THE ROLE OF ADENOSINE RECEPTOR ACTIVATION ON MATRIX METALLOPROTEINASE REGULATION

BY

MICHAEL GA-HONG WOO

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE

Department of Pharmacology and Therapeutics
Faculty of Medicine
University of Manitoba
And the Division of Neurovirology and Neurodegenerative Disorders
St.-Boniface General Hospital Research Centre
Winnipeg, Manitoba

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FACULTY OF GRADUATE STUDIES
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"An expert is a man who has made all the mistakes which can be made in a very narrow field."

-Niels Bohr

ACKNOWLEDGEMENTS

I want to thank Dr. Jonathan Geiger for his guidance and support. His hard work and dedication to science is an inspiration and I am thankful to have been a member of the Geiger Lab.

I would also like to thank my committee members Dr. Fiona Parkinson, Dr. Jim Peeling and Dr. Ian Dixon for their encouragement and support. Their contributions to this thesis are very much appreciated.

I would like to thank the faculty and staff of the Department of Pharmacology & Therapeutics for making my graduate studies a great experience.

To my parents Jim and Kitty, thank you for your love and support. Your encouragement gives me confidence in all I do. I could not have done this without you.

Finally I would like to thank the Saint-Boniface Hospital Research Centre and the Deer Lodge Hospital Foundation for their generous financial support throughout these years.

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LIST OF ABBREVIATIONS

Anti-TNF Ab	TNF-α neutralizing antibody
AC	adenylyl cyclase
ADA	adenosine deaminase
AMP	adenosine monophosphate
ANOVA	analysis of variance
AP-1	activator protein-1
ATP	adenosine triphosphate
BBB	blood-brain barrier
cAMP	cyclic adenosine monophosphate
CCPA	2-Chloro-N^6-cyclopentyladenosine
cDNA	complementary DNA
CGS 21680	2-p-(2-Carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride
CNS	central nervous system
ConA	concanavalin A
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
DTT	dithiothreitol
ECM	extracellular matrix

ELISA	enzyme linked immunosorbant assay
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
GPI	glycosyl phosphatidyl inositol
HB-EGF	heparin-binding EGF-like growth factor
IB-MECA	1-Deoxy-1-[6-[((3-Iodophenyl)methyl)amino]-9H-purin-9-yl]-N-methyl-b-D-ribofuranuronamide N^6-(3-Iodobenzyl)adenosine-5'-N-methyluronamide
IG-FBP	insulin-like growth factor binding protein
IL	interleukin
IFNγ	interferon-γ
LDL-RP	low density lipoprotein receptor-related protein
LPS	lipopolysaccharide
MMP	matrix metalloproteinase
M-MuLV	Moloney murine leukemia virus
MRS 1220	9-Chloro-2-(2-furanyl)-5-((phenylacetyl)amino)-[1,2,3]triazolo[1,5-c]quinazoline
MS	multiple sclerosis
MT-MMP	membrane-type matrix metalloproteinase
NF-kB	nuclear factor kappaB
PBS	phosphate buffered saline
PBST	phosphate buffered salline, 0.05% Tween 20
PCR	polymerase chain reaction
PDE	cyclic nucleotide phosphodiesterase
PHA	phytohemagglutinin

PI 3-kinase	phosphatidylinositol 3-kinase
PKA	protein kinase A
PMA	phorbol-12-myristate-13 acetate
PMN	polymorphonuclear cell
RECK	reversion inducing cysteine-rich protein with Kazal motifs
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SCID	severe combined immune deficiency syndrome
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCR	T-cell receptor
TNF-α	tumor necrosis factor-α
TIMP	tissue inhibitor of metalloproteinase
TSR1	neutralizing TNF-α soluble receptor 1
ZM 241385	4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

ABSTRACT

Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade extracellular matrix compounds. These enzymes serve many functions; they act as effectors of remodeling and repair, and as modulators of cell signaling seen in inflammation and cell death. Others have shown that the pro-inflammatory cytokine TNF-α increases MMP-9 activity in inflammatory cells. Recent data from our laboratory have shown that adenosine A_{2A} receptor activation in U937 pro-monocytic cells decreases TNF-α post transcriptionally. This study tested the hypotheses that activation of adenosine A_{2A} receptors decreases MMP-9 activity through the TNF-α modulated pathway, and that activation of adenosine A_1 and A_3 receptors exert similar effects because activation of these receptors also decreases TNF-α. U937 cells were treated with CGS 21680 (a selective A_{2A} receptor agonist), activated by the phorbol ester PMA, and from cell supernatants TNF-α protein levels were detected by ELISA and MMP-9 activity levels were quantified using gelatin zymography. Treatment of U937 cells with PMA/PHA resulted in a time-dependent increase in levels of TNF-α protein and MMP-9 activity. CGS 21680 decreased significantly TNF-α protein and MMP-9 activity levels. In contrast, MMP-9 gene expression as measured by RT-PCR was elevated in the presence of CGS 21680; the A_{2A} antagonist ZM 241385 blocked all three effects. Activation of adenosine A_1 and A_3 receptors elicited TNF-α and MMP-9 responses largely opposite to those observed with A_{2A} activation. In conclusion, adenosine A_{2A} receptor activation may be a useful strategy against pro-inflammatory events where TNF-α and MMP-9 are involved.

CHAPTER 1: INTRODUCTION

Matrix Metalloproteinases (MMPs)

Physiological Importance

Matrix metalloproteinases (MMPs) play key roles in tissue architecture and homeostasis. Over 23 MMPs have been identified and they are best known as effectors of cell migration, cytotoxicity and tissue remodeling, and have been implicated in processes that involve the degradation of extracellular matrix (ECM) components (6) (Table 1). MMPs are involved with normal processes in growth and development, such as nerve growth, bone formation, and angiogenesis. On the other hand, there exist many pathological conditions where ECM degradation is unregulated and this is a component of the inflammation seen in arthritis, emphysema, gastric ulcer and multiple sclerosis.

Table 1. Normal and pathological processes associated with extracellular matrix remodelling and MMP activity.

Normal Processes	Pathological Processes
Bone remodeling	Cancer
Angiogenesis	Breakdown of blood brain barrier
Immune Response	Multiple sclerosis
Inflammatory Response	Alzheimer's disease
Wound healing	Stroke
Nerve growth	Bacterial Meningitis
Apoptosis	Arthritis
Organ morphogenesis	Vascular diseases
Embryonic development	Cardiovascular disease
Blastocyst implantation	Periodontal disease
Ovulation	Corneal ulceration
Cervical dilation	Nephritis
Endometrial cycling	Skin ulceration
	Gastric ulcer
	Liver fibrosis
	Emphysema
	Liver cirrhosis
	Fibrotic lung disease
	Sorsby's fundus dystrophy
	Guillian-Barre disease
	Cardiac fibrosis

Many pathological conditions are associated with increases in MMP activity. In particular, MMP-9 is the predominant MMP secreted by monocytes in the inflammatory and immune response to *Mycobacterium tuberculosis* (7). MMP-9 is essential to leukocyte extravasation into infected sites by degrading type IV collagen in vascular basement membranes (8). As a result, the ubiquitous nature and importance of the MMPs have attracted intense interest on the part of both basic scientists and pharmaceutical companies. A basic understanding of MMP structure has resulted in the development of synthetic inhibitors to control MMP activity. Clinical trials so far have focused on using MMP inhibitors to treat cancer, a particularly exciting idea because this would target two phases of cancer progression, angiogenesis and metastasis. Synthetic inhibitors such as British Biotech's Marimastat and Batimastat have all yielded disappointing clinical results (9). Nevertheless, research in this field continues and current efforts focus on mechanisms that control MMP activity.

Historical Overview

MMPs were first described in 1962 by Jerome Gross and Charles Lapiere, while studying the metamorphosis of tadpoles (Figure 1). They discovered that during resorption of tadpole tail fins, skin would release an enzyme that degraded native collagen triple helixes in the underlying support matrix. This enzyme, the first MMP, was named interstitial collagenase (MMP-1) and was found in a wide variety of vertebrates, invertebrates and plants. Since then, MMPs have been found to be involved in almost all processes of ontogeny. MMPs regulate ovulation and uterine tissue remodeling during the menstrual cycle and also regulate tissue remodeling during

morphogenesis and growth. In adult organisms, expression of MMPs is generally low and is locally induced as seen in wound healing (6). The same functions that are beneficial in normal physiological development can become mechanisms of disease pathogenesis when regulation of MMPs becomes abnormal, as seen in cancer and overactive inflammation.

Figure 1. Interstitial collagenase (MMP-1) was first identified by Jerome Gross and Charles Lapiere in 1962 while studying the metamorphosis of a frog (*Rana clamitans*) from a tadpole (lower left) to a mature frog (upper right). Development of front and hind legs, and resorption of tail occur in order (5).

Extracellular Matrix

Tissues are made of cells. However, a large portion of tissue volume is extracellular space, which is filled by a complex network of macromolecules that make up the ECM. The ECM provides a framework on which cells grow, migrate, and differentiate, and it is essential that the ECM undergoes continuous remodeling during growth and development. ECM remodeling is complex. It is the result of multiple signals and coordinated processes, controlling numerous effectors of ECM turnover. In terms of ECM degradation, the most prominent proteases involved are the MMPs (2).

The major components of the ECM are collagen, proteoglycans and glycoproteins, and these are all secreted locally and assembled into an organized meshwork in close association with the cells that produced them (Figure 2). In most organs, the major proteinaceous component of the ECM is collagen. The fundamental higher order structure of collagen is a long thin rod-like protein composed of coiled α chains of the basic collagen molecules. Over 25 types of α chain have been identified, each coded by a different gene, composing at least 15 different types of collagen superstructures (4). Collagen types I, II and III are the most abundant and form fibrils of similar structure. Type IV collagen forms a two-dimensional reticulum and is the major component of the basal lamina. Collagen is produced and secreted by various stromal cells, predominantly fibroblasts, to form a scaffold that supports the cells that make up the tissue. Many other proteins make up the specialized ECM structure such as the basement membrane including laminin, entactin, collagen IV, and a variety of growth factors and proteases. Another class of molecules that are essential components of the ECM are the proteoglycans which have protein cores covalently bound to high-

molecular-weight glycosaminoglycans such as chondroitin, heparin, and keratan sulfates. These molecules are involved with many processes including the support of cell adhesion and binding to latent growth factors.

The major glycosaminoglycan in the ECM is hyaluronan (HA). It is primarily produced by fibroblasts, but is synthesized by other stromal cells as well. HA is part of the ECM of all organs. It is a polymer of repeating disaccharide units and it is not typically sulphated or bound to a protein core. Hyaluronan is an important regulator of numerous cellular functions including adhesion, trafficking and signaling.

The ECM has many components and it is a structure that has many functions. In addition to maintaining support for tissue integrity, ECM components regulate cell migration and provide a reservoir of cytokines and growth factors. It is an environment that undergoes constant turnover in response to a variety of cellular signals. These responses range from homeostatic resting state functions in adult organs, to full-blown tissue remodeling that occurs during normal development, wound healing, cancer, and inflammation. Alterations in ECM structure and composition are the result of various concurrent processes, which are dependent on the initial stimulus.

