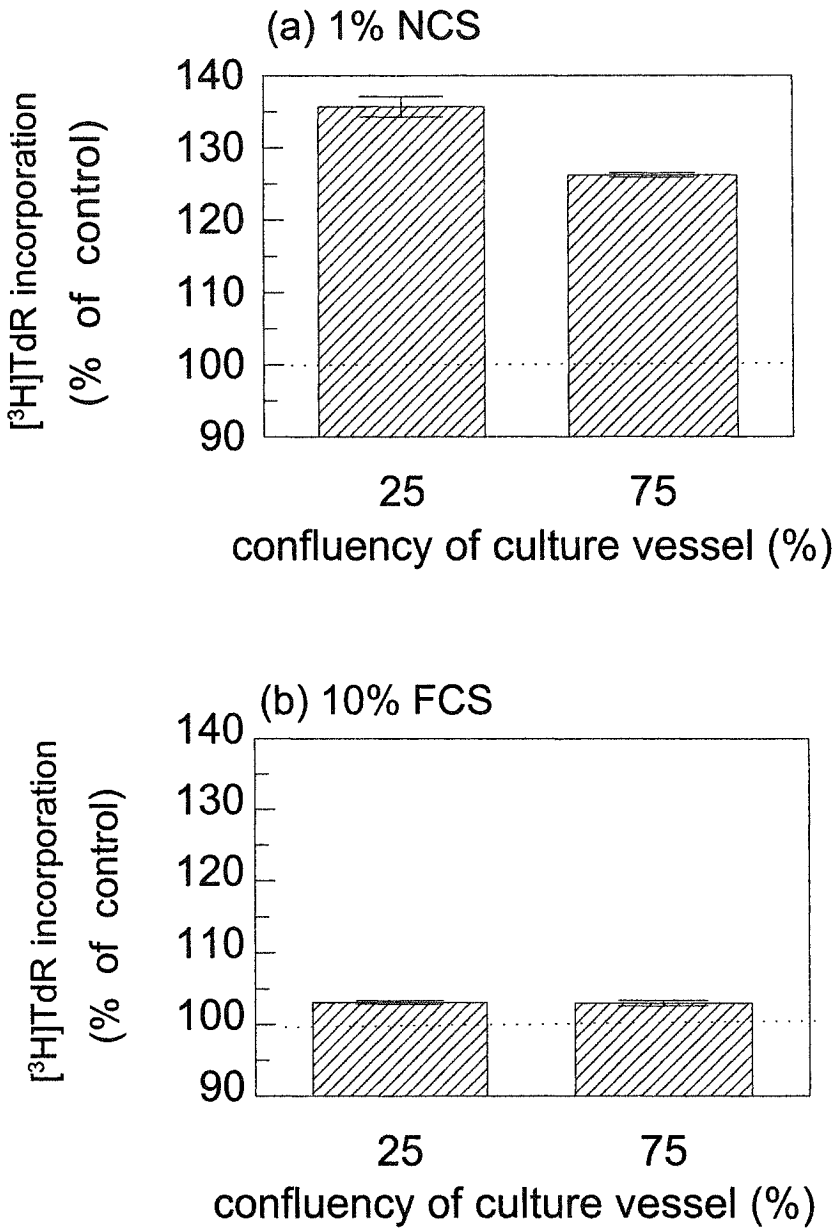

Figure 3.13: Effect of serum type and concentration in combination with cell density on the response of HT-29 cells to adenosine. (a) Low-serum conditions. High-density HT-29 cells (75% culture vessel confluency) or low-density cells (25%) were exposed to medium containing 1% NCS and treated with 30μM adenosine. (b) High-serum conditions. High-density HT-29 cells (75%) or low-density cells (25%) were exposed to medium containing 10% FCS and treated with 30μM adenosine. Data and error bars represent mean ± SEM of quadruplicate wells within a representative experiment.

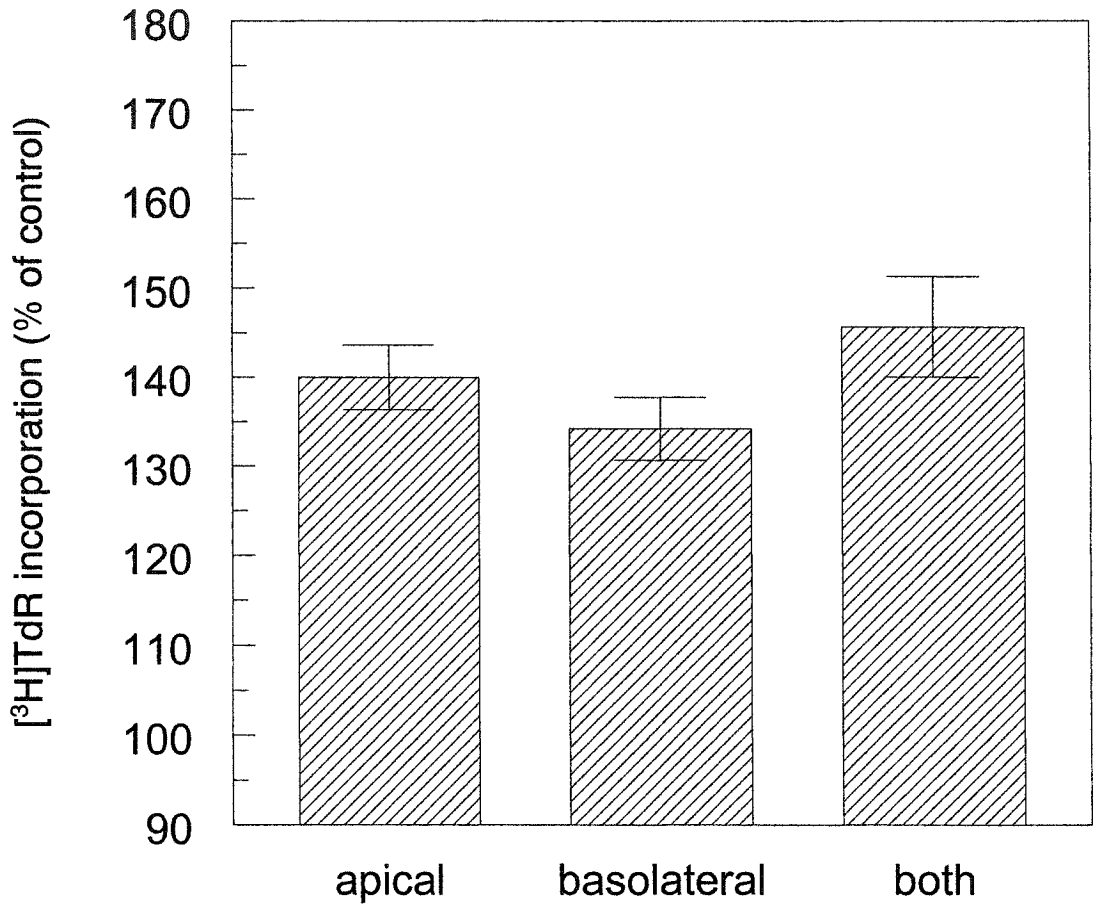

Figure 3.14: Effect of apical and/or basolateral treatment of HT-29 cells with adenosine. Cells were seeded in Transwell® culture inserts and treated with 100μM adenosine in the lower chamber (basolateral), upper chamber (apical), or both. Data points and error bars represent mean ± SEM of four independent experiments and expressed as increase over control (SFM treated cells).

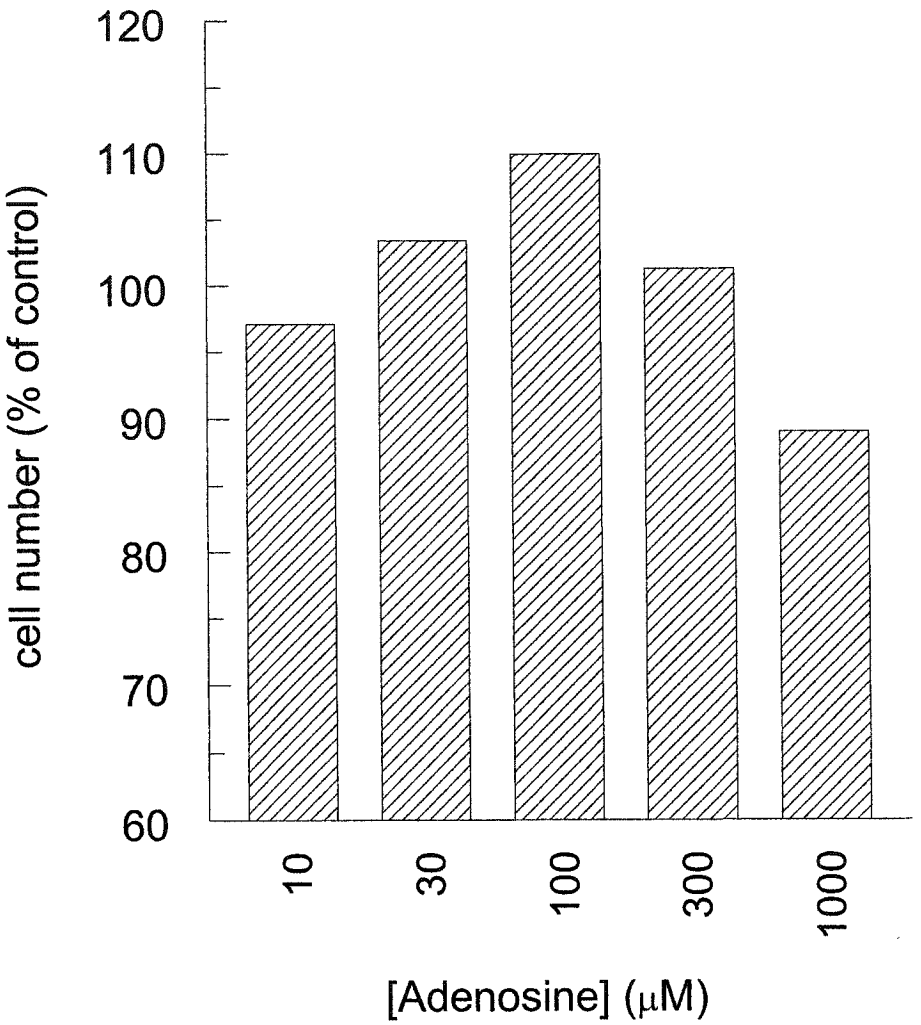

Figure 3.15: Effect of adenosine on the growth of HT-29 spheroids. Spheroids were treated with adenosine concentrations ranging from 10-1000μM. Spheroids were dissociated by trypsin and cells were counted. Bars represent mean values of two independent experiments with eight spheroids per treatment group.

