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THE A_3 ADENOSINE RECEPTOR: A LINK BETWEEN INFLAMMATION AND CANCER

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GENERAL INTRODUCTION

ADENOSINE

Adenosine is a nucleoside composed of a molecule of adenine attached to a ribose sugar molecule (ribofuranose) via a β-N9-glycosidic bond (Figure 1).

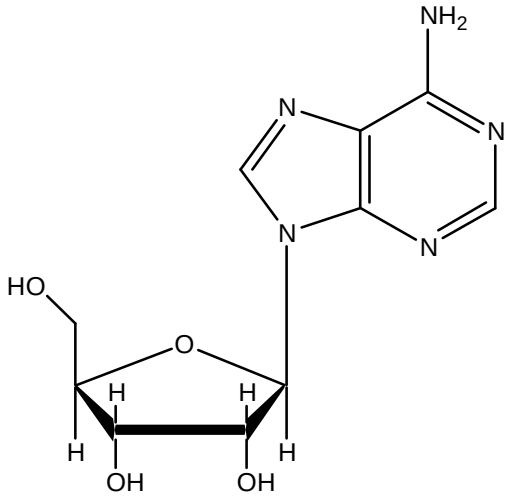


Figure 1 – Chemical structure of Adenosine

Adenosine is an endogenous nucleoside-signalling molecule, which, by acting on specific membrane receptors produces a number of physiological and pathophysiological effects in both the central nervous system and peripheral organs.

Under normal conditions, adenosine is continuously formed intracellularly as well as extracellulalry. The intracellular production is mediated either by an intracellular 5'-nucleotidase, which dephosphorylates AMP or by hydrolysis of S-adenosyl-homocysteine (Fredholm et al., 2001). Adenosine generated intracellular is transported into the extracellular space mainly via specific bi-directional transportes through facilitated diffusion that efficiently evens out the intra-and extracellular levels of

adenosine. The dephosphorylation of extracellular AMP to adenosine, mediated by ecto-5'-nucleotidase, is the last step in the enzymatic chain that catalyzes the breakdown of extracellular adenine nucleotides, such as ATP, to adenosine. Ectonucleotidases include ectonucleoside triphosphate diphosphohydrolase which can hydrolyze ATP or ADP, ectonucleotide pyrophosphatase/phosphodiesterases, alkaline phosphatases and 5'-nucleotidases (Zimmermann, 2000). When adenosine levels in the extracellular space are high, adenosine is transported into cells by means of transportes. It is then phosphorylated to AMP by adenosine kinase or degraded to inosine by adenosine deaminase. Adenosine deaminase, but not adenosine kinase, is also present in the extracellular space (Fredholm et al., 2001). Another potential source of extracellular adenosine is cAMP, which can be released from neurons and converted by extracellular phosphodiesterases into AMP and thereafter by an ecto-5'-nucleotidase to adenosine. The transport of adenosine by facilitated diffusion is equilibrative and bidirectional, meaning that the net transport of adenosine either into or out of the cell depends upon the adenosine concentration gradient in both sides of the membrane. Inhibition of adenosine transport can, therefore, inhibit either adenosine release or adenosine uptake, depending upon the intra- and extracellular levels of adenosine (Baraldi et al., 2008). However, since the extracellular formation of adenosine from released adenine nucleotides constitutes a second source of adenosine, which is not affected by transport inhibition, the transport inhibitors usually cause an icrease in the extracellular adenosine levels. Under hypoxic and ischemic conditions there is a marked increase in cytoplasmatic adenosine leading to an intense release of adenosine, which is inhibited by adenosine uptake inhibitors (Parkinson et al., 2002).

Excitatory amino acid-mediated release of adenosine is certainly involved; however, of greater importance is probably the fact that whenever intracellular levels of adenine nucleotides fall as a result of excessive energy use, the intracellular levels of adenosine

will rise dramatically (Fredholm et al., 2001). For example, following hypoxia there is a decrease of intracellular ATP, accompanied by an accumulation of 5'-AMP and subsequently adenosine: The nucleoside is thereafter transported into the extracellular space via the transporters. Furthermore, when the intracellular level of adenosine is very high, adenosine simply diffuses out of cells. Direct release of intracellular adenine nucleotides, such as ATP, that is thereafter converted extracellularly by ecto-ATPase and ecto-ATPdiphosphohydrolase (ecto-apyrase) to AMP and dphosphorylated by ecto-5'-nucleotidase to adenosine, should also be considered (Zimmermann et al., 2000). Adenosine is neither stored not released as a classical neurotransmitter since it does not accumulate in synaptic vescicles, being released from the cytoplasm into the extracellular space through a nucleoside transporter. The adenosine transportes also mediate adenosine reuptake , the direction of the transport being dependent upon the concentration gradient at both sides of the membrane (Fredlhom et al., 2001). Since it is not exocytotically released, adenosine behaves as an extracellular signal molecule influencing synaptic transmission withiìout itself being a neurotransmitter, i.e. modulates the activity of the nervous system at celluar level presynaptically by inhibiting or facilitating transmitter release, postsynaptically by hyperpolarizing or depolarizing neurons and/or exerting nn-synaptic effects. Adenosine, therefore, belongs to the group of neiromodulators.

Adenosine receptors

Four adenosine receptor (AR) subtypes (A_1, A_{2A}, A_{2B}, and A_3) have been cloned and pharmacologically characterized, all of which are G protein-coupled receptors (GPCRs). (Figure.2)

Adenosine receptors can be distinguished according to their preferred mechanism of signal transduction: A_1 and A_3 receptors interact with pertussis toxin-sensitive G proteins of the Gi and Go family; the canonical signaling mechanism of the A_{2A} and of the A_{2B} receptors is stimulation of adenylyl cyclase via Gs proteins. In addition to the coupling to adenylyl cyclase, all four subtypes may positively couple to phospholipase C via different G protein subunits (Fredholm et al, 2001; Ciruela et al, 2010). (Table.1) Furthermore it has been demonstrated that adenosine, through interaction with adenosine receptors, mediated phosphorylation of MAPK kinase family.

Considering the overall protein structure, ARs display the topology typical of GPCRs. Sequence comparison between the different GPCRs revealed the existence of different receptor families sharing no sequence similarity even if specific fingerprints exist in all GPCR classes. However, all these receptors have in common a central core domain consisting of seven transmembrane helices (TM1-7), with each TM composed of 20–27 amino acids, connected by three intracellular (IL1, IL2, and IL3) and three extracellular (EL1, EL2, and EL3) loops. Two cysteine residues (one in TM3 and one in EL2), which are conserved in most GPCRs, form a disulfide link which is possibly crucial for the packing and for the stabilization of a restricted number of conformations of these seven TMs. Aside from sequence variations, GPCRs differ in the length and function of their N-terminal extracellular domain, their C-terminal intracellular domain, and their intracellular loops. Each of these domains provides very specific properties to these receptor proteins. Particularly, consensus sites for N-linked glycosylation exist on the

extracellular regions of ARs, although the precise location of the sites for this post-translational modification varies amongst the AR subtypes. The carboxyl-terminal tails of the A_1AR, A_{2B}AR, and A_3AR, but not A_{2A}AR, possess a conserved cysteine residue that may putatively serve as a site for receptor palmitoylation and permit the formation of a fourth intracellular loop (Moro et al., 2005).

The A_1AR, A_{2B}AR, and A_3AR are very similar in regard to the number of amino acids composing their primary structure, and in general, these AR subtypes are among the smaller members of the GPCR family. For example, the human homologs of the A_1AR, A_{2B}AR, and A_3AR consist of 326, 328, and 318 amino acid residues, respectively. Conversely, the human A_{2A}AR is composed of 409 amino acids. It should be noted that the size of ARs deduced from their primary amino acid structure frequently is not consistent with the mass estimated by polyacrylamide gel electrophoresis of the expressed proteins. The post-translational glycosylation of ARs, which may vary in a cell type-dependent fashion, likely accounts for these discrepancies. The human A_1AR and human A_3AR display 49% overall sequence identity at the amino acid level, while the human A_{2A}AR and human A_{2B}AR are 45% identical (Fredholm et al, 2001).

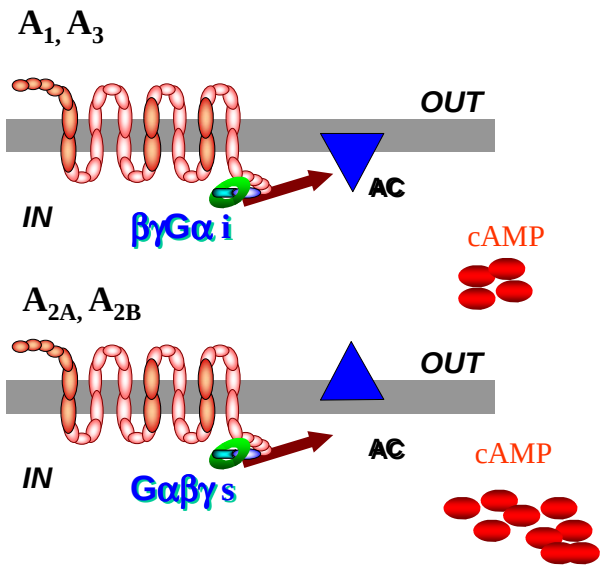


Figure.2 G protein-coupled receptors (GPCRs).

Table.1

Adenosine receptors in the brain.

Receptor	Adenosine affinity	G protein	Transduction mechanisms[a]	Physiological actions in brain
A_1R	~ 70 nM	$G_{i1,2,3}$	Inhibits AC (↓cAMP) Activates PLC (↑IP_3/DAG)	Inhibits synaptic transmission; hyperpolarizes neurons
			Activates PLA2 (↑AA) Activates PLD (↑PEtOH) Activates GIRKs Inhibits Ca^{2+} channels	
		G_o		
$A_{2A}R$	~ 150 nM	G_S[b]	Activates AC (↑cAMP)	Facilitates transmitter release; regulation of sensorimotor integration in basal ganglia
		G_{olf}	Activates AC (↑cAMP)	
		$G_{15,16}$[c]	↑IP_3 Inhibits Ca^{2+} channels	
$A_{2B}R$	~ 5000 nM	G_S[b]	Activates AC (↑cAMP) Activates Ca^{2+} channels	Increases in cAMP in brain slices
		$G_{q/11}$[c]	Activates PLC (↑IP_3/DAG)	
A_3R	~ 6500 nM	$G_{i2,3}$	Inhibits AC (↓cAMP)	Uncouples A_1R and mGlu receptors
		$G_{q/11}$	Activates PLC (↑IP_3/DAG)	

A_1 adenosine receptors

The A_1 receptor is widely expressed throughout the body, having its highest expression in the brain, spinal cord, atria and adipose tissue (Ciruela et al., 2010). Via adenosine A_1ARs, adenosine reduces heart rate, glomerular filtration rate, and renin release in the kidney; it induces bronchoconstriction and inhibits lipolysis (Elzein and Zablocki, 2008). Adenosine A_1Rs can be coupled to different pertussis toxin-sensitive G proteins, which mediate inhibition of adenylate cyclase and regulate calcium and potassium channels, as well as inositol phosphate metabolism (Fredholm et al., 2001). A_1ARs and A_{2A}ARs are primarily responsible for the central effects of adenosine (Dunwiddie and Masino, 2001). In addition to their postsynaptic locations in different brain regions, A_1ARs can be found presynaptically and modulate neurotransmitter release. Presynaptic A_1ARs are the prototype of GPCRs, the stimulation of which decreases the probability of neurotransmitter release. The main mechanism of A_1AR-mediated inhibition of exocytosis is a direct inhibitory effect on voltage-dependent Ca2+ channels (Moore et al., 2003). $A1_A$R displays two different affinities for agonist, which have classically been attributed to a different coupling to heterotrimeric G proteins. According to this two independent site model, coupled receptor–G protein complexes display high affinity for agonists and uncoupled receptors display low affinity. The reported cluster-arranged cooperative model predicts that the high- and low-affinity sites are a consequence of the negative cooperativity of agonist binding and do not seem to be related to the content of G protein-coupled or –uncoupled receptors (Franco et al., 1996). Like other GPCR members, A_1AR expression is regulated in response to agonist or antagonist stimulation. Desensitization of A_1ARs has been described in intact animals and in cell cultures. Prolonged administration of A_1AR agonists to animals leads to functional desensitization of A_1ARs in guinea pig heart, rat adipocytes, rat atrial muscle, and rat brain (Moro et al., 2006). The reduced functional response is attributable to a net loss of

A_1ARs or down-regulation, a decrease in the proportion of A_1ARs displaying the high-affinity state for agonists, and a decrease in the content of Gi proteins. The loss of binding sites on the cell membrane owing to internalization of A_1ARs is a slower event. Ser/Thr phosphorylation seems to be related to short-term clustering and desensitization, as well as long-term internalization of A_1ARs (Ciruela et al., 1997).

A_{2A} adenosine receptors

The A_{2A}AR exists in a wide variety of organs including major peripheral tissues (e.g., liver, heart, lung, and the immune system) and the central nervous system (CNS) (Lee et al., 2003). In the developing rat brain, expression of the A_{2A}AR is transiently regulated in various areas (e.g., the striatum, cortex, and hippocampus), perhaps implying a role of adenosine in neuronal development. Soon after neurogenesis, the A_{2A}AR is highly expressed by striatal neurons and co-localizes with the D_2 dopamine receptor in GABAergic striatopallidal neurons (Ferrè et al., 2008). In addition to the intense expression in the striatum, low levels of A_{2A}AR are found in many brain regions (e.g., the cortex and hippocampus) and it has been suggested that adenosine acting at the A_{2A}AR regulates important neuronal functions including neuronal protection and synaptic transmission (Ferrè et al., 2008). Regulation of A_{2A}AR gene expression is therefore likely to play an important role in neuronal development, basal ganglia activity, and many other peripheral functions. In the CNS, l-DOPA enhanced the gene expression of the striatal A_{2A}AR in 6-OHDA-lesioned rats (Tomiyama et al., 2004). Treatment with an antagonist of the NMDA receptor (memantine) was also reported to elevate the transcript level of striatal A_{2A}ARs (Marvanova and Wong, 2004). The adenosine A_{2A}AR couples primarily to members of the Gs family. Like other GPCRs it can also interact with other G proteins if the receptor is very over-expressed, but the evidence for such coupling in vivo is not compelling. In striatum the A_{2A}AR interacts

with Golf proteins (Corvol et al., 2001). It is not known if there are significant differences in receptor affinity or in signaling dependent on which of the two partners (or which variant of Gs) the receptor interacts with. There are instances where other G protein pathways have been implicated, and it will be important to determine if this alternate coupling is a regulated process, for example via phosphorylation. There is no compelling reason to assume that this GPCR coupling to members of the Gs family would signal in anything but a canonical way. Thus, most effects are probably due to activation of adenylyl cyclase and generation of cAMP. The A_{2A}AR can recruit β-arrestin via a GRK-2 dependent mechanism (Khoa et al., 2006). This is influenced by activation of cytokine receptors, which cause reduced desensitization of the A_{2A}AR (Khoa et al., 2006).

One key target of PKA is the cAMP responsive element-binding protein (CREB) which is critical for many forms of neuronal plasticity as well as other neuronal functions (Josselyn and Nguyen, 2005). Phosphorylation of CREB at Ser133 by PKA activates CREB and turns on genes with cAMP responsive elements (CRE sites) in their promoters. One important feature of CREB is that it is a point of convergence for the cAMP/PKA and MAPK pathways. Stimulation of the A_{2A}ARs counteracts the inhibition of neurite outgrowth due to MAPK blockade (Cheng et al., 2002). Stimulation of the A_{2A}AR alone also activates the Ras/Raf-1/MEK/ERK signaling through PKA-dependent and PKA-independent pathways via Src- and Sos- mediated mechanisms, respectively (Schulte and Fredholm, 2003). Interestingly, phosphorylation/activation of CREB has been shown to compete with nuclear factor-κB (NFκB) p65 for an important co-factor, CBP. Phosphorylated CREB was therefore proposed to mediate the anti-inflammatory effect of the A_{2A}AR receptor and inhibition of NFκB by A_{2A}AR activation during acute inflammation in vivo was demonstrated (Fredholm et al., 2007).

An interesting observation is that activation of A_{2A}AR receptor facilitates activities of adenosine transporters via a PKC-dependent pathway in the hippocampus, and thus reduces the level of extracellular adenosine available for A_1AR activation (Pinto-Duarte et al., 2005). In addition, PKC was shown to play a key role in mediating the enhancement of noradrenaline release by the A_{2A}AR in rat tail artery (Fresco et al., 2004). Activation of multiple signaling pathways by the A_{2A}AR appears to contribute to its diverse and complex functions in various tissues.

A_{2B} adenosine receptors

A_{2B}AR mRNA was originally detected in a limited number of rat tissues by Northern blot analysis, with the highest levels found in cecum, bowel, and bladder, followed by brain, spinal cord, lung, epididymis, vas deferens, and pituitary. The use of more sensitive reverse transcriptase-polymerase chain reaction techniques revealed a ubiquitous distribution of A_{2B}AR (Spicuzza et al., 2006). mRNA encoding A_{2B}AR was detected at various levels in all rat tissues studied, with the highest levels in the proximal colon and lowest in the liver. In situ hybridization of A_{2B}ARs showed widespread and uniform distribution of A_{2B}AR mRNA throughout the brain (Dixon et al., 1996).

Pharmacological identification of A_{2B}ARs, based on their low affinity and characteristic order of potency for agonists, also indicates a widespread distribution of A_{2B}ARs. In brain, functional A_{2B}ARs are found in neurons and glial cells. Although there is no evidence that A_{2B}AR are present in microglia, there is ample data that show that they are expressed in astrocytes and in different glioma cell lines (Fiebich et al., 1996). The expression of A_{2B}ARs in glial cells, which represent a majority of the brain cell

population, can explain the original observation that slices from all brain areas examined showed an adenosine-stimulated cAMP response.

Functional A_{2B}ARs have been found in fibroblasts and various vascular beds, hematopoietic cells, mast cells, myocardial cells, intestinal epithelial and muscle cells, retinal pigment epithelium, endothelium, and neurosecretory cells (Gessi et al., 2005). Although activation of adenyl cyclase is arguably an important signaling mechanism for A_{2A}ARs, this is not necessarily the case for A_{2B}ARs, as other intracellular signaling pathways have been found to be functionally coupled to these receptors in addition to adenyl cyclase. In fact activation of adenosine A_{2B}ARs can increase phospholipase C in human mast cells and in mouse bone marrow-derived mast cells. A_{2B}AR activation also elevates inositol triphosphate (IP3) levels, indicating this receptor can couple also to Gq-proteins. A_{2B}ARs have been implicated in the regulation of mast cell secretion and, gene expression, intestinal function, neurosecretion, vascular tone and in particular asthma (Varani et al., 2005).

A_3 adenosine receptors

The A_3 adenosine receptor (A_3AR) is the only adenosine subtype which was cloned before its pharmacological identification. It was originally isolated as an orphan receptor from rat testis, having 40% sequence homology with canine A_1 and A_{2A} subtypes (Meyerhof et al., 1991) and was identical with the A_3AR later cloned from rat striatum (Zhou et al., 1992). Homologs of the rat striatal A_3AR have been cloned from sheep and human, revealing large interspecies differences in A_3AR structure. For example, the rat A_3AR presents only 74% sequence homology with sheep and human A_3AR, while there is 85% homology between sheep and human A_3AR. This is reflected in the very different pharmacological profiles of the species homologs, especially in

terms of antagonist binding that has made characterization of this adenosine subtype difficult. Recently equine A_3AR has been cloned and pharmacologically characterized. Sequencing of the cDNA indicated that it has a high degree of sequence similarity with that of other mammalian A_3AR transcripts, including human and sheep (Brandon et al., 2006).

The A_3AR has been mapped on human chromosome 1p21-p13 (Atkinson et al., 1997) and consists of 318 aminoacid residues. Murrison et al. (1996) determined that the A_3AR gene contains 2 exons separated by a single intron of about 2.2 kb. The upstream sequence does not contain a TATA-like motif, but it has a CCAAT sequence and consensus binding sites for SP1, NF-IL6, GATA1 and GATA3 transcription factors. Involvement of the latter in transcriptional control of this gene would be consistent with a role of the receptor in immune function. The A_3AR is a G-protein-coupled receptor (GPCR) characterized by its C-terminal portion facing the intracellular compartment and 7 transmembrane spanning domains. In contrast to other adenosine receptors, the C-terminal region presents multiple serine and threonine residues, which may serve as potential sites of phosphorylation that are important for rapid receptor desensitization upon agonist application (Palmer & Stiles, 2000). Phosphorylation leads to a decrease of the number of receptors in the high-affinity state and a decrease of agonist potency to inhibit adenylyl cyclase activity. At the same time, the receptor is reversibly internalized in an agonist-dependent fashion (Trincavelli et al., 2002a).

The A_3AR has widely distributed its mRNA being expressed in testis, lung, kidneys, placenta, heart, brain, spleen, liver, uterus, bladder, jejunum, proximal colon and eye of rat, sheep and humans. However, marked differences exist in expression levels within and among species. In particular rat testis and mast cells express high concentrations of A_3AR mRNA, while low levels have been detected in most other rat tissues (Gessi et

al., 2008). Lung and liver have been found as the organs expressing high levels of A_3AR mRNA in human, while low levels have been found in aorta and brain. Lung, spleen, pars tuberalis and pineal gland expressed the highest levels of A_3AR mRNA in sheep.

The presence of A_3AR protein has been evaluated through radioligand binding, immunoassay or functional assay in a variety of primary cells, tissues and cell lines (Gessi et al., 2008). In the mouse brain a widespread, relatively low level of A_3AR binding sites was found (Jacobson et al., 1993). Similar data were obtained in the rat and in gerbil and rabbit brain. Electrophysiological and biochemical evidence suggested the presence of A_3ARs in the rat hippocampus and cortex, and functional studies also indicated its presence in the brain. In cardiomyocytes, there was no direct evidence of the presence of A_3ARs but several studies reported that it was responsible for cardioprotection in a variety of species and models, including isolated cardiomyocytes and isolated myocardial muscle preparations (Peart and Headrick, 2007). In lung parenchyma and in human lung type 2 alveolar-like cells (A549), the A_3AR was detected through radioligand binding and immunohistochemical assays (Varani et al., 2006).

The classical pathways associated with A_3AR activation are the inhibition of adenylyl cyclase activity, through the coupling with Gi proteins, and the stimulation of phospholipase C (PLC), inositol triphosphate (IP3) and intracellular calcium (Ca^{2+}), via Gq proteins (Fredholm et al., 2001). However, more recently additional intracellular pathways have been described as relevant for A_3AR signaling. For example, in the heart, A_3AR mediates cardioprotective effects through ATP-sensitive potassium (KATP) channel activation. Moreover, it is coupled to activation of RhoA and a subsequent stimulation of phospholipase D (PLD), which in turn mediates protection of

cardiac myocytes from ischemia (Mozzicato et al., 2004). In addition, in different recombinant and native cell lines, A_3AR is involved, like the other adenosine subtypes, in the modulation of mitogen-activated protein kinase (MAPK) activity (Schulte and Fredholm, 2003). A_3AR signaling in Chinese Hamster Ovary cells transfected with human A_3AR (CHO-hA3) leads to stimulation of extracellular signal-regulated kinases (ERK1/2). In particular, A_3AR signaling to ERK1/2 depends on βγ release from pertussis toxin (PTX)-sensitive G proteins, phosphoinositide 3-kinase (PI3K), Ras and mitogen-activated protein kinase kinase (Schulte and Fredholm, 2003). It has been reported that A_3AR activation is able to decrease the levels of PKA, a downstream effector of cAMP, and of the phosphorylated form of PKB/Akt in melanoma cells. This implies the deregulation of the Wnt signaling pathway, generally active during embryogenesis and tumorigenesis to increase cell cycle progression and cell proliferation (Fishman et al., 2002). Involvement of the PI3K/PKB pathway has been linked with preconditioning effects induced by A_3AR activation in cardiomyocytes from newborn rats (Germack and Dickenson, 2005). An elegant study has recently documented a role of A_3AR in cell survival signaling in resveratrol preconditioning of the heart. This study provides evidence that resveratrol preconditions the heart through the activation of adenosine A_1 and A_3AR, transmitting a survival signal through both the PI3K-Akt-Bcl2 and, only in the case of A_3AR, cAMP response element-binding protein (CREB)-Bcl2 pathways (Das et al., 2005). Subsequently it has been demonstrated that CREB phosphorylation occurs through both Akt-dependent and -independent signaling. Activation of PI3K-Akt-pBAD by A_3AR has been observed recently in glioblastoma cells leading to cell survival in hypoxic conditions (Merighi et al., 2007). Further studies indicate that A_3AR activation by interfering with PKB/Akt pathways can decrease interleukin-12 (IL-12) production in human monocytes (la Sala et al., 2005). Collectively, these findings demonstrate that several intracellular

mechanisms are involved following A_3AR stimulation, the understanding of which may be essential and crucial for explaining the different aspect of its activation.