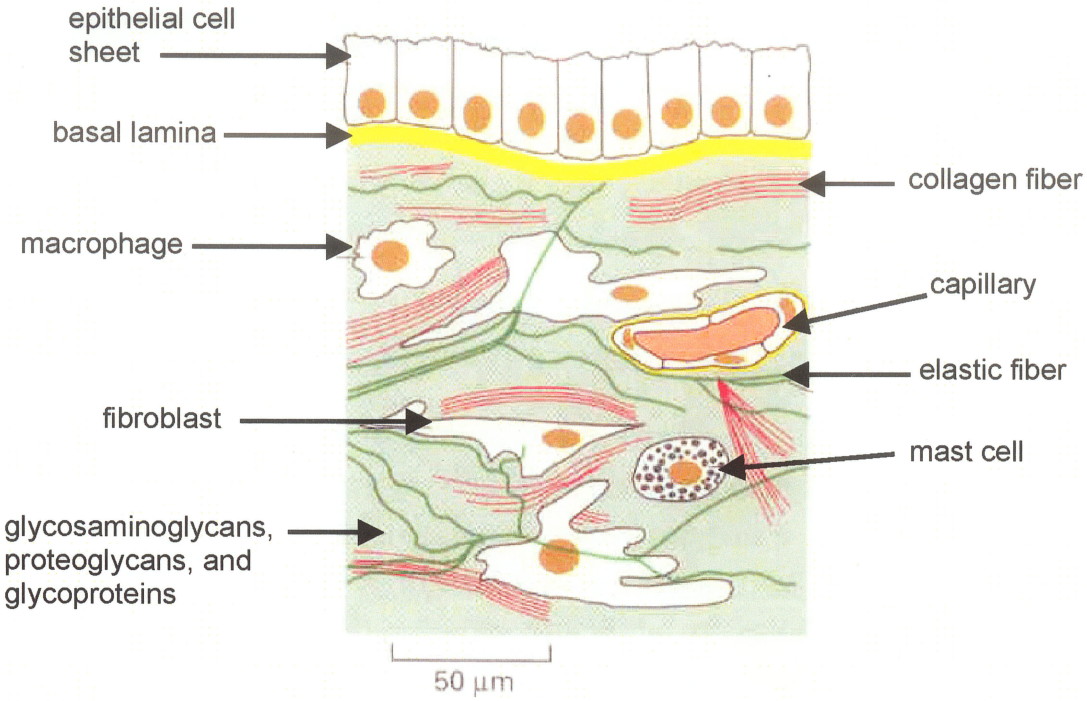

Figure 2. Components of the extracellular matrix (ECM) underlying an epithelial cell sheet (4).

Classification of MMP Subtypes

MMPs have been classified as belonging to the larger family of zinc-dependent metalloproteinases called metzincins. Other metzincin subfamilies include the ADAMS, bacterial serralysins, and the astacins (2, 6, 10, 11). Metzincins are all structurally related having a consensus motif in the catalytic domain that uses three histidine (H) residues to bind a Zn^{2+} atom in the catalytic site. This conserved sequence motif has the amino acid sequence of HExxHxxGxxHZ, where Z is unique among the subfamilies, and consists of a serine residue for MMPs (6). A second feature homologous to the metzincins is a conserved 'Met-turn" motif which is part of a distinct β-turn at the active site that is thought to be essential for activity (6).

At least 25 members of the MMP family have been identified. They are each assigned a number corresponding to the chronology in which they were discovered. Historically, MMP family members were categorized into subgroups based on perceived substrate specificities including the collagenases, gelatinases, stromelysins, matrilysins, membrane-type (MT)-MMPs, and 'other MMPs'. This historical method of nomenclature has been falling out of favor since the discovery that MMPs share a high degree of overlap in substrate specificity and are capable of cleaving a growing list of substrates that are not part of the ECM (12, 13). Non-ECM substrates include myelin basic protein (14), pro-tumor necrosis factor (15), Fas ligand (16), transforming growth factor 1-β (17), and pro-fibroblast growth factor receptor 1 (18).

As an alternative, MMPs are now classified based on structural similarities (Figure 3). Structurally, MMPs follow a basic pattern of domains upon which subtypes are classified. At the N-terminus, there is a short signal peptide ('pre-domain') that is

removed when newly synthesized MMPs travel to the cell surface (6). Also at the N-terminus is the propeptide domain which is the inhibitory sequence containing a cysteine residue that binds and inactivates the Zn^{2+} ion at the active site thereby keeping the enzyme in a latent pro-form (19). The amino-terminal catalytic domain is the core domain of MMP structure and it contains two Zn^{2+} atoms, one of which acts as the catalytic principle; hence the name "metallo". In some cases, such as with MMP-2 and MMP-9, the catalytic domain also contains three Type II fibronectin domains that although oriented away from the catalytic cleft are believed to participate in the binding of substrates and of natural inhibitors (20). A hinge region connects the amino-terminal to the carboxy-terminal domains. The carboxy-terminal hemopexin-like domain is important in substrate binding, and forms a four-bladed propeller structure that is present in all MMPs except MMP-7 and MMP-26 (2, 6). The membrane type MMPs (MT-MMPs) are unlike other MMP groups in that they are membrane bound proteins. MMP-17 and MMP-25 are anchored to the membrane by a glycosyl-phosphatidyl-inositol (GPI)-anchoring domain while the remaining MT-MMPs are transmembrane proteins (6).

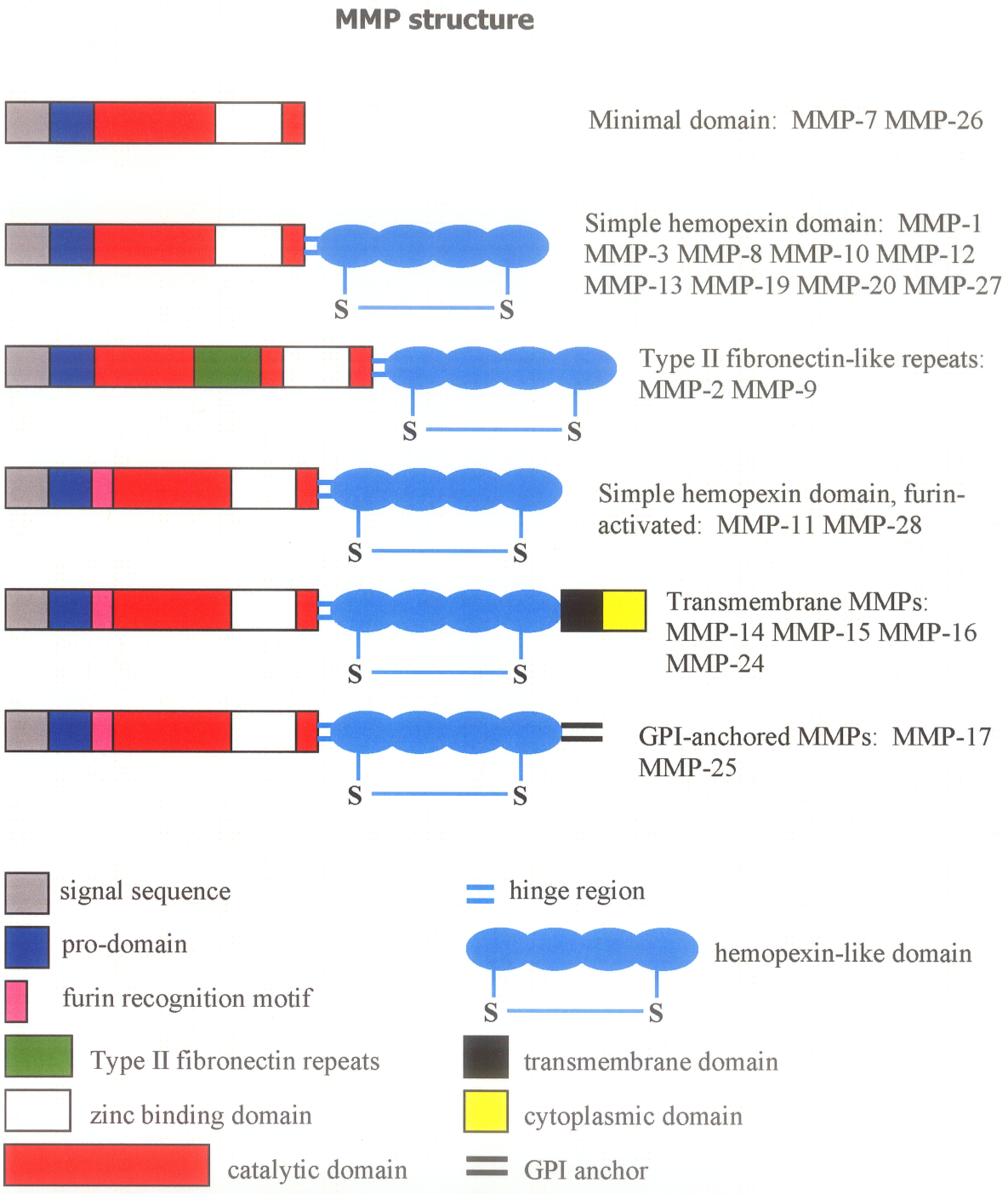

Figure 3. MMP protein structure. Structural subclasses and different MMP domains are listed for each MMP. Adapted from (2).

Regulation of MMP activity

Because of their potent proteolytic capabilities, MMPs are tightly regulated to avoid widespread tissue destruction. Regulation of these enzymes occurs at three levels (Figure 4).

The first regulatory step is at the level of transcription. At rest in most adult tissues, MMPs are not constitutively expressed and activation requires some type of signal. This signal can be in the form of inflammatory cytokines, growth factors, chemokines, oncogenes, and cell-cell or cell-matrix interactions (21). Cytokines such as IL-1 and IL-2 influence the expression and regulation of MMPs by various pathways, and TNF-α, a pro-inflammatory cytokine, has among its multiple actions the induction of MMPs (7, 22, 23). TNF-α is involved with the activation of the c-jun gene, where the gene product c-Jun combines with c-Fos. This complex then binds to the TRE/AP-1 promoter site of the MMP gene (24) resulting in gene transcription. On the other hand, MMPs and related metalloproteinases can function as sheddases or convertases to convert membrane-bound cytokines, cytokine receptors and adhesion molecules to their soluble forms (21). Many MMPs show sheddase activity *in vitro*, but it is the ADAMs (a disintegrin and metalloproteinase), which are not of the MMP family that are the more efficient proteases in releasing membrane-bound molecules.