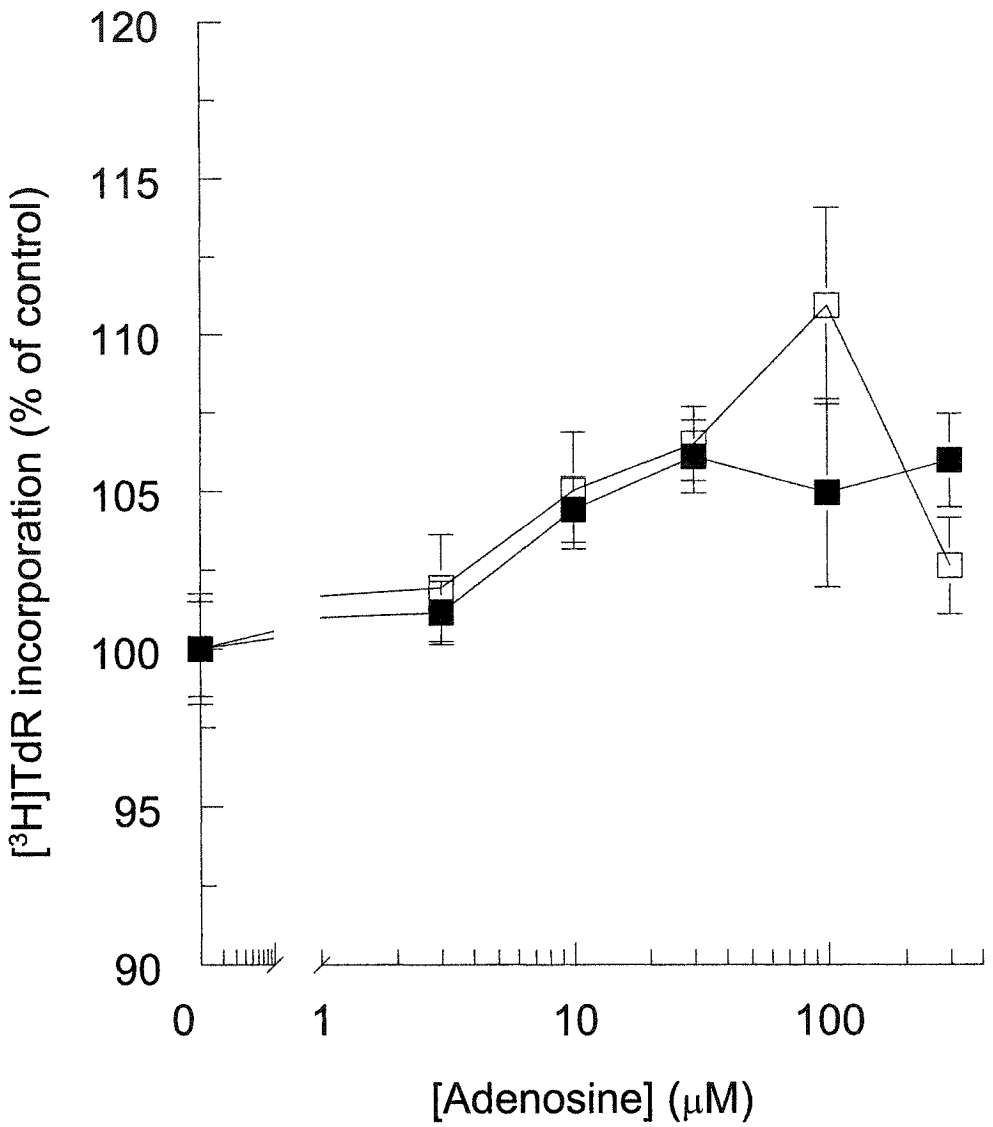

Figure 3.16: Effect of extracellular pH on the magnitude of the adenosine response in HT-29 cells. HT-29 cells were exposed to culture media at pH 7.4 (■) or pH 6.8 (□) and treated with adenosine (3-300μM). Panel is representative of three independent experiments. Data points and error bars (may be within symbols) represent mean ± SEM of four wells within a culture plate.

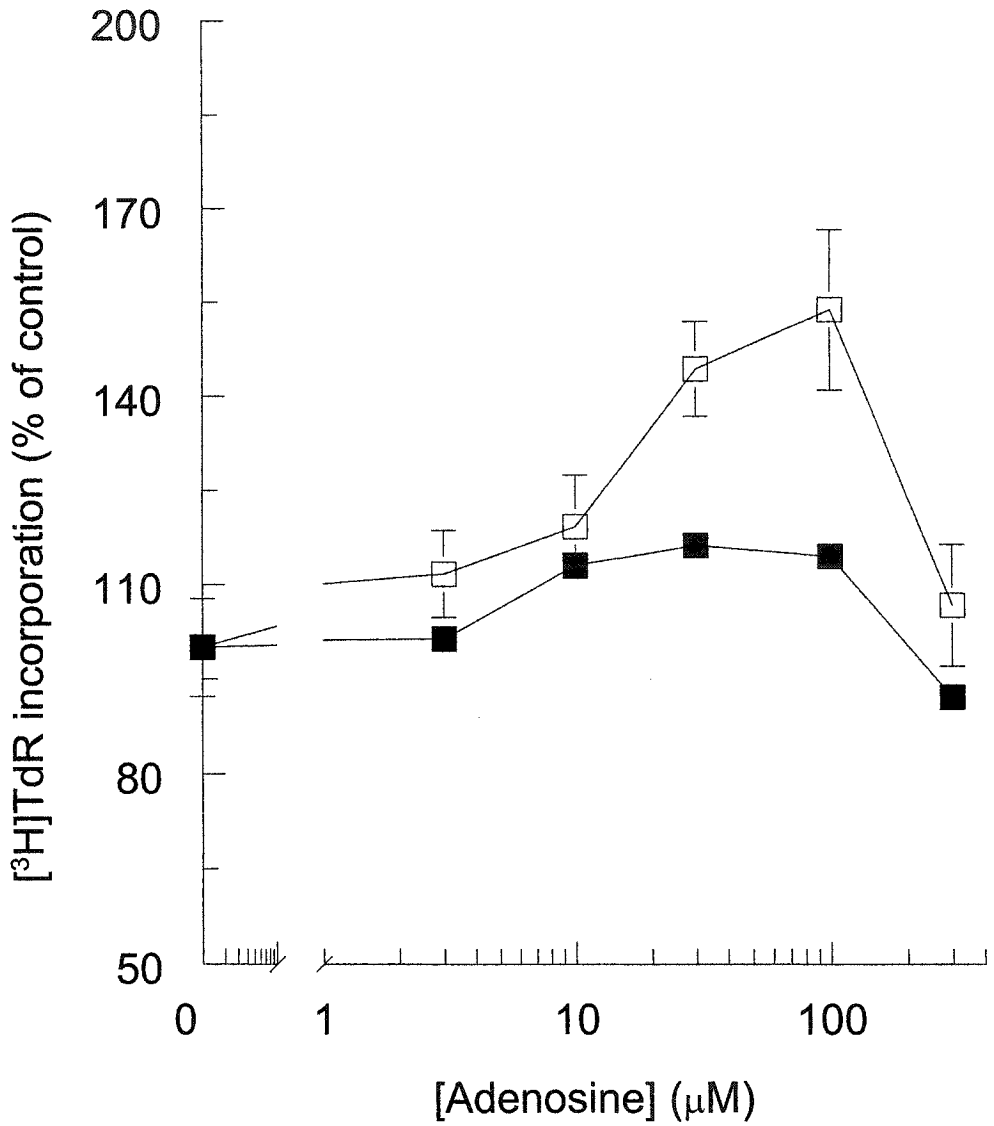

Figure 3.17: Effect of glucose concentration on the magnitude of the response of HT-29 cells to adenosine. HT-29 cells were exposed to culture medium containing either 4.5g/L of glucose (■) or glucose-free medium (□) and treated with adenosine (3-300μM). Panel is representative of five independent experiments. Data points and error bars (may be within symbols) represent mean ± SEM of four wells within a culture plate.

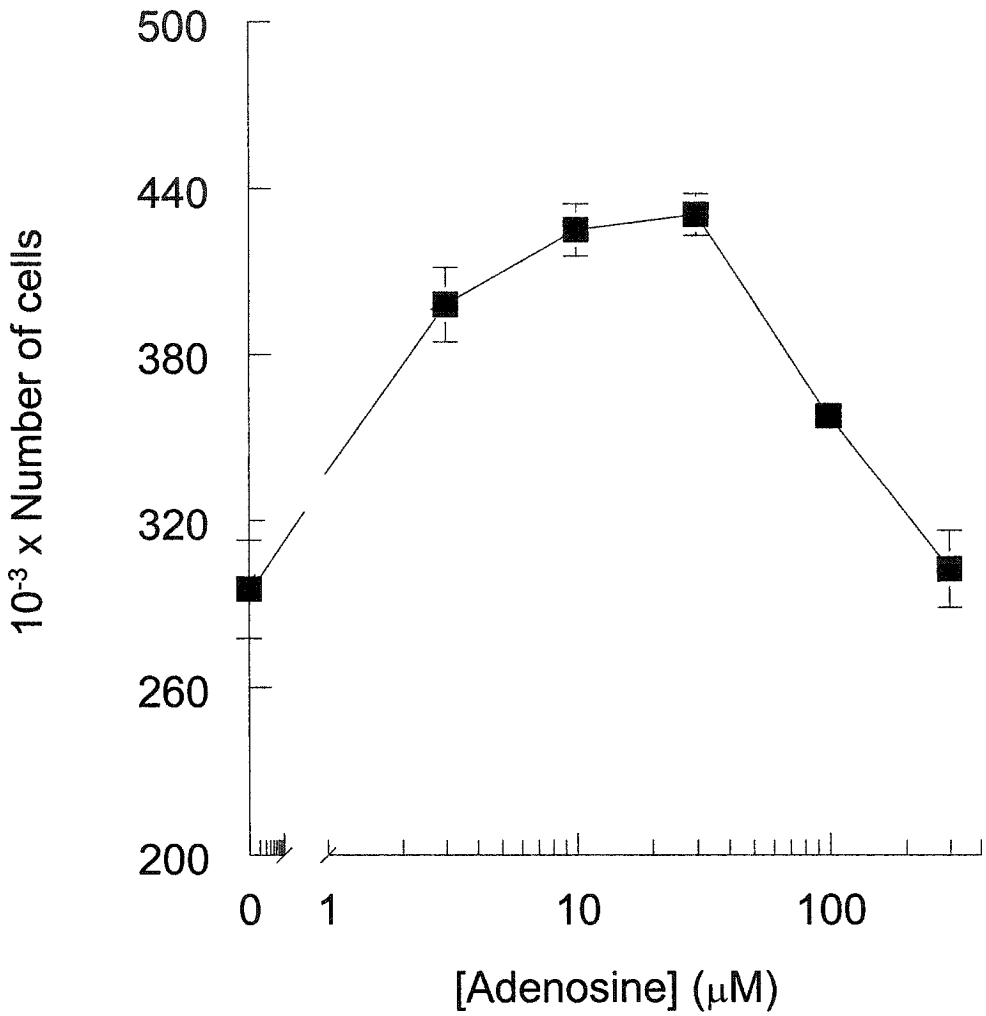

Figure 3.18: Effect of low-glucose containing media on the response of HT-29 cells to adenosine. HT-29 cells were cultured in media containing 0.5g/L glucose and exposed to adenosine ranging from 3-300μM. Data points and error bars (may be within symbols) represent mean ± SEM of quadruplicate wells within a representative experiment.