Therapeutic potential of A_3 adenosine receptors

Neuroprotection versus neurodegeneration

Considerable interest has been shown in understanding the involvement of A_3AR in normal and pathological conditions of the CNS despite its low expression in the brain (Rivkees et al., 2000). Even though the function of A_3AR in the CNS has been controversial in terms of protective versus toxic actions, actually several data point towards a neuroprotective effect. Firstly, a dual role of A_3AR was described in a model of global ischemia in gerbils where acute preischemic administration of the agonist N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide (IB-MECA) caused a severe depression of cerebral blood perfusion, worsening of neuronal damage and postischemic mortality, while its chronic administration induced a significant improvement of postischemic cerebral flow and neuron protection (Von Lubitz et al., 1994). In line with the results obtained after acute treatment, in rat cerebellar granule neurons high concentrations of Cl-IB-MECA were able to induce lactate dehydrogenase release, neuronal cell death and augmented glutamate-induced neurotoxicity through a pathway involving inhibition of cyclic AMP production (Sei et al., 1997). It was then observed that the effect of IB-MECA depended on the timing of treatment as administration of IB-MECA 20 min prior to transient middle cerebral ischemia increased the infarct size, whereas its addition 20 min after ischemia resulted in a significant decrease of damage, leading the authors to define the cerebroprotective effect of A_3AR a “right thing at a wrong time” (Von Lubitz et al., 2001). It has been speculated that the deleterious effects

caused by acute preischemic treatment with IB-MECA were the consequence of a series of adverse events triggered immediately prior to the occlusion such as release of inflammatory mediators, breakdown of the blood–brain barrier integrity and Ca^{2+} influx. In contrast, the neuroprotective effects obtained when the A_3 agonist treatment was performed following a focal insult were related to astrocyte activation or to a direct neuroprotective action (Von Lubitz et al., 2001). One of the main factors contributing to the overall neuroprotective profile of chronic treatment with A_3AR agonists was found to be the reduction in post-ischemic expression of nitric oxide (NO) synthase, the enzyme involved in NO generation (Von Lubitz et al., 1999). Other beneficial effects associated with chronic A_3AR stimulation were the increase of glial fibrillary acidic protein [(GFAP), astrocyte proliferation and preservation of the ischemia-sensitive microtubule-associated protein 2 (MAP-2) (Von Lubitz et al., 1999)]. Destructive and protective actions of A_3AR stimulation have also been demonstrated in experiments in astroglial cells where Cl-IB-MECA at nanomolar doses was responsible for "trophic effects" related to reorganization of actin cytoskeleton, while in the micromolar range was a mediator of apoptosis ([Abbracchio et al., 1997], [Abbracchio et al., 2001], [Appel et al., 2001] and [Di Iorio et al., 2002]). Such apparently opposing effects have been reconciled by hypothesizing that adenosine-induced cell death that occurs during severe metabolic stress by A_3AR activation might isolate the most damaged areas to favor those parts of the brain that still retained a chance for functional recovery, supporting the role of adenosine as a "retaliatory metabolite" (Von Lubitz, 1999). Later it was speculated that desensitization/down-regulation of the A_3AR may be the basis of cytoprotection, suggesting a role for this receptor in induction of cell death (Trincavelli et al., 2002a). Recently a study performed in primary cortical cultures demonstrated that Cl-IB-MECA antagonized the hypoxia-mediated decrease in cell viability. Moreover, when given in vivo before focal cerebral ischemia, it reduced cerebral infarction while it

was inactive in A_3 knock-out (A_3KO) mice. Furthermore A_3KO mice after ischemia presented an increase in cerebral infarction in comparison to wild-type animals suggesting that A_3AR mediate a tonic protective condition during ischemia (Chen et al., 2006b). In contrast, A_3AR activation did not affect neuronal death triggered by kainate and cyclothiazide in primary cultures of cortical neurons (Rebola et al., 2005). Contrasting results have been reported also about how A_3AR activation might influence neuronal activity in rat brain. Dunwiddie and coworkers (1997) demonstrated that in the CA1 region of the rat hippocampus A_3AR has no direct effect on synaptically evoked excitatory responses, while it induced heterologous desensitization of A_1AR, thus limiting adenosine-mediated cerebroprotection. Others suggested that A_3AR activation in cortical neurons mediated a depression of synaptic transmission by inhibiting glutamate release additionally to and independently from the A_1 receptors, thus providing neuroprotection ([Brand et al., 2001], [Lewerenz et al., 2003] and [Lopes et al., 2003b]). It was also found that activation of A_3AR by endogenous adenosine inhibited synaptic transmission during hypoxia in rat cortical neurons (Hentschel et al., 2003), and the inhibitory function of A_3AR activation was in agreement with an in vivo report showing that A_3AR has depressant effects on locomotor activity in behavioral tests (Jacobson et al., 1993). However, on the other hand it has been observed that Cl-IB-MECA facilitates epileptiform discharges in the CA_3 area of immature rat hippocampal slices, suggesting that activation of A_3AR following a rise in endogenous adenosine facilitates excitation, thus limiting the known inhibitory and neuroprotective effects of adenosine in immature brain (Laudadio & Psarropoulou, 2004). Genetic suppression of A_3AR enhanced some aspects of motor function, suppressed pain processing at supraspinal levels and showed an increase in neurodegeneration in response to repeated episodes of hypoxia, suggesting the possible use of A_3 agonists in the treatment of ischemic and degenerative conditions of the CNS (Fedorova et al.,

2003). Different evidences suggest that a part of neuroprotection induced by A_3AR derives from its modulation of the brain immune system (Haskó et al., 2005). It has been reported that functional A_3AR are expressed in mouse microglia cells, where their activation is responsible for a biphasic effect on ERK1/2 phosphorylation (Hammarberg et al., 2003) and in murine astrocytes where A_3AR stimulation induces the release of the neuroprotective chemokine CCL2 (Wittendorp et al., 2004). Moreover, in lipopolysaccharide (LPS)-treated BV2 microglial cells A_3AR activation suppresses tumor necrosis factor-α (TNF-α) production by inhibiting PI3K/Akt and nuclear factor-κB (NF-κB) activation, suggesting that selective ligands of this receptor may be of therapeutic potential for the modulation and possible treatment of brain inflammation (Lee et al., 2006a). Even though for some aspects the role of A_3AR in the CNS seems less confusing now than in the past, there are many aspects yet that need clarification before a role of A_3 agonists in therapy can be envisioned.

Cardioprotection versus cardiotoxicity

To date several pieces of evidence support the conclusion that activation of A_3AR is crucial for cardioprotection during and following ischemia–reperfusion and it has been suggested that a consistent part of the cardioprotective effects exerted by adenosine, once largely attributed to the A_1 receptor, may now be in part ascribed to A_3AR activation (Headrick & Peart, 2005). Even though there is a low expression of A_3AR in myocardial tissue, a number of studies have demonstrated that acute treatment with agonists induced protective “anti-ischemic” effects (Auchampach et al., 1997a; Tracey et al., 1997; Thourani et al., 1999a; Ge et al., 2006 and Xu et al., 2006). The molecular mechanism of A_3AR cardioprotection has been attributed to regulation of mitochondrial (mito) K_{ATP} channels (Thourani et al., 1999b; Shneyvays et al., 2004 and Peart and Headrick, 2007).

In addition Shneyvays et al. (2005) demonstrated that in cultured rat myocytes Cl-IB-MECA delayed the dissipation of the mitochondrial membrane potential ($\Delta\psi$) and decreased the elevated intracellular calcium concentrations induced by hypoxia. These effects prevented irreversible cardiomyocyte damage and confirmed previous results showing that A_3AR activation protected cardiomyocytes treated with doxorubicin via inhibition of calcium overload and prevented cardiomyocyte death during incubation in high extracellular calcium concentrations (Shneyvays et al., 2001 and Shneyvays et al., 2004). As for the timing of cardioprotection, some studies have indicated that protection occurred post-ischemia, through inhibition of neutrophil-induced reperfusion injury or inhibition of myocyte apoptotic cell death (Jordan et al., 1999 and Maddock et al., 2002), while others found that preischemic A_3AR activation was effective and necessary for cardioprotection (Thourani et al., 1999a). Auchampach et al. demonstrated that A_3 agonism was able to trigger an anti-infarct response with either pre- or postischemic treatment (Auchampach et al., 2003). Moreover, it has been reported that A_3AR activation is able to mimic or induce myocardial preconditioning, meaning that transient stimulation of the A_3AR before induction of ischemia leads to both an early and a delayed protection (Peart & Headrick, 2007). The first condition has been shown to require mito K_{ATP} channel activation through PKC, 1,2-diacylglycerol (DAG), PLD and RhoA, but also reduction of caspase 3 and increase of cell survival through MEK/ERK1/2 and PI3K pathways (Parsons et al., 2000; Sato et al., 2000; Lee et al., 2001; Nakai et al., 2001; Germack and Dickenson, 2004 and Germack and Dickenson, 2005). In addition it has been reported that resveratrol preconditions the heart through A_3AR signaling that triggers a survival effect mediated by the Akt-Bcl2-Bad signaling pathway and also by another survival signal mediated via Akt-dependent and independent CREB phosphorylation (Das et al., 2005a and Das et al., 2005b). In terms of delayed preconditioning some authors reported a role for mito K_{ATP} channels but not for

nitric oxide synthase (iNOS), while other acquired evidence of NF-κB and iNOS involvement (Takano et al., 2001 and Zhao and Kukreja, 2002). Pharmacological preconditioning (early and late) obtained after A_3AR activation is clinically important, but cardioprotection is even more relevant when it occurs at reperfusion (Auchampach et al., 2003 and Xu et al., 2006). This situation, called post-conditioning, has been demonstrated for IB-MECA through inhibition of the mitochondrial permeability transition pore (mPTP) opening via PI3K/Akt inactivation of glycogen synthase kinase (GSK-3β; Kin et al., 2005 and Park et al., 2006). The cardioprotective effects of A_3 receptors were also detected in A_3AR-overexpressing mice where after in vivo regional ischemia and reperfusion, infarct size was lower than in wild-type mice (Black et al., 2002). In these animals A_3AR overexpression decreased basal heart rate and contractility, preserved ischemic ATP and decreased postischemic dysfunction (Cross et al., 2002). On the other hand, the results obtained with mice carrying higher transgene copy numbers suggested that basal signaling was increased when the A_3AR was expressed at higher levels, leading to the development of a dilated cardiomyopathy. Paradoxically, in contrast with pharmacological evidence of A_3-induced cardioprotection the first studies carried out in mice in which the A_3AR was genetically disrupted demonstrated an improvement of cardiac function as revealed from a smaller myocardial infarct size (Guo et al., 2001). Cerniway and colleagues (2001) reported similar beneficial effects in A_3KO mice, which they initially ascribed to the absence of a proinflammatory action of A_3AR mediated through mast cell degranulation. Subsequently, it was suggested that this effect might be due to compensatory changes that developed in the KO mice due to the chronic absence of A_3AR (Harrison et al., 2002). In this respect, recent evidence obtained by using pharmacological agents and genetic methods suggests that Cl-IB-MECA protects against myocardial ischemia/reperfusion injury in mice via A_3AR activation. These conclusions were

supported by experiments with a selective A_3 antagonist and through evaluation of the A_3 agonist effects on A_3KO mice. Interestingly, in this paper by using congenic (C57BL/6) A_3KO mice, deletion of the A_3 gene itself had no effect on ischemic tolerance, suggesting that previous opposite results from the same group (Guo et al., 2001) were probably explained by differences in the genetic background of the mice rather than specific deletion of the A_3 gene. Additional studies using wild-type mice treated with compound 48/80, a condensation product of *p*-methoxyphenetyl methylamine with formaldehyde, to deplete mast cell contents excluded the possibility that Cl-IB-MECA was cardioprotective by releasing mediators from mast cells (Ge et al., 2006) and supported the idea that therapeutic strategies focusing on A_3AR subtype may represent a novel and useful approach for protection of the ischemic myocardium. The A_3-mediated cardioprotection remains a mystery if one thinks of its cellular location. Literature data reported that myocardial A_3AR expression in the mouse is very low and below the detection limits of radioligand binding or northern blot techniques (Black et al., 2002). It is also surprising that mice overexpressing the A_3AR reveal only 12 fmol/mg of protein and that animals with 66 fmol/mg of protein present negative effects like dilated cardiomyopathy, meaning that the level of A_3AR is very critical for heart function. Therefore, on the one hand it is possible to hypothesize that given the strong cardioprotective effects and the low cardiac expression, this receptor must be very efficiently coupled to protective intracellular signaling pathways. On the another hand, it is also conceivable that cardioprotection might derive at least in part from activation of A_3AR expressed in other cells (Jacobson & Gao, 2006; Fig. 2).

Anti-inflammatory versus proinflammatory effects

The interest in the elucidation of A_3AR involvement in inflammation is attested by the large amount of experimental work carried out in cells of the immune system and in a variety of inflammatory conditions. However, as in the SNC or in the cardiovascular system the A_3AR subtype appears to have a complex or "enigmatic" role, as both proinflammatory and antiinflammatory effects have been demonstrated. One of the first evidence for a role of A_3AR in increasing inflammation derived by studies in mast cells where it was found that its activation was responsible for release of allergic mediators (Ramkumar et al., 1993 and Fozard et al., 1996). In addition, it has been reported that A_3AR mRNA was higher in lung tissue of patients with airway inflammation and that A_3AR activation mediates rapid inflammatory cell influx into the lungs of sensitized guinea pigs (Walker et al., 1997 and Spruntulis and Broadley, 2001). It has been reported that A_3AR activation in RBL-2H3 mast cells inhibits apoptosis and may have a profound effect on survival of inflammatory cells expressing A_3AR in inflamed tissues, thus contributing to inflammatory cell expansion (Gao et al., 2001). Moreover, antigen-dependent degranulation of bone marrow-derived mast cells was found to be mediated by A_3AR (Reeves et al., 1997), and the ability of Cl-IB-MECA to potentiate antigen-dependent mast cells degranulation was lost by using mice lacking A_3AR, suggesting a role for antagonists as antiasthmatic agents (Salvatore et al., 2000). The involvement of A_3AR in mast cells degranulation has been further confirmed in murine lung mast cells where it was dependent from Ca^{++} elevations through G_i and PI3K coupling (Zhong et al., 2003). However, in contrast with these findings it has been demonstrated that in the rat parenchymal strip, where contraction in response to adenosine is mast cell mediated, the receptor involved shows similarities to the A_3AR but Cl-IB-MECA is a high affinity

antagonist and MRS 1523 and MRS 1191 are inactive at concentrations that substantially exceed their affinities for the rat A_3AR by adding further twist to A_3AR pharmacology (Wolber & Fozard, 2005). Moreover, it has been shown that human and canine mast cells degranulation was mediated by A_{2B}AR instead of A_3AR (Feoktistov and Biaggioni, 1995; Auchampach et al., 1997b and Ryzhov et al., 2004). This discrepancy reflects the low human and rat overall coidentity at the amino acid level of A_3AR and questions the role of the A_3AR as a target for asthma therapy. Nevertheless the high expression of A_3AR in other cells involved in allergic diseases and asthma still gives reason to suggest a role for antagonists in the treatment of these pathologies. In particular transcript levels for the A_3 subtype are elevated in the lungs of asthma and COPD patients, where expression is localized to eosinophilic infiltrates. Interestingly, similar findings were made in the lungs of adenosine deaminase deficient ($ADA^{-/-}$) mice that exhibited adenosine-mediated lung disease. Treatment of $ADA^{-/-}$ mice with MRS 1523, a selective A_3 antagonist, prevented airway eosinophilia and mucus production. Similar results were obtained in the lungs of ADA/A_3 double KO mice, suggesting that A_3 signaling plays an important role in regulating chronic lung disease and that A_3 antagonism may provide a mechanism for reducing eosinophilia (Young et al., 2004). These results are in contrast with experiments performed in human eosinophils ex vivo, where chemotaxis such as degranulation and superoxide anion production were reduced by A_3AR activation (Ezeamuzie & Philips, 1999). This discrepancy may be due to differences in mouse and human eosinophils or to differences attributed to the ex vivo nature of the chemotaxis experiments performed. Additional studies of A_3-mediated effects on mouse eosinophils ex vivo confirmed the results observed in human cells, suggesting that diminished airway eosinophilia seen in the lungs of $ADA^{-/-}$ mice following disruption of A_3AR is not a cell autonomous effect of eosinophils. Rather A_3 disruption in $ADA^{-/-}$ mice is likely to affect the expression

and activity of key regulatory molecules from other cells that present A_3AR and that affect eosinophil migration (Young et al., 2004). For example A_3AR are expressed on murine mast cells, airway macrophages and epithelial cells, all of which might affect eosinophil migration. However, levels of key regulatory cytokines such as IL-5 and IL-13, or chemokines including eotaxin I, thymus- and activation-regulated chemokine (TARC) and monocyte chemotactic protein-3 (MCP3) were not affected by A_3 removal in $ADA^{-/-}$ mice, pointing perhaps to the involvement of A_3 subtype in the regulation of other key modulators of eosinophil migration, such as cell adhesion molecules, extracellular matrix elements and proteases (Young et al., 2004). The molecular mechanisms by which A_3 signaling may affect eosinophil chemotaxis are not known, but may involve the regulation of intracellular calcium (Khono et al., 1996a). In addition to influencing chemotaxis, A_3 engagement might also affect eosinophils survival. It has been reported that A_3 subtype can protect rat mast cells from apoptosis by a pathway involving PI3K and phosphorylation of PKB. In the same way, activation of A_3AR on eosinophils may promote their survival at sites of inflammation. However, the functional role of the A_3 subtype in the pathogenesis of asthma remains controversial and differences in the pharmacology of A_3 subtype from different species render it difficult to understand whether an A_3AR agonist or antagonist could be needed to improve the treatment of asthma. At this regard, a recent paper by Rimmer and coworkers reports the effect of a novel A_{2A} agonist/A_3AR antagonist in the treatment of allergic rhinitis through a randomized, double-blind, placebo-controlled study (Rimmer et al., 2007). Unfortunately, this ligand appears to have limited clinical benefit in both the early- and late-phase response to intranasal allergen challenge, even though it reduced the release of some mediators after allergene challenge. However, as correctly pointed out by the authors, the study presented a number of shortcomings. As an example the dose of the drug was limited by the narrow therapeutic index, due to side

effects like tachycardia, raising the possibility that higher doses of new compounds with fewer side effects might be more efficacious. Alternatively, it is possible that future studies targeting a different receptor, perhaps the A_{2B}, or using dual antagonists versus A_3/A_{2B}AR will be more successful (Press et al., 2005).

Discrepancy between anti- and proinflammatory effects induced by A_3AR have been observed also in other cell types. For example, A_3AR are expressed in human neutrophils where they are involved together with A_{2A} in the reduction of superoxide anion generation (Bouma et al., 1997 and Gessi et al., 2002). However, recently an elegant study by Chen et al. reported that neutrophils rapidly hydrolyze released ATP to adenosine that then acts via A_3-subtype adenosine receptors, which are recruited to the leading edge, to promote cell migration (Chen et al., 2006a and Linden, 2006).

In addition to a role of A_3AR in increasing inflammation, evidence that A_3AR decrease inflammation have also been reported in literature. As an example, it has been shown that A_3AR suppress TNF-α release induced by endotoxin CD14 receptor signal transduction pathway from human monocytes and murine J774.1 macrophages (Le Vraux et al., 1993 and McWhinney et al., 1996). Moreover, in a macrophage model the A_3AR was the prominent subtype implicated in the inhibition of LPS-induced TNF-α production (Sajjadi et al., 1996). This effect was associated with changes in stimulation of the activator protein-1 (AP-1) transcription factor, whereas it was independent of MAPK and NF-κB, PKA, PKC and PLC. This was not confirmed in BV2 microglial cells where A_3-mediated inhibition of LPS-induced TNF-α expression was associated with the inhibition of LPS-induced activation of PI3K/Akt and NF-κB pathway (Lee et al., 2006a). The inhibitory effect induced by A_3AR on TNF-α production was also assessed in A_3KO mice where the A_3 agonist was unable to reduce TNF-α levels in contrast with its effect in wild-type animals (Salvatore et al., 2000). Recently, it has

been reported that in mouse RAW 264.7 cells the A_3 subtype inhibits LPS-stimulated TNF-α release by reducing calcium-dependent activation of NF-κB and ERK 1/2 (Martin et al., 2006) while in peritoneal macrophages, isolated from A_3KO mice, the ability of IB-MECA to inhibit TNF-α release was not altered in comparison to wild-type mice (Kreckler et al., 2006). In this study, the inhibitory effect was exerted through the activation of A_{2A} and A_{2B} agonists as recently demonstrated also in human monocytes (Zhang et al., 2005 and Haskó et al., 2007). The discrepancy observed among these papers might not depend on species differences, being in both cases mouse cells, but by other factors including the source of the cells, and/or the inflammatory stimulus used. However in spite of these contrasting results, one of the best potential therapeutic applications of the regulatory role of A_3 activation on TNF-α release has been found in the treatment of arthritis. A_3AR agonists exert significant antirheumatic effects in different autoimmune arthritis models by suppression of TNF-α production (Baharav et al., 2005). The molecular mechanism involved in the inhibitory effect of IB-MECA on adjuvant-induced arthritis included receptor down-regulation and deregulation of the PI3K–NF-κB signaling pathway (Fishman et al., 2006 and Madi et al., 2007). Previous studies also demonstrated that A_3AR activation inhibited macrophage inflammatory protein (MIP)-1α, that is a C-C chemokine with potent inflammatory effects, in a model of collagen-induced arthritis, providing the first proof of concept of the adenosine agonists utility in the treatment of arthritis (Szabò et al., 1998).

In agreement with an antiinflammatory role for the A_3AR, it has been recently demonstrated that A_3AR activation decreases mortality and renal and hepatic injury in murine septic peritonitis (Lee et al., 2006b). Higher levels of endogenous TNF-α were observed in A_3KO mice after sepsis induction, in comparison to wild-type animals and IB-MECA significantly reduced mortality in mice lacking the A_1 or A_{2A} but not the

A_3AR, demonstrating specificity of the A_3 agonist in activating A_3 subtype and mediating protection against sepsis-induced mortality (Lee et al., 2006b). A similar mortality reduction associated with a decrease of IL-12 and interferon-γ (IFN-γ) production induced by A_3AR activation was previously observed by Haskó et al. (1998) in endotoxemic mice. In addition other investigators reported reduced inflammation and increased survival following A_3 activation in 2 murine models of colitis (Mabley et al., 2003). Furthermore, a protective role for A_3AR in lung injury following in vivo reperfusion has been recently reported (Matot et al., 2006). In contrast, it has been demonstrated that A_3AR activation exacerbates renal dysfunction and mice lacking A_3AR have been found to have better renal function following renal ischemia reperfusion injury (Lee and Emala, 2000 and Lee et al., 2003). A_3 receptors have been found to be up-regulated in ocular ischemic diseases and in conditions associated with oxidative stress. Their activation lead to the regulation of chloride channels in nonpigmented ciliary epithelial cells, suggesting that A_3 agonists would increase aqueous humor secretion and thereby intraocular pressure in vivo, while antagonists may represent a specific approach for treating ocular hypertension (Mitchell et al., 1999; Okamura et al., 2004 and Schlotzer-Schrehardt et al., 2005).

Antitumor versus tumorigen effects

A very interesting area of potential application of A_3 ligands concerns cancer therapies. The possibility that A_3 adenosine receptor plays a role in the development of cancer has aroused considerable interest in recent years (Fishman et al., 2002 and Merighi et al., 2003 and Gessi et al., 2008). The A_3 subtype has been described in the regulation of the cell cycle and both pro- and antiapoptotic effects have been reported depending on the

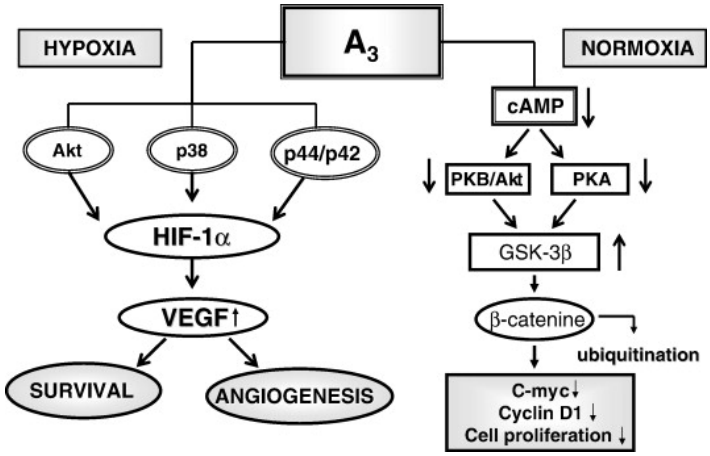
level of receptor activation (Jacobson, 1998; Yao et al., 1997; Gao et al., 2001; Merighi et al., 2005a; Nakamura et al., 2006 and Gessi et al., 2007). Starting from the observation that muscle tissues are resistant to tumor metastases, it was reported that one of the active components of muscle cell conditioned medium was adenosine, that exerted a differential effect on tumor and normal cell growth (Fishman et al., 1998); this inhibition was removed when the A_3AR was blocked, while it was mimicked following A_3AR agonist stimulation. The mechanism was found to involve inhibition of telomerase activity and arrest in the G_0/G_1 phase of the cell cycle, leading to a cytostatic effect (Fishman et al., 2000). In addition, it was demonstrated that A_3AR inhibited tumor growth by regulation of the WNT pathway (Fishman et al., 2004). The WNT pathway, active during embryogenesis and tumorigenesis, mediates cell cycle progression and cell proliferation. A key modulator of this pathway is represented by GSK-3β that is crucial for β-catenin phosphorylation. β-Catenin induces the transcription of genes fundamental for cell cycle progression such as c-*myc* and cyclin D_1. Upon exposure of tumor cells to the A_3 agonist, a decrease in the protein expression level of A_3AR and the downstream effectors PKA and PKB was observed. Consequently, the GSK-3β protein level increased, resulting in the destabilization of β-catenin and the subsequent suppression of cyclin D_1 and c-myc expression (Fig. 4). IB-MECA treatment also induced down-modulation of the expression of NF-κB, known to regulate the transcription of cyclin D_1 and c-Myc (Fishman et al., 2003 and Fishman et al., 2004). Moreover A_3AR agonist treatment induced inhibition of tumor growth both in vitro and in vivo, gave a synergistic effect in combination with chemotherapy and exhibited a myelostimulatory effect by inducing G-CSF production by mononuclear cells, thus leading to the development of A_3 agonists in clinical trials for colon carcinoma (Jacobson & Gao, 2006). Other authors found inhibition of cell proliferation or induction of apoptosis by treating cells with the A_3 agonist, but the effects generally

were observed only at micromolar doses and the involvement of the A_3 subtype was questioned (Wen and Knowles, 2003; Panjehpour and Karami-Tehrani, 2004; Merighi et al., 2005a and Nakamura et al., 2006). In this respect several observations may be underlined: (i) it has been demonstrated that Cl-IB-MECA at micromolar doses inhibits cell proliferation and this effect is reduced by blocking the receptor, supporting a role for the A_3 subtype (Merighi et al., 2005a); (ii) it has been previously reported that IB-MECA, at micromolar doses in breast cancer cells, inhibits cell proliferation through interaction with receptors different from the adenosine subtypes such as estrogen receptor α (Lu et al., 2003); (iii) at micromolar doses Cl-IB-MECA loses its selectivity for A_3 receptors and the complicating presence of interaction with other adenosine subtypes might be involved in the final response; (iv) the difference between the effects induced by low and high doses of Cl-IB-MECA could be attributed to the receptor desensitization of A_3 receptors that has been demonstrated by other authors in various cell systems (Trincavelli et al., 2002a). Conversely, it has been demonstrated that A_3AR in retinal ganglion cells was obligatory for life (Zhang et al., 2006), and it has been recently observed in colon cancer cells that after treatment with ADA, Cl-IB-MECA increased cell proliferation through the activation of A_3 subtype and involvement of ERK1/2 (Gessi et al., 2007). It is important to underline that all these experiments have been performed in normoxic conditions. From another point of view, hypoxia that is typical of solid tumors (Vaupel et al., 1989), creates conditions that, on one hand, are conducive to the accumulation of extracellular adenosine and, on the other hand, stabilize hypoxia-inducible factors, such as HIF-1α ([Blay et al., 1997], [Semenza, 2000], [Hockel and Vaupel, 2001], [Linden, 2001], [Minchenko et al., 2002], [Fredholm, 2003] and [Sitkovsky et al., 2004]). HIF-1, the most important factor involved in the cellular response to hypoxia, is up-regulated across a broad range of cancer types and is involved in key aspects of tumor biology, such as angiogenesis,

invasion and altered energy metabolism (Semenza, 2003). HIF-1 is a heterodimer composed of an inducibly expressed HIF-1α subunit and a constitutively expressed HIF-1β subunit (Epstein et al., 2001). HIF-1α and HIF-1β mRNAs are constantly expressed under normoxic and hypoxic conditions (Wiener et al., 1996). The unique feature of HIF-1 is the regulation of HIF-1α expression: it increases as the cellular O_2 concentration is decreased (Semenza, 2000 and Minchenko et al., 2002). During normoxia, HIF-1α is rapidly degraded by the ubiquitin proteasome system, whereas exposure to hypoxic conditions prevents its degradation (Minchenko et al., 2002). A growing body of evidence indicates that HIF-1 contributes to tumor progression and metastasis (Welsh and Powis, 2003 and Hopfl et al., 2004). Immunohistochemical analyses have shown that HIF-1α is present in higher levels in human tumors than in normal tissues (Zhong et al., 1999). Interestingly, it has been demonstrated that A_3AR are also overexpressed in cancer tissues in comparison to normal mucosa (Gessi et al., 2004a). Furthermore, attention has been paid to responses to chronic hypoxia that involve adenosine-induced changes in the transcription regulator HIF-1 expression. In particular, the correlation between adenosine receptor stimulation and HIF-1α expression modulation in hypoxia has been investigated. Adenosine increases HIF-1α protein expression in response to hypoxia in human melanoma, glioblastoma and colon cancer cells (Merighi et al., 2005b; Merighi et al., 2006 and Merighi et al., 2007b). These results indicate that the cell surface A_3AR transduces extracellular hypoxic signals into the cell interior. Increased HIF-1α protein synthesis through the activation of the Akt or MAPKinase pathways is a common theme accounting for the up-regulation. To evaluate how A_3AR accumulates HIF-1α in hypoxia, the signaling pathway generated by A_3AR stimulation has been investigated and it was found that MAPKinase activity is required for the HIF-1α expression increase induced by A_3AR activation (Fig.3). Furthermore, as HIF-1α plays a key role in inducing angiogenesis, we

have also studied the role of adenosine in mediating the production of VEGF in tumor cells. Activation of the A_3AR in glioblastoma and colon cancer cells stimulates VEGF expression (Merighi et al., 2006 and Merighi et al., 2007b), whereas this receptor subtype promotes VEGF downregulation in PC12 pheochromocytoma cells (Olah & Roudabush, 2000). It has been proposed that the effect of VEGF on new capillary formations is facilitated by the concomitant stimulation of A_{2B} and A_3 receptors that induce the expression of angiopoietin-2 (Feoktistov et al., 2003). Indeed, the activation of A_3 receptors results in increased expression of angiopoietin-2 in mast and melanoma cells (Feoktistov et al., 2003 and Merighi et al., 2005b). Although adenosine may contribute rather little to the increase in VEGF induced by hypoxia, it may contribute as much as 50% to angiogenesis (Adair, 2005). This could mean that adenosine acts also independently of VEGF, something that is not unlikely given the involvement of multiple cell types and multiple angiogenetic factors. Recent studies indicate that pharmacological inhibition of HIF-1α and particularly of HIF-regulated genes, that are important for cancer cell survival, may be more advantageous than therapeutic approaches based on HIF-gene inactivation. In this regard, A_3AR antagonists are able to block HIF-1α, angiopoietin-2 and VEGF protein expression accumulation in hypoxia, indicating a new approach for the treatment of cancer, based on the cooperation between hypoxic and adenosine signals.