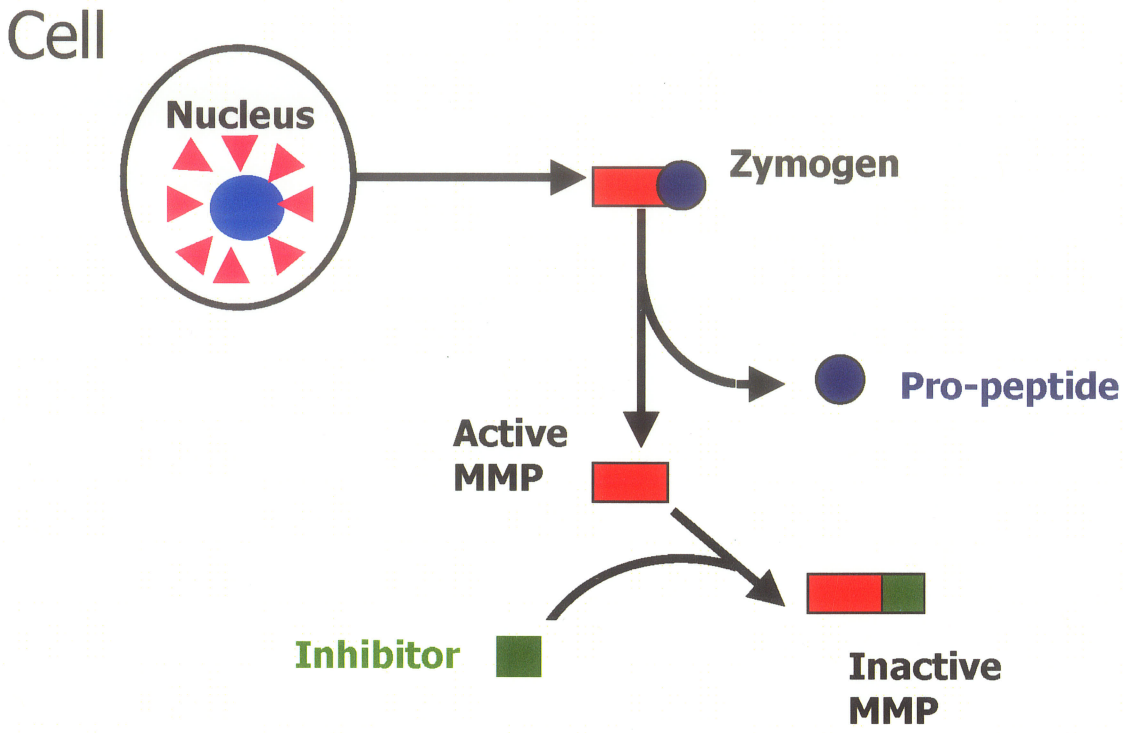

Figure 4. Regulatory steps controlling MMP activity. The first step is at the level of gene transcription. The second step involves removal of the propeptide domain from the zymogen to activate the MMP. The third and last step involves inactivating the active MMP by binding to an endogenous inhibitor.

The second level of regulation occurs post-transcriptionally. MMPs are expressed as inactive zymogens. They are expressed with a propeptide sequence that binds to and inactivates the catalytic site using a cysteine residue to chelate the catalytic Zn^{2+} ion. To activate MMPs, the cysteine/ Zn^{2+} interaction must be disrupted (known as the 'cysteine switch' mechanism (Figure 5)) and full activation of these molecules requires the removal of the propeptide region (25). *In vivo*, activating factors include the plasminogen plasmin cascade as well as other MMPs. Non-proteolytic activating factors include sulfhydryl-reactive agents and denaturants such as urea (6, 26). Activation of the membrane type (MT)-MMPs is somewhat different. This subset of MMPs has a cleavage site for FURIN-like prohormone convertases between the propeptide and catalytic domain, and is activated during secretion to the cell membrane where they are attached in the active form (6).

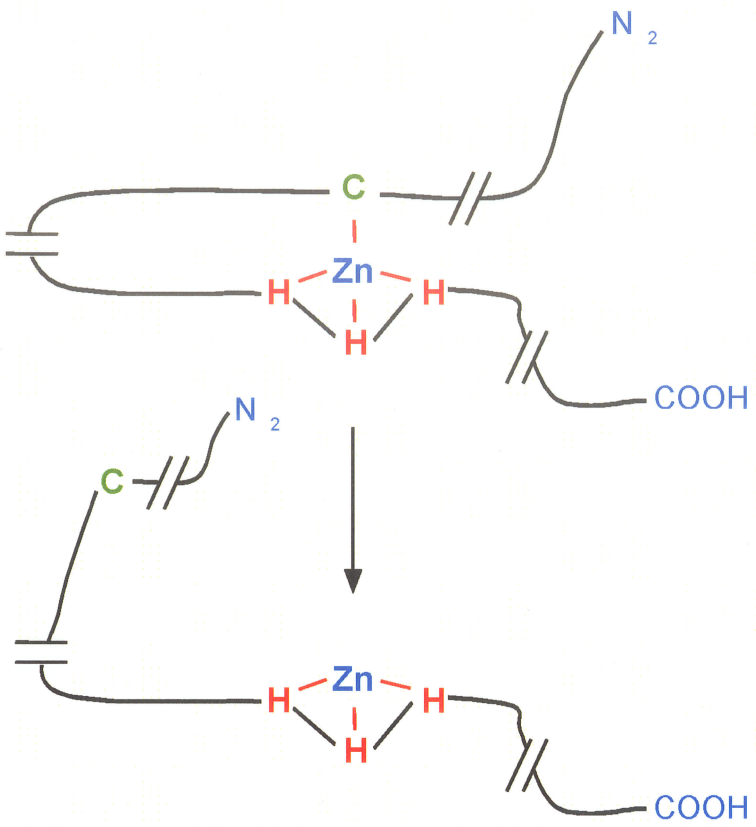

Figure 5. Activation of matrix metalloproteinases involves a cysteine switch mechanism. A reduction in the sulphydryl-zinc bond of the propeptide exposes the catalytic site.

The third (and last) regulatory mechanism involves inhibition of active MMPs by a variety of endogenous inhibitors. The main plasma proteinase inhibitor is α_2-macroglobulin, a 772 kDa protein that can inhibit all endoproteinases including the MMPs and ADAMS but not the astacins (27). These α_2-macroglobulins are produced mainly by liver hepatocytes, but production in other cell types such as macrophages has been reported. α_2-Macroglobulin binds to MMPs in a 1:1 stoichiometric fashion and inhibition occurs through a novel mechanism involving the presentation of a 'bait' region, where once it is proteolytically cleaved, causes a conformational change that irreversibly traps the proteinase by transacylation. These covalent complexes are then bound and cleared by scavenger receptors (28, 29). Although known as the major plasma inhibitor of MMPs, its role and importance in MMP regulation in the pericellular environment is still unclear. It has been reported that α_2-macroglobulin-serine-proteinase complexes bind to α_2-macroglobulin receptors, known as the low density lipoprotein receptor-related protein (LDL-RP). Binding to LDL-RP occurs prior to internalization and degradation (30), and the same mechanism seems to exist for MMPs. This was shown to occur with MMP-13 in osteoblastic cells (31), and for the MMP-9/TIMP-1 complex in mouse embryonic fibroblasts (32).

The major cellular inhibitors of MMPs are the tissue inhibitors of metalloproteinases (TIMPs) (27). Currently, four TIMPs have now been cloned, purified and characterized. The four TIMPs each share basic similarities; however they also exhibit distinct structural features, differences in biochemical properties, and different expression patterns. These findings suggest that TIMPs have unique roles *in vivo* and

further underscore the findings that MMP regulation is complex and this complexity includes regulation of inhibitors.

The biochemical properties of TIMPs are well documented. The four TIMP genes are located intragenically in intron 5 of the synapsin genes (33). The molecular weights of TIMPs are roughly 21- 28 kDa and they are variably glycosylated. Structurally, these molecules have six disulfide bonds which contribute to a three-loop N-terminal domain that interacts with a three-loop C-subdomain. Experiments have attributed most of their biological functions to sequences within the N-terminal domain, although it is the C-subdomains that interact with some MMPs and the hemopexin domains of MMP-2 and MMP-9 (34).

Like the MMPs, TIMPs are secreted proteins. Binding to membrane-bound proteins such as the MT-MMP can also localize them to the cell surface, and TIMP-3 is unique in that it is sequestered to the ECM by binding to heparin-sulphate-containing proteoglycans and chondroitin-sulphate-containing proteoglycans (35).

Inhibition of MMPs occurs by binding to the catalytic site. Structural work has demonstrated that during inhibition, the N-terminal group of the TIMP fills the fourth coordination site of the active site zinc (36). All TIMPs have the ability to form tight binding-noncovalent complexes with multiple members of the MMP family. They exhibit different efficacies against different MMP members and preferentially target either the pro or active form of MMPs (37). Also, they show different tissue expression patterns and modes of regulation. TIMPs are also involved with activation of some MMPs. ProMMP-2 is activated by the membrane-bound MT-MMP. TIMP-2 binds with both MT-MMP and proMMP-2 to facilitate activation of MMP-2. To complicate

matters, MMP-2 activation is facilitated only by low concentrations of TIMP-2 whereas at higher concentrations TIMP-2 inhibits proMMP-2 activation (38).

The importance of TIMPs in biological development has been described for TIMP-$1^{-/-}$, TIMP-$2^{-/-}$ and TIMP-$3^{-/-}$ knockout mice. Although viable and fertile, these knockout mice showed disturbances in phenotype that depends on proper ECM development. TIMP-1 for example, is a multifunctional protein, and has been implicated in many reproductive processes. TIMP-1 knockouts have altered reproductive parameters including decreased levels of serum progesterone and abnormal uterine morphology which contributes to a reduced reproductive lifespan (39). These changes are associated with significant increases in MMP activity, where the absence of TIMP-1 causes an imbalance in MMP regulation. Increases in MMP activity can have many negative biological effects on the uterus and the reproductive process since MMPs also regulate the bioavailability of cytokines (eg. IL-1 β, TNF-α) (40, 41) and growth factors (eg. IG-FBP-3, HB-EGF) (42, 43), which will influence cell proliferation and cell death in the uterus. In TIMP-2 null mice, the only reported disturbance is in the activation of pro-MMP-2 (44), which reflects its unique role in pro-MMP-2 activation by MT1-MMP. Gene expression of MMP-2 in adults is generally low if not undetectable and it is thought that MMP-2 plays a larger role in early developmental processes such as in angiogenesis. For this reason, MMP-2 knockouts have been studied extensively in angiogenesis models in cancer research. Such studies have shown that growth and vascularization of transplanted tumours in MMP-2-null mice are reduced (45). TIMP-3 knockout mice at 2 weeks of age show spontaneous lung air space enlargement that progresses until the animal becomes moribund after 1 year (46). This phenotype is characteristic of

emphysema-like conditions where the balance of TIMP/MMP causes an increase in collagen turnover in the alveolar interstitium.

The last category of MMP inhibitor is the reversion inducing cysteine-rich protein with Kazal motifs (RECK). This molecule is the only known membrane bound inhibitor of MMPs; it attaches to the cell membrane through a carboxy-terminal GPI-modification (47). It is a 110 kDa glycoprotein that is widely expressed in human tissues with low expression in tumour cells. RECK appears to be essential for normal development because mice lacking a functional RECK gene do not survive beyond embryonic day 11.5 (48). Histological examination of RECK null embryos reveals severe disruption to the ECM around blood vessels and the neural tube. There is partial destruction of the basal lamina, disruptions in the patterns of fibronectin and laminin, and almost complete loss of collagen fibers as determined by electron microscopy (48). The absence of RECK expression may result in deregulation of the MMPs found in these areas (such as MMP-2, MMP-9 and MT1-MMP). Consequently, excess MMP activity would lead to excess degradation of the ECM, and disruption of the integrity of the blood vessels and surrounding tissues.