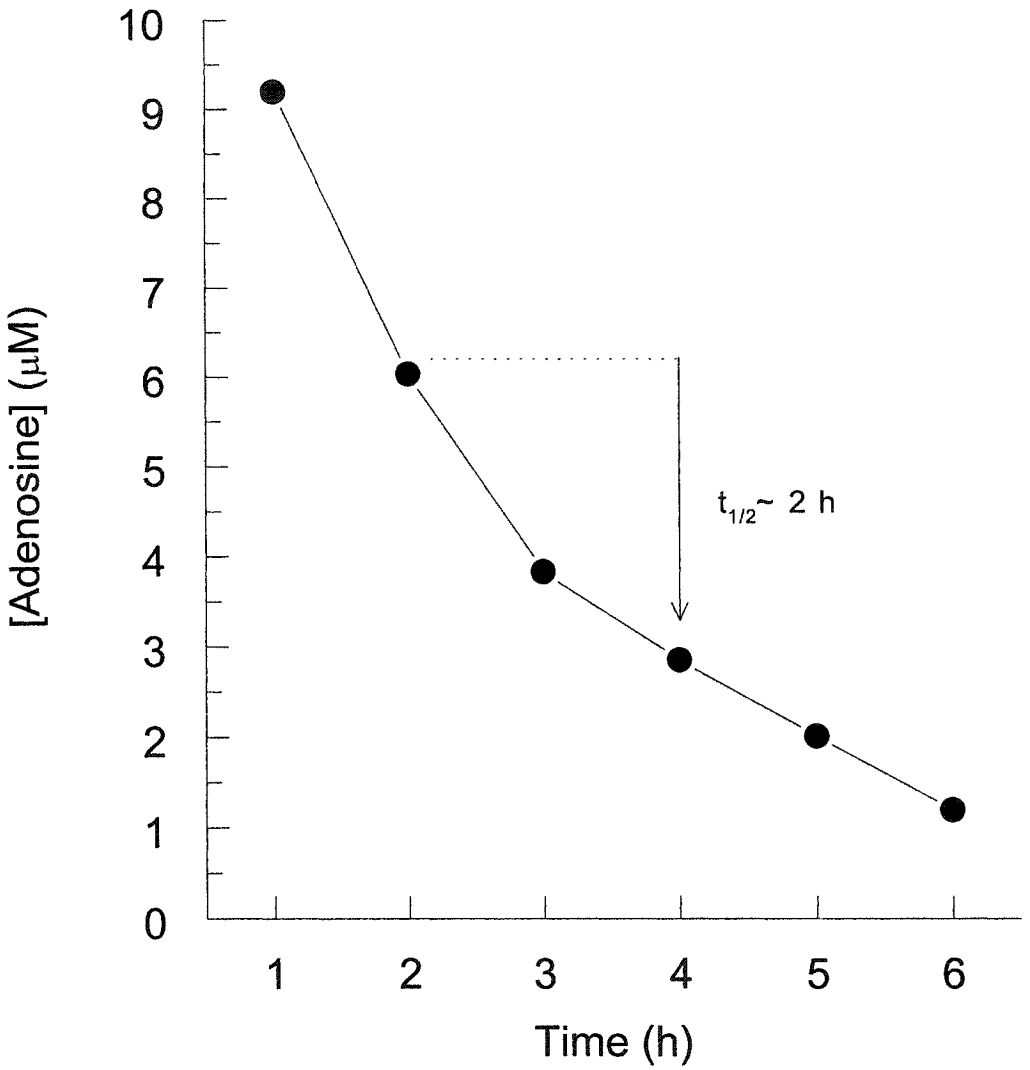

Figure 3.19: Rate of metabolism of adenosine by HT-29 cells in culture. HT-29 cells were exposed to a single initial adenosine dose of 10μM. Data points are means of duplicate cultures.

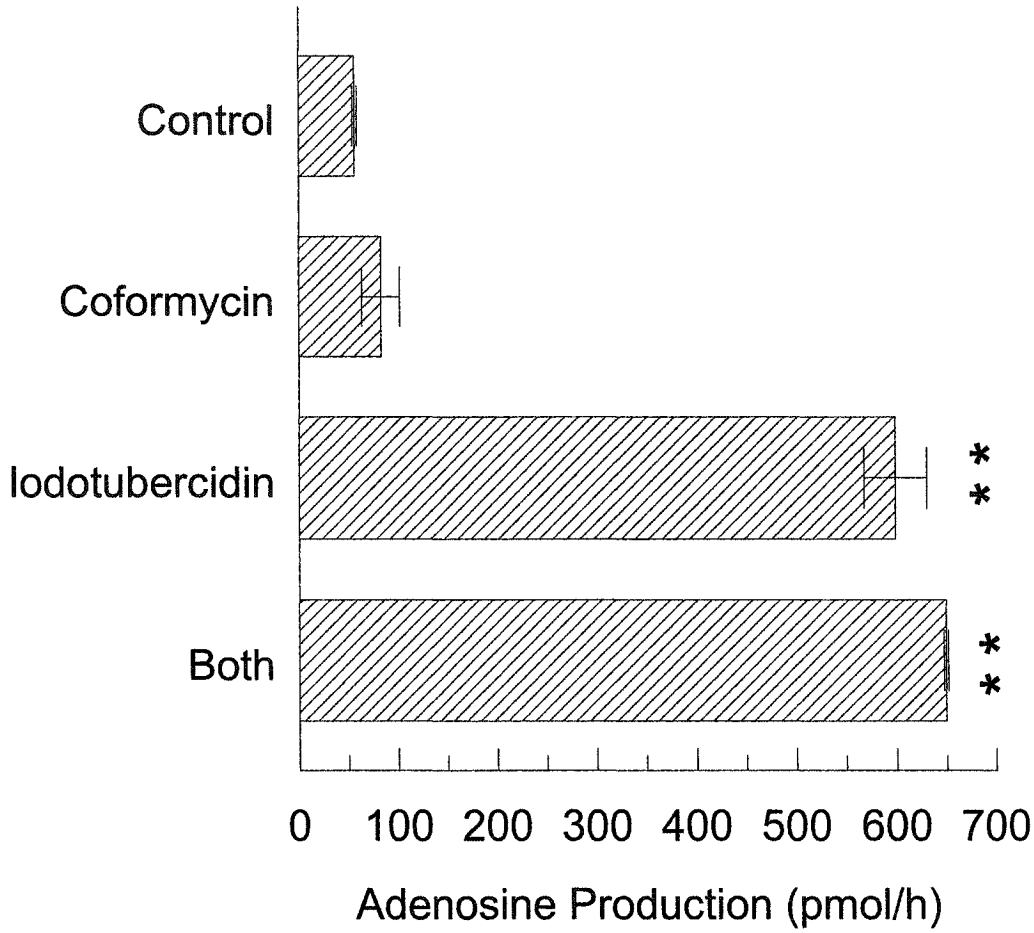

Figure 3.20: Involvement of adenosine deaminase and adenosine kinase in the production of adenosine by HT-29 cells. Data and error bars represent mean ± SEM of determination from triplicate cultures. Significantly different from control: $*P < 0.05$; $**P < 0.01$.

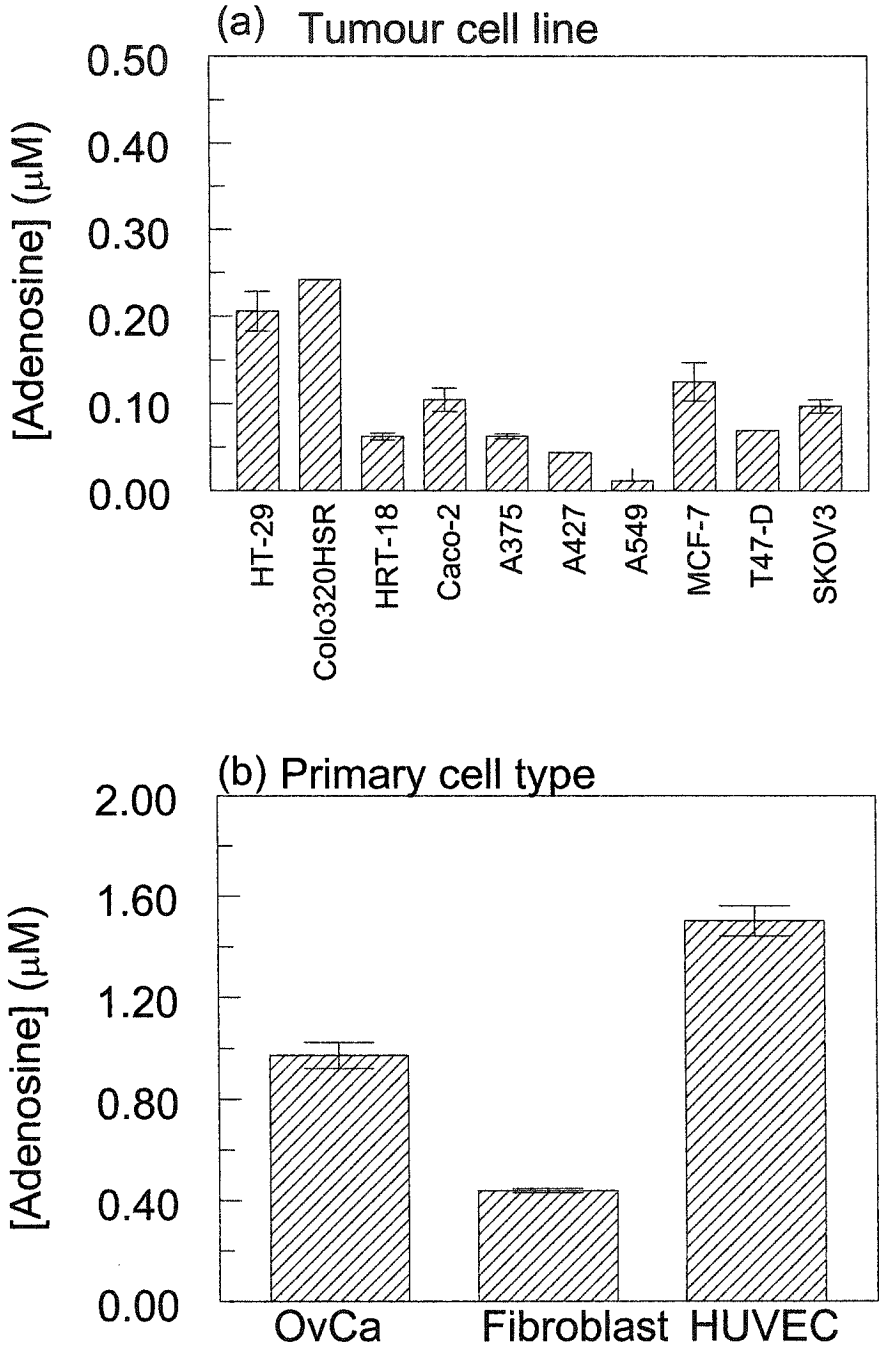

Figure 3.21: Concentration of adenosine measured in culture media of human cells exposed to hypoxia. (a) Human tumour cell lines [colorectal (HT-29, HRT-18, Colo320*HSR*, and Caco-2); melanoma (A375); lung (A427 and A549); breast (MCF-7 and T47D); and ovarian (SKOV3)]; (b) primary cells [ovarian (OvCa), fibroblasts, and HUVECs] were exposed to hypoxia for 6h and adenosine measurements made thereafter. Data and error bars (may be within symbols) represent mean ± SEM of determinations from triplicate cultures.

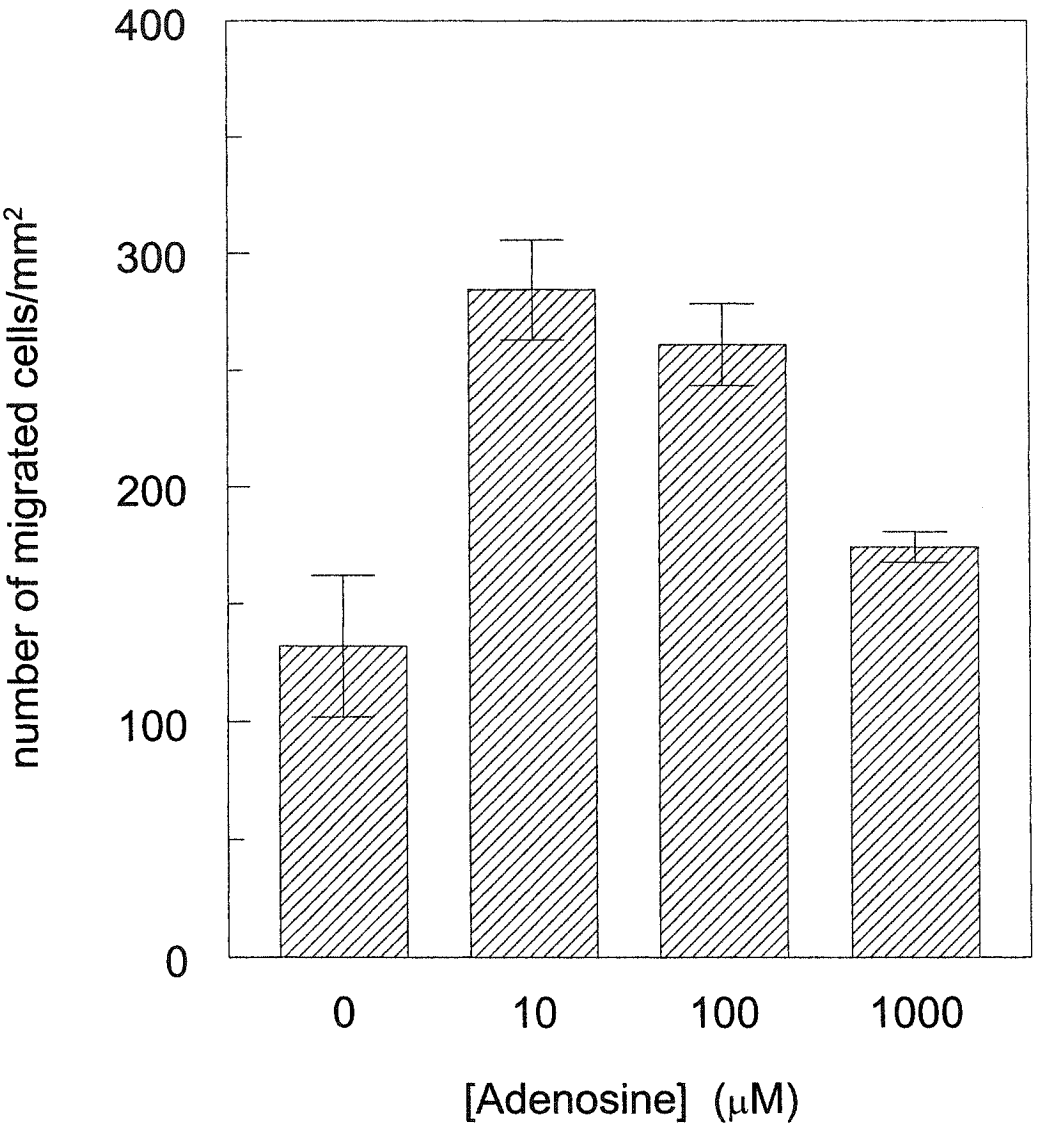

Figure 3.22: Effect of adenosine on HRT-18 cell migration. HRT-18 cells were seeded in the upper chamber of Transwell® culture inserts. Adenosine (10-1000μM) was added to the bottom chamber. The average number of migrated cells/mm^2 determined. Data and error bars represent mean ± SEM of four independent experiments.