Figure.3



Immunosuppressive versus immunostimulating effects

The ability of immune cells to fight tumor cells is fundamental for successful host defense against cancer. Adenosine, whose concentration increases within hypoxic regions of solid tumors, may interfere with the recognition of tumor cells by cytolytic effector cells of the immune system (Blay et al., 1997 and Merighi et al., 2003). Adoptive immunotherapy with lymphokine-activated killer (LAK) cells has shown some promise in the treatment of certain cancers that are unresponsive to conventional treatment approaches. However, colon adenocarcinomas tend to respond poorly to LAK therapy, possibly as a result of tumor-induced immunosuppression. It has been demonstrated that colon adenocarcinoma cells inhibited anti-CD3-activated killer cell induction through the production of a tumor-associated soluble factor that was distinct from transforming growth factor beta or prostaglandins (Hoskin et al., 1994a). Therefore, adenosine was indicated as a possible inhibitor of killer T-cell activation in the microenvironment of solid tumours (Hoskin et al., 1994b and Hoskin et al., 1994c). Indeed, evaluating the adhesion of murine spleen-derived anti-CD3-activated killer (AK) lymphocytes to syngeneic MCA-38 colon adenocarcinoma cells it was found that adenosine reduced adhesion by up to 60% (MacKenzie et al., 1994). The inhibitory effect of adenosine was exerted on AK cells and not on the MCA-38 targets and the agonist potency profile indicated that the A_3 receptor subtype might be responsible for the inhibition of adhesion. The authors suggested that this mechanism of immunosuppression, secondary to tissue hypoxia, may be important in the resistance of colorectal and other solid cancers to immunotherapy. In addition the same authors demonstrated that adenosine plays a strong inhibitory effect on the induction of mouse cytotoxic T cells (Hoskin et al., 2002). Diminished tumoricidal activity correlated with

reduced expression of mRNAs coding for granzyme B, perforin, Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL). IL-2 and IFN-γ synthesis by AK-T cells was also inhibited by adenosine. The inhibitory effect of adenosine on AK-T cell proliferation was also blocked by an A_3 receptor antagonist suggesting that adenosine acts through A_3 receptors to prevent AK-T cell induction. Tumor-associated adenosine may act through the same mechanism to impair the development of tumor-reactive T cells in cancer patients. Therefore, the suppression of T-killer cell function suggests that adenosine may act as a local immunosuppressant within the microenvironment of solid tumors. Subsequently, it was reported that adenosine partially inhibits the interaction of T lymphocytes with tumor cells by blocking the function of integrin α4β7 that is the major cell adhesion molecule involved in the adhesion of T cells to syngeneic MCA-38 adenocarcinoma cells (MacKenzie et al., 2002). The effect of adenosine has been investigated on the expression of costimulatory molecules by T cells in resting and activated conditions. One of the most important costimulatory molecules present on the T cells surface are CD2 and CD28 acting in concert to achieve optimal costimulation of T lymphocytes during interaction with antigen presenting cells. It has been demonstrated that adenosine interfered with activation-induced expression of the costimulatory molecules CD2 and CD28 in a way IL-2 dependent but not involving the accumulation of intracellular cAMP, possibly by activating the A_3 subtype (Butler et al., 2003). However, recent data obtained from studies using adenosine receptor KO mice examined the capability of adenosine and its analogues to inhibit the ability of LAK to defeat tumor cells. This work demonstrated that adenosine and adenosine A_{2A} ligands suppress the cytotoxicity of LAK cells in parallel with their ability to increase cAMP levels. These effects were produced by interfering with both perforin-mediated and Fas ligand-mediated killing pathways. Studies with LAK cells generated from A_1 and A_3AR KO mice indicated the lack of any involvement of these adenosine subtypes in the

inhibitory effect exerted by adenosine, whereas LAK cells obtained from A_2AR KO mice were resistant to the inhibitory effect of the nucleoside. Only very high concentrations of the non selective agonists 5-*N*-ethylcarboxamide adenosine (NECA) or 2-chloroadenosine (CADO) produced mild inhibition of LAK cytotoxicity that were possibly induced through A_{2B} activation, suggesting the predominant role of the A_{2A} subtype in inhibition of LAK cell toxicity (Raskovalova et al., 2005). Therefore, the authors indicate the use of A_{2A} antagonists to increase the efficacy of immunotherapy (Fredholm, 2007). In contrast to the immunosuppressive role of adenosine in the environment of solid tumors, it has been reported that A_3AR activation stimulates the proliferation of bone marrow cells in vitro. This effect was induced through the adenosine-mediated G-CSF production by peripheral blood mononuclear cells (PBMC). In vitro studies were also confirmed by in vivo experiments, which revealed an increase in leukocyte and neutrophil numbers, when adenosine was administered before chemotherapy (Bar-Yehuda et al., 2002). The molecular mechanism at the basis of G-CSF production included the upregulation of PI3K, PKB/Akt and NF-κB. In addition, it has been observed that Cl-IB-MECA potentiates the activity of NK cells in naïve and tumor bearing mice through the induction of IL-12 production; this effect was dependent on inhibition of cAMP levels and PKA expression. IL-12 is a potent stimulant of NK cells and is a cytotoxic factor that exerts a potent anti-tumor effect in vivo. It induces IFN-γ production by activated T and NK cells and augments cytotoxic activity of these cells via perforin, Fas and Trail-dependent mechanisms. Therefore, A_3AR activation enhances NK cell activity and probably NK cell-mediated destruction of tumor cells (Harish et al., 2003). This antitumor effect played in immune cells is in line with other findings of the same group demonstrating a direct inhibitory action of A_3 receptor activation on tumor cell growth (Fishman et al., 2003)

GLIOBLASTOMA TUMORS

Introduction and Histological Classification

The most common primary brain tumor is the glioma. Histologically, gliomas can resemble astrocytes, oligodendrocytes, or ependymal cells; thus, on the basis of their morphologic appearance they are classified as astrocytomas, oligodendrogliomas, or ependymomas, respectively (1–5). Astrocytomas express glial fibrillary acidic protein, an intermediate filament found in astrocytes that is routinely used as an aid in classifying a glioma as an astrocytoma.

Primary brain tumors account for 1.4% of all cancers and 2.4% of all cancer deaths in the United States, and approximately 20,500 newly diagnosed cases and 12,500 deaths are attributed to primary malignant brain tumors each year. The risk factors for the development of a glioma are not clear, but occupational exposure to organic solvents or pesticides appears to be a predisposing factor. It has also been suggested that cytomegalovirus (CMV) infection may play a role in the etiology or progression of some gliomas, based on detection of CMV RNA in glioblastoma (GBM) tumors (7). There are two peak incidences of gliomas, one in the age group of 0 to 8 years (8) and the second in the age group of 50 to 70 years (5), and there is a slight male predominance (9).

The symptoms of patients presenting with a glioma depend on the anatomical site of the glioma in the brain and can include headaches; nausea or vomiting; changes in speech, vision, hearing, or balance; mood and personality alterations; seizures or convulsions; and memory deficits .The time frame of the onset of symptoms depends in part on the grade of the glioma; with GBM tumors the onset of symptoms is typically rapid. Surgical biopsy is necessary to determine whether the tumor is a primary brain tumor and to diagnose the tumor type and grade.

Glioma tumors are histologically separated into Grades I through IV according to the World Health Organization (WHO) criteria. Grade I tumors typically have a good prognosis and more frequently occur in children (5, 8), and Grade II tumors are characterized on histologic examination by

hypercellularity: These Grade II tumors have a 5–8-year median survival. Grade III astrocytoma tumors (anaplastic astrocytoma tumors) are characterized on histologic examination according to hypercellularity, as well as nuclear atypia and mitotic figures (see Figure 4). Anaplastic astrocytoma has a 3-year median survival (10–14). Grade IV gliomas, also known as GBMs, are characterized on histologic examination according to hypercellularity, nuclear atypia, mitotic figures, and evidence of angiogenesis and/or necrosis (see Figure 5). The median survival for patients with GBM tumors is 12–18 months, and older patients (>60 years of age) typically have a survival that is somewhat shorter than the median.

Oligodendroglioma tumors are histologically separated into Grades II and III according to the WHO criteria. The Grade II tumors exhibit hypercellularity and bland nuclei on histologic examination (see Figure 6), and the Grade III tumors (anaplastic oligodendrogliomas) exhibit the additional histologic features of prominent mitotic figures and evidence of angiogenesis.

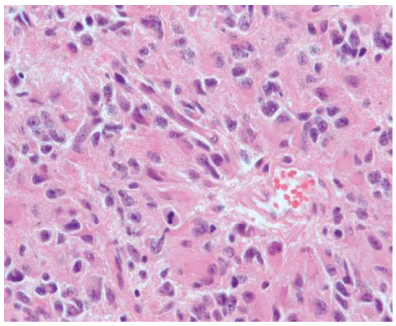


Figure 4

Anaplastic astrocytoma (World Health Organization Grade III). A mitotic figure is shown in the bottom center of the photomicrograph, and tumor nuclei are pleomorphic. Both are typical of an anaplastic astrocytoma.

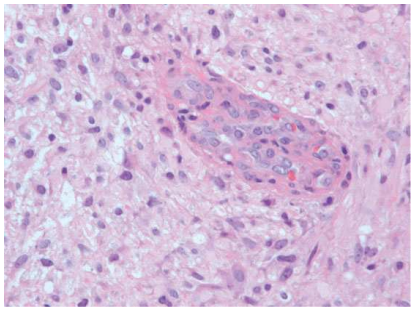


Figure 5

Glioblastoma tumor (World Health Organization Grade IV). Endothelial cell proliferation (angiogenesis) is shown in the center of this photomicrograph.

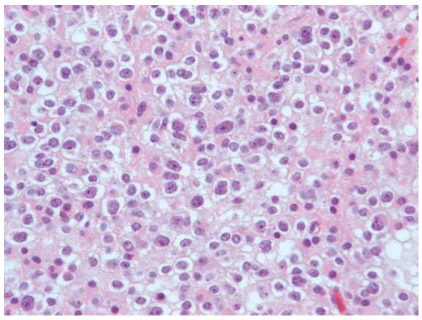


Figure 6

Oligodendroglioma (World Health Organization Grade II). The cleared cytoplasm and bland monomorphic nuclei typical of an oligodendroglioma are shown in this photomicrograph.

Major Genetic Alteration

The ongoing characterization of the genetic alterations in glioma tumor cells is revealing considerable variability among tumors of the same type and grade. This heterogeneity may contribute to the current limitations in predicting patient survival on the basis of histologic analysis of glioma type and grade alone and suggests that classification of certain types and grades of gliomas according to their genetic phenotype will lead to a more accurate prediction of survival and response to therapy. Grade I tumors, which are frequently benign, typically do not progress to Grade II, III, or IV tumors, and their genetic alterations are different from those found in the Grade

II–IV tumors; thus, they are not discussed herein. Oligodendroglioma (WHO Grade II) and anaplastic oligodendroglioma tumors (WHO Grade III) frequently exhibit loss of heterozygosity (LOH) on chromosomes 1p and 19q (observed in 40%–90% of biopsies, depending on the study) .This is the most common genetic alteration found in oligodendroglioma tumors and predicts a favorable response to certain chemotherapeutic agents, a favorable response to radiation therapy, and longer survival even after recurrence. Glioma biopsy tissue can be routinely tested for LOH on chromosome 1p and 19q by fluorescence in situ hybridization (FISH) or by Southern blotting in the pathology laboratory. It is not yet known which genes at the 1p and 19q loci are involved in the promotion of growth of the oligodendroglioma tumors nor how the loss of these genes contributes to a more favorable therapeutic response and a more favorable prognosis; however, at least one of these genes may be involved in the initiation of oligodendroglial tumorigenesis .Another common genetic alteration in oligodendroglial tumors is downregulation of the tumor suppressor and lipid phosphatase *PTEN* gene. Downregulation of this gene has been found in 50% of these tumors, and this downregulation appears to be a consequence of methylation of the promoter region.

Amplification of platelet-derived growth factor receptor alpha (PDGFRα) occurs in approximately 7% of oligodendroglial tumors. Astrocytoma tumors (WHO Grade II) frequently (3%–33%) exhibit amplification of the *PDGFRα* and/or *PDGFRβ* genes and of the genes encoding their ligands, *PDGF-A* and *-B* or *C* and *-D*. The amplification of the *PDGFRα* gene may result from amplification of chromosome 4q12. These genetic alterations probably play an important role in gliomagenesis, given that retroviral expression of PDGF-B in neural progenitor cells can initiate gliomagenesis in newborn mice and in adult rats.

In astrocytomas that do not express high levels of PDGF-A and -B, expression of PDGF-C and -D may be increased and is thought to substitute for the protumorigenic role of PDGF-B. Loss of *p53* is also a common genetic event in astrocytoma tumors (WHO Grade II). In the more malignant form of astrocytoma, anaplastic astrocytoma (WHO Grade III), loss of the gene that encodes the cell-cycle progression regulator Rb, which occurs as a consequence of the deletion of chromosome

13q13, is detected in approximately 30% of tumors. Downregulation or mutation of the tumor-suppressor gene *p16INK4A*/*CDKN2A* occurs in approximately 50% of these tumors. The downregulation can occur as a result of either hypermethylation of the promoter region or loss of the chromosome 9p region. The *p16INK4A* and *ARF* genes are encoded by a single genetic locus known as *INK4a*/*ARF*, which is located at chromosome 9p21 and encodes the precursor of p16INK4A and ARF. Approximately 50% of anaplastic astrocytoma tumors have a mutation of the *p53* gene. In addition, the gene encoding the endogenous p53 inhibitor, MDM2 (on chromosome 12q), is amplified in 13% to 43% of these tumors. As a consequence of the alterations in the *Rb1*/*CDK4*/*p16INK4A* and *p53*/*p14ARF* genes, signals that negatively regulate the cell cycle are interrupted, resulting in deregulated cell proliferation. Loss of chromosome 22q and gain of chromosome 7q are also found in approximately 20% of anaplastic astrocytoma tumor samples, but the identity of the gene(s) or loci that contribute to anaplastic astrocytoma tumorigenesis or progression is not yet known. GBMtumors (WHO Grade IV) can be subdivided into primary and secondary tumors on the basis of the patient's age at presentation and the genetic alterations in the tumor. Primary GBM tumors present de novo in older patients (typically >60 years of age) without a preexisting lower-grade glioma, and they account for approximately 90% of all GBM tumors. SecondaryGBMtumors arise from a preexisting Grade II or III astrocytoma or from a mixed glioma (oligoastrocytoma). In primary GBM tumors, amplification and/or mutation of the gene encoding epidermal growth factor receptor (EGFR), found on chromosome7, occurs in up to 60% of tumors. The most common mutation is a gain-of-function mutation due to an in-frame deletion of exons 2–7; this mutation results in the constitutive activation of EGFR, which can promote glioma cell proliferation and invasion. Deletion of the lipid phosphatase gene, *PTEN*, due to LOH of chromosome 10q or mutation, is also a common genetic occurrence in the primary GBM tumors; this deletion results in increased AKT/mTOR activity, which promotes cell survival, proliferation, and invasion. Both amplification of the *EGFR* gene and LOH of the *PTEN* gene can be readily detected by FISH or Southern blotting in the pathology laboratory. Several other potential

tumorsuppressor gene candidates on chromosome 10q, such as *DMBT1* (deleted in malignant brain tumors 1) and the Myc antagonist *Mxi1*, have been proposed. Also, the *MDM2* gene (an inhibitor of p53 on chromosome 12q) is amplified in approximately 10% to 15% of GBM tumor samples. Hypermethylation of the promoter of the gene encoding the DNA-repair enzyme, MGMT, occurs in both primary GBM(36%) and secondary GBM (75%) tumors and indicates a better response to temozolomide therapy. Heterogeneity in glioma tumors is also found within individual tumors. For example, certain areas of a glioma tumor may experience hypoxic conditions. Hypoxia results in the activation of proangiogenic genes and a focally increased angiogenic response. Also, breakdown of the blood-brain barrier can occur focally within a glioma tumor, resulting in leakage of serum-derived extracellular matrix proteins into certain areas of the tumor. Focal expression of serum-derived extracellular matrix proteins can alter integrin signaling and the motility of the glioma cells.

Molecular Mechanisms Contributing to the Proliferative and Invasive Phenotype

Like other malignant tumors, glioma tumors proliferate rapidly. This highly proliferative phenotype is due to the loss of multiple cell cycle inhibitors as well as to increased signaling from multiple growth factor receptors that act through downstream effectors to exert positive effects on the regulation of the cell cycle. The growth factor receptors that initiate a proliferative signal in these tumors include EGFR and PDGFR. Frequently, expression of both the ligand and the receptor is increased in glioma tumors, suggesting that there exists an autocrine or paracrine loop that amplifies signaling. Importantly, the EGFR and the PDGFR growth factor receptors cooperate or coordinate with cell adhesion receptors, such as integrins and Eph kinases, resulting in an amplification of the growth factor receptor signal. Growth factor receptors and cell-adhesion receptors typically rapidly activate focal adhesion kinase (FAK), a cytoplasmic nonreceptor tyrosine kinase. FAK is a major positive regulator of cell-cycle progression and acts by increasing extracellular signal–regulated kinase (ERK) activity and cyclin D1 transcription, as well as by inhibiting expression of p27Kip1.

Gliomas are invasive tumors. For the malignant gliomas, the invasive phenotype is a highly characteristic feature; others have referred to this phenotype as a signature feature. As with the proliferative phenotype, growth factor receptor signaling plays a major role in promoting the invasive phenotype in cooperation with, or in coordination with, cell-adhesion receptors and proteases. Multiple growth factor receptors have been shown to promote glioma cell migration and invasion, including c-Met, EGFR, and PDGFR. Typically, there is increased expression of both the growth factor receptor and ligand in the tumor, again suggesting that an autocrine or paracrine loop that promotes signaling is in place. Members of several different families of celladhesion receptors, including members of the integrin family, the Eph/Ephrin family, and the CD44 family, have been shown to promote glioma cell migration and invasion. In some instances, expression of cell-adhesion receptors, such as integrins alpha v beta 3 and alpha v beta 5, is increased in malignant glioma tumors. The integrin receptors provide the interaction with the cytoskeleton of the cell that generates the traction that enables the cell to pull itself forward. Regarding the Eph/Ephrin family, current data indicate that the Ephrin-B3 ligand and the Eph-B3 receptor promote glioma cell invasion. Cell-surface receptors from different classes or families probably cooperate or coordinate signaling events in a context dependent manner that is also regulated temporally. Signaling molecules in the glioma cells act downstream of the cell-surface growth factor receptor and cell-adhesion receptor to amplify and propagate the proinvasion signal. These signaling molecules include cytoplasmic tyrosine kinases, adaptor molecules, and cytoskeletal proteins. For example, both the tyrosine kinase FAK (72, 86, 98, 99) and another member of this family, Pyk2, can promote glioma cell migration and invasion in a contextdependent manner. The Src family tyrosine kinases also are necessary for glioma cell invasion. Adaptor molecules from the Crk-associated substrate (CAS) family, such as HEF1 and p130CAS, promote glioma invasion, and members of the Crk family of adaptor molecules act downstream of HEF1 or CAS proteins in this process. Two signaling molecules that regulate glioma cell survival and proliferation, phosphatidylinositol-3-kinase (PI3K) and PTEN, also regulate glioma cell migration and invasion. PI3K positively

regulates glioma cell migration and invasion (105). PTEN appears to negatively regulate these processes; thus, the loss of PTEN function in malignant gliomas can promote glioma cell invasion. Glioma cell invasion most likely requires protease degradation of the extracellular matrix. Several families of proteases, including the serine proteases, cathepsins, matrix metalloproteinases (MMPs), and the ADAMTS family of metalloproteases, have been shown to play a role in glioma cell migration and invasion. Protease activity can be regulated by multiple factors in a tumor. One important aspect of this regulation is the localization of protease function in specific regions of the tumor cell membrane. An example of this process is the localization of the serine protease, urokinase. Urokinase expression is increased inGBM tumors in vivo, and downregulation either of urokinase or of its receptor (the urokinase receptor) inhibits glioma cell invasion. The binding of urokinase to its receptor localizes this protease to specific areas of the cell membrane and promotes its activity in these areas because the binding of urokinase to its receptor is necessary for optimal protease activity. Also, the receptor colocalizes with specific integrin receptors on the cell membrane, further specifying the membrane region that exhibits protease activity. A second example is the binding of MMP-2 to integrin alpha v beta 3 on the cell surface, which both localizes and enhances the activity of this protease. Thus, proteases act in concert with cell-surface receptors and downstream signaling molecules to promote glioma cell invasion.

Treatmen of Glioblastoma tumor

Treating glioblastoma is a complex process, requiring a variety of techniques and procedures. The initial treatment often includes ***steroid medications*** to reduce swelling and inflammation of brain tissue, as well as ***anticonvulsant medications*** to prevent and control seizures if you have

experienced them. If fluid has built up in the brain, a physician may insert a ***shunt***–a long, thin tube that draws excess fluid from the brain. Common treatments to remove or reduce the size of glioblastoma include a combination of ***surgery, radiation therapy,*** and ***chemotherapy.*** For all operable tumors, surgery followed by radiation therapy and chemotherapy is recommended to improve patients' survival. Surgeons work to remove as much of the glioblastoma as possible while trying to minimize damage to healthy tissue. Some tumors can be removed completely while others only partially or not at all. To gain access to the tumor, surgeons may cut bone from the skull in a procedure called ***craniotomy*** and replace the bone after the procedure. Some surgeons use a highpowered microscope (microsurgery) or computer programs that create 3-D maps of the tumor's location; these maps help surgeons to remove tumors with minimal damage to healthy tissue and can reduce your pain and recovery time. In some situations, ultrasonic waves can be used to break apart the tumor, with the fragments removed by suction, in a procedure called ***ultrasonicaspiration***. Sometimes, to prevent cancer from coming back, surgeons place chemotherapy coated wafers in the space where a tumor has been removed. Radiation therapy–pinpointed high-energy beams–can shrink tumors or destroy cancer cells remaining after surgery. This treatment is also an option if surgery is not possible. Radiation therapists sometimes use 3-D maps similar to what surgeons use to deliver radiation in the exact size and shape of the tumor.

A common treatment regimen involves seven weeks of targeted radiation treatment combined with a chemotherapy drug called ***temozolomide (Temodar®)***, which makes the tumor more sensitive to the radiation therapy. Because of the difficulty of treating glioblastoma, you should consider enrolling in a ***clinical trial*** testing new treatments. These treatments are highly experimental in nature but may be an option, especially for advanced cancers. Some trials may involve biologic therapy, which uses the natural defenses of the immune system to fight cancer.

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AIM OF THE THESIS

Adenosine is an endogenous and ubiquitous nucleoside that exerts many biological functions through interaction with 4 distinct subtypes of G protein-coupled receptors divided into A_1, A_{2A}, A_{2B}, and A_3. This nucleoside plays an important role in modulating inflammation and tumorigenesis. In the past most of the anti-inflammatory effects of this nucleoside were thought to be due to the activation of the A_{2A} subtype, however more recently, the involvement of the A_3 subtype has been also considered relevant for the outcome of inflammation.

Cells involved in inflammation are important substrates on which adenosine plays multiple physiological functions. In the chapter 1 we will summarize the status of the art on the role of the A_3 receptor in different types of immune cells including neutrophils, eosinophils, lymphocytes, monocytes, macrophages and dendritic cells.

Several studies in literature and obtained in our laboratory have shown that adenosine exert important modulatory function in the growth of tumors, giving an essential role in this to the A_3 receptor. In particular local invasive growth is one of the key features of primary brain tumors. Glioma is the most common primary adult brain tumor with poor prognosis because of the aggressive invasion of the surrounding normal brain. Although our understanding of glioma oncogenesis has steadily improved, the molecular mechanisms that mediate glioma invasion are still poorly understood. The degradation of extracellular matrix (ECM) which exerts biochemical and mechanical barriers to cell movement has been shown to be an important biological process in tumor invasion and metastasis [1]. The metalloproteinases (MMPs) are a family of enzymes able to degrade and remodel the extracellular matrix. They are involved in many physiological and pathological processes, including inflammation and tumor growth. In particular, the degradation of extracellular matrix (ECM), which exerts a mechanical and biochemical barrier to cell movement, was demonstrated to be an important biological process in the invasion and the tumor metastatic process. In particular, it was demonstrated that MMP9 facilitates in vitro invasion of glioblastoma

cells and the up-regulation of this metalloproteinase is associated with the progression of malignant glioma in vivo.