MMPs in Neurodegenerative Disease

The study of MMPs in the development of neurodegenerative disease is still relatively new when compared to MMP research in heart disease and cancer. However, study in this field has been attracting more attention with reports of exciting findings in models of multiple sclerosis (MS) and other neuro-inflammatory diseases. In the context of neuro-inflammatory diseases, MMPs have been implicated in the development and progression of many processes such as the breakdown of the blood-brain barrier (BBB) and opening of the blood-nerve barrier, invasion of neural tissue by blood-derived immune cells, shedding of cytokines and cytokine receptors, and direct neurotoxicity (19).

MMPs in Multiple Sclerosis

Multiple sclerosis (MS) is a chronic disease of the CNS characterized by multifocal inflammation and destruction of myelin. Viral infection, genetic predisposition, environmental factors, and autoimmunity are all considered to contribute to the pathogenesis of the disease (49-51). This disease is characterized by blood-brain barrier (BBB) breakdown that results in an influx of T cells and monocytes that contribute to lesion formation in the CNS.

In brain, the endothelial cell wall strengthened by tight junctions along with increased numbers of mitochondria and carrier-mediated transport mechanisms defines the blood-brain barrier (BBB) (52). The basement membrane that underlies the vasculature plays a critical role in maintaining the integrity of the BBB by providing

structural support to the endothelial cell wall. The BBB functions to protect the central nervous system from invasive agents such as inflammatory cells and bacteria as well as from chemical agents.

For over 20 years, proteinases have been detected in the cerebrospinal fluid (CSF) of MS patients. Current technologies have allowed us to identify these proteinases to include members of the MMP family; of particular interest is MMP-9, which is not present in the CSF of normal healthy individuals. Further examination of MMP profiles in serum and in immune cells has revealed increases in MMP-9 gene expression in leukocytes and elevated serum MMP-9 levels in MS patients when compared to healthy individuals (19). In addition, the ratio of MMP-9:TIMP-1 in the serum of MS patients is increased, further suggesting that MMP-9 over-activity plays a role in MS (19).

Animal models of neuroinflammation generally involve increasing cytokine and free radical production by intracerebral injections of LPS or TNF-α. Intracerebral injection of TNF-α into rat brain induces MMP-9 production after 24 hours and is associated with disruption of the BBB. This opening of the BBB is blocked by the addition of an MMP inhibitor Batimastat (BB-94) (53). Intracerebral injection of LPS induces similar results, causing BBB breakdown and an increase in MMP-9 production after 8 hours (54). This effect was blocked with another hydroxymate-type MMP inhibitor, BB-1101.

MMPs in Stroke

Other compelling evidence linking MMPs to brain injury involves models of stroke. Ischemic stroke is a major public health problem in Western society and it is one

of the leading causes of death and disability among the elderly (55). Ischemic stroke is defined as a clinical syndrome of permanent brain dysfunction, which can improve or worsen over time as a result of an infarction. The extent of the lesion is an important determinant of patient outcome. The ischemic lesion is typically composed of a necrotic core characterized by irreversible tissue damage. However, the final size of the tissue damage depends on the fate of the penumbra region surrounding the core, which can survive as long as the blood flow remains at 20% of normal (56). The cellular pathophysiological mechanisms involved in the damage to this penumbra region are not fully understood (57). It is important to characterize the mechanisms that are involved with the initial event as well as the mechanisms that lead to neuronal death once the blood circulation is compromised.

The disruption of blood circulation leads to oxygen and glucose deprivation. This initiates an acute inflammatory response that is characterized by recruitment of polymorphonuclear cells (PMN) and monocytes/macrophages into the ischemic lesion. Whether or not this response provides a net detriment or benefit to the patient is not fully understood as this response may be related to both neuronal damage and recovery. Inflammation associated with stroke can aggravate brain damage by several mechanisms, such as plugging the surrounding capillaries, producing harmful free radicals and activating destructive proteases. Combined, these events lead to edema, tissue destruction and brain damage. Leukocytes and their secreted products are also implicated in this process. A correlation between the extent of leukocyte infiltration and final lesion volume has been demonstrated (58, 59). Also, cytokines and MMPs secreted by inflammatory cells contribute to the development of an inflammatory reaction by up-

regulating adhesion molecules, promoting leukocyte-endothelium interactions, inducting chemokine expression, promoting proteolysis of the basal membrane and weakening the BBB (57). Weakening of the BBB is an event that involves various contributors including proteases and free radicals. MMPs are of particular importance due to their capability to loosen the connections between cells. T cells from the blood secrete MMP-9 in order to cross the BBB, while pericytes and microglia secrete MMP-2, -3, and -9 (60).

In the case of stroke, there is a breakdown of the BBB with an increase in vascular permeability. Damage to the BBB often results in hemorrhage, edema and inflammation resulting in neuronal cell death (54). Inflammation in the CNS behaves differently than in the rest of the body as the process involves resident microglial cells and mononuclear cells and neutrophils recruited from the blood (61). For this reason, it is difficult to separate the contributions of the blood-borne cells from the cells endogenous to the nervous system. Brain injury after focal stroke is mainly a function of decreased blood flow and of energy depletion caused by the occlusion of a cerebral blood vessel. The neuronal tissue becomes infracted as a result of these events with contributions from excitotoxicity, enzyme activation, edema, and inflammation.

The involvement of MMPs in stroke was proposed based on findings that MMP-2 and MMP-9 gene expression is up-regulated after focal cerebral ischemia in rats (62). In addition, MMPs have long been shown to play an important role in vascular and cardiac disease (63-65). MMP inhibitors and MMP-neutralizing antibodies reduced edema and infarct size in rat and mouse models of stroke (62, 66, 67). MMP-9 may be more important than MMP-2 in stroke-induced brain injury because MMP-9 but not MMP-2

knockout mice have significantly smaller lesion volumes compared to wild-type mice (68, 69). Further, studies on human brain have shown that MMP-9 protein levels are increased within days of infarction and levels remain elevated even after death, suggesting a role in tissue destruction. From the combination of animal and human studies, a consistent pattern of protease-mediated cell damage is emerging.

Adenosine

Biochemistry of adenosine

Adenosine is an adenine nucleoside that is a ubiquitous compound in the body. It is a component of nucleic acids and is a metabolic breakdown product of ATP, the body's main energy source (70). As such, levels of adenosine intra- and extra-cellularly can rise when energy demand exceeds energy supply and fluctuating levels are dependent on adenosine production, metabolism and transport (Figure 6). Enzymes such as 5'-nucleotidase increase adenosine production from adenosine monophosphate (AMP), whereas adenosine kinase and adenosine deaminase can catalyze the metabolism to AMP and inosine, respectively (71). Transport of the nucleoside can also control adenosine levels intra- and extra-cellularly, as movement is dependent on passive diffusion, equilibrative transporters, and sodium-dependent concentrative transporters (72, 73). Once produced, adenosine can act on four subtypes of cell surface receptors; A_1, A_{2A}, A_{2B} and A_3 (Table 2).

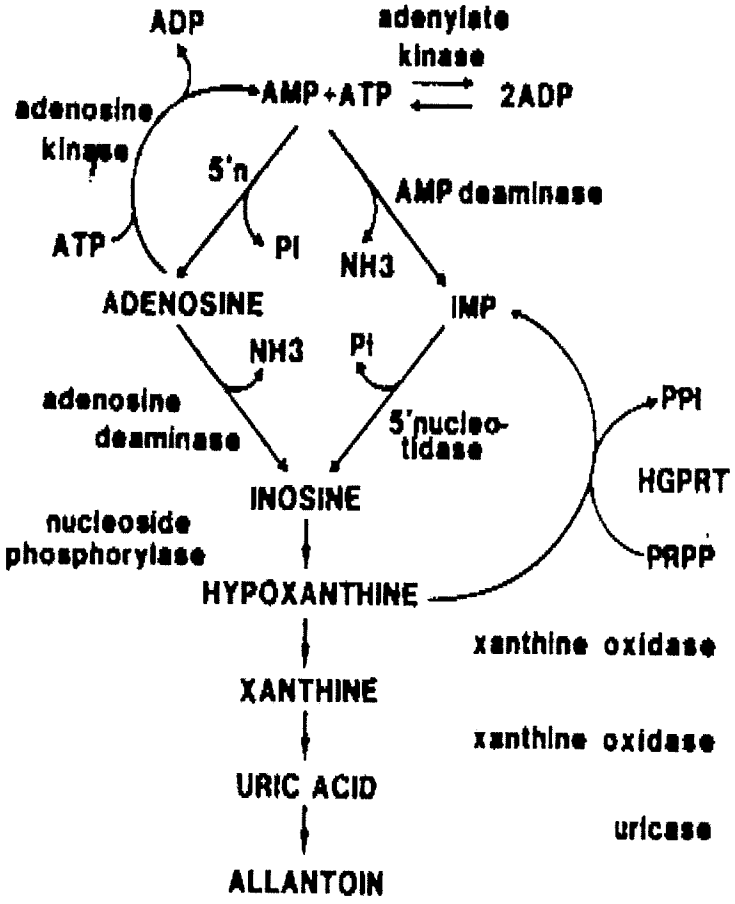

Figure 6. Adenosine metabolism and production (1).

Adenosine signalling and cAMP

Following the cloning of the adenosine receptors, significant insight was made regarding the characterization of adenosine-mediated signaling and adenosine receptor-mediated immunosuppression. The adenosine receptors are associated with G-protein signaling pathways. The A_1R and A_3R are linked to the inhibitory G-protein G_i and G_o and the $A_{2A}R$ and $A_{2B}R$ with the stimulatory G-protein G_s (70). Thus, the A_1R and A_3R are negatively coupled to adenylyl cyclase with activation resulting in decreased cyclic adenosine monophosphate (cAMP) (74). Conversely, the $A_{2A}R$ and $A_{2B}R$ are positively coupled to adenylyl cyclase so that activation increases cAMP levels (74).

Cyclic AMP is a ubiquitous second messenger that influences many cell functions primarily through its ability to phosphorylate proteins through the activation of cAMP-dependent protein kinase A (PKA). cAMP levels are regulated by the activity of adenylyl cyclase (AC) that catalyzes its synthesis from ATP and cyclic nucleotide phosphodiesterases (PDEs) that catalyze its degradation. The roles of cAMP in specific cellular functions are inferred from the use of agents that activate adenylyl cyclase directly or through receptor mediated activation. Agents such as cholera toxin, forskolin, hydrophobic cAMP analogues, and inhibitors of PDEs such as methylxanthines have all been used to examine effects of intracellular cAMP levels. The anti-inflammatory properties of these drugs are related to the increase in intracellular cAMP levels, since the cAMP/PKA pathway is recognized as a powerful suppressor of inflammatory cell function. Increases in intracellular cAMP inhibits release of reactive oxygen species and proinflammatory mediators such as TNF-α and IFN- γ blocks the proliferation of T-cells and enhances the production of the anti-inflammatory cytokine IL-10 (75). In addition,

cAMP-elevating ligands such as catecholamines, prostaglandins, dopamine and histamine have all shown immunosuppressive and anti-inflammatory properties *in vivo* (76, 77).