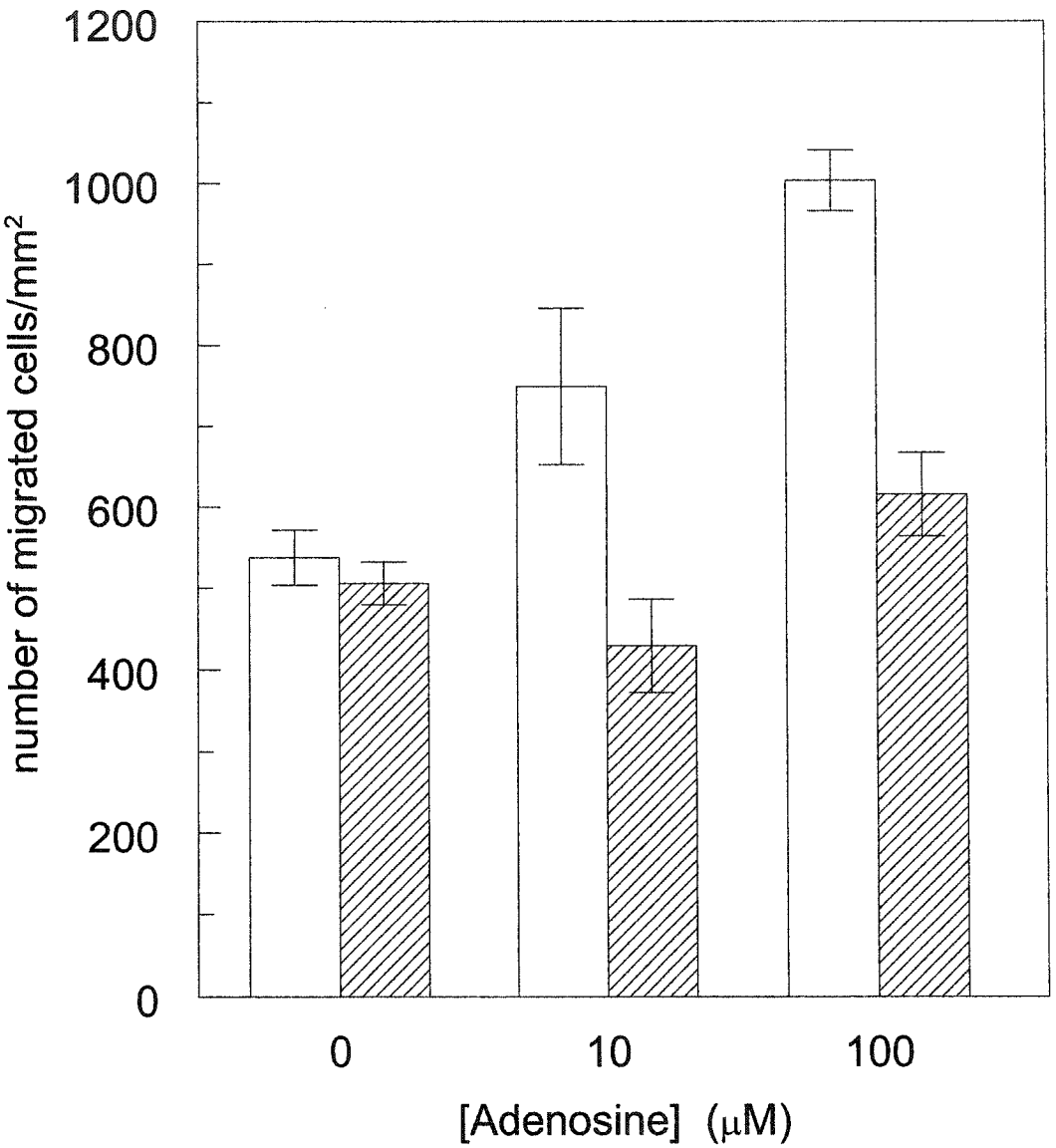

Figure 3.23: Effect of adenosine deaminase on adenosine-induced migration. HRT-18 cells were seeded in the upper chamber of Transwell® culture inserts. Additions of adenosine (10-1000μM) in presence (hatched bars) or absence (open bars) of ± 0.5U ADA were made to the bottom chamber. The average number of migrated cells/mm^2 was determined. Data and error bars represent mean ± SEM of three culture inserts within a representative experiment.

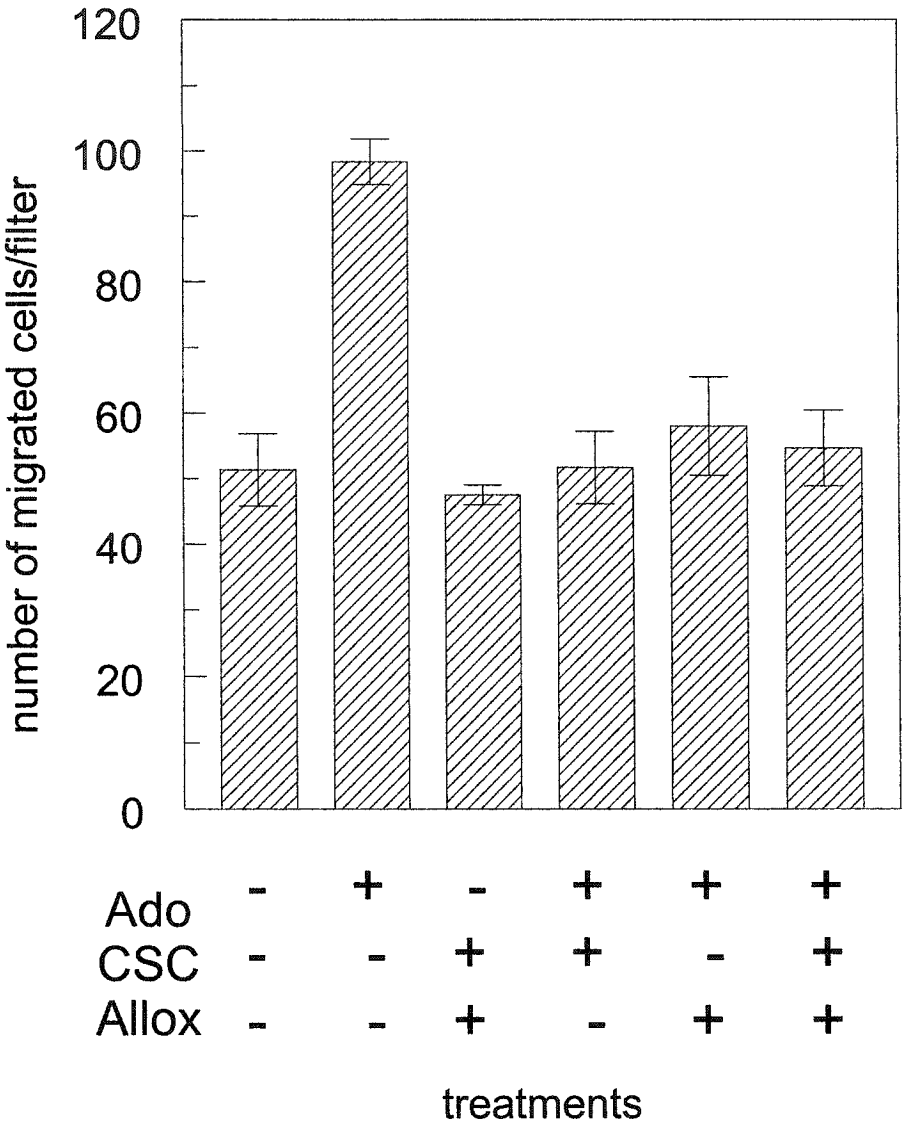

Figure 3.24: Effect of adenosine A_2 receptor antagonism on adenosine-induced migration. HRT-18 cells were pre-treated with CSC (1μM), alloxazine (5μM), or both for 30 min and seeded in the upper chamber of Transwell® culture inserts. Adenosine (10μM) was added to the bottom chamber. The number of migrated cells/mm^2 was determined. Data and error bars represent mean ± SEM of three culture inserts within a representative experiment.