Therefore the aim of chapter 2 will be to evaluate the involvement of adenosine on the regulation of metalloproteinases and in particular of MMP9 in U87MG glioblastoma cells.

In chapter 2, we showed that adenosine is able to increase both MMP9 mRNA and protein levels through the activation of the A_3 adenosine receptor using real time RT-PCR and Western blotting.

We noted that the A_3 receptor stimulation led to increased levels of MMP9 protein in cellular extracts of U87MG cells, through phoshorylation of ERK1/2, JNK, Akt/PKB and the transcription factor AP-1.

The A_3 receptor activation also led an increase in extracellular levels of MMP9 in the supernatants of glioblastoma cells as evaluated by ELISA and gelatine zymography assays. Finally, as for the physiological relevance of the A_3 receptor-mediated stimulation of MMP9 we found that the A_3 agonist was responsible for an increase of the invasive ability of U87MG cells.

CHAPTER 1:

A_3 adenosine receptor regulation of cells of the immune system and modulation of inflammation

Abstract

The interest in the elucidation of A_3 adenosine receptor involvement in inflammation is evident from the large amount of experimental work carried out in peripheral blood cells of the immune system and in a variety of inflammatory conditions. Following a detailed analysis of the literature the A_3 adenosine receptor subtype appears to play a complex role as both pro and anti-inflammatory effects have been demonstrated depending not only on the cell types investigated but also on the model of inflammation used and the species considered (Gessi et al., 2008). This chapter will discuss developments in our understanding of the role of adenosine A_3 receptor activation in the function of the different types of cells of the immune system including neutrophils, eosinophils, lymphocytes, monocytes, macrophages and dendritic cells.

A_3 adenosine receptor effects on neutrophil function

Neutrophils represent a larger percentage of circulating leukocytes than any other cell type. They are the first white blood cells to arrive at an injured or infected site. Neutrophils arise in the bone marrow and then must traverse the vasculature to arrive at the sites of injury. They leave the circulation at the level of the postcapillary venules following specific interaction with endothelium. Once in the extravascular space, neutrophils follow a gradient set up by chemoattractants, such as activated complement components, cytokines, lipids or bacterial products by means of specific cell surface receptors. Although the primary role of the neutrophil is to rid the body of injurious organisms and clean up the debris after tissue injury, the extracellular release of any of the contents of the phagolysosome or the generation of toxic oxygen metabolites into the extracellular space can lead to destruction of normal, uninjured cells surrounding the infected site. It is the destruction of the surrounding tissue by overactive neutrophils that adds so greatly to tissue destruction in the setting of reperfusion injury. Adenosine, acting through its cell surface receptors, is a potent regulator of neutrophil function.

The first report implicating a role for A_3 receptors in human neutrophils came in 1997 following investigations into the effect of adenosine and its more selective analogues on neutrophil degranulation in human whole blood (Bouma et al., 1997). Adenosine inhibited concentration-dependently the LPS- and TNF-alpha-induced release of the azurophilic granule proteins with an IC_{50} in the µmolar range. The inhibitory effects of adenosine were partially blocked by the A_2 receptor antagonist 3,7-dimethyl-1-propargylxanthine, the A_1/A_2 antagonist 8(p-sulfophenyl)theophyline, and the A_1/A_3 antagonist xanthine amine congener, but not by the A_1 antagonist 1,3-dipropyl-8-cyclopentylxanthine. The highly selective A_3 agonist N6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide and the nonselective agonist 2-chloroadenosine reduced degranulation more potently than the A_1 agonist N6-cyclopentyladenosine. The inhibitory effects of N6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide and 2-chloroadenosine were strongly reversed by xanthine amine congener, but were not affected by 8(p-sulfophenyl)theophyline. These data suggest that adenosine acted via A_2 as well as A_3 receptors to inhibit neutrophil degranulation. However, activation of A_3 receptors in canine neutrophils did not attenuate superoxide anion production but reduced platelet-activating factor-stimulated neutrophil adherence to coronary endothelium suggesting that it might be a novel target for treatment of myocardial ischemia and reperfusion (Jordan et al., 1999).

Subsequent binding and functional studies showed that human neutrophils expressed A_3 receptors which were coupled to the inhibition of adenylyl cyclase and calcium signalling (Gessi et al., 2002). However in the case of calcium the high micromolar doses of the A_3 agonist 2-chloro-N^6-(3-iodobenzyl)adenosine-5'-*N*-methylcarboxamide (Cl-IB-MECA) and the A_3 antagonist 5-*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2(2furyl)-pyrazolo-[4,3e]-1,2,4-triazolo[1,5-c] pyrimidine (MRE 3008F20) needed to stimulate or block Ca^{2+} mobilization respectively, were not completely consistent with the involvement of an A_3 receptor. Similar effects of Cl-IB-MECA in mobilizing Ca^{2+} have been found in several cell systems a finding that is difficult to reconcile with the high affinity of this selective A_3 agonist in binding and cAMP inhibition assays (Kohno et al.,

1996a,b; Jacobson, 1998; Reeves et al., 2000; Reshkin et al., 2000; Shneyvays et al., 2000; Gessi et al., 2001; Suh et al., 2001; Merighi et al., 2001). The reason why high, nonselective doses of Cl-IB-MECA are needed to stimulate Ca^{2+} mobilization remains unknown. A contribution of other mechanisms other than A_3 receptor stimulation cannot be excluded. Importantly, for the first time it was suggested that both A_3 and A_{2A} receptors contribute to the inhibition of oxidative burst, an indication of anti-inflammatory activity (Gessi et al., 2002). Using this readout, alterations of A_3 adenosine receptors in human neutrophils exposed to low frequency, low energy pulsing electromagnetic fields (PEMFs) has been reported. There is considerable interest in the use of PEMFs in clinical practice since the date correlate well with inflammatory conditions. Saturation experiments after treatment with PEMFs revealed that the A_3 receptor density in human neutrophils was increased. Consistent with this in functional assays Cl-IB-MECA and N^6-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IB-MECA) were able to inhibit cyclic AMP accumulation and their potencies were increased after exposure to PEMFs. These results indicated that in human neutrophils treated with PEMFs there are significant alterations in the A_3 adenosine receptor density and functionality (Varani et al., 2003). The upregulation cannot be ascribed to the synthesis of new receptors since the duration of PEMF treatment was too short. The upregulation of A_3 adenosine receptors is most likely due to a translocation of this receptor subtype to the membrane surface. It is of interest that PEMFs treatment also modified the binding parameters of the A_{2A} adenosine receptors but not those of α_2, β_2 adrenergic and μ, k opioid receptors suggesting a relationship between adenosine receptor-mediated anti-inflammatory effects and PEMF exposure (Varani et al., 2002).

An up-regulation of the A_3 adenosine receptor has also been observed in neutrophils obtained from patients with colorectal cancer in comparison with healthy subjects. This overexpression was found to reflect at peripheral level the same up-regulation found in the tumoral tissue from the colon in comparison to healthy mucosa, suggesting that peripheral A_3 adenosine receptors in neutrophils might represent potential marker for revealing colorectal cancer (Gessi et al., 2004a). It was also

found that in a small cohort of subjects A_3 receptor expression of circulating blood cells normalizes after surgical treatment, consistent with the negative results of follow-up evaluation with carcinoembryonic antigen (CEA), computed tomography scan, and colonoscopy. Hence, the improved health of patients after surgical resection seems to be associated with restoration of a normal adenosinergic system, at least in terms of A_3 receptor expression. These findings might be used for clinical applications. In particular, examination of neutrophil A_3 expression (for example, in addition to CEA determination) could play a role in the screening of high-risk individuals or in the follow-up of patients after surgical resection.

Recently Chen et al., reported that migrating human neutrophils secrete ATP at the leading edge, which signals via P2Y2 receptors to amplify chemoattractant signals (Chen et al., 2006a). Neutrophils rapidly hydrolyze released ATP to adenosine which then acts via A_3 receptors, which are recruited to the leading edge, to promote cell migration. In resting cells, A_3 receptors appear to be located primarily in intracellular compartments associated with granules (Chen et al., 2006a). Upon cell stimulation with chemoattractant, A_3 receptors are rapidly mobilized at the leading edge to promote chemotaxis. Thus, ATP release and autocrine feedback through P2Y2 and A_3 receptors provides signal amplification and controls gradient sensing and migration of neutrophils. Interestingly, chemotaxis of neutrophils obtained from A_3 receptor knockout (KO) animals is inhibited. In contrast, A_{2A} receptors are uniformly distributed across the cell surface and cell polarization does not seem to change this distribution pattern (Chen et al., 2006a). This suggests that the inhibitory A_{2A} receptors may function to globally suppress pseudopod formation across the entire cell surface of neutrophils, except at the leading edge, where A_3 adenosine receptor counteract the suppressive action of A_{2A} receptors (Chen et al., 2006a; Linden, 2006; Junger, 2008). However, these findings were questioned recently by Hoeven et al. (2008) who demonstrated that A_3 adenosine receptor activation is responsible for inhibition of superoxide production and chemotaxis of mouse bone marrow neutrophils, suggesting that the A_3 receptor may contribute to the anti-inflammatory actions of adenosine. Although there are many differences between this study and the

earlier work, including the species difference (mouse vs human), the pharmacological agents used to stimulate the A_3 adenosine receptor (CP-532,903 versus IB-MECA), the methods used to isolate/culture murine neutrophils, the stimulation protocols (including the time and duration of pretreatment of cells with agonists), and the state of cell priming, a definite explanation for the differences in results obtained in these studies remains unclear.

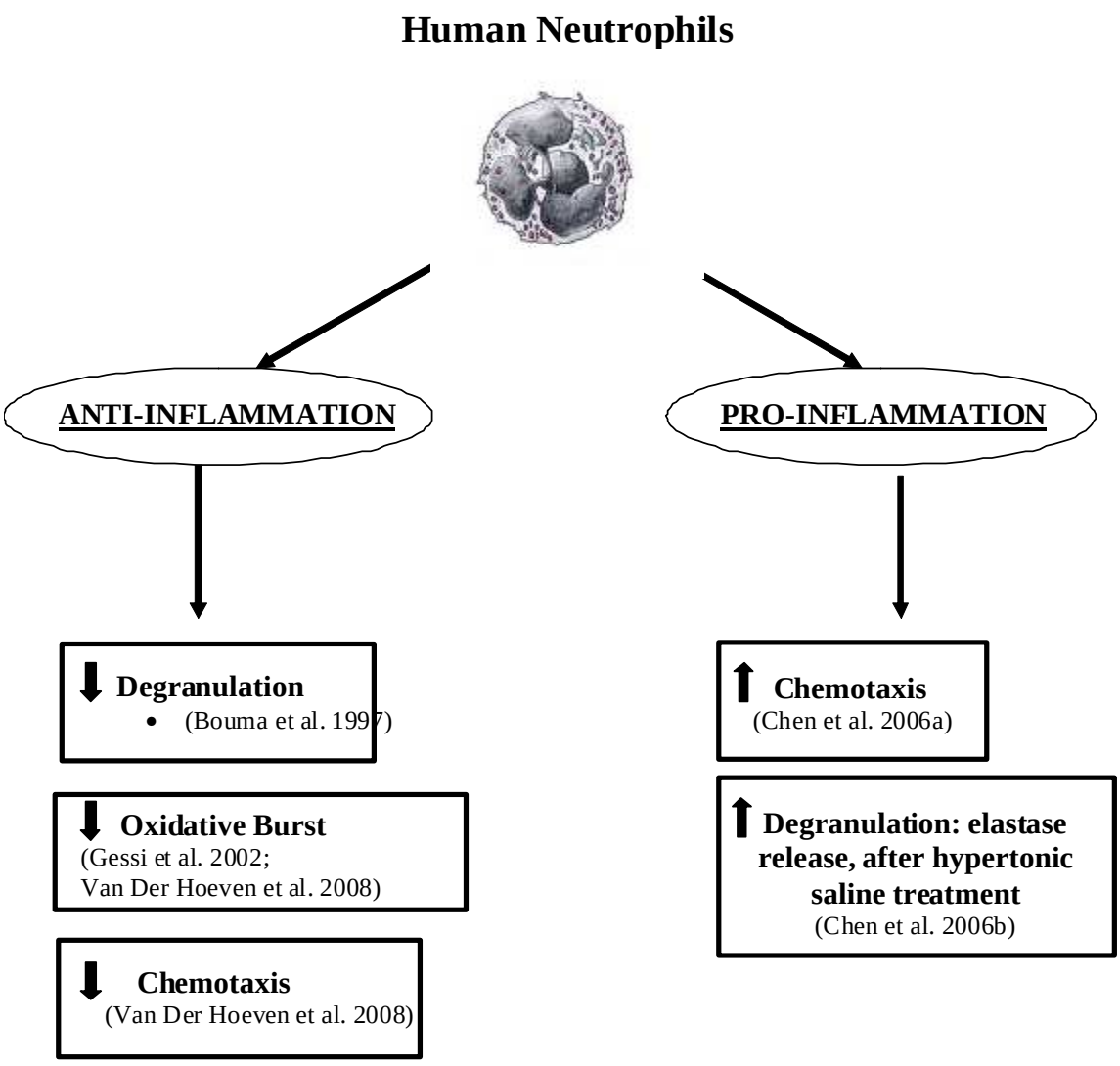
Consistent with a pro-inflammatory role of A_3 adenosine receptors in human neutrophils it has been demonstrated that A_3 receptors together with P2Y subtypes mediate neutrophil elastase release induced by hypertonic saline (Chen et al., 2006b). Hypertonic saline holds promise as a novel resuscitation fluid for the treatment of trauma patients because it inhibits polymorphonuclear neutrophil activation and thereby prevents host tissue damage and associated post-trauma complications. However, under certain conditions of cell activation, hypertonic saline can increase neutrophil degranulation, which could exacerbate tissue damage in trauma victim (Chen et al., 2006b). The cellular mechanism by which hypertonic saline increases degranulation involves elastase release and ERK and p38 MAPK activation when hypertonic saline is added after submaximal activation of neutrophils with formyl peptide (fMLP) or phorbol ester (PMA). Agonists of P2 nucleotide and A_3 adenosine receptors mimicked these enhancing effects of hypertonic saline, whereas antagonists of A_3 receptors or removal of extracellular ATP with apyrase diminished the response to hypertonic saline suggesting that hypertonic saline upregulates degranulation via ATP release and positive feedback through P2 and A_3 receptors. It has been hypothesized that these feedback mechanisms can serve as potential pharmacological targets to fine-tune the clinical effectiveness of hypertonic saline resuscitation (Chen et al., 2006b). In this context, it has been shown that A_3 receptor activation may diminish the efficacy of hypertonic saline in a mouse model of acute lung injury after sepsis (Inoue et al., 2008a). Acute lung injury in wild-type mice treated with hypertonic saline 60 min after sepsis induction, through cecal ligation and puncture (CLP), was significantly greater than in wild-type mice pretreated for 5 and 15 min with hypertonic saline. Parallel experiments aimed at evaluating the expression of A_3 receptors in human neutrophils treated

with hypertonic saline either 10 min before or after stimulation with formyl methionylleucyl-phenylalanine (fMLP) reveal that in the first condition A_3 receptor expression was reduced whilst in the second one it was markedly increased. These findings show that the opposing effects of hypertonic saline *in vivo* correlate with differences in the cell surface expression of A_3 receptors, suggesting that the enhancing effects of hypertonic saline are a result of increased A_3 receptor expression of stimulated neutrophils. The aggravating effect of delayed hypertonic saline treatment was absent in A_3 receptor knockout (KO) mice. Similarly, mortality in wild-type mice with delayed hypertonic saline treatment was significantly higher than in animals treated with hypertonic saline before CLP. Mortality in A_3 receptor KO mice remained at only 50% regardless of timing of hypertonic saline administration. These findings suggest that A_3 antagonists could improve the efficacy of hypertonic saline resuscitation by reducing side effects in patients whose polymorphonuclear neutrophils are activated before hypertonic saline treatment. The role of A_3 and P2Y2 receptors in neutrophil sequestration in the lungs in a mouse model of sepsis has also been demonstrated (Inoue et al., 2008b). Sepsis was induced by CLP using wild type mice, homozygous A_3 receptor KO mice, and P2Y2 receptor KO mice. The data suggest that A_3 and P2Y2 receptors are involved in the influx of neutrophils into the lungs after sepsis. Neutrophil sequestration in the lungs reached a maximum 2 h after CLP and remained significantly higher in wild type mice compared with A_3 KO and P2Y2 KO mice. Survival after 24 h was significantly lower in WT mice than in A_3 KO or P2Y2 KO mice. Thus, pharmaceutical approaches that target these receptors might be useful to control acute lung tissue injury in sepsis.

It has been recognized that the inflammatory response to infection depends on the coordinated interaction of the adenine nucleotides, ATP, ADP and adenosine released by damaged tissue (Linden, 2006). Therefore the contribution of A_3 receptors expressed in neutrophils, must be in concert with the other purinergic receptors to allow neutrophil adhesion, extravasation and chemotaxis. Neutrophils express predominantly A_{2A} and A_3 receptors which have opposite effects on these cells. In this chapter we have described how neutrophils following gradients of ATP and

adenosine initiate and increase the speed of chemotaxis via P2Y and A_3 receptors, respectively and that the A_{2A} may amplify gradient signals by inhibiting chemotaxis at membrane region distant from the leading edge where the A_3 receptor predominates and increases chemotaxis. This seems possible given that the affinity of adenosine for the A_{2A} receptor is several orders of magnitude higher than its affinity for the A_3 receptors. Thus, differences in external adenosine concentrations in the environment surrounding migrating neutrophils may contribute to the regulation of chemotaxis (Chen et al., 2006a). A coordinated activity of A_{2A} and A_3 receptors has also been found with respect to degranulation and superoxide anion production in human neutrophils where both receptors cooperate to fine-tune the inflammatory response (Bouma et al., 1997; Gessi et al., 2002). However, it is important that the inhibitory effect exerted by A_{2A} and A_{2B} receptors on chemotaxis and adhesion to endothelial cells, respectively can overcome the stimulatory effect exerted by A_3 when excessive influx of neutrophils damages host tissues (Zhang et al., 2006). After activation of A_3 receptors opposite effects on inflammation have been reported depending essentially on the response considered, the experimental conditions and the species used. It is relevant to underline that by comparing the studies performed in human neutrophils both anti and proinflammatory effects have been demonstrated (Figure 1). Therefore caution should be used before proposing A_3 agonists as anti or proinflammatory agents until a more definite role of this receptor has been defined.

Fig. 1-Effects of A_3 adenosine receptors in neutrophils.



A_3 adenosine receptor effects on eosinophil function

Eosinophils are one of the immune system components responsible for combating infection. Along with mast cells, they also control mechanisms associated with allergy and asthma. Eosinophils develop and mature in the bone marrow. They differentiate from myeloid precursor cells in response to the cytokines interleukin 3 (IL-3), interleukin 5 (IL-5), and granulocyte macrophage colony-stimulating factor (GM-CSF). Eosinophils produce and store many secondary granule proteins prior to their exit from the bone marrow. After maturation, eosinophils circulate in blood and migrate to inflammatory sites in tissues, in response to chemokines such as CCL11 (eotaxin-1), CCL24 (eotaxin-2), CCL5 (RANTES), and leukotriene B4 (LTB4). At these infectious sites, eosinophils are activated by Type 2 cytokines released from a specific subset of helper T cells (T_h2); thus IL-5, GM-CSF, and IL-3 are important for eosinophil activation as well as maturation. Following activation, eosinophils release the contents of small granules within the cellular cytoplasm, which contain many chemical mediators, such as histamine and proteins such as eosinophil peroxidase, RNase, DNases, lipase, plasminogen, and major basic protein that are toxic to both parasite and host tissues (Gleich and Adolphson, 1986).

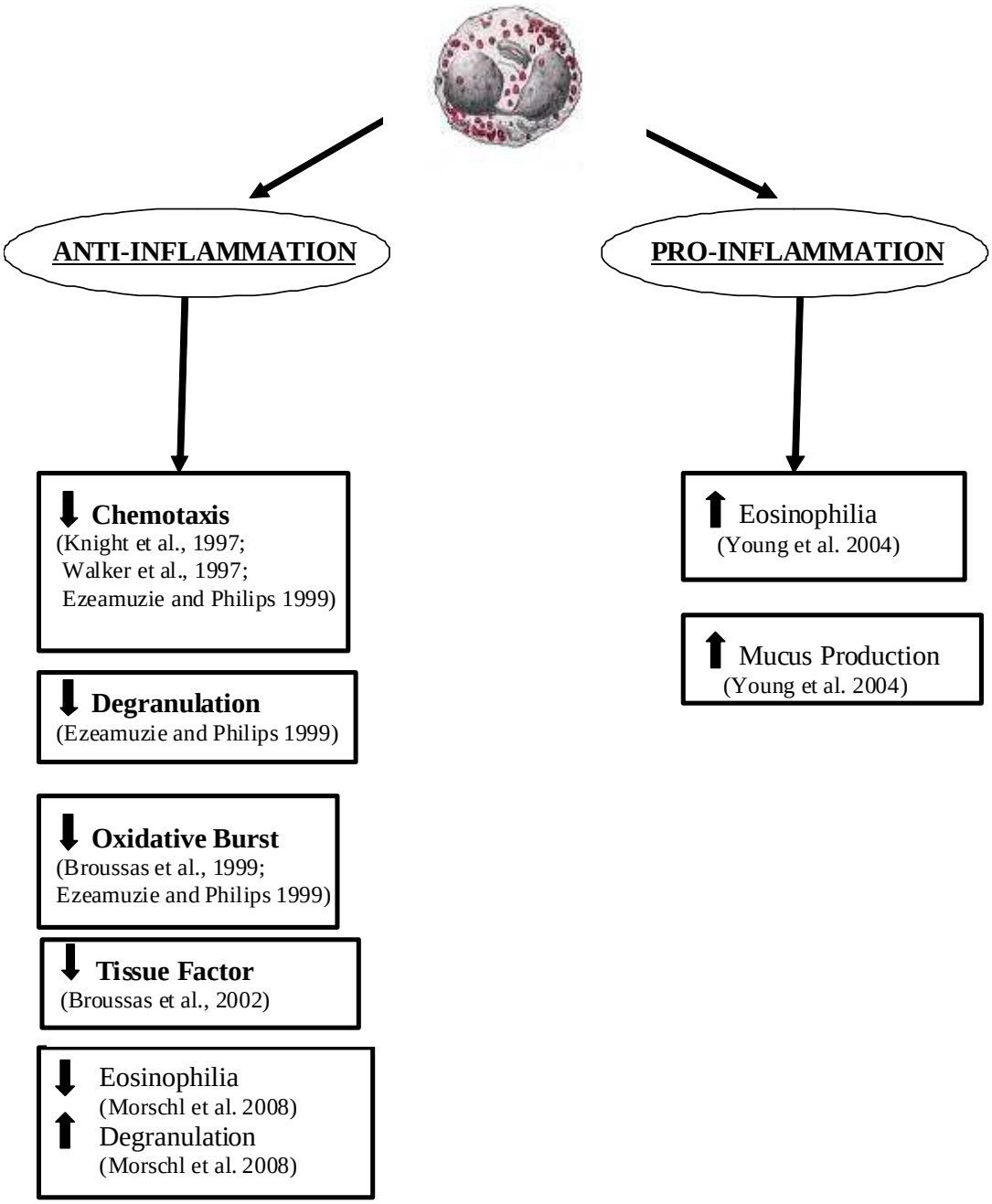
A_3 receptors are present on human eosinophils and couple to signalling pathways that lead to cell activation (Kohno et al., 1996a; Reeves et al., 2000). Despite this it has not proven easy to demonstrate the functional consequences of activation of these sites (Reeves et al., 2000). Nevertheless, the chronic inflammation in asthma is characterised by extensive infiltration of the airways by activated eosinophils (Holgate, 1999; Pearlman, 1999) and it remains possible that the elevated adenosine concentrations associated with asthma would contribute to eosinophil activation through stimulation of A_3 receptors. In addition, it has been speculated that activation of A_3 receptors may protect eosinophils from apoptosis (Gao et al., 2001). Thus, blockade of A_3 receptors may reduce the numbers of eosinophils and their activation thereby reducing the pro-inflammatory burden in the lung. Consistent with this, following 6 weeks treatment of mild asthmatics with

theophylline there was a significant reduction in the number of activated eosinophils beneath the epithelial basement membrane (Sullivan et al., 1994). Significantly, the average blood levels in this study (37 µM) were within the range of the affinity of theophylline for the human A_3 receptor. Moreover, it has been reported that activation of A_3 receptors mediates inhibition of eosinophil chemotaxis (Knight et al., 1997). The authors argue that since adenosine levels are highest at the site of inflammation, A_3 receptor activation would be pro-inflammatory by inhibiting eosinophil migration away from the sites of inflammation. Clearly, however, inhibition of chemotaxis could be pro- or anti-inflammatory. In line with a pro-inflammatory role, a high expression of A_3 receptor transcripts has been found in eosinophilic infiltrates of the lungs of patients with asthma and chronic obstructive pulmonary disease (COPD) (Walker et al., 1997). Interestingly, similar findings were seen in the lungs of adenosine deaminase deficient ($ADA^{-/-}$) mice that showed adenosine-mediated lung disease. Treatment of $ADA^{-/-}$ mice with MRS 1523, a selective A_3 receptor antagonist, prevented airway eosinophilia and mucus production. Similar results were obtained in the lungs of ADA/ A_3 receptor double KO mice, suggesting that A_3 receptor signalling plays an important role in regulating chronic lung disease and that A_3 receptor antagonism may be useful for reducing eosinophilia (Young et al., 2004). However these results contrast with those from experiments performed in human eosinophils *ex vivo,* where chemotaxis, degranulation and superoxide anion production were reduced by A_3 receptor activation (Knight et al., 1997; Walker et al., 1997; Ezeamuzie and Philips, 1999). This discrepancy was later attributed to the *ex vivo* nature of the chemotaxis experiments and implied that diminished airway eosinophilia seen in the lungs of $ADA^{-/-}$ mice following disruption of A_3 receptor is not a direct effect on the eosinophils but be due to the modulation of key regulatory molecules from other cells that express A_3 receptors and that affect eosinophil migration (Young et al., 2004). For example A_3 receptors are expressed on murine mast cells, airway macrophages and epithelial cells, all of which might affect eosinophil migration. However levels of key regulatory cytokines such as IL-5 and IL-13, or chemokines including eotaxin I, thymus- and activation-regulated chemokine (TARC) and monocyte chemotactic protein-3

(MCP3) were not affected by A_3 receptor deletion in $ADA^{-/-}$ mice, pointing perhaps to the involvement of A_3 receptor in the regulation of other key modulators of eosinophil migration such as cell adhesion molecules, extracellular matrix elements and proteases (Young et al., 2004). In contrast to a pro-inflammatory role of the A_3 subtype implied by the work of Young and colleagues cited above, the involvement of the A_3 adenosine receptor in a bleomycin model of pulmonary inflammation and fibrosis seems to indicate an anti-inflammatory effect (Morschl et al., 2008). Analysis of A_3 adenosine receptor KO mice revealed enhanced pulmonary inflammation including an increase in eosinophils and a selective up-regulation of eosinophil related chemokines and cytokines in the lungs of A_3 adenosine receptor KO mice exposed to bleomycin. This increase in eosinophil numbers was accompanied by a decrease in the eosinophil peroxidase activity in lavage fluid from A_3 adenosine receptor KO mice exposed to bleomycin, an observation suggesting the A_3 adenosine receptor is necessary for eosinophil degranulation in this model. Together these results suggest that the A_3 adenosine receptor mediates anti-inflammatory functions in the bleomycin model, and is also involved in regulating the production of mediators that can impact fibrosis (Morschl et al., 2008). The effects obtained in human eosinophils after A_3 receptor activation including inhibition of chemotaxis, degranulation, oxidative burst and the effects obtained from *in vivo* models of lung disease such as eosinophilia and mucus production are summarized in Figure 2.