Protective roles of adenosine

Endogenous adenosine is another cAMP-elevating stimulus and the potential physiological 'off' signal protecting against excess inflammatory tissue damage. One of the first reports demonstrating this link was by Sattin and Rall (78), who showed that adenosine was one of the most potent stimulators of cAMP accumulation in brain slices of guinea pig.

The importance of adenosine as an immunosuppressor was later described from studies involving adenosine deaminase (ADA)-deficient patients who suffered from severe combined immune deficiency syndrome (SCID) (79, 80). The need to understand the pathogenesis of SCID resulted in multiple studies targeting the immunosuppressive effects of adenosine on various immune cell types including mast cells, neutrophils, T cells (81) and monocytes/macrophages (82). Results demonstrated that activation of adenosine receptors on immune cells suppressed the production of proinflammatory mediators including TNF-α (82, 83) and MMPs (84). In human and murine monocytes/macrophages, activation of adenosine receptors (particularly the A_{2A} receptors) by adenosine or its analogues has been shown to modulate the production of inflammatory cytokines including TNF-α (85), IL-10 (86) and IL-12 (87). IL-12, a proinflammatory cytokine and a central inducer of Th1 responses and cell-mediated immunity, is suppressed by adenosine whereas production of IL-10, a protective cytokine that suppresses IL-12 and TNF-α release, is enhanced by adenosine and A_{2A} receptor

agonists in both *in vitro* and *in vivo* models (83, 86, 87). Reports also indicate that adenosine causes strong inhibition of T-cell receptor (TCR)-triggered CD25 upregulation and T-cell proliferation, and also suppresses virtually all tested T-cell functions, including cytotoxicity and FasL production (88).

Further evidence suggesting a physiological role for endogenous adenosine as an immunosuppressor and anti-inflammatory agent has been provided by studies on the release of adenosine from cells. Cells exposed to various stimuli found in inflamed areas and in anoxia or hypoxia, have been shown to increase adenosine release in sufficient concentrations to affect other cells and physiological processes (71).

The most conclusive evidence highlighting anti-inflammatory effects of adenosine were obtained in genetic *in vivo* models of acute inflammation. In a study by Ohta and Sitkovsky (89), the importance of the adenosine A_{2A} receptor in regulating inflammation was demonstrated in mice. Using adenosine A_{2A} receptor knockout mice (Adora2a $^{-/-}$ N7), the physiological feedback mechanism of the adenosine system was demonstrated in tissue-specific and systemic inflammatory responses. Intravenous injection of concanavalin A (ConA) was used as an inflammatory stimulus, which is a well-described *in vivo* inflammation model of viral and autoimmune hepatitis. ConA-induced liver injury involves a host of inflammatory mediators such as T cells, macrophages and cytokines such as TNF-α, interleukin (IL)-4 and interferon (IFN)- γ (90, 91). Ohta and Sitkovsky showed that sub-threshold doses of ConA caused little tissue damage in wild-type mice but resulted in extensive tissue damage and a more prolonged and higher production profile of pro-inflammatory cytokines in mice deficient in the A_{2A} adenosine

receptors. The inflammatory damage seen in the Adora2a $^{-/-}$ N7 mice was so severe that 2 out of 4 mice in the group died within 8 hours (89).

Table 2. Summary of adenosine receptor signaling. Abbreviations: phospholipase C (PLC), phospholipase A_2 (PLA_2), inositol trisphosphate (IP_3), diacylglycerol (DAG), phospholipase D (PLD) (3).

Receptor	G protein	Effector
A_1	$G_{i\ 1/2/3}$, G_o	↓ cAMP ↑ PLC (IP_3/DAG) ↑ PLA_2 ↑ PLD ↑ K^+ channels ↓ Ca^{2+} channels
A_{2A}	G_s, G_{olf}, $G_{15/16}$	↑ cAMP ↓ Ca^{2+} channels ↑ IP_3 ($G_{15/16}$)
A_{2B}	G_s	↑ cAMP ↑ PLC (IP_3/DAG)
A_3	G_{i3}, $G_{q/11}$	↓ cAMP ↑ PLC (IP_3/DAG) ↑ $[Ca^{2+}]_i$

CHAPTER 2: HYPOTHESIS

Adenosine acting through adenosine A_{2A} receptors decreases MMP-9 activity levels through a TNF-α modulated pathway.

CHAPTER 3: OBJECTIVES

Objective #1: Optimize treatment conditions of U937 cells with phorbol-12-myristate-13 acetate (PMA) and phytohemagglutinin (PHA) to measure MMP-9 protein production by gelatin zymography, TNF-α protein levels by ELISA, and MMP-9 gene expression.

Objective #2: Determine the degree to which PMA/PHA-induced increases in TNF-α production mediates increases in MMP-9 activity.

Objective #3: Determine the degree to which agonists of three separate subtypes of adenosine receptors affect levels of TNF-α protein and MMP-9 activity.

Objective #4: Determine the effect of adenosine A_{2A} receptor activation on MMP-9 gene expression.

Objective #5: Determine the effect of adenosine A_{2A} receptor activation on cytosolic and membrane bound MMP-9 protein levels.

CHAPTER 4: MATERIALS AND METHODS

Cell culture

Human pro-monocytic U937 cells obtained from American Type Culture Collection (Rockville, MD) were maintained in RPMI 1640 (Gibco) growth media, pH 7.2, supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% antibiotic/antimycotic (Gibco). Because FBS was found to contain various MMP contaminants (Figure 7), cells were washed 3-times with AIM V serum-free media (Gibco) prior to plating of cells and cells were maintained in this serum-free media for all experiments. U937 cells (200 µl) were aliquoted into 96 well plates (Nunc) at a density of $1x10^6$ cells/ml for all experiments unless stated otherwise. Cells were incubated at 37°C with 5% CO_2 and 95% O_2.

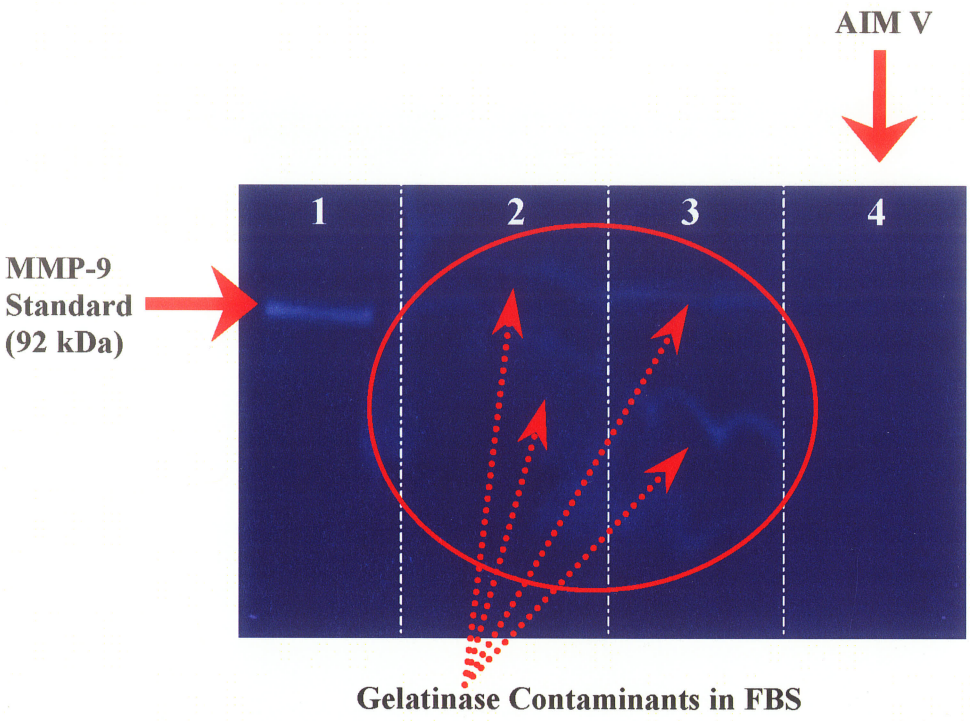

Figure 7. Gelatinase contaminants in fetal bovine serum (FBS) as detected by gelatin zymography. U937 cells were maintained in RPMI 1640 supplemented with 10% FBS. Our experimental procedure required cells to be washed and plated in AIM V serum free media to remove all gelatinase contamination. (Lane 1) MMP-9 Standard (BioMol) (Lane 2) 15 µl 100% FBS (Lane 3) 15 µl RPMI media with 10% FBS (Lane 4) 15 µl AIM V media (GIBCO).

TNF-α detection by enzyme linked immunosorbant assay (ELISA)

To determine the effects of adenosine receptor activation on levels of TNF-α protein production, cell supernatants were collected and analyzed by ELISA. The "capture" antibody used was a purified anti-human TNF-α primary antibody (BD PharMingen) made up to a final concentration of 2 μg/ml in a 0.05 M carbonate-bicarbonate buffer (0.8 g/500 ml Na_2CO_3, 1.47 g/500 ml $NaHCO_3$, pH 9.6). Corning EIA/RIA 96 well plates were coated overnight at 4°C with 50 μl/well of 2 μl/ml primary antibody solution. The following day, plates were washed 3-times with PBS-Tween wash buffer (10 mM PBS pH 7.4, 0.05% Tween-20). Plates were then treated for 1 hour with 100 μl/well of blocking buffer (1% BSA in PBS). Plates were then washed 3-times with PBS-Tween wash buffer and loaded with 50 μl/well of TNF-α protein standards or cell supernatants; all samples were assayed in triplicate. TNF-α standards (500 ng/μl in 1% BSA) were prepared at concentrations of 5000, 2500 1250, 625, 312, 156, 78 and 39 pg/ml in PBS. Plates were plastic-wrapped and stored overnight at 4°C. Following overnight incubation, plates were washed 3-times with PBS-Tween wash buffer, and were then treated at room temperature for 30 minutes with a labeled secondary antibody (the "detection" antibody) consisting of 50 μl of 0.5 μg/ml of biotin mouse anti-human TNF-α (BD PharMingen) prepared in dilution buffer (0.5% BSA, 0.05% Tween in PBS). Plates were then washed 3-times in PBS-Tween wash buffer and subsequently treated with 50 μl/well of enzyme linked Streptavidin-alkaline phosphatase (BD PharMingen) prepared in dilution buffer (1:1000 dilution) for 30 minutes at room temperature. Plates were then washed for a final 3-times with PBS-Tween wash buffer and wells were

developed for 15 minutes at room temperature with 50 µl of phosphatase substrate solution prepared from 1 mg/ml p-nitrophenyl phosphate (Sigma) in substrate buffer (100 mg/L $MgCl_2 \bullet 6H_2O$, 10% diethanolamine, pH 9.8). After 15 minutes of development, absorbance values from ELISA plates were determined using a Molecular Devices absorbance reader using Softmax Pro Version 4.0 software. Absorbance values were taken at 405-620 nm. TNF-α protein levels were determined based on TNF-α standard curves.