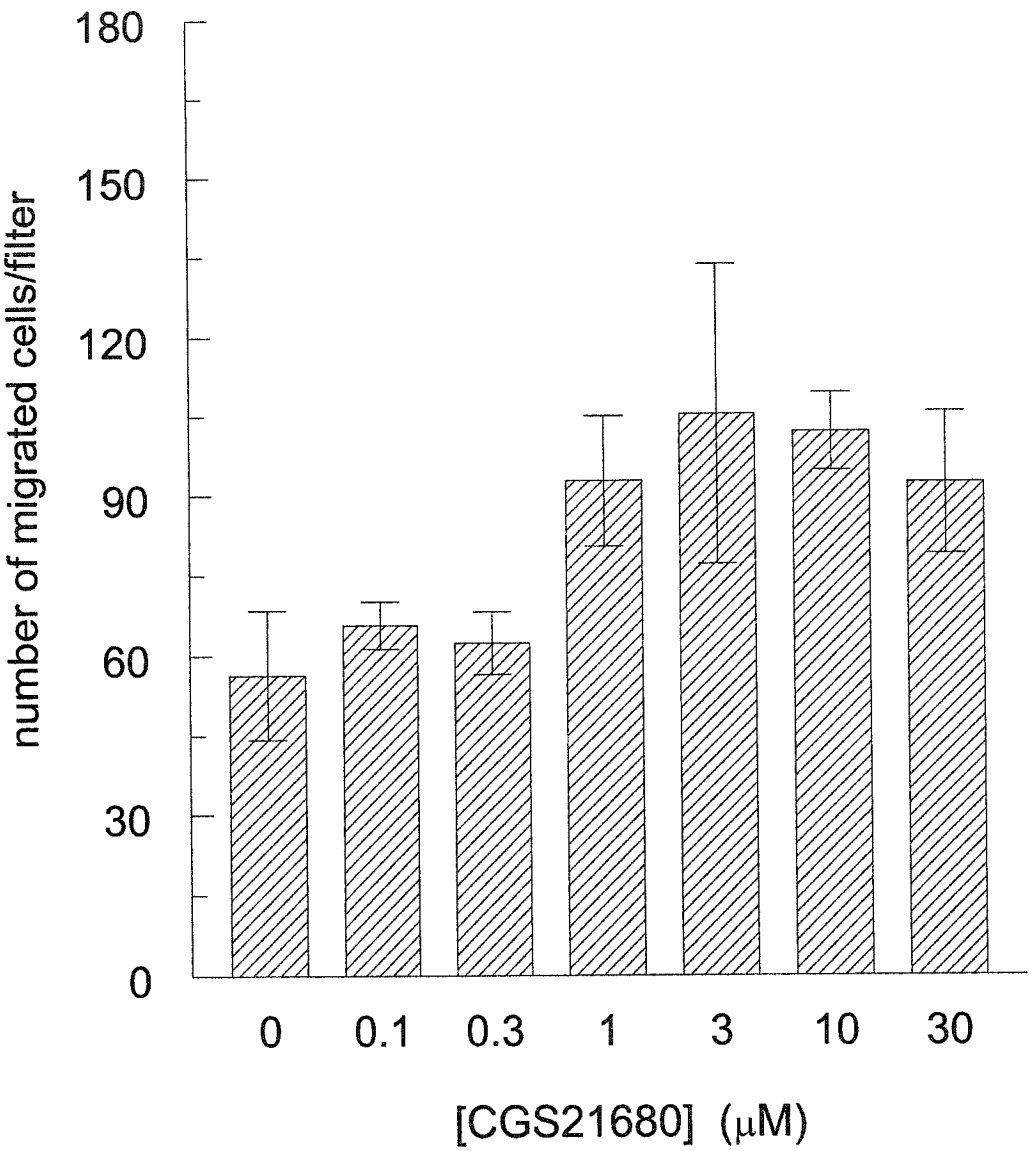

Figure 3.25: Effect of the adenosine A_2-selective agonist CGS21680 on migration. HRT-18 cells were seeded in the upper chamber of Transwell® culture inserts. CGS21680 (0.1-30μM) was added to the bottom chamber. The number of migrated cells/mm^2 was determined. Data points and error bars represent mean ± SEM of three culture inserts within a representative experiment.

Table 3.1: Potency of adenosine in stimulating DNA synthesis in human colorectal carcinoma cells

Cell line	EC_{50} (μM) (mean ± SEM)
HT-29	5.7 ± 0.6 (n = 6)
T84	3.8 ± 1.0 (n = 4)
Colo320*HSR*	6.8 ± 0.4 (n = 4)
Caco-2	30.0 ± 7.7 (n= 4)
HRT-18	17.5 ± 1.3 (n=4)

Cultures of carcinoma cells were treated with single doses of adenosine over the concentration range 1-300μM and the effect on DNA synthesis was determined. Individual dose-response curves were determined using four to eight replicate cultures at each adenosine concentration. The EC_{50} within each experiment was defined as the concentration of adenosine that produced a half-maximal stimulation of DNA synthesis. The table shows mean ± SEM of values for each cell line derived from four to six completely separate experiments.

Table 3.2: Effect of single adenosine receptor antagonists on the adenosine-mediated stimulation of proliferation in colon carcinoma cell lines

Receptor Antagonist	HT-29	T84	HRT-18	Colo320*HSR*	Caco-2
DPCPX	(1/6)	-	-	-	-
CSC	(1/6)	(1/4)	-	-	(1/3)
Alloxazine	(2/6)	(1/4)	(3/4)	(1/4)	(1/3)
MRS1191	(1/6)	(1/4)	(1/4)	-	-
MRS1220	(1/6)	-	-	-	-
MRS1523	-	-	(1/4)	-	-

Receptor antagonists were added at a final concentration of 20μM with the exception of alloxazine, which was added at a final concentration of 50μM.

Data are expressed as # of experiments where an inhibition ($P < 0.05$) was observed/total number of experiments.

" – " no inhibition of the adenosine response

Table 3.3. Effect of adenosine receptor antagonists in combination on the adenosine-mediated stimulation of proliferation in HT-29 cells

Receptor Antagonist	# of observed inhibitions of the adenosine response/ total # of experiments
D+C	0/6
D+A	2/6
D+M	3/6
C+A	3/6
C+M	2/6
A+M	2/6
D+C+A	3/6
D+C+M	2/6
D+A+M	2/6
C+A+M	2/6
D+C+A+M	4/6

Adenosine was added at a final concentration of 10-30μM. Receptor antagonists were added at a final concentration of 10μM (DPCPX, CSC, and MRS1191) and 50μM for alloxazine.

Inhibition of the adenosine response ($P < 0.05$).

Table 3.4: Adenosine receptor expression in five human colorectal carcinoma cell lines

Cell line	A_1	A_{2a}	A_{2b}	A_3
HT-29	+	++	+++	++
T84	+	++	+++	++
HRT-18	+	+	+++	+
Colo320*HSR*	-	+	++	+
Caco-2	-	+	++	+

RT-PCR was performed on carcinoma cell cultures as described in "Materials and Methods".

" – " not detectable
" + " detectable expression
" ++ " moderate expression
" +++ " strong expression

Table 3.5: Checkerboard analysis of adenosine-induced HRT-18 cell migration. HRT-18 cells were seeded onto Transwell® culture inserts. Different concentrations of adenosine (1-100μM) were added to both top and bottom chambers. The average number of migrated cells/mm^2 was determined. Values represent means of three determinations.

		Upper Chamber			
		0	1μM	10μM	100μM
Lower Chamber	0	582	543	700	761
	1μM	465	487	616	549
	10μM	857	739	829	543
	100μM	846	762	756	610

4. DISCUSSION

4.1 What effect does adenosine have on the growth of colon carcinoma cells?

Recently there have been several reports suggesting that adenosine has a differential effect on the growth of tumour and non-tumour cells and that adenosine may act to selectively kill tumour cells (Fishman *et al.,* 2000; Ohana *et al.*, 2001). Moreover, the same group has proposed the use of specific adenosine agonists as chemotherapeutic agents for the treatment of colon cancer (Fishman *et al.,* 2001; Ohana *et al.*, 2003). Given the extent of the controversy on the role for adenosine in tumour cell growth (Introduction, Section 1.7), this suggestion may be premature. Indeed several groups have found adenosine to be growth-promoting to cancer cells (Orrico *et al.,* 1991; Lelièvre *et al.*, 1998a & b; Mujoomdar *et al.*, 2004).

The first objective of this work was to determine if adenosine increased or decreased the growth of colon carcinoma cells in culture, using a panel of cell lines and paying careful attention to the assay protocol.

4.1.1 Adenosine consistently stimulates DNA synthesis in five colon carcinoma cell lines

As previously discussed, there exists extensive controversy in the literature regarding the effect of adenosine on cell growth. The reported effects of adenosine on normal cells vary considerably. Firstly, adenosine has been found to increase DNA synthesis or cell growth in mammary epithelial cells (Yuh and Sheffield, 1998), arterial

endothelial cells (Dubey *et al.*, 2002), COS-7 monkey kidney fibroblasts (Lemmens *et al.*, 1996), human umbilical vein endothelial cells (Ethier *et al.*, 1993; Sexl *et al.*, 1995), and chick astrocytic cells (Rathbonee *et al.*, 1992). Conversely, adenosine has been shown to inhibit the growth of certain normal cells including 3T3 murine fibroblasts (Ishii and Green, 1973), peripheral blood lymphocytes (van der Krann, *et al.*, 1986), and smooth muscle cells (Dubey *et al.*, 1997; 2000).

The reported effects of adenosine on tumour cell growth are equally contradictory. Adenosine has been shown to promote the growth of HL-60 leukemic cells (Orrico *et al*, 1991), human colon carcinoma cells (Lelièvre *et al.,* 1998a; 1998b), and human breast carcinoma cells (Mujoomdar *et al.*, 2004). In complete contrast, it has been suggested that adenosine acts specifically via the A_3 receptor to inhibit the growth of tumour cells (Fishman *et al.*, 2000; Ohana *et al.*, 2001) and that A_3 agonists may therefore be potential selective chemotherapeutic agents (Fishman *et al.*, 2001; Ohana *et al.*, 2003).

The present study examined the effect of adenosine on the growth of five human colon carcinoma cell lines. Throughout all experiments, we failed to find evidence of an inhibitory or cytotoxic effect of adenosine. Moreover, the typical response of the colon carcinoma cells to adenosine was one of increased proliferation. The adenosine-mediated stimulation of DNA synthesis was typically observed at concentrations above 3μM. Across all cell lines, low micromolar concentrations of adenosine were growth-promoting. Low micromolar concentrations of adenosine are pathophysiologically relevant. Such levels are expected to be present in the microenvironment of solid tumours due to tumour hypoxia (Blay *et al.*, 1997).

The overall shapes of the adenosine dose-response curves varied between the cell lines studied, although biphasic response curves were obtained for T84, HRT-18, and Caco-2 cells, with a decline in the adenosine stimulation at concentrations greater than 100μM (Figure 3.5a, b, and d). Despite the decline in DNA synthesis at high adenosine concentrations, the level of DNA synthesis was still greater than control. It was possible that this decrease in the adenosine response at high concentrations was due to cell death superimposed upon the growth stimulation. The trypan blue dye exclusion test was therefore used to examine the effect of adenosine on cell viability. No evidence for adenosine-induced cell death was observed across the concentration range used in the present study (data not shown). Similarly, there was no effect observed in the MTT assay, which measures mitochondrial succinate dehydrogenase activity and provides sensitive measurements of cell viability and metabolic activity at adenosine concentrations up to 300μM. This agrees with our previous finding in studies of breast carcinoma cells, in that we failed to observe an inhibitory effect of adenosine on breast carcinoma cells at concentrations less than 600μM using the MTT assay and the JAM assay (which measures the fragmentation of pre-labelled DNA a hallmark of apoptotic cell death) (Mujoomdar *et al.*, 2004). Although more sensitive methods such as TUNEL may detect early apoptotic events, our data suggest that it is unlikely that adenosine at concentrations used in the present study cause cell death. Adenosine has been shown to induce tumour cell apoptosis (Bajaj *et al.*, 1983; Tanaka *et al.*, 1994), but the required concentration of adenosine typically significantly exceeds 100μM. Lower concentrations of adenosine have been shown to induce apoptosis only in the presence of ADA inhibitors (Tanaka *et al.*, 1994; Barry and Lind, 2000). The concentration of adenosine

within the solid tumour microenvironment is in the low micromolar range (Blay *et al.*, 1997) and although likely higher in hypoxic foci and necrotic areas, is unlikely to approach the concentrations needed to cause cell death.

If not cell death, then what is responsible for the decline in DNA synthesis at adenosine concentrations greater than 100μM? It may be that in the presence of a high concentration of adenosine there are changes in the expression or distribution of adenosine receptors. Like other G-protein-coupled receptors (GPCR), adenosine receptors are subject to desensitization, internalization, and down-regulation (Trincavelli *et al.*, 2002). Desensitization is evident when receptor signaling processes plateau and then diminish despite continued presence of agonist and is associated with the uncoupling of the GPCR from its associated G-protein (Bunemann and Hosey, 1999). Removal of the agonist reverses desensitization. Desensitization has been reported for all four adenosine receptor subtypes (Mundell *et al.*, 2000; Trincavelli *et al.*, 2000; Escriche *et al.*, 2003). Desensitization can occur in minutes, whereas receptor down-regulation is induced by chronic exposure to agonists and occurs over hours to days. Down-regulation involves decreased total receptor levels through increased intracellular degradative pathways and/or modulation of mRNA transcription/translation (Trincavelli *et al.*, 2002). It is possible that a high concentration of adenosine may induce changes in receptor function by altering sensitivity or receptor number through desensitization and down-regulation, respectively. In addition, the five cell lines used herein differ with respect to their phenotype, cell-substratum contacts, basal growth rate, and differentiation status. These characteristics may influence their responses to growth-modulating agents, including adenosine.