Fig. 2-Effects of A_3 adenosine receptors in eosinophils

Given the important species differences in the location and functional response to A_3 receptors, only the effects obtained in human are in bold.



A_3 adenosine receptor effects on lymphocyte function

The ability of immune cells to fight tumor cells is fundamental for successful host defence against cancer. Adenosine, whose concentration increases within hypoxic regions of solid tumors, may interfere with the recognition of tumor cells by cytolytic effector cells of the immune system (Blay et al., 1997; Merighi et al., 2003). Adoptive immunotherapy with lymphokine-activated killer (LAK) cells has shown some promise in the treatment of certain cancers that are unresponsive to conventional treatment approaches. However, colon adenocarcinomas tend to respond poorly to LAK therapy, possibly as a result of tumor-induced immunosuppression. It has been demonstrated that colon adenocarcinoma cells inhibited anti-CD3-activated killer cell induction through the production of a tumor-associated soluble factor that was distinct from transforming growth factor beta or prostaglandins (Hoskin et al., 1994a). As a result, adenosine was suggested as a possible inhibitor of killer T-cell activation in the microenvironment of solid tumours (Hoskin et al., 1994b; Hoskin et al., 1994c). Indeed, evaluating the adhesion of murine spleen-derived anti-CD3-activated killer (AK) lymphocytes to syngeneic MCA-38 colon adenocarcinoma cells it was found that adenosine reduced adhesion by up to 60% (MacKenzie et al., 1994). The inhibitory effect of adenosine was exerted on AK cells and not on the MCA-38 targets and the agonist potency profile indicated that the A_3 receptor subtype might be responsible for the inhibition of adhesion. The authors suggested that this mechanism of immunosuppression, secondary to tissue hypoxia, may be important in the resistance of colorectal and other solid cancers to immunotherapy. In addition the same authors demonstrated that adenosine plays a strong inhibitory effect on the induction of mouse cytotoxic T cells (Hoskin et al., 2002). Diminished tumoricidal activity correlated with reduced expression of mRNAs coding for granzyme B, perforin, Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL). Interleukin-2 (IL-2) and interferon-γ (IFN-γ) synthesis by AK-T cells was also inhibited by adenosine. The inhibitory effect of adenosine on AK-T cell proliferation

was also blocked by an A_3 receptor antagonist suggesting that adenosine acts through A_3 receptors to prevent AK-T cell induction. Tumor-associated adenosine may act through the same mechanism to impair the development of tumor-reactive T cells in cancer patients. Therefore the suppression of T-killer cell function suggests that adenosine may act as a local immunosuppressant within the microenvironment of solid tumors. Subsequently it was reported that adenosine partially inhibits the interaction of T lymphocytes with tumor cells by blocking the function of integrin $\alpha 4\beta 7$ which is the major cell adhesion molecule involved in the adhesion of T cells to syngeneic MCA-38 adenocarcinoma cells (MacKenzie et al., 2002). The effect of adenosine has been investigated on the expression of costimulatory molecules by T cells in resting and activated conditions. The most important costimulatory molecules present on the T cells surface are CD2 and CD28 acting in concert to achieve optimal costimulation of T lymphocytes during interaction with antigen presenting cells. It has also been demonstrated that adenosine interferes with activation-induced expression of the co-stimulatory molecules CD2 and CD28 by an IL-2 dependent mechanism but not involving the accumulation of intracellular cAMP and possibly by activating the A_3 subtype (Butler et al., 2003). Subsequently the inhibitory effect mediated by adenosine on the ability of LAK cells to kill tumor cells was attributed essentially to the cAMP-elevating A_{2A} receptor whilst no evidence of the involvement of cAMP inhibitory A_1 or A_3 subtypes in the regulation of the cytotoxic activity of LAK cells was found (Raskovalova et al., 2005). Indeed, it has been suggested that hypoxic cancerous tissues may be protected by the same hypoxia→adenosine→A_{2A} receptor pathway that was recently shown to be critical and nonredundant in preventing excessive damage of normal tissues by overactive immune cells *in vivo* (Ohta et al., 2001).

In contrast to the immunosuppressive role of adenosine in the environment of solid tumors, it has been reported that A_3 receptor activation stimulates the proliferation of murine bone marrow cells *in vitro*. This effect was induced through the G-CSF production by human peripheral blood mononuclear cells (PBMC) mediated by adenosine. The finding was confirmed in *in vivo*

experiments, which revealed an increase in leukocyte and neutrophil numbers when adenosine was administered before chemotherapy (Fishman et al., 2000). The molecular mechanisms underlying G-CSF production included the upregulation of the PI3K, PKB/Akt and NF-kB pathways (Bar-Yehuda et al., 2002). In addition, it has been observed that Cl-IB-MECA increases the activity of NK cells in naïve and tumor bearing mice through the induction of IL-12; this effect was dependent on inhibition of cAMP levels and PKA expression. IL-12 is a potent stimulant of NK cells and is a cytotoxic factor that exerts a potent anti-tumor effect *in vivo*. It induces IFN-γ production by activated T and NK cells and augments cytotoxic activity of these cells via perforin, Fas and Trail-dependent mechanisms. Therefore, A_3 receptor activation enhances NK cell activity and probably NK cell-mediated destruction of tumor cells (Harish et al., 2003). The expression of A_3 receptor was also investigated in resting and activated lymphocytes (Gessi et al., 2004b). Activated human lymphocytes undergo a rapid induction of both transcript and protein of A_3 receptors. The kinetics of this up-regulation revealed that even at earlier time points, the increase was present only in $CD4^+$ cells, whereas it was not changed in $CD8^+$ cells. Therefore, it is possible that in humans, as in mice (Hoskin et al., 2002), A_3 receptors play an immunosuppressive role in $CD8^+$ T cells, but their up-regulation in $CD4^+$ cells strongly suggests that they might also be implicated in T helper cell activities. One method of increasing the number of A_3 receptors on the cell membrane is to increase the accumulation of mRNA encoding the A_3 subtypes. As evaluated by means of real-time RT-PCR experiments, activation of T cells with PHA rapidly increased the level of A_3 message in the $CD4^+$ subset, but not in the $CD8^+$ cells. This increase in A_3 receptor mRNA, which could occur as a result of an increase in transcription and/or an increase in mRNA stability, is likely to be responsible for the increased synthesis of receptor proteins as detected by means of binding and Western blot studies. The rapid up-regulation of A_3 receptors functionally coupled to adenylyl cyclase in activated T cells may indicate another potential example of biological significance for adenosine-mediated responses in T cells.

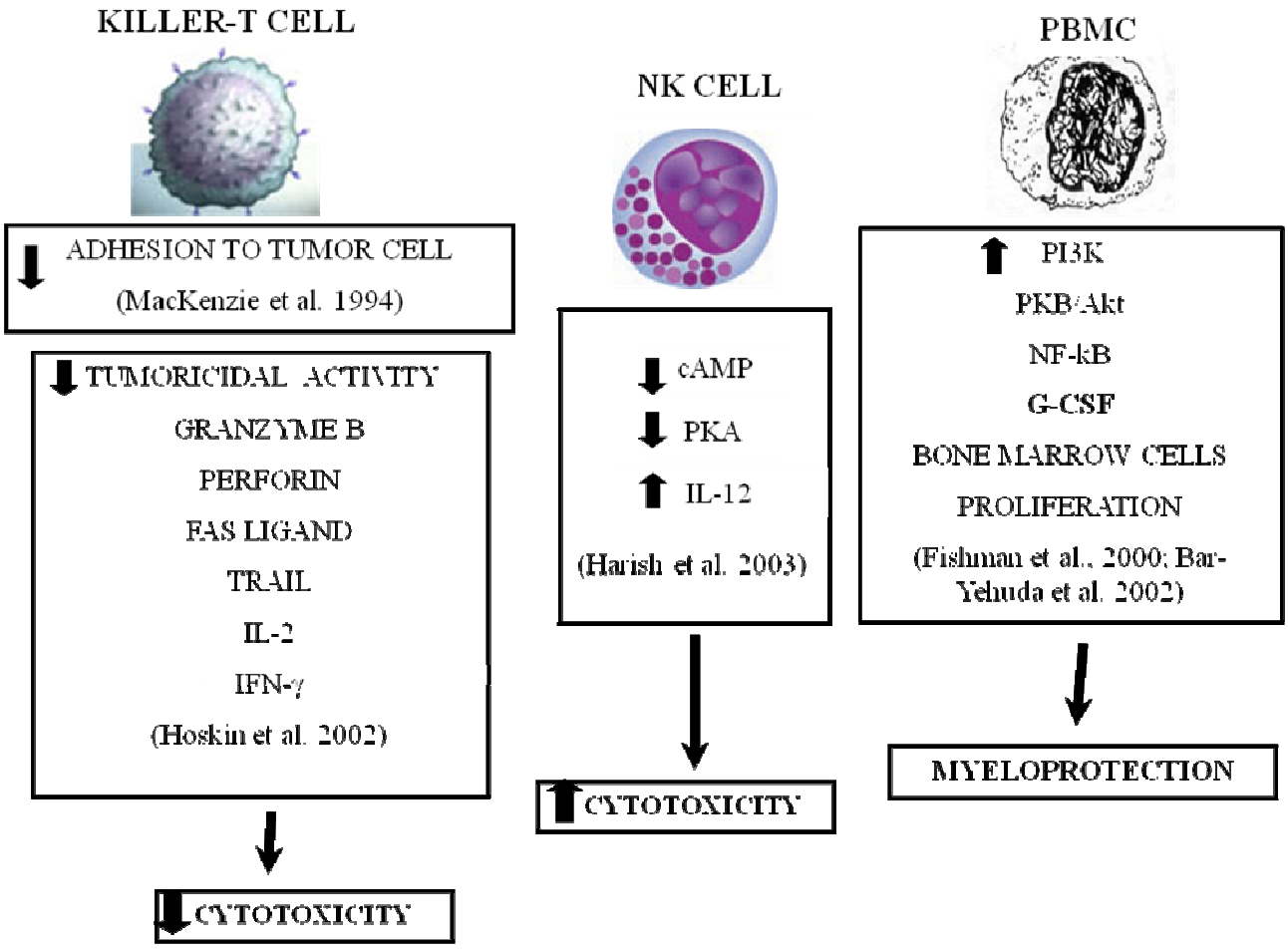
An overexpression of A_3 receptors has also been detected in lymphocytes of patients with colorectal cancer. Interestingly, the existence of A_3 receptors was previously demonstrated on Jurkat cells, a human leukemic cell line, where they were associated with inhibition of adenylyl cyclase activity and calcium modulation (Gessi et al., 2001). Blood lymphocytes obtained from 30 colorectal cancer patients showed a >3-fold overexpression of A_3 receptors compared with blood cells from healthy donors, in line with the data found in tissues. No association was found with stage of the disease, tumor site, patient age, or gender. Even though the mechanism of this up-regulation are not known it is interesting that binding data from tissues, as in circulating blood cells, discriminate between small-sized adenomas and cancer, suggesting that A_3 receptor may be a requirement for colorectal tumor progression. These receptors may represent, like those in neutrophils, tumoral markers due to their higher expression in comparison to that observed in healthy subjects. This suggests that peripheral blood cells mirror at the peripheral level the higher levels of the A_3 receptor found in colorectal cancer. However the selectivity of the A_3 receptor as a tumoral marker may be of only limited value because a similar phenomenon has been confirmed in patients with rheumatoid arthritis. Thus the A_3 receptor was overexpressed in PBMC of patients with rheumatoid arthritis compared to healthy subjects and was directly correlated to an increase in NF-kB in the same cells (Madi et al., 2007). Similar data were found in phytohemagglutinin and lipopolysaccharide-stimulated PBMC from healthy subjects suggesting that receptor upregulation is induced by inflammatory cytokines controlling the expression of the A_3 adenosine receptor transcription factor NF-kB (Madi et al., 2007). It seems that the A_3 adenosine subtype found in PBMC obtained from peripheral blood may not represent a specific tumoral marker but more generally a marker for inflammation.

In conclusion, it is well established that extracellular adenosine has the potential to be an important inhibitor of tumor cell destruction by NK and LAK cells within the microenvironment of solid tumors by signaling primarily through A_{2A} and A_3 adenosine receptors on the surface of T cells (Hoskin et al., 2008). However after the demonstration that

genetic deletion of immunosuppressive A_{2A} and A_{2B} receptors or their pharmacological inactivation can prevent the inhibition of anti-tumor T cells by the hypoxic tumor and facilitate full tumor rejection, several reviews focused on the relevance of A_{2A} and in minor part of A_{2B} adenosine subtypes to improve the effectiveness of immune-based cancer therapies (Ohta et al., 2006; Lukashev et al., 2007; Sitkovsky et al., 2008a,b). In contrast to the well described mechanisms by which A_{2A} adenosine receptor signaling blocks T cell activation and effector function, little is known about the mechanism of A_3 adenosine receptor-mediated T cell inhibition. Moreover, while the importance of A_{2A} adenosine receptor signaling in adenosine-mediated suppression of T cell responses has been confirmed using A_{2A} adenosine receptor-deficient mice (Lukashev et al., 2003), similar confirmatory studies have not yet been performed with A_3 adenosine receptor deficient mice. Additional studies need to be performed in human lymphocytes as almost all the functional effects attributed to A_3 receptor activation are derived from studies carried out in mice species (Figure 3). The identification of adenosine receptor subtypes and/or signal transduction pathways through which adenosine exerts its inhibitory effects on cell-mediated anti-tumor immune responses may allow for the development of novel “anti-adenosinergic” approaches that increase the effectiveness of therapeutic cancer vaccines and other immune-based cancer therapies.

Fig. 3-Effects of A_3 adenosine receptors in lymphocytes

Given the important species differences in the location and functional response to A_3 receptors, only the effects obtained in human are in bold.



A_3 adenosine receptor effects on monocyte-macrophage function

In vivo and *in vitro* studies in animal systems led to the concept of the mononuclear-phagocyte system as a cell system involved in host defenses, phagocytosis, and antigen presentation and processing (Douglas, 1999). Following Metchnikoff's development of phagocyte theory, Wright described opsonins as factors in serum that facilitated phagocytosis. Aschoff defined the reticuloendothelial system as a cellular system in which tissue macrophages and monocytes share important functional characteristics, namely, phagocytic ability and adhesiveness to glass. Subsequently, the histologic development of silver stains by Del Rio-Hortega defined a type of macrophage-related cell in the brain, the microglia. In the mid-1960s, the late Zanvil Cohn and his collaborators carried out seminal studies of mononuclear phagocytes leading to concepts of macrophage differentiation, activation, secretion, and the relationship of macrophages to antigen presentation and processing (for further details see Douglas, 1999).

Adenosine has been investigated as an endogenous regulator of monocyte-macrophage functions. The effects produced by A_3AR activation of macrophages seem to indicate an anti-inflammatory effect of this adenosine subtype. For example, the A_3ARs suppress TNF-α release induced by the endotoxin CD14 receptor signal transduction pathway from human monocytes and murine J774.1 macrophages (Le Vraux et al., 1993; McWhinney et al., 1996). Moreover in a macrophage model the A_3AR was the prominent subtype implicated in the inhibition of LPS-induced TNF-α production (Sajjadi et al. 1996). This effect was associated with changes in stimulation of the activator protein-1 (AP-1) transcription factor, whereas it was independent of MAPKs, NF-kB, PKA, PKC and PLC. The inhibitory effect induced by the A_3AR on TNF-α production was also assessed in A_3KO mice where the A_3 agonist was unable to reduce TNF-α levels in contrast to its effect in wild type animals (Salvatore et al., 2000). In contrast, in BV2 microglial cells the A_3-mediated inhibition of LPS-induced TNF-α expression was associated with the inhibition of LPS-induced activation of the PI3-kinase/Akt and NF-kB pathways (Lee et al., 2006). Recently it has been reported that in mouse

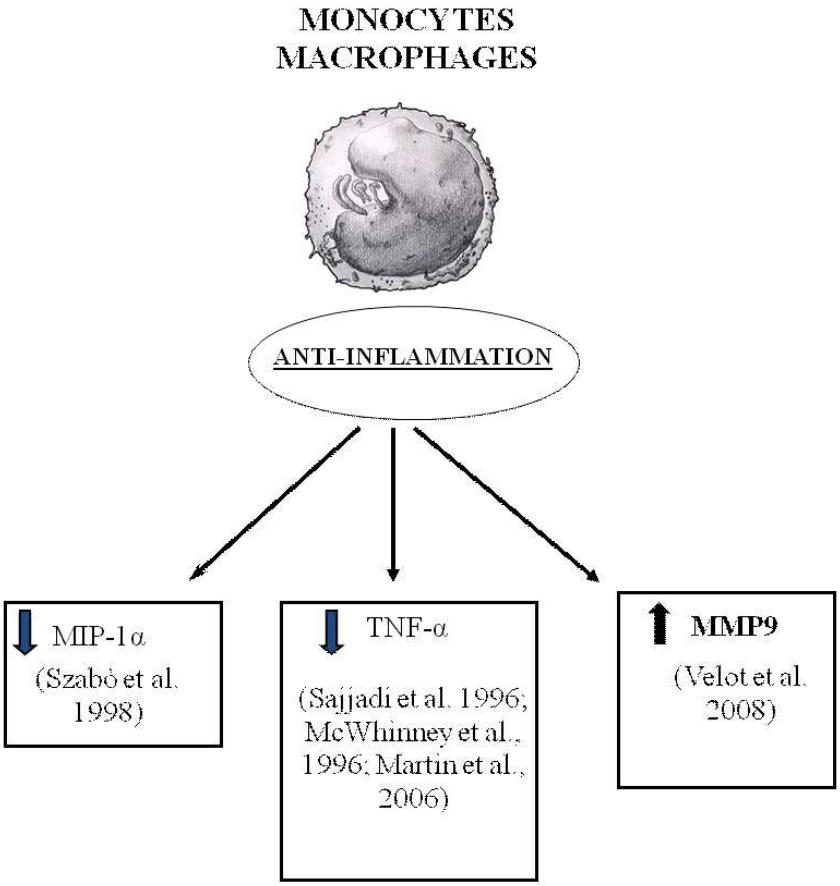
RAW 264.7 cells the A_3 subtype inhibits LPS-stimulated TNF-α release by reducing calcium-dependent activation of NF-kB and ERK 1/2 (Martin et al., 2006). In contrast, in peritoneal macrophages, isolated from A_3 KO mice, the ability of IB-MECA to inhibit TNF-α release was not altered in comparison to wild type mice (Kreckler et al., 2006). In this study, the inhibitory effect was exerted through the activation of A_{2A} and A_{2B} agonists as has been recently demonstrated in human monocytes (Zhang et al., 2005; Haskó et al., 2007). The discrepancy observed among these papers cannot be the consequence of species differences, since in both cases mouse cells were used. Other factors, including the source of the cells and/or the inflammatory stimulus used, may be responsible. However in spite of these contrasting results, one of the most likely therapeutic applications of the regulatory role of A_3 activation on TNF-α release is in the treatment of arthritis. More recent studies show that A_3AR agonists exert significant effects in different autoimmune arthritis models by suppression of TNF-α production (Baharav et al., 2005). The molecular mechanisms involved in the inhibitory effect of IB-MECA on adjuvant-induced arthritis include receptor downregulation and de-regulation of the PI3K-NF-kB signalling pathway (Fishman et al., 2006; Madi et al., 2007). Thus, A_3AR activation by IB-MECA inhibited macrophage inflammatory protein (MIP)-1α, a C-C chemokine with potent inflammatory effects, in a model of collagen-induced arthritis, providing the first proof of concept of the adenosine agonists utility in the treatment of arthritis (Szabò et al., 1998). Other anti-inflammatory effects involving A_3 receptors activation include inhibition of fMLP-triggered respiratory burst and tissue factor expression by human monocytes (Broussas et al., 1999, 2002). Recently, it has been reported that, adenosine may be involved in ventricular remodeling by stimulating Matrix metalloproteinase-9 (MMP-9) production in human macrophages following A_3 receptor activation (Velot et al., 2008). MMP-9 plays an important role in ventricular remodelling after acute myocardial infarction (MI). Adenosine enhanced MMP-9 production when macrophages were activated by hypoxia or Toll-like receptor-4 ligands such as lipopolysaccharide, hyaluronan, and heparan sulfate. The effect of adenosine was replicated by the A_3 agonist IB-MECA and inhibited by silencing the A_3AR through the use of RNA

interference. Interestingly, it was found that MMP-9 expression was higher in blood cells from patients with acute MI compared with healthy volunteers with important implications for therapeutic strategies targeting adenosine receptors in the setting of MI (Velot et al., 2008).

In conclusion as for the role of A_3 receptors in the inhibition of TNF-α production in macrophages discrepant results have been obtained and not only due to the different species considered. For example some studies attributed reduction of TNF-α to A_3 receptors either in human and mouse species (Sajjadi et al., 1996; McWhinney et al., 1996), whilst other found this effect to be mediated essentially by A_{2A} and in minor part by A_{2B} without the involvement of the A_3 receptors again in both human and mouse species (Zhong et al., 2005; Kreckler et al., 2006). Therefore it is difficult in this case to verify the relevance of the A_3 receptor-induced cellular response when other adenosine subtypes like A_{2A} and A_{2B} are also activated. As for the effects exerted by the A_3 subtype in human monocytes and macrophages it is possible to find support for an anti-inflammatory role for this receptor as attested by reduction of tissue factor, oxidative burst and perhaps TNF-α release. Also the recent discovery of an increase in MMP9 supports a role for A_3 agonists in the therapy of myocardial infarction (Velot et al., 2008) (Figure 4).

Fig. 4-Effects of A_3 adenosine receptors in monocytes-macrophages

Given the important species differences in the location and functional response to A_3 receptors, only the effects obtained in human are in bold.



A_3 adenosine receptor effects on dendritic cell function

Dendritic cells are antigen-presenting cells specialized to activate naive T lymphocytes and initiate primary immune responses (Steinman, 1991; Hart, 1997; Banchereau,et al., 1998). Dendritic cells originate from hemopoietic stem cells and migrate into peripheral tissues. Dendritic cells reside in an immature form in unperturbed tissue, where they are capable of taking up antigens but weak at stimulating T cells. Under the influence of a variety of so-called danger signals including pathogens; dying cells; soluble CD40 ligand; cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6), or bacterial products such as LPS dendritic cells undergo a process of differentiation known as maturation. Thereafter, they migrate to the T-cell areas of secondary lymphoid organs. This maturation process is associated with reduced phagocytic and endocytic activity, increased membrane expression of major histocompatibility complex and co-stimulatory molecules, production of cytokines such as interleukin 12 (IL-12), and acquisition of potent T-cell-stimulating functions. Depending on the conditions, dendritic cells can stimulate growth of a variety of T-cell subsets. In the presence of IL-12, they support the growth of Th1 cells, whereas with IL-4 dendritic cells induce Th2-cell differentiation. In recent years it has become clear that A_3 adenosine receptors play a role in regulation of various activities of dendritic cells.