Analysis of MMP-9 Gene Expression by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

To determine the effects of adenosine receptor activation on MMP-9 gene expression, RT-PCR was used. U937 cells in AIM V serum-free media were plated on 24 well plates (Nunc) at a concentration of 1 x 10^6 cells/ml, with a final volume of 2 ml/well. After 6 hours of drug treatment, cells were collected by gently rinsing plates with supernatant after which the cell/supernatant suspension was collected and spun at 1396 x g for 5 minutes. The supernatant was removed and the cell pellet was resuspended in PBS followed by another 5-minute spin at 1396 x g. The supernatant was discarded and the cell pellet was resuspended in PBS and centrifuged once more. Cell pellets were frozen at –80°C until taken for use. RNA extraction was performed using the GenElute Total Mammalian RNA Isolation Kit (Sigma) and total RNA was obtained according to the manufacturer's instructions. Cells were lysed in 250 µl lysis buffer (1% 2-mercaptoethanol), transferred to filtration columns, and centrifuged at 14,500 x g for 2

minutes to remove cellular debris and to shear DNA. Equal volumes of 99% ethanol were added to the filtrate to precipitate nucleic acids. This solution was then transferred to binding columns where nucleic acid was isolated and purified using chemical washes to remove salts, proteins, and other soluble impurities. Binding columns were washed once with wash solution 1 followed twice by wash solution 2. Between each wash, columns were spun for 15 seconds at 1396 x g. After the final wash, binding columns were spun for 2 minutes to dry. Binding columns were transferred to fresh collection tubes and total RNA was eluted with 50 µl of elution solution. Total RNA was stored at –80°C or until used for cDNA synthesis.

Synthesis of cDNA was performed using the First Strand cDNA Synthesis Kit (MBI Fermentas) according to the manufacturer's instructions. Total RNA (10 µl) was mixed with 1 µl of 10X reaction buffer (Sigma DNase kit) and 1 µl of Amplification Grade DNase I for 15 minutes at room temperature. Stop Solution (1 µl) was then added to chelate Ca^{2+} and Mg^{2+} and to inhibit DNase I activity. Samples were heated to 70°C for 10 minutes to denature DNase I and to elongate RNA strands. While hot, oligo d(T) primer was added (1 µl of 0.1 µg/µl) and samples were immediately placed on ice. Samples were then mixed with 4 µl of 5X reaction buffer, ribonuclease inhibitor (1 µl of 20 U/µl) and dNTP mix (2 µl of 10mM) and incubated at 37°C for 5 minutes. M-MuLV reverse transcriptase (2 µl of 20 U/µl) was then added to samples and incubated at 37°C for 1 hour followed by a 10-minute incubation at 70°C that ended the reverse transcriptase reaction. cDNA was stored at –20°C or until used for PCR.

PCR amplification was performed using a Taq PCR Master Mix Kit (Qiagen) as per manufacturer's instructions. If cDNA was stored at –20°C, samples were heat

denatured at 95°C for 5 minutes. For each PCR reaction, 2 µl of cDNA was combined with 23 µl of PCR Master Mix solution. Master Mix solution per PCR reaction was composed of 12.5 µl Taq PCR Master Mix, 2.5 µl of the 3'and 5' primer solution (1 µM final concentration) and 4.5 µl RNase free H_20. PCR primers for MMP-9 (5'-CACTGTCCACCCCTCAGAGC, 3'-GCCACTTGTCGGCGATAAGG) yielding a PCR product of 263 base pairs in length, and GAPDH (5'-TGATGACATCAAGAAGGTGGTGAAG, 3'-TCCTTGGAGGCCATGTGGGCCAT) yielding a 240 base pair product were purchased from BioMol. The parameters for the PCR reaction included an initial 5 minute denaturing step at 95°C followed by 30 cycles of denaturing/annealing/extending at 94°C for 30 sec., 58°C for 1 min., and 72°C for 50 sec. The reaction was completed by incubating samples for 7 minutes at 72°C; samples could then be kept at 4°C indefinitely. PCR products were stored at –20°C. PCR products were combined with 6X loading dye (Fermentas) at a 5:1 volume ratio and 10 µl of each sample was loaded onto 1.2% agarose gels and detected under UV light using ethidium bromide staining. Densitometric analysis of electrophoretic bands was performed using a BioRad Gel Documentation System.

MMP-9 detection by gelatin zymography

U937 cell culture media was collected and stored at –20°C until used. Aliquots of each sample were subjected to SDS-PAGE in 10% polyacrylamide gels containing 1.2 mg/ml of Gelatin Type B (Sigma). The method of Laemmli (92) was followed, excluding any reducing agents or boiling products. Aliquots of conditioned media (15

μl) were added to 5 μl of non-reducing sample buffer containing 2% wt./vol. SDS, 10% glycerol, 50 mM of Tris-HCl (pH 6.8), and 0.005% bromophenol blue. Samples were mixed and added to the gel without heating. After electrophoresis at 30 mV, gels were washed twice in 2.5% Triton X-100 at 4°C for 30 minutes to remove the SDS and renature the proteins. The gels were then incubated in substrate buffer (100 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$) overnight at 37°C. The gels were then stained with 0.1% Coomassie Brilliant Blue R-250 diluted in 40% methanol and 10% acetic acid for 1 hour, and destained until bands with diminished staining appeared on a uniformly stained background. The clear bands represent the positions of matrix metalloproteinases having gelatinase activity. The molecular weight of the gelatinase was estimated using prestained molecular weight standards (BioRad). Gels were analyzed and bands were quantified using a BioRad Gel Documentation System. To account for the differences in band intesity as a result of digestion, staining, destaining, and imaging variables, results between zymograms were normalized by representing the data as a ratio of the PMA/PHA control present in each gel.

Immunofluorescent assay

U937 cells were collected after incubations were completed by centrifuging at 1396 x g for 3 minutes. Cell pellets were resuspended twice in 1X PBS each time centrifuging samples at 1396 x g for 3 min. After the third wash, cell pellets were resuspended in 100 μl of PBS and 2 drops of the cell solutions were smeared on glass slides (Fisher Superfrost). Cell densities were adjusted with the addition of PBS so that

cell staining could be identified more easily. Samples from control and experimental treatments were smeared on the same slide for comparison of staining. Spots were air dried, fixed with ice cold acetone for 3 minutes at –20°C before being traced with a Pap pen and allowed to dry. Slides were then treated with blocking solution (Roche) at room temperature for 1 hour, followed by two 5-minute washes of PBST. Slides were treated for 40 minutes at 37°C with the primary antibody (Anti-MMP-9, Catalytic Domain; BioMol) that was diluted at 1:2000 with 1X PBST. The primary antibody was removed by washing slides twice with 1X PBST for 5 minutes. Cells were incubated at 37°C for 35 minutes with secondary antibody (goat antimouse IgG; Alexa 546) diluted 1:400 with 1X PBST. The secondary antibody was removed by washing slides twice with 1X PBST for 5 minutes. Finally, cells were treated with Hoechst 33342 stain (diluted 1:5000 in 1X PBST) for 10 seconds in the dark at room temperature followed by a wash in PBS. Mounting medium and cover slips were added and slides were allowed to dry; cells were imaged immediately, stored at 4°C for imaging the next day, or stored at –20°C for analysis later. Slides were imaged using a Zeiss upright fluorescence microscope at 630X magnification running the Zeiss Axio Vision Software.

Western blot

For all western blot experiments, cells were added to 6 well plates (Nunc) at a density of 1 x 10^6 cells/ml and a final volume of 4 ml/well. Samples were collected by gently rinsing plates with supernatant, collecting the cell/supernatant suspension, and

centrifugation at 1396 x g for 5 minutes. Supernatants were removed and cell pellets were resuspended in PBS and re-centrifuged as above twice. Cell pellets were frozen at –80°C until taken for assay. Whole cell lysates were prepared by boiling cell pellets for 15 minutes in 100 µl of Laemmli buffer (0.5 M Tris base (pH 6.8), 4% SDS) followed by centrifugation for 30 seconds at 14,500 x g. A syringe fitted with a 27.5 gauge needle was used to break up pellets and samples were re-centrifuged and whole cell lysates were transferred to a new tube. Protein content was determined using the BioRad DC Protein Assay Kit, a method compatible with samples containing detergent. Aliquots (50 µg) of protein were loaded into wells at a volume of 30 µl. Samples were prepared in 2X gel loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 20% glycerol and 0.2% bromophenol blue). Samples were loaded in 1.0 mm thick, 10% acrylamide gels along with 15 µl of BioRad broad range pre-stained protein standard (205 kD - 6 kD). Composition of resolving gels consisted of 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 10% acrylamide/bisacrylamide, 0.05% ammonium persulfate, and 0.05% Temed. Stacking gels consisted of 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 3% acrylamide/bisacrylamide, 0.1% ammonium persulfate, and 1% Temed. Gels were run in cold running buffer (5 mM Tris-base, 50 mM glycine, 0.02% SDS pH 8.3) at 50 volts for 15 minutes followed by 150 volts until loading dye approached the bottom edge of gel. Proteins were subsequently transferred onto a nitrocellulose membrane in cold transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol pH 8.3) using the BioRad Mini Trans-Blot wet transfer apparatus running at 20 volts overnight at 4°C. Following the transfer, nitrocellulose membranes were incubated with blocking buffer (1% Roche Blocking Reagent in TBS) overnight at 4°C. Primary antibody (Anti-MMP-9 Catalytic

Domain (BioMol)) was diluted 1:2000 in 0.5% Roche Blocking Reagent in TBS and incubated with nitrocellulose membranes overnight at 4°C on a rocking platform. The following day, membranes were washed 3-times with TBS-Tween (1% Tween), rinsed once with TBS, and incubated for 1 hour with horseradish peroxidase conjugated secondary antibody. The secondary antibody was an IgG H+L antibody obtained from Jackson ImmunoResearch Laboratories and was prepared in 0.5% Roche Blocking Reagent in TBS at a concentration of 200 ng/ml. Bands were visualized using ChemiGlow West Substrate (Alpha Innotech, San Leanardo, CA). Membranes were incubated for 5 minutes in working solution consisting of equal volume of ChemiGlow West luminal/enhancer solution and stable/peroxide solution. Protein bands were then imaged and quantified using a BioRad FluorS Max imaging system.

Statistical Analysis

Statistical analysis of all data was performed using GraphPad Prism version 2.01 (San Diego, CA). Analysis was done using one-way ANOVA, followed by a Student-Newman-Keuls multiple comparison post test. Statistical differences were considered significant at the $p < 0.05$ level.

CHAPTER 5: RESULTS

Optimization of experimental model

Before we could test our hypothesis that activation of adenosine A_{2A} receptors could decrease MMP-9 activity, experimental conditions first had to be optimized. Cells were seeded at a density of 1,000,000 cells/ml and treatment concentrations of PMA/PHA were held constant for all zymography and gene expression experiments. To induce TNF-α production and to mimic an inflammatory event, U937 cells were treated with 10 ng/ml phorbol-12-myristate-13-acetate (PMA) and 5 μg/ml phytohemagglutinin (PHA). PMA, a phorbol ester, is a known activator of protein kinase C (PKC), an established mediator of inflammatory cell signaling pathways (93-96). The effect of PMA on the expression pattern and distribution of PKC isozymes in U937 cells have previously been described by Kwon *et al.* (97). PHA causes monocyte activation by stimulating proliferation and agglutination (98). Selection of PMA and PHA concentrations were chosen based on reports demonstrating their ability to significantly increase levels of TNF-α in U937 and other immune cells (99, 100). In addition, PMA has been reported to increase levels of MMP-9 in U937 cells (101) and astrocytes (94).