4.1.2 Pathophysiological concentrations of adenosine stimulate DNA synthesis in colon carcinoma cells

The EC_{50} value for adenosine stimulation (single dose-concentration of adenosine added at t=0 that produced one half-maximal response) was typically below 20μM (Table 3.1). These values fall within the concentration range of adenosine expected to be present *in vivo* (Blay *et al.*, 1997). The Caco-2 cell line was the only line that had an EC_{50} value greater than 20μM and whose value varied considerably. Interestingly, Caco-2 cells are unique compared with the other cell lines used in that Caco-2 cells are capable of undergoing spontaneous differentiation in culture. It may be that changes in cell topology as a result of differentiation may alter the response of cells to adenosine, for example, by altering cell-surface adenosine receptor expression and/or distribution.

Usually, a single addition of adenosine to cultures was made at t=0 and the resulting effect on DNA synthesis was measured at t=48h. The half-life ($t_{1/2}$) of adenosine in our culture system has been measured previously and it was found to range from 40min to 3h depending on the cell line used (Mujoomdar *et al.*, 2003; data not shown). Given that a single dose of adenosine was sufficient to stimulate DNA synthesis, coupled with our information regarding the relatively rapid $t_{1/2}$ of adenosine in culture, this suggested that this concentration range probably represents an overestimation of the minimum pro-stimulatory concentration of adenosine. Although the concentration of adenosine in the tumour microenvironment is in the range of 3-10μM, the concentration of adenosine likely varies between regions of the solid tumour. Furthermore, *in vivo*, a persistent and sustained concentration of adenosine would be present. It was possible

that a lower steady-state concentration of adenosine would stimulate DNA synthesis. Repeated additions of lower concentrations of adenosine to HT-29 cell cultures over a 12h period produced a similar dose-response curve for growth promotion as single dosing, but with a leftward shift indicating an increase in the potency (with a decrease in the EC_{50} of nearly one order of magnitude) of adenosine in stimulating DNA synthesis. These data indicate that tumour cells are sensitive to lower concentrations of adenosine when the latter is continually present over a longer period. Therefore, even low levels of adenosine produced by metabolically active cells in the presence of mild hypoxia may promote tumour cell growth.

Despite observing an increase in the potency of adenosine at a lower steady-state concentration, the single-dosing approach was used in future experiments to ensure consistency in dosing between experiments. Adenosine given as a single dose is overall representative of the concentration of adenosine *in vivo*. A concentration corresponding to an EC_{50} of approximately 6μM for adenosine stimulation of HT-29 cells (with a $t_{1/2}$ of approximately 90 min to 2h) using single dosing (Table 3.1) would translate into an hourly dose of 500nM over a 12h period.

4.1.3 Adenosine analogues may not fully represent native adenosine

Some studies that evaluated the effect of adenosine on cell growth have used adenosine analogues (van der Krann *et al.*, 1986; Tey *et al.,* 1992; Tanaka *et al.*, 1994; Imura and Shimohama, 2000; Fishman *et al.*, 2000; Merighi *et al.*, 2002; Trincavelli *et al.,* 2003). When comparisons with native adenosine were made, frequently only a single concentration of adenosine was used to examine the effect. For example, Tanaka and

colleagues (1994) found that adenosine at a concentration of 1mM induced apoptosis of HL-60 human leukemic cells. Similarly, work by Imura and Shimohama (2000) demonstrated that the same concentration of adenosine was also cytotoxic to glial cells. We also demonstrated that adenosine at millimolar concentrations can have cytotoxic effects (Mujoomdar *et al.*, 2004; data not shown) but; in the present study, much lower (and more reasonable) concentrations of adenosine were examined. We also chose to examine a range of adenosine concentrations to thoroughly study the effect of adenosine on tumour cell growth and to detect potential biphasic effects. Surprisingly, many other studies relied exclusively on the use of adenosine analogues (Dubey *et al.*, 2000; Fishman *et al.*, 2001; Dubey *et al.*, 2002; Ohana *et al.,* 2003); the effects of these analogues were then attributed to adenosine. Caution should be taken when interpreting data obtained from the use of adenosine analogues only. Adenosine analogues are typically more potent at certain receptor subtypes. Therefore, signaling through a particular receptor subtype(s) that was activated by the analogue may result in a cellular response different than what would have been obtained had native adenosine been used. Adenosine, at an appropriate concentration can concurrently activate all adenosine receptor subtypes that are expressed on a particular cell type.

The most common argument for using adenosine analogues is that adenosine is metabolized rapidly. Therefore, a sustained concentration of adenosine that would be capable of exerting a variety of cellular effects including modulation of the cellular growth response cannot be maintained. Often, these reports suggest that the use of analogues is advantageous because they are more stable than adenosine, although the majority of reports do not provide any data directly demonstrating rapid metabolism of

adenosine in their particular cellular system. Indeed, we have found that the rate of adenosine metabolism is variable even between cell lines of the same origin (data not shown). Although the metabolism of adenosine is rapid (seconds) in some biological systems (Moser *et al.*, 1989), in our cellular system, the rate of adenosine is considerably slower. In addition, our approach of approximating a steady-state adenosine concentration by providing repeated doses of adenosine was neither performed in the various other studies nor was it even suggested as an alternative to using analogues.

Some adenosine analogues may have effects that could be adenosine-independent. For example, Ceruti and co-workers (2000) used 2-chloroadenosine (2-CA), an adenosine analogue, to investigate the effect of adenosine on astrocytoma cell death. They found that 2-CA at a concentration of 100μM (which correlated to the EC_{50} value) induced apoptosis. At a concentration of 100μM, adenosine receptor activation was not the mechanism of 2-CA-induced cell death. It is unclear why such a high concentration of 2-CA was used to study adenosine-like effects. Only a low micromolar concentration of 2-CA is needed to activate all four adenosine receptor subtypes (Fredholm *et al.*, 1994). Interestingly, we have observed dramatic changes in tumour spheroids treated with 2-CA at a concentration as low as 1μM, that are not seen with adenosine. 2-chloroadenosine caused a dramatic reduction in spheroid cell number, which is in contrast to spheroids treated with adenosine. We observed that treatment of tumour spheroids with 2-CA also rendered the spheroids more sensitive to dissociation by trypsin (unpublished observations). This may be due to 2-CA-induced changes in cell-cell contacts and cell adhesion properties, which may, in turn, lead to cellular apoptosis through anoikis. Rufini and colleagues (1997) demonstrated that 2-CA induced cell detachment and

apoptosis in C2C12 myoblastic cells. They reported that the cytoskeleton was a downstream target for 2-CA action and that apoptosis was preceded by disarrangement of the main cytokeletal component, the actin microfilaments. These data give strength to the argument that the actions of some adenosine analogues may not truly represent the actions of adenosine.

Adenosine studies also often make use of agents that inhibit its metabolism or conversion, thereby sustaining its concentration, e.g. ADA inhibitors. However, routinely inhibiting ADA activity to sustain adenosine levels and prevent its metabolism may lead to misrepresentation of effects by adenosine because ADA itself has signaling capabilities through binding to cell-surface proteins including DPPIV (also known as ADAcp and CD26) and to A_1 and A_{2b} adenosine receptors (Saura *et al.*, 1996; Herrera *et al.*, 2001).

Clearly it is important to study native adenosine. Any possible non-adenosine actions of adenosine analogues did not confound the interpretation of our results. In our hands, a single dose of adenosine, in the absence of inhibitors of metabolism, consistently increased DNA synthesis.

4.1.4 The [^{3}H]TdR incorporation assay is an appropriate tool for measuring the response of cells to adenosine

To ensure that adenosine treatment not only stimulated DNA synthesis in human colon carcinoma cells, but also resulted in an increase in cell proliferation, HT-29 cell number was measured in response to adenosine treatment. Adenosine stimulated cell

proliferation and showed concentration dependence with the maximal effect reached at a dose of 30μM adenosine with an EC_{50} of approximately 10μM, similar to that of the DNA synthesis assay system. Given that we found that adenosine stimulated DNA synthesis using the [^{3}H]TdR incorporation assay and this increase in DNA synthesis correlated with an increase in cell number, we chose to use the [^{3}H]TdR incorporation assay in the majority of our future experiments. However, in spite of the parallel response in cell number, and despite the fact that the [^{3}H]TdR incorporation assay has been used in previous studies investigating adenosine, validate its use in our particular system under our methodological conditions. Moreover, we wanted to determine if variation in the methodology of the [^{3}H]TdR incorporation assay used in different studies could account for the discrepancies noted in the literature regarding adenosine's effect on cell growth.

Our standard [^{3}H]TdR incorporation assay protocol involves addition of [^{3}H]TdR at a final concentration of 1μCi/ml, added simultaneously with adenosine. No more than 48h later (prior to cytokinesis), incorporation of [^{3}H]TdR into newly formed DNA is measured. This approach to labeling ensures that all cells traversing S phase are captured by the assay and avoids misinterpretation due to alteration in cell cycle kinetics under different culture conditions. The length of exposure of cells to [^{3}H]TdR can vary between assay protocols used by different workers. A shorter 'pulse-label' is sometimes favoured (e.g. 4 hours was used by Dubey *et al.*, 2000). Pulse-labeling involves the addition of [^{3}H]TdR either at the start of S-phase or during S-phase. Therefore, information regarding the timing of the cell cycle is critical. For example, if the cells were labeled when they were not in S-phase or when only a small proportion of cells remained in S-

phase, a stimulation by adenosine may have been either missed or under interpreted. We chose not to pulse-label in these experiments because cumulative labeling eliminates concerns about appropriate pulse label timing.

As mentioned, adenosine and [^{3}H]TdR were added to cell cultures at the same time in our standard assay protocol. In order to exclude the possibility that the exogenously added adenosine was interfering with thymidine transport into the cell, the addition of [^{3}H]TdR was delayed 24h after adenosine treatment. The response of HT-29 cells was once again one of increased growth and showed a very similar pattern to our previous results. Addition of [^{3}H]TdR to HT-29 cell cultures at 24h post adenosine treatment provides time for adenosine to undergo approximately 16 degradation half-lives, after which the exogenous adenosine will certainly have been depleted and unable to interfere with thymidine transport and its subsequent fate.

To ensure that any thymidine and/or adenosine in the calf serum used to supplement the culture medium was not present in an amount that could alter either the specific activity of the [^{3}H]TdR or the concentration of adenosine that was present in the cultures, the calf serum was dialyzed thereby eliminating any appreciable amounts of either nucleoside. We found that there was no difference in the ability of culture media containing either dialyzed or undialyzed sera to support the adenosine response in HT-29 cells. This was consistent with the finding by Traut (1994) that the level of thymidine present in serum is low. These data also argue against the adenosine response being modulated by serum-derived adenosine. This follows given that we found that adenosine levels present in newborn and fetal calf sera, that are used to supplement our cultures, range from 1-3μM (data not shown). Our [^{3}H]TdR incorporation protocol involves a

'serum-downshift' step in which the culture medium is changed to contain reduced serum (0.5-1%), meaning the amount of adenosine that could be present would be less than 20nM. The cultures are then incubated for a further 48h prior to adenosine/[^{3}H]TdR addition, corresponding to approximately 32 degradation half-lives of adenosine for HT-29 cell cultures. Any adenosine that would have been present would have been completely eliminated by that time. Furthermore, the amounts of thymidine present in serum are unlikely to have confounded our results. No effect on the adenosine-mediated increase in DNA synthesis was observed when either the specific activity of [^{3}H]TdR was either increased or decreased.

4.2 Can cell culture parameters modulate the adenosine response?

4.2.1 Cell density and serum supplementation can modulate the adenosine response

We observed that the magnitude of the adenosine response was greatly influenced by cell culture parameters such as cell density and serum supplementation. HT-29 cells showed a progressive decline in the adenosine response with increasing cell density. The first possible explanation for this is that the adenosine response weakens under conditions during which adenosine is more rapidly metabolized, i.e. at a higher cell culture density where more cells are present and capable of degrading adenosine. However, differences simply in adenosine metabolism could not explain the altered adenosine response because no rightward shift in the adenosine response curves for increasing cell densities was observed. This would be predicted if adenosine was being metabolized more rapidly by a

larger number of cells because at a high cell density a higher initial concentration of adenosine would be required to exert the same effect. This was not the case. Cell density may function as a mechanism through which cells sense their extracellular environment and modulate their responses to mitogenic stimuli accordingly.