The expression and function of adenosine receptors in human dendritic cells has been investigated by using reverse transcriptase-polymerase chain reaction and functional experiments (Panther et al., 2001). mRNA expression of the A_3 receptor has been detected in immature dendritic cells together with A_1 and A_{2A} receptors. Adenosine, IB-MECA and also the A_1 agonist CHA, induced Ca^{2+} transients as well as actin polymerization and chemotaxis but only in immature dendritic cells. These findings suggest that adenosine may control proinflammatory activities of dendritic cells and regulate their accumulation at target sites. Maturation of dendritic cells is accompanied by a loss of the adenosine responses such as Ca^{2+} transients, actin polymerization, and migration. Unequivocal evidence of cell surface expression of the A_3 receptor in immature dendritic cells was obtained from

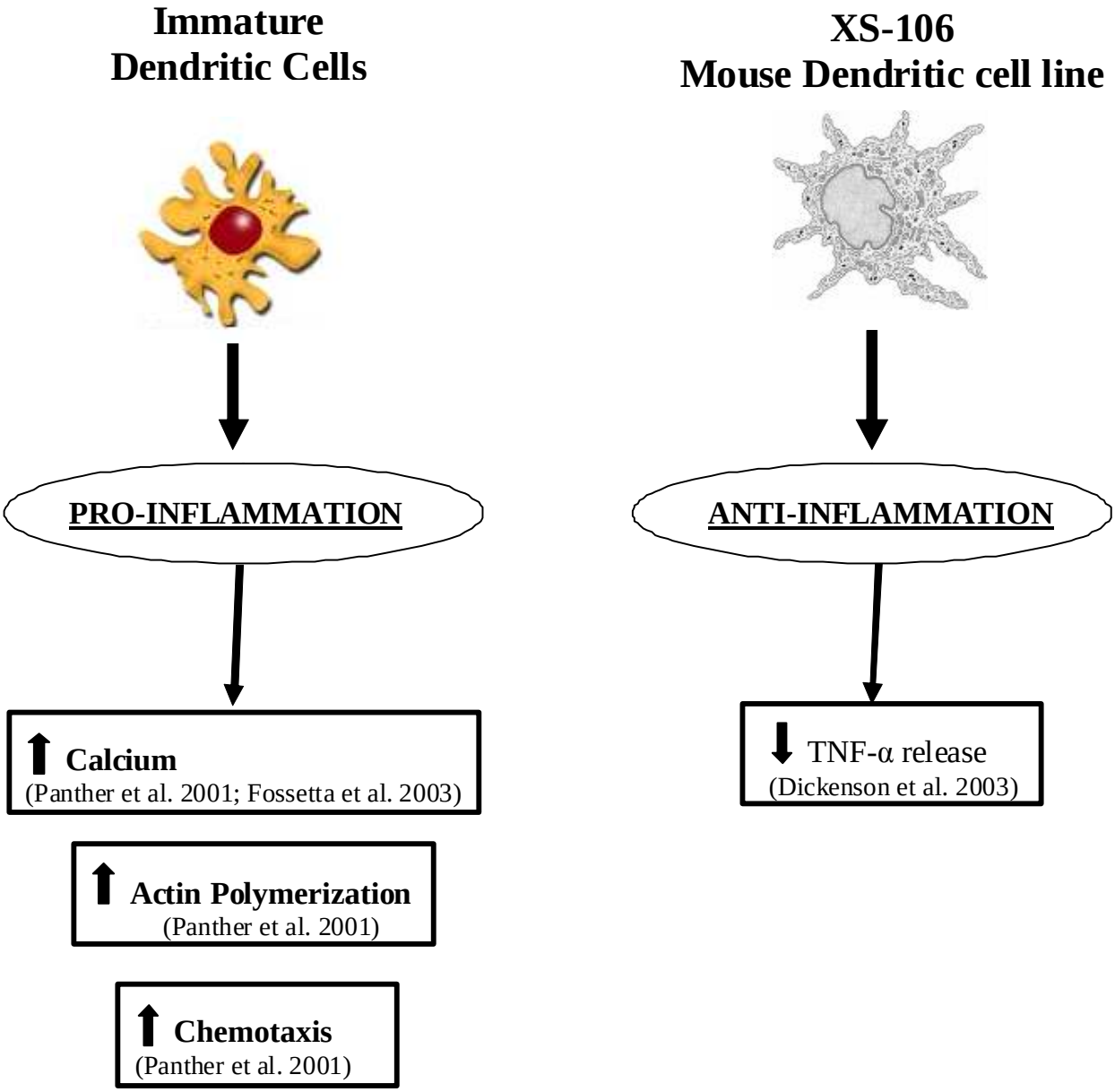
[^{125}I]ABMECA binding experiments. Saturation isotherms indicated a Bmax of approximately 300 fmol/mg membrane protein, and competition for the radioligand of a variety of adenosine receptor ligands categorically identified the binding site as the A_3 receptor (Fossetta et al., 2003). Moreover through fluorometric imaging plate reader (FLIPR)-based analysis of calcium mobilization it was shown that the A_3 adenosine receptor is coupled to calcium mobilization in a pertussis toxin-dependent way. Interestingly these authors demonstrated that adenosine is much more potent at the A_3 receptor than had been appreciated, being active in the low nanomolar range. Generally, adenosine has been regarded as a low potency agonist of the A_3 receptor, with apparent affinities ranging from 300 nM to 1 μM (Fredholm et al., 2001). The presence of functional A_3 receptors has been observed in XS-106, a mouse dendritic cell line, where they were coupled negatively to adenylyl cyclase and to stimulation of p42/p44 mitogen-activated protein kinase phosphorylation. Adenosine A_3 receptor activation also inhibits lipopolysaccharide-induced TNF-α release from XS-106 cells as already reported in macrophages (McWhinney et al., 1996; Dickenson et al., 2003). At present, the signal transduction pathway involved in adenosine A_3 receptor-mediated inhibition of TNF-α release from XS-106 cells (and see above macrophages) is unclear. Inhibition of TNF-α release is usually associated with Gs-protein-coupled receptor-mediated cyclic AMP production. Interestingly, adenosine A_3 receptors have been shown to induce an increase in intracellular calcium and potentiate Ca^{2+} currents via protein kinase A activation in A6 renal cells (Reshkin et al., 2000) and hippocampal CA_3 pyramidal neuronal cells (Fleming and Mogul, 1997). In addition, activation of the adenosine A_3 receptor stimulates cyclic AMP production in human eosinophils (Ezeamuzie and Philips, 2003). However, in XS-106 cells, Cl-IB-MECA did not stimulate cyclic AMP accumulation indicating that the adenosine A_3 receptor is not directly coupled to Gs-protein/cyclic AMP accumulation in XS-106 cells. Finally, the transcript for the A_3 adenosine receptor was elevated more than 100-fold in immature dendritic cells compared with monocyte precursors. A_3 receptor transcript was substantially diminished by LPS-induced maturation of immature dendritic cells. The strict dependence of A_3 receptor expression on the immature cells suggests that the A_3

receptor could also be involved in the maintenance of the immature phenotype, and its abrupt disappearance may be crucial for transition to a fully activated dendritic cell (Fossetta et al., 2003).

The relevance of the A_3 receptor over the other adenosine subtypes in immature human dendritic cells is attested to by different studies demonstrating a role for this receptor in the increase of intracellular calcium, actin polymerization and chemotaxis (Panther et al., 2001; Fossetta et al., 2003) (Figure 5). However a loss of the A_3 and an increase of the A_{2A} receptor has been reported during maturation of dendritic cells. This switch has been interpreted as a protective effect of adenosine in the context of tissue injury as A_{2A} activation plays an inhibitory role on dendritic cells migration. In this way adenosine could counterbalance inflammatory stimuli by delaying the arrival of mature dendritic cells to lymph nodes, thereby impairing the initiation of immune responses and reducing the potentially detrimental effects of chronic cell activation responsible for tissue damage and disease.

Fig. 5-Effects of A_3 adenosine receptors in dendritic cells

Given the important species differences in the location and functional response to A_3 receptors, only the effects obtained in human are in bold.



Conclusion

The data summarized in this chapter show that A_3 receptors are present in immune cells and are indeed involved in the physiopathologic regulation of inflammatory and immune processes. However results from *in vitro* and *in vivo* studies in experimental animals suggest activation of the A_3 subtype can be both pro or anti-inflammatory depending on:

i) the cell type examined e.g. neutrophil, eosinophil, macrophage, T cell, dendritic cell;

ii) the cellular model used e.g. in vitro or ex vivo; transgenic animals;

iii) the response investigated e.g. degranulation, oxidative burst, migration, maturation, cytokine production;

iv) the species considered e.g. human or animal;

v) the presence and functional roles of other adenosine receptor subtypes.

Even though it seems that in each cell type examined contrasting effects have been reported, the results reviewed here offer the background for possible new therapeutic strategies for a number of inflammatory conditions such as sepsis, asthma and autoimmune disorders including rheumatoid arthritis, Crohn's disease and psoriasis. Indeed at the moment there are A_3AR agonists in clinical development for rheumatoid arthritis. Unfortunately there are no A_3AR antagonists in clinical development but a number of molecules are in biological testing as therapeutic agents for asthma and COPD, glaucoma, and stroke, waiting to enter the clinical arena (Baraldi et al. 2008). Future studies aimed at elucidating new effects of the A_3 subtype in the modulation of important inflammatory responses in the different peripheral blood cells are likely to reveal exciting new potential therapeutic applications of A_3 agonists and/or antagonists.

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CHAPTER 2:

Modulation of MMP-9 in U87MG glioblastoma cells by A_3 adenosine receptors

INTRODUCTION

Local invasive growth is one of the key features of primary brain tumors. Glioma is the most common primary adult brain tumor with poor prognosis because of the aggressive invasion of the surrounding normal brain. Although our understanding of glioma oncogenesis has steadily improved, the molecular mechanisms that mediate glioma invasion are still poorly understood. The degradation of extracellular matrix (ECM) which exerts biochemical and mechanical barriers to cell movement has been shown to be an important biological process in tumor invasion and metastasis [1]. ECM degradation and remodelling require the action of extracellular proteinases, among which the matrix metalloproteinases (MMPs) have been shown to play an essential role. Indeed MMPs are cation-dependent endopeptidases which have been implicated in the malignancy of gliomas [2]. In particular it has been shown that MMP-9 facilitates the invasion of glioblastoma cells *in vitro* and MMP-9 overexpression correlates with the malignant progression of gliomas *in vivo* [3], [4], [5] and [6]. On the basis of reports from several different laboratories, it has been generally concluded that the basal levels of MMP-9 in most cancer cell lines are usually low and that its expression can be induced by treatment of growth factors, cytokines, tumor promoters and oncogenes, through the activation of its gene promoter [7]. Several studies have identified signal transduction pathways involved in the regulation of MMP-9 expression in tumor cells [8]. A major mechanism through which signals from extracellular stimuli are transmitted to the nucleus involves activation of extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 mitogen-activated protein kinase (MAPK) [7], [9], [10] and [11]. Furthermore a critical role for PI3K/Akt signalling in the MMP-9 modulation has been described [9] and [12]. The human MMP-9 promoter contains several cis-acting regulatory elements that participate in the regulation of the MMP-9 gene expression, including sites that bind the transcription factors activator protein 1 (AP-1), nuclear factor-kappa B (NF-kB) and Sp1. In particular the AP-1 transcription complex appears to play an essential role in stimulating transcriptional activation of MMP-9 [10], [11] and [13].

Adenosine (Ado) is a purine nucleoside which is released from metabolically active cells or is generated extracellularly by degradation of released ATP. It regulates a wide variety of physiological processes interacting with one or more of four known cell surface receptors named A_1, A_{2A}, A_{2B} and A_3 [14]. The development of potent A_3 agonists and selective antagonists revealed that the A_3 subtype plays a pivotal role in inflammation, in the ado-induced modulation of tumor cells biology [15], [16] and [17] and the A_3 subtype has been found up-regulated in colorectal cancer [18]. Recently, we have demonstrated that ado stimulates proliferation of colon cancer cells and up-regulates under hypoxia the transcription factor hypoxia-inducible factor-1α (HIF-1α); this led to an increase in vascular endothelial growth factor (VEGF) and angiogenesis in melanoma, glioblastoma and colon carcinoma cells through A_3 receptor activation [19], [20], [21] and [22]. This effect was also observed in pro-inflammatory and pro-atherosclerotic foam cells [23]. However, the involvement of ado in the regulation of MMP-9 in tumor cells have not been investigated by now. In this study we will use U87MG human glioblastoma cells as an *in vitro* model to evaluate the role of ado in the modulation of MMP-9 and the intracellular pathways involved. The main finding of this work is that ado increases MMP-9 protein levels in cellular extracts of U87MG cells through A_3 receptors activation and phosphorylation of ERK1/2, JNK, Akt and AP-1. Furthermore it also increases extracellular MMP-9 levels in supernatants from U87MG cells; this effect is responsible for an increase of glioblastoma cell invasion.

MATERIAL AND METHODS

Materials

U87MG glioblastoma cancer cells were purchased from American Type Culture Collection (Manassas, VA, USA). [^{3}H]DPCPX (specific activity 120 Ci/mmol), was purchased by NEN Research Products, (Boston, MA, USA). [^{3}H]ZM 241385 (specific activity 20 Ci/mmol), was

furnished by Tocris, (Boston, MA, USA). [^{3}H]MRE 2029F20 (specific activity 123 Ci/mmol) and [^{3}H]MRE 3008F20 (specific activity 67 Ci/mmol), were synthesized at Amersham International (Buckinghamshire, UK). MRE 2029F20, MRE 3008F20, DPA23 were synthesized by Prof. P.G. Baraldi (Department of Pharmaceutical Sciences, University of Ferrara, Italy). U0126 and SB 202190 were from Promega (Milan, Italy). SH-5 was from Vinci-Biochem (Florence, Italy). Adenosine A_3 receptors and MMP-9 small interfering RNA (siRNA) were from Santa Cruz Biotechnology, D.B.A. ITALIA s.r.l., (Milan, Italy). RNAiFect Transfection Kit was from Qiagen (Milan, Italy). NDGA was obtained from Calbiochem Inalco S.p.A. (Milan, Italy). Unless otherwise noted, all other reagents were purchased from Sigma (Milan, Italy).

Cell cultures

U87MG glioblastoma cancer cells were maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12 medium) with 20% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, at 37 °C in 5% CO_2/95% air. All treatments to the cells with ado were carried out in the presence of the adenosine deaminase (ADA) inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) 5 μM and those with ado agonists were performed in the presence of ADA. Cells viability was assessed through trypan blue assays and was not modified after treatments with ado or drugs.

Real-time RT-PCR

Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method. Quantitative real-time RT-PCR assay [19] of MMP-9 mRNA was carried out using gene-specific fluorescently labelled TaqMan MGB probe (minor groove binder) in a ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington Cheshire, UK). For the real-time RT-PCR of

A_1, $A_{2A,}$ A_{2B} and A_3 ado subtypes the assays-on- demand™ Gene expression Products Hs00181231_m1, Hs00169123_m1, Hs00386497_m1, Hs00181232_m1, were used, respectively (Applied Biosystems, Monza, Italy). Moreover curves of ado receptors cDNA plasmid standards with a range spanning at least six orders of magnitude (10^{-11}–10^{-16} g/μl) were generated. These standard curves displayed a linear relationship between Ct values and the logarithm of plasmid amount [19]. Quantification of ado receptor messages in cancer cells was made by interpolation from standard curve of Ct values generated from the plasmid dilution series. For the real-time RT-PCR of MMP-9 and TIMP-1 the assay-on-demand™ Gene expression Products Hs00234579_m1, Hs00171558_m1 were used, respectively. For the real-time RT-PCR of the reference gene the endogenous control human β-actin kits was used, and the probe was fluorescent-labeled with VIC™ (Applied Biosystems, Monza, Italy).

Binding studies

Binding assays were carried out according to Gessi et al. [19]. Saturation experiments of antagonist radioligands [^{3}H]DPCPX (0.2–20 nM), [^{3}H]ZM 241385 (0.2–20 nM), [^{3}H]MRE 2029F20 (0.4–40 nM) and [^{3}H]MRE 3008F20 (0.3–30 nM) to label A_1, A_{2A}, A_{2B} and A_3 ado receptors, respectively, were carried out in U87MG cells. 100 μl of membrane homogenate (80–100 μg of protein assay^{-1}) were incubated in duplicate, in a final volume of 250 μl in test tubes containing 50 mM Tris–HCl buffer (10 mM $MgCl_2$ for A_{2A}, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM benzamidine for A_{2B} and 10 mM $MgCl_2$, 1 mM EDTA for A_3) pH 7.4, with 10–12 different concentrations of each selective radioligand. Non-specific binding, defined as binding in the presence of 1 μM DPCPX, 1 μM SCH 58261, 1 μM MRE 2029F20, 1 μM MRE 3008F20 for A_1, A_{2A}, A_{2B} and A_3 ado receptors, respectively, at the K_D value for each radioligand was ≈30–35% of total binding. Bound and free radioactivity were separated, after an incubation time of 120 min at 4 °C, by filtering the assay mixture through Whatman GF/B glass-fiber filters using a cell harvester

(Packard Instrument Company, CT, USA). The filter bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Scint 20.

Western blotting analysis

Whole cell lysates, prepared as described previously [21], were resolved on a 10% SDS gel and transferred onto the nitrocellulose membrane. Western blot analyses were performed as described previously using antibody against MMP-9 (Calbiochem Inalco, Milan, Italy) (1:200 dilution) in 5% non-fat dry milk in PBS 0.1% Tween-20 overnight at 4–8 °C. Recombinant active MMP-9 (83 kDa) was used as positive control (Calbiochem Inalco, Milan, Italy). Aliquots of total protein sample (50 μg) were analyzed using antibodies specific for phosphorylated (Thr183/Tyr185) p44/p42 MAPK (1:5000 dilution) (Promega, Milan, Italy), phosphorylated (Thr180/Tyr182) p38 MAPK (1:1000 dilution) (Cell Signaling Technology, Milan, Italy), phosphorylated (Ser473) Akt/PKB (protein kinase B) (1:1000 dilution), phosphorylated (Thr183/Tyr185) SAPK/JNK, phosphorylated (Ser73) c-Jun (Cell Signaling Technology, Milan, Italy), and for A_3 receptor (Aviva Antibody Corporation, Milan, Italy) (1 mg/ml dilution). Filters were washed and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies against mouse and rabbit IgG (1:2000 dilution). Specific reactions were revealed with the Enhanced Chemiluminescence Western blotting detection reagent (Amersham Corp., Arlington Heights, Ill.). Tubulin (1:250) was used to ensure equal protein loading.

Densitometry analysis

The intensity of each band in immunoblot assay was quantified using a VersaDoc Imaging System (Bio-Rad, Milan, Italy). Mean densitometry data from independent experiments were normalized to the results in control cells. The data were presented as the mean ± S.E.

Treatment of cells with small interfering RNA (siRNA)

U87MG cells were plated in six-well plates and grown to 50–70% confluence before transfection. Transfection of ado A_3 receptor or MMP-9 siRNA, was performed at a concentration of 100 nM using RNAiFectTM Transfection Kit for 72 h [21]. A non-specific control ribonucleotide sense strand (5′-ACU CUA UCU GCA CGC UGA CdTdT-3′) and antisense strand (5′-dTdT UGA GAU AGA CGU GCG ACU G-3′) were used under identical conditions.

Enzyme-linked immunosorbent assay (ELISA)

The levels of MMP-9 protein secreted by the cells in the medium were determined by an ELISA kit (RayBio Elisa Kit, Tebu-bio, Milan, Italy). In brief, subconfluent cells were changed into fresh serum-free medium in the presence of solvent or various concentrations of ado and ado ligands for 24 h. The medium was collected, centrifuged for 5 min at 900 g to remove floating cells and assayed for MMP-9 content by ELISA according to the manufacturer's instructions. The data were presented as mean ± SE from three independent experiments.

Gelatin zimography

Cells were incubated in serum-free medium for 24 h and the respective supernatants were used for the experiments. Twenty micrograms were mixed with sample buffer (0.5 M Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue) and separated on 10% SDS-polyacrylamide gels containing either gelatine from porcin skin (1 mg/ml). After electrophoresis, gels were washed for 1 h in renaturing buffer (2.5% Triton X-100) and subsequently incubated overnight at 37 °C in 50 mM Tris–HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij 35 pH 7.5). Gels were stained with 0.3% Coomassie Brilliant blue and destained with 7% acetic acid methanol, 1% methanol and 7%

Invasion assay

Invasion was measured by assessment of the U87MG cells migration rate by using Cell Invasion Assay kit (Chemicon International, Milan, Italy). 2×10^5 U87MG cells were plated into the upper

well of the chamber in serum-free medium for 72 h, while the lower well was filled up to the top with MEM plus 10% FBS as chemoattractant. Treatment of the cells with ado, Cl-IB-MECA and inhibitors was carried out for 48 h. After incubation stained invasive cells were quantitated by dissolving them in 10% acetic acid and reading OD at 560 nm according to the manufacturer's instructions.

Protein inhibitors

U0126, SB202190, SH-5 specific inhibitors of ERK 1/2, p38 and Akt were used at a concentration of 1 μM according to previous experiments showing their specificity carried out in cancer cells [22]. SP600125, the JNK inhibitor was used at a dose of 1 μM according to its affinity and selectivity data [24]. Nordihydroguaiaretic acid (NDGA), the AP-1 inhibitor was used at a dose of 10 μM according to literature data [25]. MMP-9 inhibitor I was used at a dose of 50 nM [26].

Data analysis

Binding studies were analyzed with the program LIGAND [27]. Statistical analysis was performed by means of analysis of variance (ANOVA) and the Dunnett's test. $P < 0.05$ was considered significant.

RESULTS

Expression of ado receptors mRNA and protein in human U87MG glioblastoma cells

First of all we examined the pattern of expression of ado receptors in U87MG glioblastoma cells. The mRNA level of ado receptors was examined through real-time RT-PCR experiments. As shown in Fig. 1A U87MG cells expressed all ado subtypes with the following rank order $A_{2A} > A_{2B} > A_1 > A_3$. At a protein level saturation assays with the A_1 receptor antagonist [^{3}H]DPCPX revealed the presence of A_1 receptors with a K_D value of 2.5 ± 0.3 nM and a B_{max} value of 28 ± 1 fmol/mg of protein; saturation studies with the A_{2A} antagonist [^{3}H]ZM 241385 showed A_{2A} receptors with a K_D value of 2.0 ± 0.1 nM and a B_{max} value of 110 ± 13 fmol/mg of protein; saturation experiments with the A_{2B} antagonist [^{3}H]MRE2029F20 detected A_{2B} receptors with a K_D value of 3.8 ± 0.5 nM and a B_{max} value of 85 ± 9 fmol/mg of protein; saturation assays with the A_3 antagonist [^{3}H]MRE3008F20 revealed A_3 receptors with a K_D value of 2.2 ± 0.40 nM and a B_{max} value of 102 ± 10 fmol/mg of protein (Fig. 1B).

Figure.1

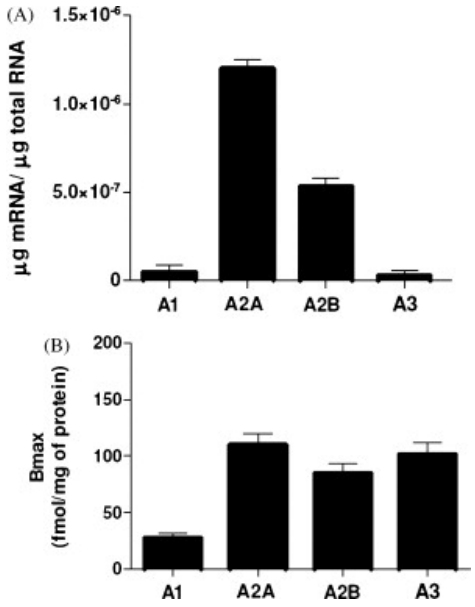

Fig. 1. mRNA and protein expression of ado receptors in human U87MG glioblastoma cells. (A) Bar graph showing µg RNA/µg total RNA of human A_1, A_{2A}, A_{2B} and A_3 ado receptors evaluated through real-time RT-PCR experiments. (B) Bar graph showing B_{max} (fmol/mg of protein) of human A_1, A_{2A}, A_{2B} and A_3 ado receptors measured by means of binding experiments. Experiments were performed as described in Section 2. Values are the means and vertical lines S.E. of the mean of three separate experiments performed in triplicate.

Ado effect on MMP-9 mRNA in U87MG glioblastoma cells

Initial experiments were carried out to evaluate the effect of ado on MMP-9 transcript levels in U87MG cells. After treatment of 6, 12, 24 and 48 h with the nucleoside (100 μM) we observed a time-dependent increase in MMP-9 mRNA of 1.5 ± 0.2, 1.8 ± 0.2, 2.5 ± 0.3 and 3.5 ± 0.4 fold, respectively (Fig. 2A). In order to identify which receptor subtype was involved in the ado-mediated MMP-9 increase we incubated U87MG glioblastoma cells with high affinity A_1, A_{2A}, A_{2B} and A_3 ado analogues. The A_1, A_{2A} and A_{2B} agonists, CHA, CGS 21680 and DPA23 respectively, did not affect MMP-9 increase whilst the A_3 agonist Cl-IB-MECA 100 nM was able to induce a raise of 2.0 ± 0.3 fold of MMP-9 mRNA level, after 24 h of treatment, in U87MG glioblastoma cells. These results indicated that ado may increase MMP-9 mRNA through the involvement of A_3 receptors (Fig. 2B). Furthermore ado 100 μM and the A_3 agonist Cl-IB-MECA 100 nM induced a modest stimulation of 1.6 ± 0.2 and 1.4 ± 0.2 fold of increase, respectively, of tissue inhibitor metalloproteinase-1 (TIMP-1) (Fig. 2C). The relative affinities of adenosine agonists as well as antagonists used in this study for the different adenosine receptor subtypes are provided in Table 1.

Figure.2

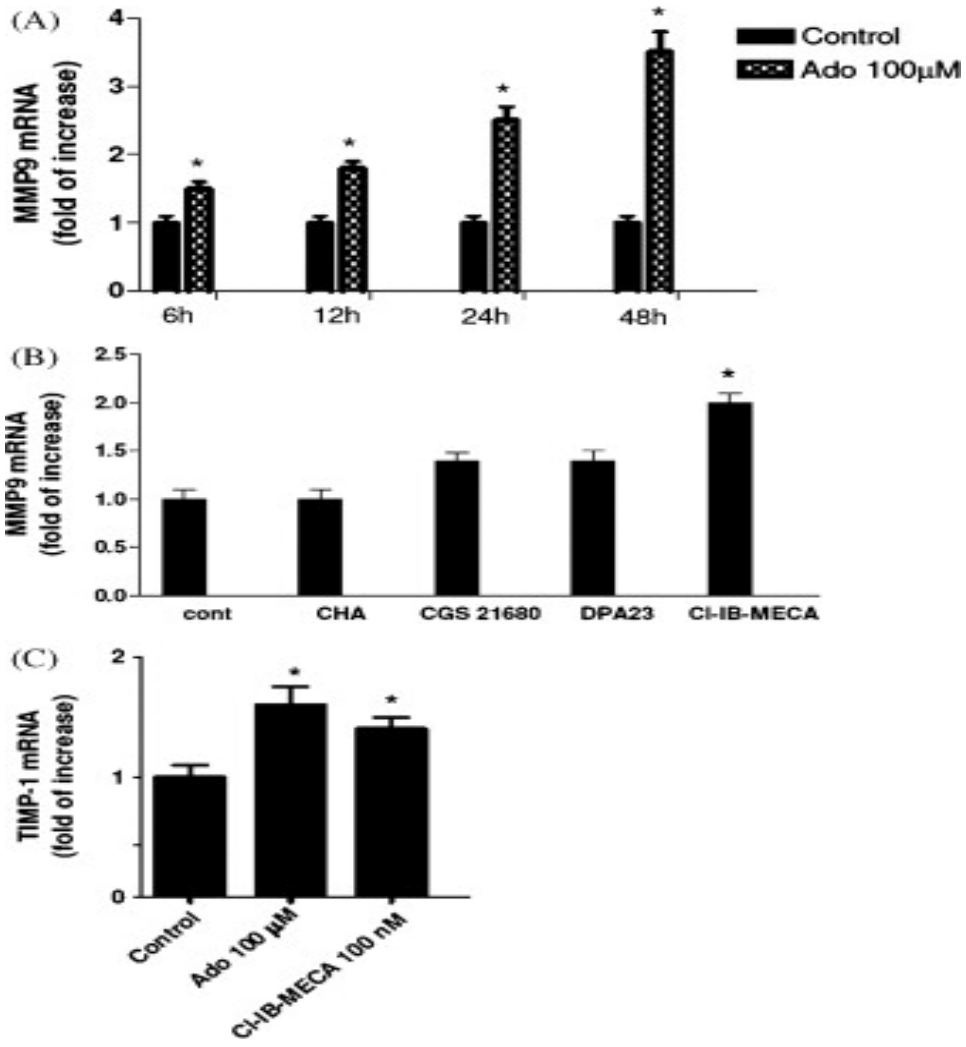

Fig. 2. Modulation of MMP-9 mRNA by ado in U87MG glioblastoma cells. (A) Time course of MMP-9 mRNA expression in U87MG cells after treatment with ado 100 μM. (B) Effect of ado receptor agonists, 100 nM CHA, 100 nM CGS 21680, 100 nM DPA23, 100 nM Cl-IB-MECA on MMP-9 mRNA induction in U87MG cells. (C) Effect of ado and Cl-IB-MECA on TIMP-1 mRNA expression in U87MG glioblastoma cells. Experiments were performed as described in Section 2. Values are the means and vertical lines S.E. of the mean of three separate experiments performed in triplicate. $P < 0.05$ compared with the control (cells incubated in the absence of treatment). MMP-9 mRNA control values at 6, 12, 24 and 48 h were arbitrarily defined as 1.