Optimal treatment time of U937 cells with PMA/PHA for induction of MMP-9 activity was determined by zymography. U937 cells were treated with 10 ng/ml PMA and 5 μg/ml PHA and cell supernatants were collected every hour for up to 8 hrs. MMP-9 activity in supernatants increased significantly in a time-dependent manner (Figure 8A); when compared to 1-hr controls MMP-9 activity increased to 170 % at 2 hr, 259 % at 3

hr, 337 % at 4 hr, 385 % at 5 hr, 497 % at 6 hr, 536 % at 7 hr and 598 % at 8 hr. Zymographic bands were most easily identified (Figure 8A) at 8 hours and this time point was chosen for all subsequent zymographic experiments. In the absence of PMA/PHA, only very faint bands of MMP-9 activity were observed (data not shown).

Preliminary experiments looking at gelatinase activity at 0, 4, 8 and 12 hr time points were also done to confirm that all the gelatin was not completely degraded at 8 hrs. Results clearly demonstrated an increase in zymographic band intensity at 12 hrs compared to 8 hrs (data not shown).

Cell supernatants from the same experiments were analyzed by ELISA for protein levels of TNF-α. Significant time dependent increases in TNF-α protein levels were detected in supernatants of cells treated with PHA/PHA with a time course similar to that observed for MMP-9 activity (Figure 8B). Compared to 1 hr controls (31 pg/ml), TNF-α protein levels increased to 293 pg/ml at 2 hr, 609 pg/ml at 3 hr, 864 pg/ml at 4 hr, 1046 pg/ml at 5 hr, 1194 pg/ml at 6 hr, 1396 pg/ml at 7 hr, and 1622 pg/ml at 8 hr.

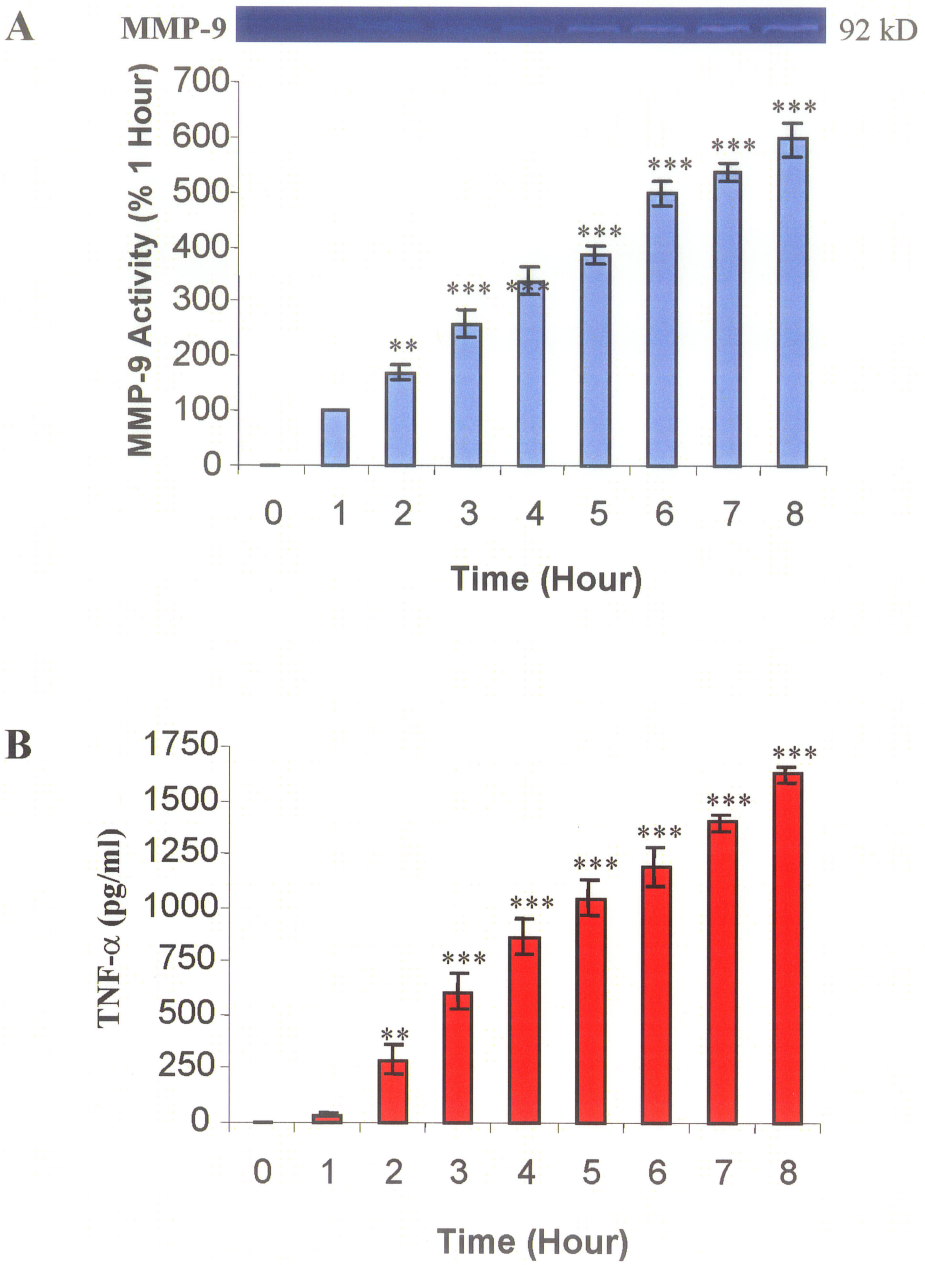

Figure 8. MMP-9 activity and TNF-α protein levels are increased in a time dependent fashion after stimulation of U937 cells with 10 ng/ml PMA and 5 μg/ml PHA. (A) Upper figure: Representative zymogram showing MMP-9 activity. Lower figure: Densitometric analysis of MMP-9 activity using gelatin zymography. (B) TNF-α levels as quantified by ELISA. Results shown represent the mean ± standard error of three experiments (Student-Newman-Keuls, $^{**}p < 0.01$, $^{***}p < 0.001$ v. 1 hour).

Time course experiments were also performed to examine MMP-9 gene expression by RT-PCR. MMP-9 gene expression in U937 cells was increased significantly ($p < 0.05$) 6 and 8 hrs after treatment with 10 ng/ml PMA and 5 μg/ml PHA (Figure 9); in relation to values at 0 hr, the increase at 6 hr was 81% and at 8 hr was 77%. Based on these results, 6 hrs was chosen as the PMA/PHA treatment duration for all subsequent gene expression studies.

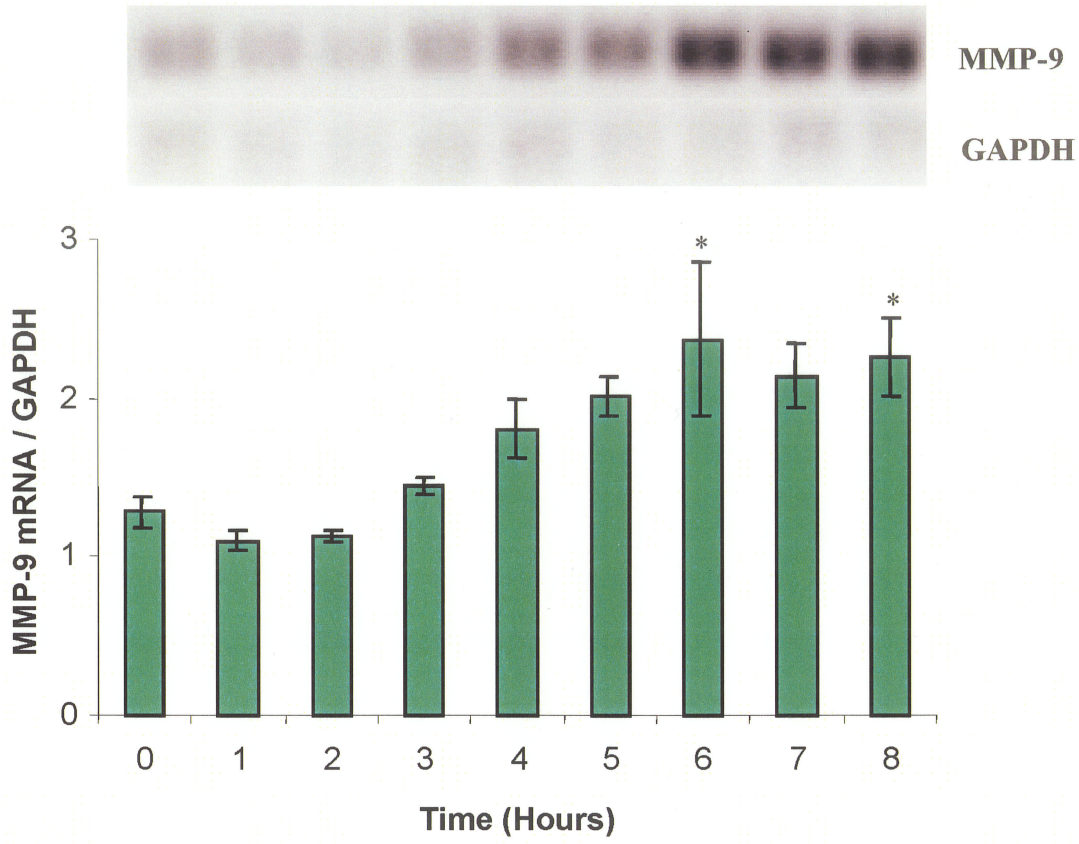

Figure 9. MMP-9 gene expression as measured by RT-PCR is highest 6 hours after treatment of U937 cells with 10 ng/ml PMA and 5 μg/ml PHA. The upper figure is a representative RT-PCR analysis depicting increases in MMP-9 mRNA by PMA/PHA with time. In the lower figure, MMP-9 PCR products were normalized to GAPDH and results shown represent the mean ± standard error of three experiments (Student-Newman-Keuls, *$p < 0.05$ v. 0 hour).

PMA/PHA induced TNF-α production increases MMP-9 activity

To test our hypothesis that adenosine can regulate MMP-9 activity through a TNF-α mediated pathway, we first determined the extent to which TNF-α was inducing MMP-9 activity in our model of inflammation. This was accomplished using either a neutralizing TNF-α soluble receptor 1 or a TNF α-neutralizing antibody. U937 cells were treated with a neutralizing TNF-α soluble receptor 1 (TSR1) just prior to being treated with 10 ng/ml PMA and 5 μg/ml PHA for 8 hrs. Concentration dependent decreases in MMP-9 activity were observed after treatment with TSR1; changes in MMP-9 activity compared to PMA/PHA controls were +5%, -4%, -14%, -13%, -28% and -43% with 32, 63, 125, 250, 500 and 1000 ng/ml TSR1, respectively (Figure 10A). When the samples were analyzed for TNF-α protein levels, the results were less clear (Figure 10B); TNF-α protein levels did not decrease in a concentration-dependent manner with TSR1 treatment.