Serum supplementation, type and concentration, also had a profound influence on the magnitude of the adenosine response. To this end, the most robust adenosine response occurred in cultures that were exposed to media containing a low concentration of newborn calf serum (NSC) rather than fetal calf serum (FCS). Although the adenosine response was observed under nearly all serum conditions, thereby excluding the possibility that only serum-deprived cultures are sensitive to the mitogenic effects of adenosine, its magnitude decreased as serum concentration increased and was least robust when cells were cultured in the presence of FCS. It may be that the rate of cell replication in the presence of 10% FCS was near maximal and addition of adenosine did not potentiate it to the extent of cells cultured in NCS.

We do not as yet know for certain how and why serum supplementation impacts upon the adenosine response. We can however exclude the more obvious explanation that changes in adenosine metabolism occur due to differences in deaminase activity in serum because the abilities of NCS (1 and 10%) and FCS (10%) to degrade adenosine are low and showed no differences (Mujoomdar *et al.*, 2004). In addition, our laboratory has not detected significant levels of intact immunoreactive ADA in NCS or FCS by Western blotting (Tan and Blay, unpublished data). At this time, our most likely explanation is that serum protein factors are capable of modulating the proliferative signal of adenosine through cell-cell and cell-extracellular matrix (ECM) contacts. Indeed adenosine has

been shown to modulate cell adhesion to the substratum (Abbrachio *et al.*, 1997) and integrin function (MacKenzie *et al.*, 2002). Modulation of these cellular interactions by adenosine might influence either the inherent response of cells to adenosine (whether it is pro- or anti-mitogenic) or the magnitude of adenosine's proliferative signals.

4.2.2 Tumour microenvironmental conditions support the adenosine response

Standard cell culture conditions differ dramatically from those to which a tumour cell would likely be exposed to *in vivo*. As discussed, the TM is relatively harsh because of its hypoxic, glucose-deprived, and acidic conditions. The next objective in this study was to determine if cells exposed to TM-like conditions, specifically acidic and low-glucose conditions, responded similarly to adenosine as standard-cultured cells. Would the TM-conditions support the adenosine response? Would the adenosine response be different under TM-like conditions? I found that HT-29 cells cultured across a wide pH range responded to adenosine with increased DNA synthesis. HT-29 cells exposed to low-glucose conditions also responded to adenosine with increased DNA synthesis and cell number. Although the magnitude of the adenosine response did not differ significantly between standard and TM conditions, there was a suggestion that standard culture conditions may not be optimal to support the adenosine response and that the response might be more potent under TM conditions. For example, the adenosine-mediated increase in HT-29 cell number under low-glucose conditions was robust and evident at lower concentrations of adenosine than cells treated with adenosine under standard culture conditions. Interestingly, the growth of C6 rat glioma multicellular

spheroids was enhanced under low glucose conditions and was inhibited under high glucose conditions (Acker *et al.*, 1992). Further experimentation would be necessary to confirm the observation made in our cellular system. However, importantly these experiments confirm the adenosine response in TM-like conditions and suggest that cells *in vivo* exposed to increased levels of adenosine would likely respond with increased cell proliferation.

4.3 Are the growth promoting effects of adenosine mediated by cell-surface adenosine receptors?

4.3.1 Adenosine receptor antagonists are not capable of consistent blockade of the adenosine response: changes in receptor expression may influence antagonism of the adenosine response

The results of using single selective adenosine receptor antagonists in each of five human colon carcinoma cell lines in an effort to block the adenosine response were somewhat variable, despite repeated and careful experimentation. There was a strong sense that receptor subtype expression and/or coupling was dynamic. However, in all cell lines the adenosine response was blocked in at least one experiment by the A_{2b} antagonist alloxazine. In fact, alloxazine blocked the adenosine response in one-third of all experiments. The A_{2b} receptor has been implicated in the modulation of vascular smooth muscle cell growth (Dubey *et al.*, 1998; 2000; 2002). It may be that the A_{2b} receptor works in concert with additional receptor subtypes to mediate the adenosine response in colon carcinoma cells. Co-expression of functional A_{2a} and A_{2b} receptors has

been demonstrated in the NG108-15 neuroblastoma/glioma cell hybrid line (Mundell and Kelly, 1998). I therefore determined whether combinations of the four receptors antagonists would block the adenosine response. However, the multiple antagonist experiments failed to produce a clear effect even though all but one of the combinations was capable of blocking the adenosine response in at least one of the three experiments. Perhaps receptor activation in the presence of adenosine follows a temporal and/or spatial pattern that cannot be inhibited by receptor antagonist using our current approach. Furthermore, the inability to block adenosine-induced proliferation consistently with a combination of all four antagonists strongly suggests that one or more additional, non-classical receptors may be involved.

T84 cells have been shown to respond differentially to adenosine and its analogues when added to either the apical or basolateral surfaces of a monolayer culture. For example, apical stimulation of T84 cells was less potent at inducing chloride secretion than basolateral addition. In addition, there were differences in adenosine-stimulated cAMP production between the two surfaces (Barrett *et al.*, 1989). Moreover, it has been shown that adenosine receptors on the apical surface of canine tracheal epithelial cells are able to stimulate chloride secretion (Pratt *et al.*, 1986). I examined the effect of adenosine given apically and/or basolaterally to HT-29 cell monolayers and found no difference in their sensitivities to adenosine, although I observed an increased response when both apical and basolateral surfaces were exposed to adenosine concurrently. This model was not studied extensively. It is possible that the apparently complete HT-29 monolayers were in fact 'leaky' and allowed adenosine to pass through the cell layer. However, it is feasible that distinct receptor patterns for apical and

basolateral sides do not occur in HT-29 cells and therefore, differential sensitivities to adenosine may not occur.

The primary mechanism by which adenosine exerts its effect on cell proliferation may be through a non-adenosine receptor pathway, which has been described for other adenosine-mediated cellular responses (Mirabet *et al.*, 1997; Apasov *et al.*, 2000; Peart *et al.*, 2003). An alternative explanation for the ambiguous response to adenosine receptor antagonists is that the growth stimulation is not mediated by adenosine itself, but by one of its precursors or its metabolite, inosine. Adenosine is metabolized to inosine via ADA. Inosine has been found to modulate several biological responses such as inflammatory cytokine production (Haskó *et al.*, 2000) and mast cell degranulation (Jin *et al.*, 1997). However, both of these effects were mediated by the A_3 receptor subtype. Therefore, the action of inosine at A_3 receptors should have been blocked by the A_3 antagonists. Additionally, I have shown that two other adenine-containing molecules, ATP and AMP, can induce cell proliferation in HT-29 cells. It cannot however be ruled out that these effects by AMP and ATP may be in part due to their breakdown in culture since no inhibitors of their metabolism were included in the assay. This adenosine-like effect of AMP and ATP may help to explain the only partially-blocking effects by adenosine receptor antagonists, in that during the antagonist assays, inhibitors of AK are not provided thereby freely allowing the conversion of adenosine to AMP. ATP and the other adenine containing nucleotides bind with higher affinity than adenosine to ATP receptors, or P2 receptors, which are expressed by HT-29 cells (Höpfner *et al.*, 1998). It has also been shown that adenosine (or P1) receptors and P2 receptors can associate. These heteromeric P2-like adenosine receptors have novel pharmacological and

functional characteristics and may provide an additional mechanism for increased diversity of purine signaling (Yoshioka *et al.*, 2001). It is possible that unique receptor expression patterns and/or associations may exist in our cellular system and may influence our ability to antagonize the adenosine response.

Adenosine receptors have been shown to be expressed by many cancer cells and have been implicated in their cell growth processes (see section 4.3). Adenosine receptor expression was examined by RT-PCR in all five human carcinoma cell lines and more extensively in HT-29 and HRT-18 cells. Despite repeated careful experimentation, there were inconsistencies in the results (appended to this thesis) that have made interpretation difficult. However, these data do bear resemblance to the antagonist data. Despite this variability in expression, all four receptor subtypes were expressed by HT-29 and HRT-18 cells. The A_{2b} subtype was consistently expressed and had the strongest level of expression relative to the other subtypes.

Given that cell culture parameters had a profound influence upon the adenosine response, I investigated the effect of cell density, serum supplementation, and treatment with adenosine on adenosine receptor expression in HT-29 and HRT-18 cells. I am unable to conclude whether or not cell density had an effect on the mRNA expression of adenosine receptors. Expression of the A_1 and A_3 subtypes was only detected at cell densities of 10^5 cells/ml and greater and not at the lower cell densities. This is intriguing given that I found the most potent adenosine response occurred at low cell densities ($< 10^5$ cells/ml). It may be that at low cell density adenosine's effects are predominately through a non-adenosine receptor-mediated pathway. It is also possible that at low cell densities receptor number is too low to permit detection by the present methodology.

Expression of cell-surface A_{2b} protein on HT-29 cells could not be detected by radioligand binding assay using a polyclonal antibody to the human A_{2b} receptor subtype (Tan and Blay, unpublished observations). It may be that surface receptor number is not high enough to detect by this method. Alternative methods such as immunoprecipitation and Western blotting may permit A_{2b} receptor detection. The pharmacological data presented herein do however support the expression of the A_{2b} receptor and the functional involvement of this subtype in the mediation of the adenosine response. Currently, agonists with A_{2b} selectivity are not available. Studies that implicate the A_{2b} receptor typically do so by exclusion using receptor agonists with other selectivities. To date, NECA remains the most potent A_{2b} agonist with an EC_{50} value of 2μM (Feoktistov and Biaggioni, 1997). Responses elicited by NECA at concentrations in the low micromolar range, but not by agonists with greater A_1, A_{2a}, and A_3 selectivity, would be characteristic of A_{2b} receptors. Alternatively, the cellular response to adenosine could be measured in the presence of highly selective receptor antagonists for the other three receptor subtypes. If antagonists for these three subtypes failed to block the adenosine response, this too would suggest the participation of the A_{2b} receptor subtype. Presently there are also few potent A_{2b} receptor antagonists available. Alloxazine and enprofylline are most often used. Brackett and Daly (1994) reported that alloxazine was 9-fold more selective as an antagonist at A_{2b} receptors than at A_{2a} receptors. Enprofylline, although not potent, has been shown to be 22-fold more selective for A_{2b} versus A_1, 5-fold versus A_{2a}, and 6-fold versus A_3 in stably-transfected CHO cells (Robeva *et al.,* 1996). There is one recently published study using an A_{2b} antagonist that was not used in this study, MRS1706 (Trincavelli *et al.,* 2003). To date, this is the most potent A_{2b} antagonist commercially

available with K_i of 1.39nM. This compound may help to elucidate the involvement of the A_{2b} receptor in both the proliferative and migratory responses.

Cell density has been shown to modulate numerous responses including mitogenic responses and growth factor interactions. For example, Richardson and colleagues (1999) observed a 3- to 5-fold reduction in bFGF binding per cell as the density of a stromal fibroblast culture was increased. They also found that the binding affinity of bFGF was 10-20 fold higher at low cell densities and that bFGF maximally stimulated cell proliferation at intermediate cell densities. Cultures of varying densities would differ dramatically in cell-cell and cell-ECM interactions which may, in turn, have an effect on gene expression including cell-cycle regulatory proteins (Nakatsuji and Miller, 2001).

Unlike its effect on the biological response of cells to adenosine, serum supplementation did not dramatically alter the expression patterns of the adenosine receptors in HT-29 and HRT-18 cells (Appendix: Table A.2). All four receptor subtypes were expressed under growth conditions (DMEM containing 10% NCS) and only the A_1 receptor was differentially expressed by both HRT-18 and HT-29 cells when the cells were exposed to low-serum (1% NCS) and fetal calf serum (10%) supplementation.