Table 1.

Affinity (Ki, nM) of selected adenosine receptor agonists and antagonists to A_1, A_{2A}, A_{2B} and A_3 adenosine receptors.

	A_1	A_{2A}	A_{2B}	A_3	Ref.
Agonists					
CHA	3.5	812	>1000	83	[60]
CGS 21680	289	27	>10,000	67	[61]
DPA23	8.5	>1000	7.3	38.4	[62]
Cl-IB-MECA	220	5360	>100,000	1.4	[61]
Antagonists					
DPCPX	3.9	129	51	1100	[15]
SCH 58261	549	1.1	>10,000	>10,000	[15]
MRE 2029F20	200	>1000	5.5	>1000	[15]
MRE 3008F20	1200	141	2100	0.82	[15]
ZM 241385	774	1.6	75	0.82	[61]

Ado effect on MMP-9 protein levels in U87MG glioblastoma cells

Using an anti-MMP-9 antibody, that recognized both the pro and active forms of MMP-9 as bands migrating at 92 kDa and ≈78 kDa respectively, we found that Ado 100 μM induced a time-dependent increase of both pro and active MMP-9 levels starting from 6 h and reaching a peak after 12–24 h of incubation (Fig. 3A and B). U87MG glioblastoma cells showed constitutive expression of the ≈78 kDa form of MMP-9, that was the active MMP-9 protein, as further verified by comparison with the recombinant active MMP-9, used as positive control (Fig. 3C). As the effect induced by ado on active MMP-9 was most evident we focused our attention on it. Treatment of the cells with ado in the range 0.01–100 μM for 24 h resulted in a dose-dependent increase of active MMP-9 with an EC_{50} of 3.5 ± 0.2 μM (Fig. 4A and B). By using a series of selective antagonists of A_1, A_{2A}, A_{2B} and A_3 ado receptors we observed that the nucleoside effect was abrogated in the presence of the A_3 blocker MRE 3008F20, whilst was unaffected by 100 nM DPCPX, SCH 58261 and MRE 2029F20, A_1, A_{2A}, and A_{2B} antagonists, respectively (Fig. 4C and D). To further verify the involvement of A_3 receptors in the modulation of MMP-9 protein expression, we treated U87MG glioblastoma cells with the high affinity A_3 receptor agonist Cl-IB-MECA, in western blotting experiments (Fig. 5A). A_3 receptor activation by Cl-IB-MECA, in the range of concentration 1–500 nM, produced a stimulatory effect on MMP-9 protein levels, with an EC_{50} of 10 ± 1 nM (Fig. 5B). Furthermore increasing concentrations of MRE 3008F20 (0.05–100 nM) were able to inhibit MMP-9 protein increase, induced by a maximal dose (100 nM) of Cl-IB-MECA, with an IC_{50} of 1.3 ± 0.1 nM (Fig. 5C). To demonstrate more conclusively a role for A_3 receptor in the adenosine-induced MMP-9 protein accumulation, we tried to knockdown A_3 receptor expression using small interfering-(si)-RNA leading to a transient knockdown of the A_3 receptor gene (siRNAA_3). U87MG glioblastoma cells were transfected with non-specific random control ribonucleotides or with siRNAA_3. As expected 72 h post

transfection, A_3 receptor mRNA and protein levels of U87MG glioblastoma cells were significantly reduced (Fig. 5D and E, respectively). Therefore, at 72 h from the siRNA transfection we found that the stimulatory effect induced by a maximal dose of the A_3 agonist was strongly reduced, suggesting that inhibition of A_3 receptor expression is responsible for the block of Cl-IB-MECA-mediated MMP-9 increase (Fig. 5F and G)

Figure.3

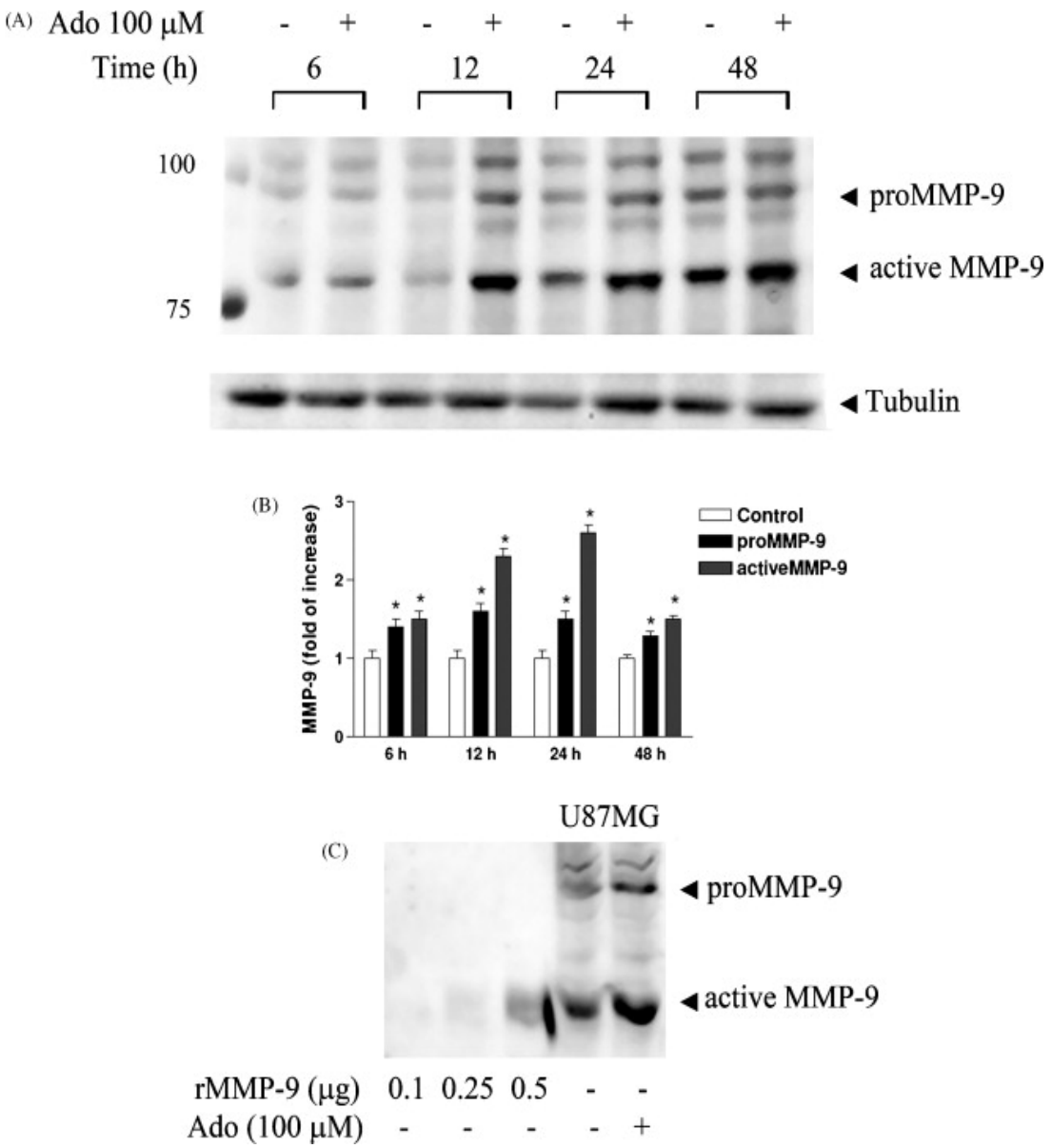

Fig. 3. Modulation of pro and active MMP-9 protein levels by ado in U87MG glioblastoma cells. (A) Time course of MMP-9 protein levels in U87MG cells after treatment with ado 100 µM. On the left the 75 and 100 kDa bands of the molecular weight marker are shown. (B) Densitometric quantification of MMP-9 western blot is the mean ± S.E. values ($N = 3$); $P < 0.05$ compared with the control. MMP-9 protein control values at 6, 12, 24 and 48 h were arbitrarily defined as 1. (C) Increasing concentrations (0.1, 0.25, 0.5 µg) of recombinant active MMP-9 (83 kDa) and endogenous pro and active MMP-9 in U87MG cells in the absence or presence of ado.

Figure.4

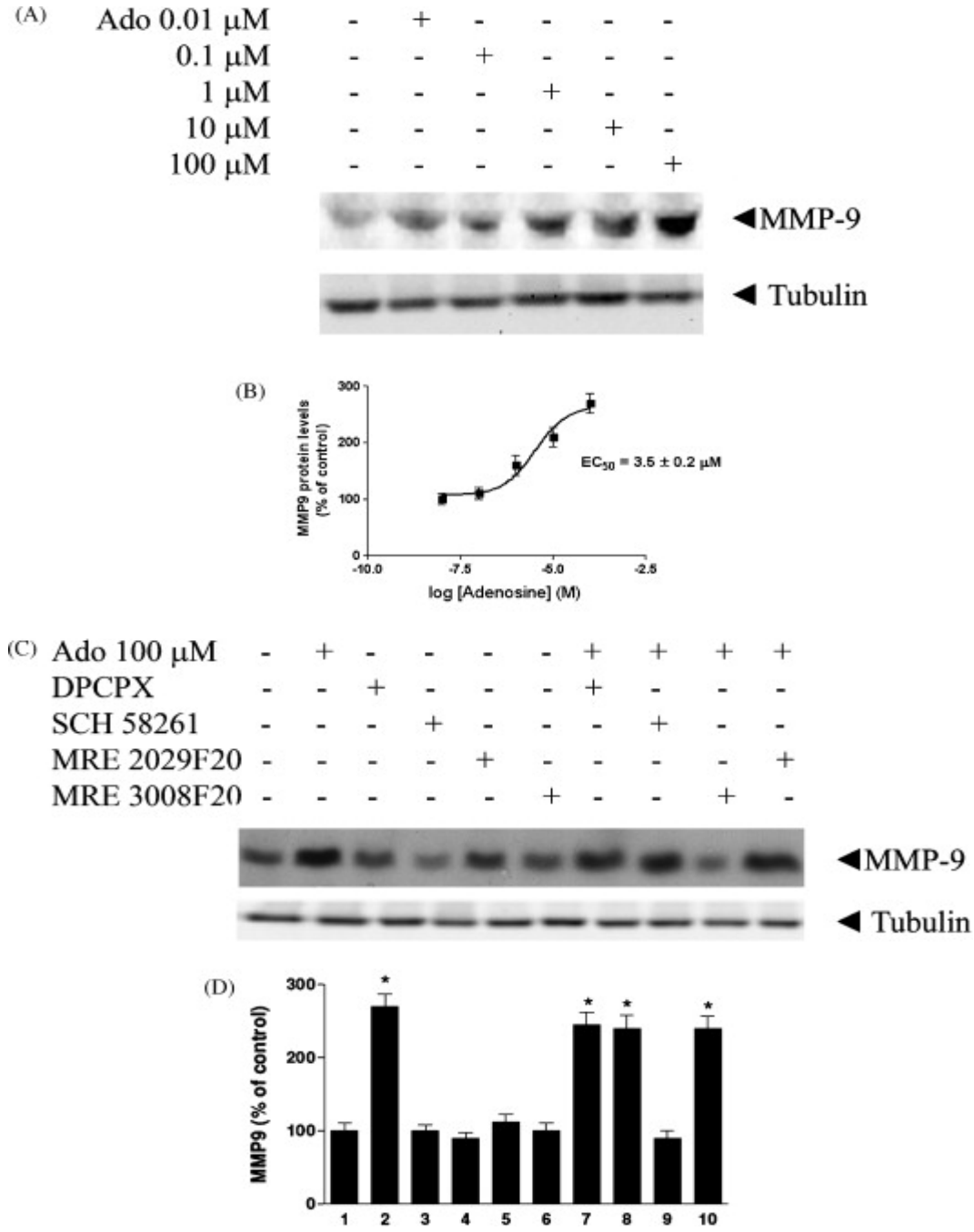

Fig. 4. Effect of ado and ado antagonists on MMP-9 protein levels in U87MG glioblastoma cells. (A and B) Immunoblot and relative dose-response curve of ado (0.01–100 µM) on MMP-9 protein levels, respectively. (C) Effect of ado 100 µM on MMP-9 protein levels and antagonism by 100 nM DPCPX, SCH 58261, MRE 3008F20 and MRE 2029F20. (D) Densitometric quantification of MMP-9 western blot is the mean ± S.E. values ($N = 3$); $P < 0.05$ compared with the control (cells incubated in the absence of treatment).

Figure.5

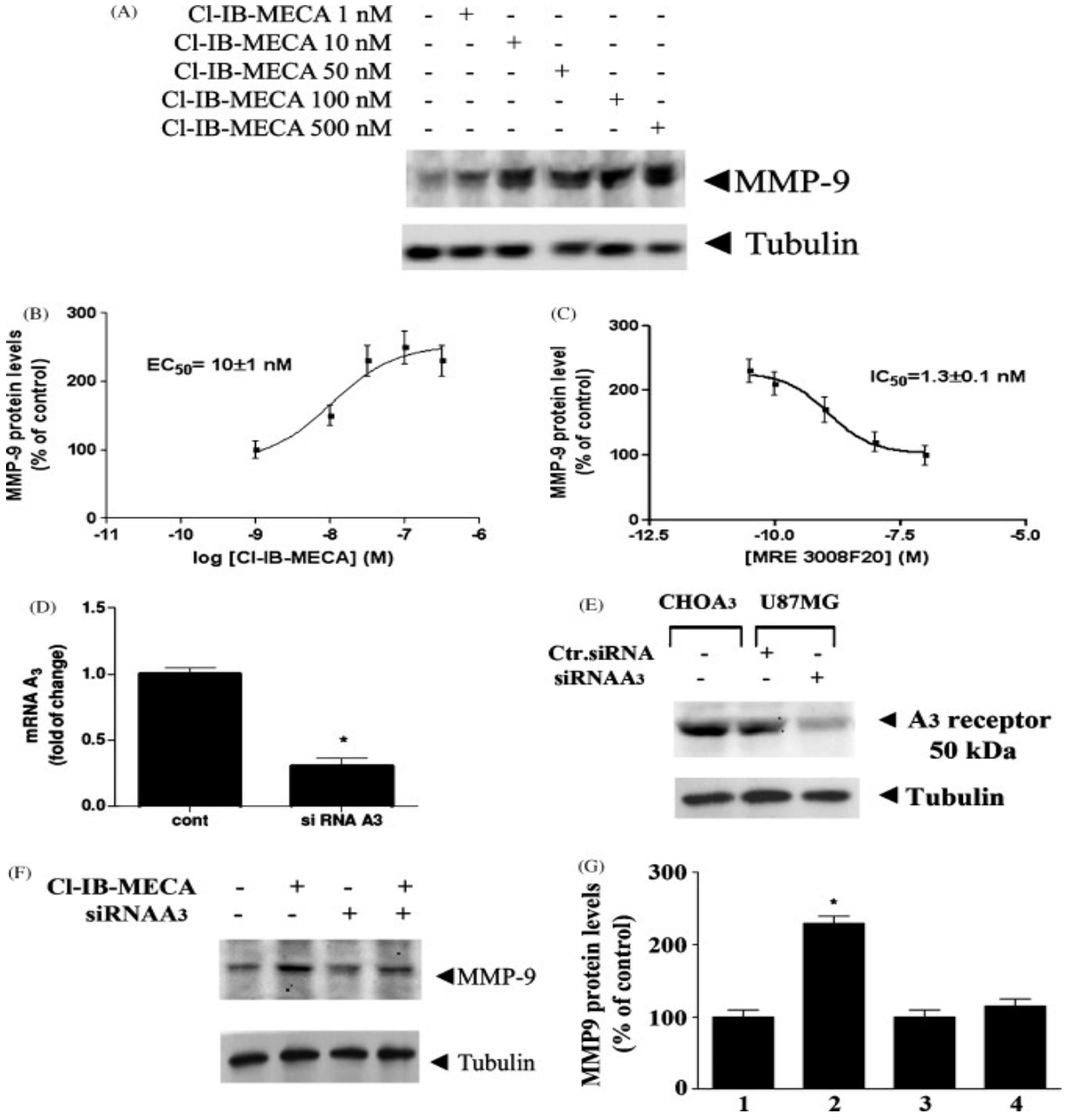

Fig. 5. Involvement of A_3 receptor on ado-induced MMP-9 protein levels modulation in U87MG glioblastoma cells. (A and B) Immunoblot and relative dose-response curve of Cl-IB-MECA (1–500 nM) on MMP-9 protein levels, respectively. (C) Dose-response curve of antagonism by MRE 3008F20 (0.05–100 nM) on 100 nM Cl-IB-MECA effect. Values are the means and vertical lines SE of the mean of three separate experiments performed in triplicate. (D) Relative A_3 ado receptor mRNA quantification, related to β-actin mRNA, and (E) A_3 receptor protein level in U87MG cells after transfection with A_3 siRNA for 72 h. (F) Effect of A_3 receptors siRNA treatment on Cl-IB-MECA (100 nM) induction of MMP-9 in U87MG cells. (G) Densitometric quantification of MMP-9 western blot is the mean ± S.E. values ($N = 3$); $P < 0.05$ compared with the control.

Intracellular pathways activated by ado A_3 receptor to increase MMP-9 levels in U87MG glioblastoma cells

To establish the intracellular pathways triggered by A_3 receptors to stimulate MMP-9 protein increase in U87MG cells the effect of the A_3 receptor agonist on MAPK and Akt activation was investigated. To this aim glioblastoma cells were pretreated for 30 min with 1 μM U0126, SB202190, SP600125, SH-5, specific inhibitors of ERK1/2, p38, JNK and Akt, respectively and subsequently stimulated with Cl-IB-MECA. As reported in Fig. 6A and B, U0126, SP600125 and SH-5 inhibitors at a concentration of 1 μM were able to inhibit the effect induced by the A_3 agonist Cl-IB-MECA on MMP-9 protein levels whilst SB202190 did not, suggesting that ERK1/2, JNK and Akt but not p38 pathway were required for MMP-9 protein increase induced by A_3 receptor activation. Furthermore, we observed that treatment of glioblastoma cells with Cl-IB-MECA for 6, 12 and 24 h induced a time-dependent increase of ERK1/2, JNK and Akt phosphorylation (Fig. 6C–E). Similar results were obtained with ado (Fig. 7A–C) and its effect was antagonized by MRE 3008F20 and siRNAA_3 supporting a role for A_3 receptors in kinases phosphorylation (Fig. 7D–F).

Figure.6

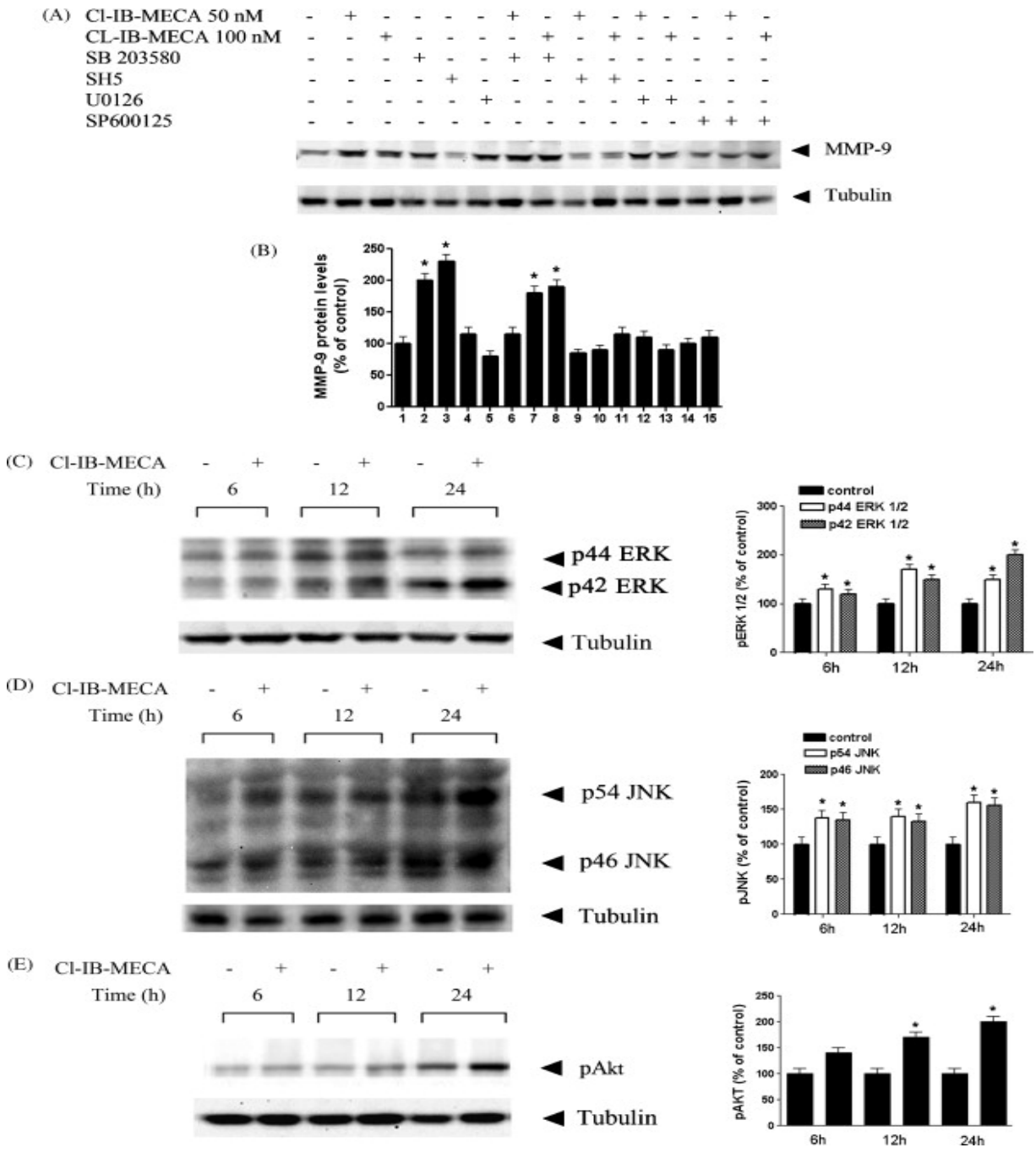

Fig. 6. Involvement of MAPK and Akt kinases in A_3 receptor-induced MMP-9 protein levels in U87MG cells. (A) Role of p38, Akt, ERK 1/2 and JNK inhibitors in MMP-9 modulation induced by Cl-IB-MECA 50–100 nM. (B) Densitometric quantification of MMP-9 western blot is the mean ± S.E. values ($N = 3$); (C–E) Time course of Cl-IB-MECA 100 nM on ERK1/2 (C), JNK (D) and Akt (E) phosphorylation. Densitometric quantification of western blots is the mean ± S.E. values ($N = 3$); $P < 0.05$ compared with the control (cells incubated in the absence of treatment).

Figure.7

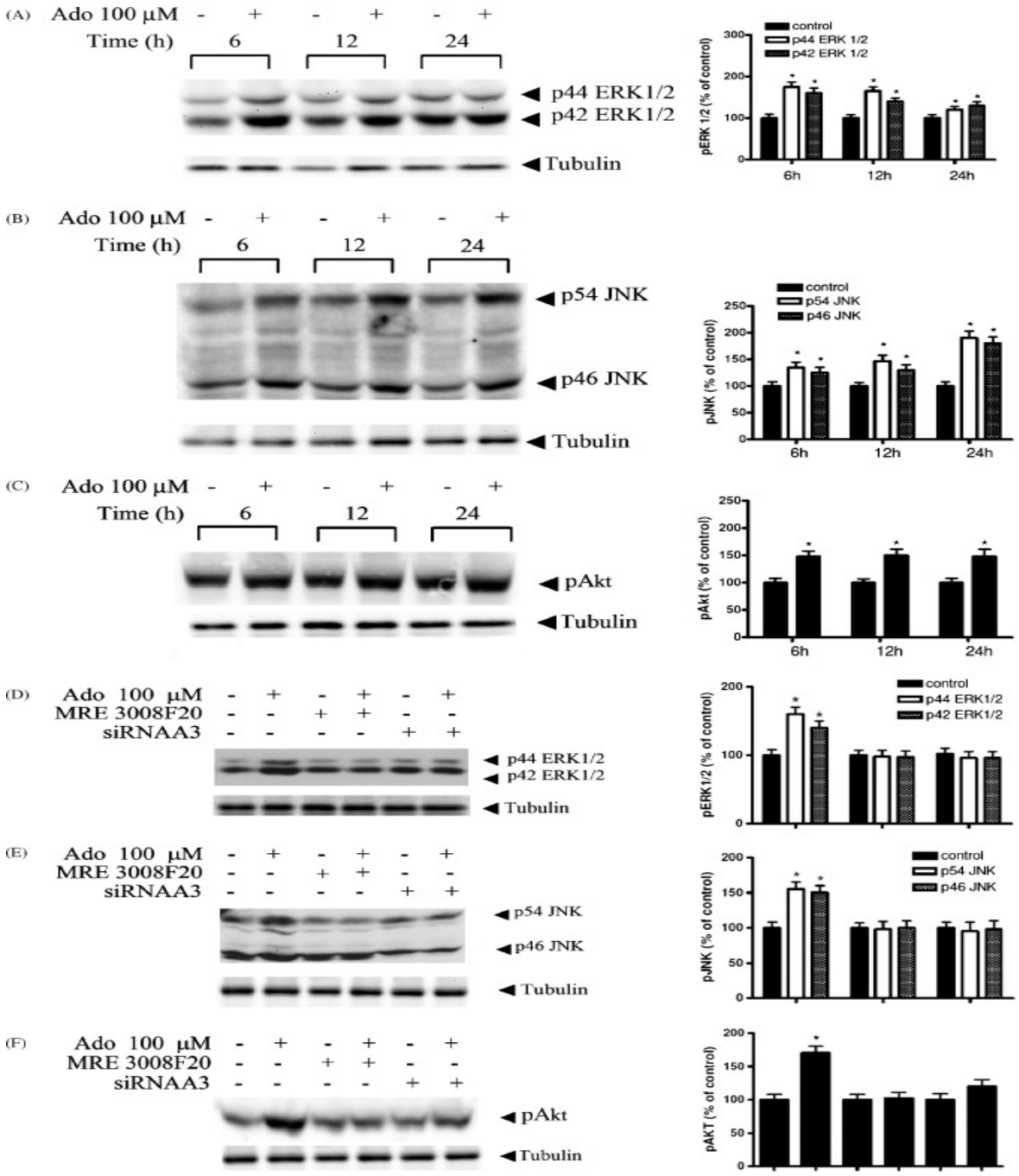

Fig. 7. Time course of ado 100 μM on ERK1/2 (A), JNK (B) and Akt (C) phosphorylation and antagonism by MRE 3008F20 and siRNA of A_3 receptors on the ado effect (12 h) on ERK1/2 (D), JNK (E) and Akt (F) phosphorylation. Densitometric quantification of western blots is the mean ± S.E. values ($N = 3$); $P < 0.05$ compared with the control (cells incubated in the absence of treatment, or with MRE 3008F20, or with siRNAA_3 alone, defined as 100).

AP-1 involvement in the A_3 receptor-mediated increase of MMP-9 levels in U87MG glioblastoma cells.

The activator protein-1 (AP-1) transcription complex appears to play an essential role in stimulating transcriptional activation of MMP-9. In order to verify the involvement of AP-1 in the A_3 ado receptor-induced MMP-9 protein increase, we performed western blotting experiments in the presence of ado 100 μM and the A_3 agonist Cl-IB-MECA 500 nM, in combination with the AP-1 antagonist NDGA 10 μM. As reported in Fig. 8A and B the AP-1 inhibitor was able to reduce the ado A_3 receptor-mediated effect on MMP-9 levels. Furthermore, to evaluate in more detail the effect of A_3 receptor activation on the phosphorylation of c-Jun, a major subunit of AP-1, glioblastoma cells were treated with increasing concentrations of ado and Cl-IB-MECA. As shown in Fig. 8C-F both ado and Cl-IB-MECA were able to raise p-c-Jun protein levels in a dose-dependent way with an EC_{50} of 4.5 ± 0.3 μM and 9.0 ± 0.8 nM, respectively. When the A_3 receptor subtype was blocked with the A_3 antagonist MRE 3008F20, the stimulatory effect induced by Cl-IB-MECA on p-c-Jun was reverted, suggesting again the involvement of A_3 receptors in this effect (Fig. 8G and H).

Figure.8

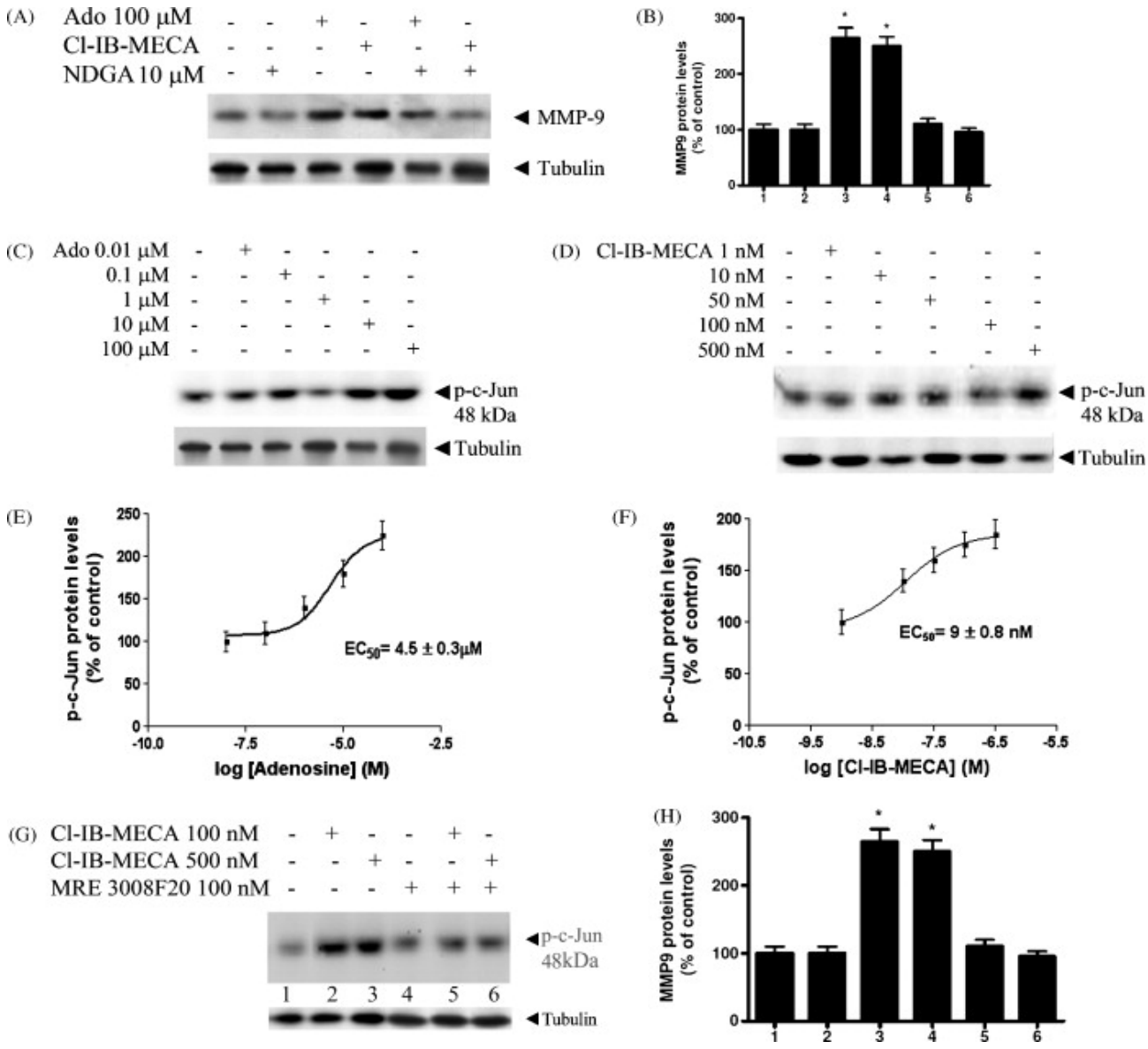

Fig. 8. Involvement of AP-1 in A_3 receptor-induced MMP-9 protein levels in U87MG cells. (A) Detection of MMP-9 protein by western blotting experiment in the presence of ado (100 μM) or the A_3 agonist Cl-IB-MECA (500 nM) in combination with the AP-1 antagonist NDGA 10 μM. (B) Densitometric quantification of western blots is the mean ± S.E. values (N = 3); $P < 0.05$ compared with the control. (C–F) Immunoblots and relative dose-response curves of ado (0.01–100 μM) and Cl-IB-MECA (1–500 nM) on p-c-Jun protein levels, respectively. (G) Effect of Cl-IB-MECA 100 and 500 nM on p-c-Jun levels and antagonism by MRE 3008F20 100 nM on Cl-IB-MECA effect. (H) Densitometric quantification of MMP-9 western blot is the mean ± S.E. values (N = 3); $P < 0.05$ compared with the control.

MMP-9 levels in supernatants from U87MG glioblastoma cells by ELISA and gelatin zimography assays

In order to investigate whether A_3 receptor activation was also able to increase MMP-9 protein levels in the supernatants from human U87MG glioblastoma cells, ELISA assays, recognizing both the latent and active MMP-9 forms, were performed. After 24 h of treatment ado and the A_3 agonist Cl-IB-MECA, were able to induce a stimulatory effect of 162 ± 14% and 175 ± 15% on MMP-9 levels in cancer cells that was antagonized by both A_3 antagonist and siRNA treatment (Fig. 9A). The A_1, A_{2A} and A_{2B} adenosine analogues did not modulate MMP-9 levels. Furthermore, MMP-9 activity was evaluated in the conditioned medium using zymography. A band corresponding to the active 82 kDa MMP-9 form was detected and was increased after exposure of U87MG glioblastoma cells to the A_3 agonist Cl-IB-MECA 100–500 nM and Ado 10–100 μM for 24 h (Fig. 9B and C).

Figure.9

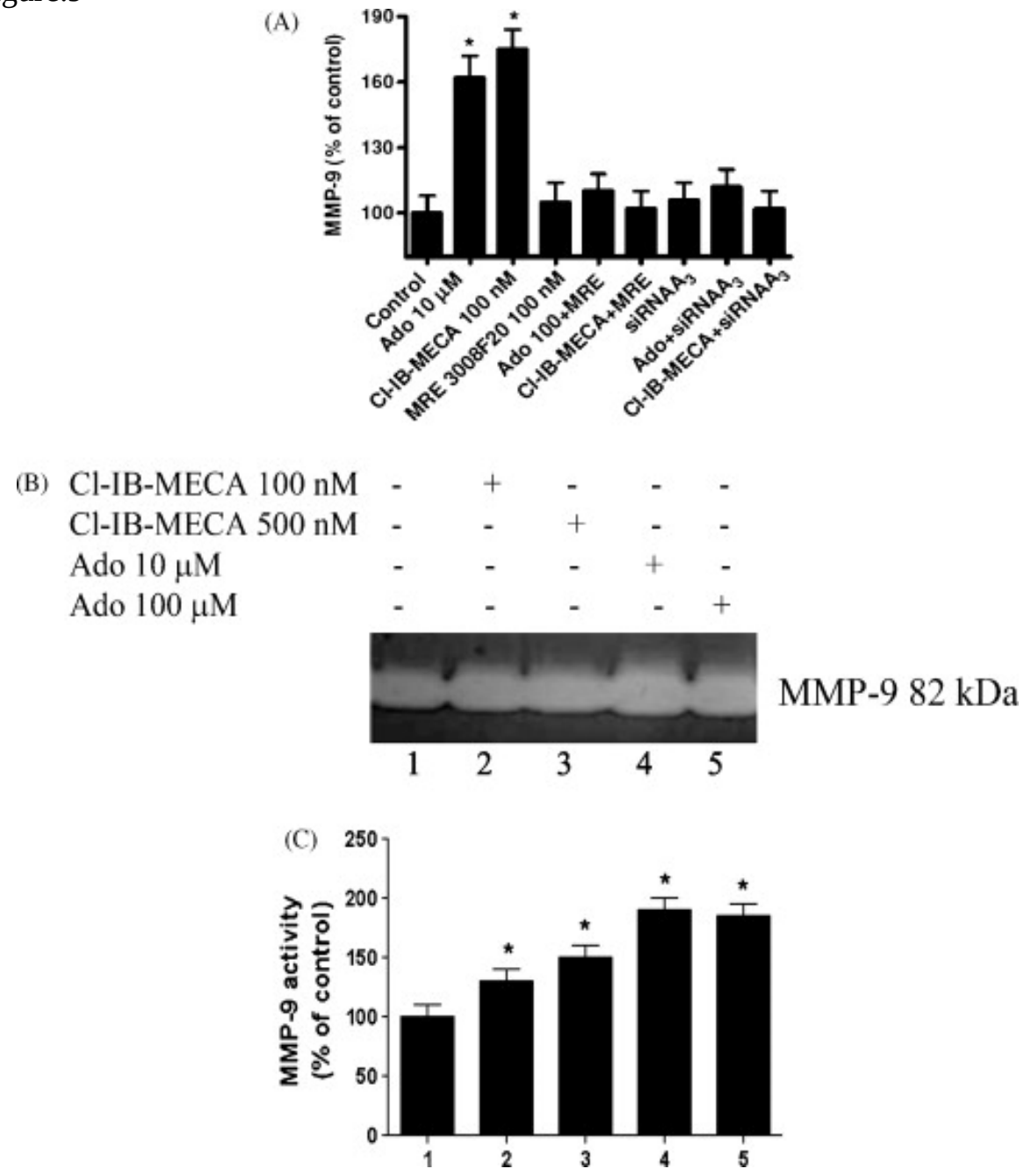

Fig. 9. A_3 receptor-mediated increase of MMP-9 in supernatants of U87MG cells. (A) Effect of ado, Cl-IB-MECA, A_3 antagonists and A_3 silencing on MMP-9 levels by ELISA assay. Values are the mean ± S.E. values (N = 3); $P < 0.05$ compared with the control. (B) Effect of 100, 500 nM Cl-IB-MECA and 10, 100 µM ado on MMP-9 activity by gelatin zymography. Experiments were performed as described in Section 2. (C) Densitometric quantification of gelatine zimography. Values are the means and vertical lines S.E. of the mean of three separate experiments performed in triplicate. $P < 0.05$ compared with the control.

A_3 receptor effect on invasiveness of U87MG glioblastoma cells

To assess whether increased production of MMP-9 protein by A_3 receptor activation resulted also in increased invasion of tumor cells, an *in vitro* cell invasion assay was done. We cultured U87MG glioblastoma cells on invasion chambers in the absence and in the presence of Ado 100 μM and the A_3 agonist Cl-IB-MECA 100 nM. After 48 h of cultures in the presence of Ado and the A_3 agonist, the invasive capacity of U87MG cells was highly increased (160 ± 20% and 180 ± 30% of control, respectively) and the effect was reduced by the addition of the A_3 antagonist MRE 3008F20, A_3 silencing, MMP-9 inhibitor I and MMP-9 silencing. This suggests that A_3 receptors and MMP-9 play a role in modulating glioblastoma invasiveness (Fig. 10A). The stimulatory effect induced by both Ado and Cl-IB-MECA was also antagonized by U0126, SH-5 and SP600125 indicating the involvement of ERK1/2, Akt and JNK in the invasion of U87MG cells (Fig. 10B).

Figure.10

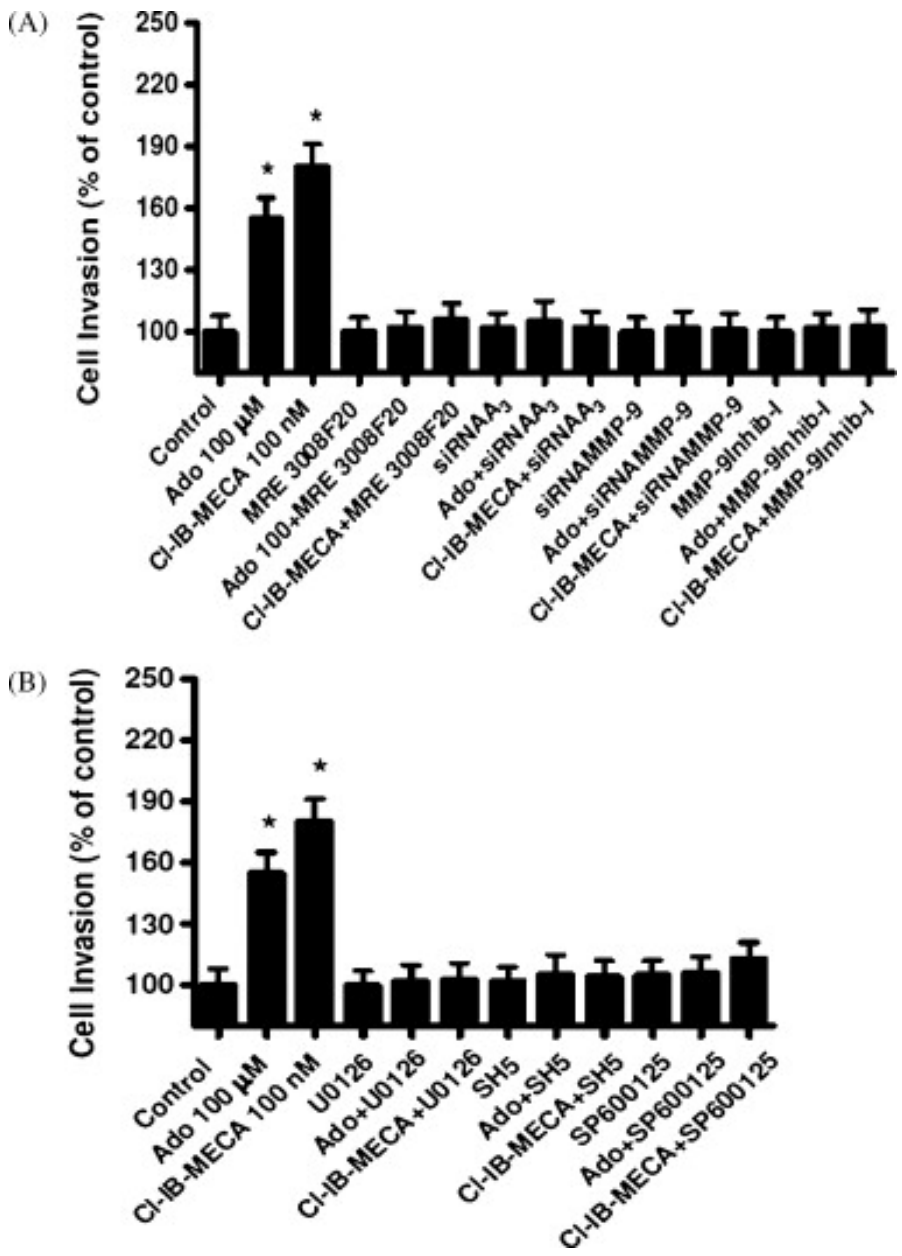

Fig. 10. Modulation of U87MG cell invasion. (A) Effect of 100 μM ado and 100 nM Cl-IB-MECA on U87MG cell invasion and antagonism by 100 nM MRE 3008F20, A_3 receptor silencing, MMP-9 silencing and 50 nM MMP-9 inhibitor I. (B) Effect of ERK 1/2, Akt and JNK inhibitors in A_3 receptor-mediated cell invasion. Experiments were performed as described in Section 2. Values are the means and vertical lines S.E. of the mean of three separate experiments performed in triplicate. $P < 0.05$ compared with the control.

Discussion

MMPs play a major role in promoting tumor metastasis. In particular overexpression of MMP-9 has been shown to be associated with the progression and invasion of several tumors including gliomas [3]. Although malignant gliomas rarely metastasize outside of the central nervous system, they are extremely invasive tumors. Several works have reported that glioblastomas produce significantly higher levels of MMP-9 than do lower-grade tumors and normal brain tissue [7] and [28].

Ado depending on the extracellular concentration and expression of different ado receptor subtypes is known as a modulator of cell proliferation, apoptosis and angiogenesis (for review see Ref. [16]). Only few experimental works have focused on the effects of ado in MMP modulation and have been performed in cells of the immune system. In fact it has been reported that ado inhibits neutrophil-produced MMP-9 via A_{2A} receptors, it increases MMP-9 in macrophages through A_3 subtypes [29] and [30] and it suppresses MMP-9 production under hypoxic conditions in human monocyte-derived dendritic cells via A_{2B} receptors [31]. In the current study, we sought to determine the involvement of ado in the regulation of MMP-9 production in U87MG glioblastoma cells. The main finding of this work is that ado increases MMP-9 levels through activation of A_3 receptors, ERK 1/2, JNK, Akt, AP-1 and is responsible for an increase in cell invasion. First of all we evaluated mRNA and protein levels of ado receptors in U87MG cells. Our results from real-time RT-PCR experiments showed that all ado receptors were expressed in glioblastoma cells with the following order $A_{2A} > A_{2B} > A_1 > A_3$. This mRNA order of expression did not match exactly the protein order found by binding assays that was $A_{2A} > A_3 > A_{2B} > A_1$. This was due to the low mRNA level of A_3 receptors in comparison to its high protein amount, already observed in different cell systems [19] and [32]. This supports the emerging evidence that mRNA expression patterns are necessary but are by themselves insufficient for the quantitative description of biological systems. This evidence includes discoveries of post-transcriptional mechanisms controlling the protein translation rate or the half-lives of specific proteins or mRNAs [33] and [34]. However it is also

known that the gene promoter region of A_3 receptors is rich in putative binding sites for ubiquitous transcription factors including AP-1, SP-1, EF-1A. These ubiquitous factors may be responsible for the widespread, low level of A_3 mRNA expression observed [35]. In this particular case it could be speculated that c-Jun, stimulated by A_3 receptors in U87MG cells, may be involved in negative feedback mechanisms to regulate A_3 mRNA expression in response to high MMP-9 and cell invasion levels. Other transcription factors such as NF-kB and CREB interacting with the A_3 gene promoter have been demonstrated to be involved in the overexpression of A_3 receptors in peripheral blood cells of patients with rheumatoid artritis [36].

As uncontrolled activation of MMP-9 is potentially dangerous in a cellular environment**,** enzyme expression is tightly regulated at the transcriptional and/or translational level. Therefore we started to evaluate the effect of ado on MMP-9 transcription in U87MG cells. We found that the nucleoside was able to increase in a time-dependent manner the expression of MMP-9 mRNA starting from 6 h, with the maximal effect observed after 12–24 h. The effect of ado was replicated by the A_3 ligand Cl-IB-MECA, but not by A_1, A_{2A} and A_{2B} agonists suggesting the involvement of A_3 receptors. Furthermore we found that both ado and the A_3 agonist induced a modest increase of TIMP-1 expression levels, suggesting the existence of a regulatory loop to compensate for MMP-9 production. This was observed also in THP-1 macrophages by Velot et al. [30]. However like in THP-1 cells also in U87MG cells its low increase is probably insufficient to counterbalance the raised expression of MMP-9, induced by A_3 receptor activation [30]. A reduced level of both MMP-9 and TIMP-1 levels have been detected also in lungs and in the bronchoalveolar lavage fluid of A_3 receptors deficient mice suggesting that the A_3 plays a regulatory role in their production [37]. Like most MMPs, MMP-9 is secreted as a latent zymogen, requiring activation in the extracellular milieu or on the cell surfaces to be catalytically competent [38] and [39]. However it has been also reported that some cellular types e.g. microvascular endothelial cells or tumor cells are capable of accumulating active gelatinase B in the cytoplasm or in membrane vesicles [40], [41] and [42] or

that specific cell-MMP interactions may occur such as binding of MMP-9 to CD44 [43]. Evidence of constitutive activation of MMP-9 in U87MG cells was suggested by expression of the active form of MMP-9 in cellular extracts of glioblastoma cells. Similar results have been obtained in different types of cancer cell lines including DU145 (prostate androgen-independent carcinoma), SK-NEP-1 (adult-derived anaplastic Wilms' tumor), A431 (squamous cell carcinoma), Wit 49 (Wilms' tumor cell line), A549 (non-small cell lung carcinoma) and LNCaP (prostate androgen-dependent carcinoma) [42]. Ado increased, in glioblastoma cells, the intracellular levels of both latent and active MMP-9 with a major effect on the second one. The conclusion that the effects of ado on MMP-9 protein were mediated through A_3 receptors was supported by the observation that the stimulatory effects of this nucleoside on MMP-9 protein were mimicked by the A_3 receptor agonist Cl-IB-MECA, and inhibited by the A_3 receptor antagonist MRE 3008F20. In particular, the potencies of these drugs were in agreement with their inhibitory equilibrium binding constants (K_i) observed in binding experiments for the ado A_3 receptor [44]. Furthermore, the A_3 receptor-induced MMP-9 protein increase was blocked following the inhibition of A_3 receptor expression at the mRNA and protein levels, by the RNA interference approach.

MMP-9 gene expression can be activated via a number of signal transduction pathways including those involving ERK1/2, p38, JNK and PKB/Akt [7] and [9]. Ado has been demonstrate to activate some of these signalling molecules in different systems and cellular models through the activation of A_3 receptors (for review see Ref. [45]). In particular an increase in ERK1/2 phosphorylation has been reported initially by Schulte and Fredholm [46] in transfected CHO cells and then it has been confirmed in other systems like microglial and colon carcinoma cells [19] and [47]. Several studies have also indicated the ability of A_3 receptors to activate PKB/Akt. This has been observed in rat basophilic leukemia 2H3 mast cells, in cardiomyocytes, in melanoma and colon carcinoma cells [20], [22], [48] and [49]. As for p38 a link with ado A_3 receptors has been observed in rat basophilic leukemia 2H3 cells, colon carcinoma cells and rat hearts [22], [50] and [51]. In contrast the effect of

A_3 receptor activation on JNK has not been investigated. Our results show that stimulation of MMP-9 by A_3 receptor activation was abrogated by inhibitors of ERK1/2, JNK and Akt but not of p38, suggesting that more than one signalling pathway is involved in the MMP-9 modulation induced by A_3 receptor activation. Indeed, Cl-IB-MECA and ado-mediated an increase in the phosphorylation of ERK1/2, JNK and Akt kinases confirming that they belong to the signaling pathways utilized by the nucleoside following A_3 receptor stimulation. In particular it has been reported that JNK is necessary to phosphorylate c-Jun proteins and it is well known that the AP-1 transcription complex appears to play an essential role in stimulating transcriptional activation of MMP-9 [7] and [13]. In its active form AP-1 complex may comprise homodimers of c-Jun or heterodimers between c-Fos, c-Jun and ATF2 [52]. Furthermore activation of ERK1/2 has been shown to induce c-Jun expression and phosphorylation, indicating cross-talk between ERK1/2 and JNK pathways in the regulation of c-Jun activity [8] and [53]. We found that MMP-9 regulation by A_3 ado receptor activation was blocked after treatment with the AP-1 inhibitor; c-Jun, was also increased by ado and Cl-IB-MECA and this effect was antagonized by MRE 3008F20 suggesting the involvement of A_3 ado receptor. We demonstrated that A_3 receptor activation induced an increase of active MMP-9 as shown by ELISA and gelatine zymography experiments carried out on the supernatants obtained from U87MG cells, according to data showed by Velot et al. in macrophages [30].

Finally, as for the physiological relevance of the A_3 receptor-mediated stimulation of MMP-9 we found that Cl-IB-MECA was responsible for an increase of the invasive ability of U87MG cells. This effect was dependent by modulation of MMP-9, exerted through A_3 receptors activation and ERK1/2, JNK, Akt and AP-1 phosphorylation. Interestingly it was reported that MMP-9 inhibition might be useful for treating the invasiveness of brain tumors. Furthermore the antisense MMP-9 vector or adenovirus expressing antisense MMP-9 decreased the PMA-induced migration and

invasion of glioblastoma cells [54]. Interestingly the increase induced by adenosine in cell invasion has been previously reported also in breast cancer cells [55] and [56].

It is well known that the A_3 receptor plays an important role in regulating normal and tumor cell growth [16]. Elegant *in vivo* studies by Fishman's group showed the efficacy of A_3 agonists in various tumor-bearing animals, supporting the utilization of A_3 agonists to treat cancer [57]. In particular melanoma, colon, prostate and hepatocellular carcinomas were reduced in *in vivo* animal models by IB-MECA or Cl-IB-MECA [17], [57] and [58]. Importantly in these studies, the combined treatment of IB–MECA with the specific agents cyclophosphamide, 5-fluorouracil and taxol respectively, resulted in an enhanced antitumor effect. On the other hand, it has been reported that in U87MG glioblastoma cells under hypoxic conditions, Cl-IB-MECA induced up-regulation of hypoxia-inducible factor 1 (HIF-1) alpha and VEGF supporting a role for A_3 antagonists as a novel approach for the treatment of glioblastomas [21]. A possible explanation for these different results may be that cell response to a given A_3 agonist is determined by a plethora of factors, including agonist concentration and affinity, receptor density, interaction between different adenosine receptors expressed on the cell surface, cell type and the cell microenvironment. Furthermore ado effects on MMP-9 and cell invasion not necessarily shall to occur during carcinogenesis. For example, it has been demonstrated that the *in vivo* administration of Ado reversed cirrhosis and liver dysfunction and that one of the mechanisms involved in this effect was through an increase in collagenolytic activity [59]. Accordingly the results of this work, revealing a role of A_3 receptor in the increase of glioblastoma cell invasion by stimulation of MMP-9, adds a new function to the complex role of A_3 receptors in tumor biology and need to be verified in *in vivo* experiments.

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