The presence of all four adenosine receptors subtypes (as here in three of five cell lines tested) has also been shown to occur in A375 human melanoma cells (Merighi *et al.*, 2001) and differential expression of the four subtypes has been demonstrated in the human enteric nervous system (Christofi *et al.*, 2001). These findings beg the question, why would a cell express four types of adenosine receptors? Perhaps the net outcome of signal transduction through multiple receptors would likely determine if cell proliferation or cell death occurs. In the TM, the concentration of adenosine is high enough to activate

all four receptor subtypes. Therefore, the number of each receptor subtype and its associated second-messenger systems will greatly influence the cellular response to adenosine.

Although the adenosine response was not consistently blocked by adenosine-receptor antagonists, the response was capable of being antagonized by these agents. This therefore implicates adenosine receptors in the mechanism by which adenosine stimulates DNA synthesis and cell proliferation. As previously discussed, there are several valid explanations for the inability of the antagonists to fully and consistently abrogate the response including the lack of a potent A_{2b} receptor antagonist and the dynamic nature of adenosine receptor expression. Newer approaches, including antisense technology and small inhibitory RNAs (siRNAs), may help to clarify the involvement of particular receptor subtypes.

4.4 What is the contribution of some cells types comprising solid tumour tissue to the production of adenosine?

Our laboratory (Blay *et al.*, 1997) and others (Melani *et al.*, 2003) have found that the extracellular fluid of solid tumours *in vivo* contains elevated levels of adenosine. The concentrations of adenosine are sufficient to exert immunosuppressive effects (Haskó and Cronstein, 2004) as well as mitogenesis, as I have demonstrated. All cells in a tumour are potential sources of adenosine, but my objective was to determine if colon carcinoma cells produced significant amounts of adenosine.

It is well documented that production of adenosine is increased due to hypoxia, so it was not surprising that the rate of adenosine production by HT-29 cells under standard culture conditions was low, approximately 100pmol/h. Inhibition of AK by 5'-iodotubercidin significantly increased the rate of adenosine production. This is consistent with the activity of the enzyme, for which the K_m is well below that of ADA. However, examination of cell lines from numerous origins including colorectal, melanoma, lung, breast, and ovarian revealed that they too produced only small amounts of adenosine when exposed to hypoxia. Interestingly, a single sample of primary tumour cells produced at least four times more adenosine that any other of the cell lines. It is not yet known why the primary cancer cells produced more adenosine than the cell lines. It may be that there are differences in the enzymes involved in adenosine production and/or metabolism, such as 5'-NT and ADA, although additional work would be needed to confirm this. It would be interesting to examine additional primary cancer cells from other origins to determine if they too had a high capacity for adenosine production.

Solid tumour tissue is made up not only tumour cells, but also stromal fibroblasts, blood vessels composed of endothelial and smooth muscle cells, as well as infiltrating cells amongst others. The ability of fibroblasts and endothelial cells to produce adenosine in response to hypoxia was examined. Primary human umbilical vein endothelial cells (HUVEC) produced the most adenosine of all the cell types examined including primary tumour and fibroblast cells, although the primary tumour cells were significant contributors. Vascular endothelial cells have been shown to release ATP (Bodin and Burnstock, 2001) and the release of adenosine from pig aortic endothelial cells during hypoxia and glucose deprivation has been noted (Shryock *et al.*, 1988).

Therefore, it is likely that endothelial cells and perhaps tumour cells are potent producers of adenosine. The adenosine produced, in turn may promote the growth of all cell types comprising a solid tumour.

4.5 In addition to increasing tumour cell growth, does adenosine have any additional tumour-promoting roles?

Adenosine is a known immunosuppressant and has potent inhibitory effects on the immune response to cancer (Introduction, Section 1.6). Adenosine also has a role as a pro-angiogenic molecule (Introduction, Section 1.5). These two roles for adenosine, coupled with my findings that adenosine is mitogenic to tumour cells, provide a solid rationale that adenosine, in the tumour microenvironment, acts as a tumour promoter. My final objective in this study was to determine if adenosine had additional tumour-promoting roles. Specifically, does adenosine promote tumour cell migration? Cell migration is an integral part of the multi-step processes of invasion and metastasis.

4.5.1 HRT-18 cells respond to adenosine with a dose-dependent increase in migration

Adenosine, at concentrations present in the tumour microenvironment and at concentrations that promoted tumour cell growth, stimulated HRT-18 cell migration. This stimulatory effect was observed beginning at concentrations of adenosine as low as 1μM. Maximal stimulation typically occurred at an adenosine concentration of 10μM and persisted at concentrations up to 300μM. The average maximal stimulation was over 170% of control. Adenosine has been shown to be a chemoattractant or a modulator of

migration for non-tumour cells. Adenosine enhances chemotaxis of neutrophils to formyl methionyl peptide (Cronstein *et al.*, 1990) and stimulates migration of endothelial cells in the presence of FCS (Meininger *et al.*, 1988). Meininger and colleagues (1988) observed a two-fold stimulatory effect at an adenosine concentration of 5μM. Pro-migratory effects in response to adenosine have been documented for melanoma cells (Woodhouse *et al.*, 1998). Melanoma arises from the squamous epithelium; to our knowledge, this study is the first to demonstrate that adenosine stimulates motility of a cancer arising from columnar epithelial cells. In addition, this is also the first demonstration that adenosine directly stimulates the motility of colon carcinoma cells in the absence of additional stimulators.

Checkerboard analysis was performed to distinguish between random (chemokinetic) and directed (chemotactic) motility responses to adenosine. Adenosine was both chemotactic and chemokinetic for HRT-18 cells. Woodhouse and co-workers (1998) also found adenosine to be both chemotactic and chemokinetic for melanoma tumour cells.

4.5.2 Adenosine receptors are responsible for adenosine-induced migration

Adenosine receptor antagonists were used to distinguish between a receptor-mediated response and effects through other mechanisms. DPCPX, CSC, alloxazine, MRS1220, and MRS1523 were used in attempt to block the pro-migratory response of HRT-18 cells to adenosine. Both CSC (A_{2a}) and alloxazine (A_{2b}) abrogated the adenosine response, thereby implicating these receptor subtypes in the adenosine response.

The A_{2a} agonist CGS21680 further confirmed the involvement of adenosine and A_2 receptors. CGS21680 stimulated the migration of HRT-18 cells in a dose-dependent manner, initially occurring at 10μM. A biphasic response of HRT-18 cells to CGS21680 was observed. Biphasic responses are typical of migration dose-response curves and relate to the inability of the cell to sense a gradient. For chemotaxis to occur, a cell must be able to distinguish a spatial difference in concentration of the attractant at the cell's leading edge versus its trailing end. This allows the cell to become polarized and sense the concentration gradient. At high concentrations, all receptors are saturated and no gradient can be sensed. These data suggest that the A_{2a} receptor is involved in the adenosine-induced increase in HRT-18 migration. Due to the lack of availability of a selective agonist for the A_{2b} receptor, the involvement of the A_{2b} receptor cannot be ruled out and further experimentation is required to fully examine its involvement.

What is the consequence of increased motility in response to adenosine? The increase in level of extracellular adenosine in the hypoxic regions of solid tumours might stimulate the migration of cells comprising the tumour mass. This stimulation of migration might promote dissemination of tumour cells and the invasion/metastatic process. Also, the angiogenic process in tumours is dependent upon the proliferation and migration of endothelial cells. This process is stimulated by adenosine. Therefore, adenosine in the tumour microenvironment might promote invasion and metastasis by increasing tumour cell and endothelial cell migration.

5. SUMMARY, SIGNIFICANCE, AND FUTURE WORK

5.1 Summary and conclusions

In summary, my data demonstrate that adenosine, at concentrations that would be present in the tumour microenvironment *in vivo* (due to tumour hypoxia), stimulates the growth of human colon carcinoma cells *in vitro*. I observed this mitogenic effect of adenosine using two distinct assay methods, the [^{3}H]TdR incorporation assay and by measurement of cell number. Tumour microenvironment-like conditions supported the adenosine response, and my data may suggest a more pronounced response under TM conditions; further experimentation is required to confirm this observation. The adenosine response in culture was most pronounced when cells were cultured in lowserum-containing medium, but cells exposed to normal culture conditions also responded to adenosine. The adenosine response was also influenced by cell density. Growing cell populations at low cell density were most susceptible to the mitogenic effects of adenosine. Although I do not yet know the mechanism(s) by which serum type/concentration and cell density impact upon the proliferative signals of adenosine, I hypothesize that the modulation of cell-cell and cell-ECM contacts and communication is integral. It dos not depend on differences in the rate of adenosine metabolism

The adenosine-mediated increase in cell growth was not consistently blocked by adenosine receptor antagonists, but my existing data do not rule out their involvement. It is likely that the involvement of adenosine receptors is dynamic and can therefore be modulated by factors known to influence the adenosine response (e.g. the serum and cell density factors mentioned above) and by adenosine itself. My data also suggest the possible existence of other receptor(s) for adenosine.

Tumour cell lines of various origins including colorectal carcinoma produced only low levels of adenosine. Interestingly, primary tumour cells and primary endothelial cells

were significant producers of adenosine. It is likely that these cell types, *in vivo*, contribute to the majority of tumour adenosine production.

In addition to promoting cell growth, adenosine also promoted the migration of HRT-18 cells at similar adenosine concentrations that increased cell growth. This effect was abrogated by adenosine A_2 receptor antagonists.

5.2 Significance and implications of findings

Although it is well documented that adenosine has robust immunosuppressive effects, much controversy exists as to whether adenosine has a growth-promoting role in solid tumours. Conflicting reports have suggested that adenosine either has several important tumour-promoting roles (Spychala, 2000) or that adenosine agonists might be used as chemotherapeutic agents in the treatment of colorectal cancer (Fishman *et al.*, 2001; Ohana *et al.*, 2003). Our data clearly indicate that adenosine consistently promotes the growth of colon carcinoma cells *in vitro*. I have also demonstrated that adenosine promotes migration of another tumour cell type, specifically HRT-18 colon carcinoma cells. Our experimental approach relied on endogenous adenosine itself rather than adenosine analogues. This negates concerns that the effects of adenosine analogues may not be truly representative of adenosine. I have validated the use of the [^{3}H]TdR incorporation assay for measuring the effect of adenosine, and perhaps other nucleosides and nucleotides, on cell growth.

The increase in cell migration, coupled with my data very clearly indicating that adenosine indeed stimulates the growth of colon carcinoma cells, further supports the argument that adenosine has a pro-growth role in solid tumours.

Given that the leading cancers in Canada grow as solid tumours (lung, colon, breast, and prostate), and that the response of solid tumours to treatment is poor, decreasing levels of adenosine in the microenvironment of solid tumours, or interfering with its action, may constitute a therapeutic strategy in the treatment of solid tumours.

5.3 Future directions

To further examine the involvement of adenosine receptors in the adenosine response, additional approaches such as antisense or siRNA technology could be used. Alternatively, adenosine receptor knock-out mice could be examined to confirm or exclude the involvement of particular receptor subtypes.

Solid tumours could be grown in mice using cells that overexpress ADA and the effect on tumour growth could be determined. Alternatively, the local concentration of adenosine could be decreased pharmacologically by local delivery of ADA and/or AK.

Appendix A: Additional adenosine receptor expression data in support of Results Section 3.4.

Table A.1: Effect of cell density on adenosine receptor expression in HT-29 human colorectal cell cultures

Cell density (cells/well)	A_1	A_{2a}	A_{2b}	A_3
12 500	-	-	-	-
50 000	-	-	-	-
100 000	+	-	-	+

RT-PCR was performed on cell cultures as described in "Materials and Methods"

" - " not detectable
" + " detectable expression

Table A.2: Effect of serum type and concentration on adenosine receptor expression in HT-29 and HRT-18 human colorectal cell cultures

	HT-29			
	A_1	A_{2a}	A_{2b}	A_3
10% FCS	-	+	+	+
10% NCS	+	+	+	+
0.5% NCS	-	+	+	+

	HRT-18			
	A_1	A_{2a}	A_{2b}	A_3
10% FCS	-	+	+	+
10% NCS	+	+	+	+
0.5% NCS	-	+	+	+

RT-PCR was performed on cell cultures as described in "Materials and Methods"

" - " not detectable
" + " detectable expression

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