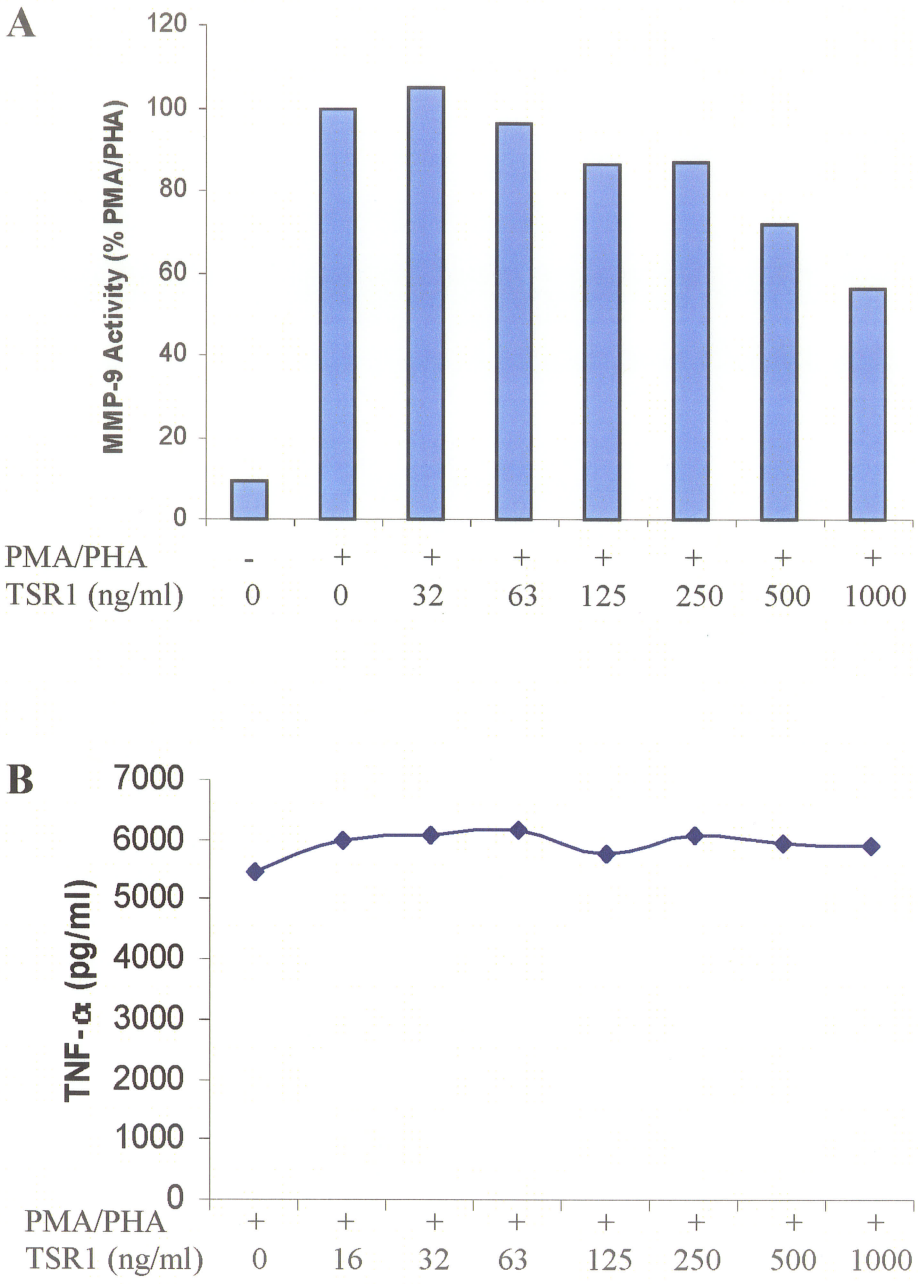

Figure 10. TNF-α soluble receptor 1 (TSR1) decreases PMA/PHA induced MMP-9 activity in U937 cells but show no effect on TNF-α protein levels. Cells were stimulated with 10 ng/ml PMA and 5 μg/ml PHA for 8 hrs. Results illustrated are from a single experiment. (A) Densitometric analysis of MMP-9 activity as determined by gelatin zymography. (B) TNF-α levels as quantified by ELISA.

The second strategy involved the use of a TNF-α neutralizing antibody (anti-TNF Ab). U937 cells were treated with anti-TNF Ab just prior to the application of 10 ng/ml PMA and 5 μg/ml PHA for 8 hrs. Concentration dependent decreases in MMP-9 activity were observed with the antibody treatment (Figure 11A). Using 125 ng/ml of the TNF-α Ab, MMP-9 activity levels decreased 20% compared to control. Use of 250 ng/ml, 500 ng/ml and 1000 ng/ml resulted in decreases of 34%, 40% and 57%, respectively (Figure 11A). Analysis of the same samples for TNF-α protein levels showed a similar concentration dependent decrease (Figure 11B). Statistically significant decreases in levels of TNF-α protein and MMP-9 activity were observed with antibody concentrations of 125, 250, 500 and 1000 ng/ml.

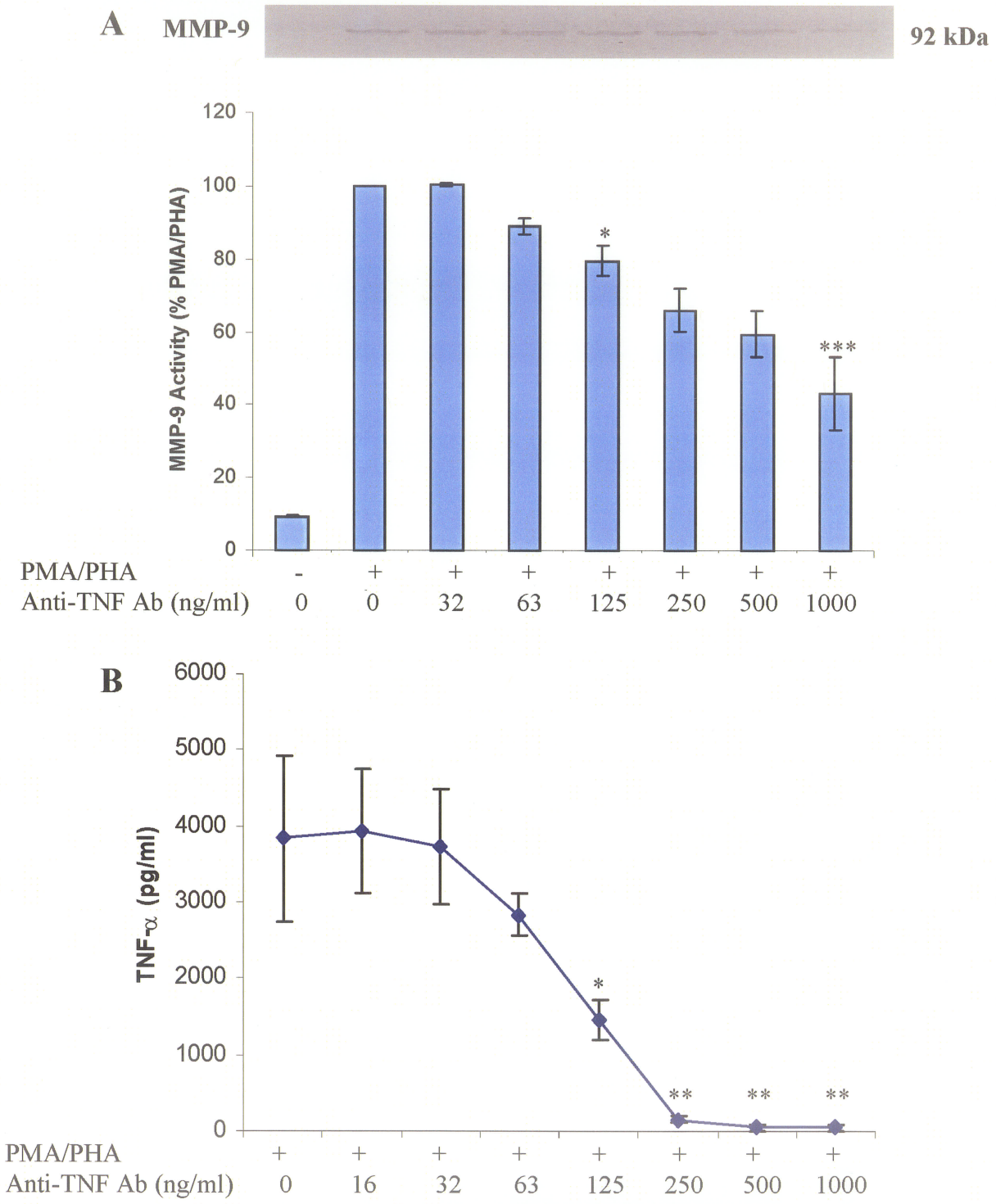

Figure 11. Anti-TNF-α neutralizing antibody (Anti-TNF Ab) decreases PMA/PHA induced MMP-9 activity and TNF-α protein levels in a concentration dependent fashion. U937 cells were stimulated with 10 ng/ml PMA and 5 μg/ml PHA for 8 hrs. Results represent the mean ± standard error of three experiments (Student-Newman-Keuls, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ v. PMA/PHA control.) (A) Densitometric analysis of MMP-9 activity as determined by gelatin zymography along with a representative zymogram. (B) TNF-α protein levels as quantified by ELISA.

Effects of adenosine receptor activation on MMP-9 activity

Previous findings have shown that activation of adenosine A_{2A} receptors decreases TNF-α levels (83, 100, 102, 103). Using pharmacological techniques, our next step was to determine if adenosine had any effect on MMP-9 activity.

Adenosine A_{2A} Receptor

Activation of adenosine A_{2A} receptors with 100 nM of the selective adenosine A_{2A} receptor agonist CGS 21680 resulted in a statistically significant ($p < 0.01$) 32% decrease in MMP-9 activity compared to PMA/PHA controls (Figure 12A). Pretreatment of cells for 30 min with the adenosine A_{2A} receptor antagonist ZM 241385 (100 nM) abolished the effect of CGS21680 on levels of MMP-9 activity. Pretreatment of cells with ZM 241385 alone resulted in a statistically significant ($p < 0.01$) 23% increase in MMP-9 activity. Activation of adenosine A_{2A} receptors decreased PMA/PHA induced TNF-α protein production in these same samples (Figure 12B). 30 minute pretreatment with 100 nM CGS 21680 decreased significantly ($p < 0.05$) TNF-α protein levels by 26% compared to the PMA/PHA control; levels decreased from 2905 pg/ml to 2142 pg/ml. This effect was specific to adenosine A_{2A} receptor activation because pre-pretreatment with ZM 241385 abolished the effects of CGSA 21680 (Figure 12B). Pretreatment of cells with ZM 241385 alone did not result in a statistically significant change in TNF-α protein levels (as was the case with MMP-9), but there appeared to be a trend towards an increase (Figure 12B).

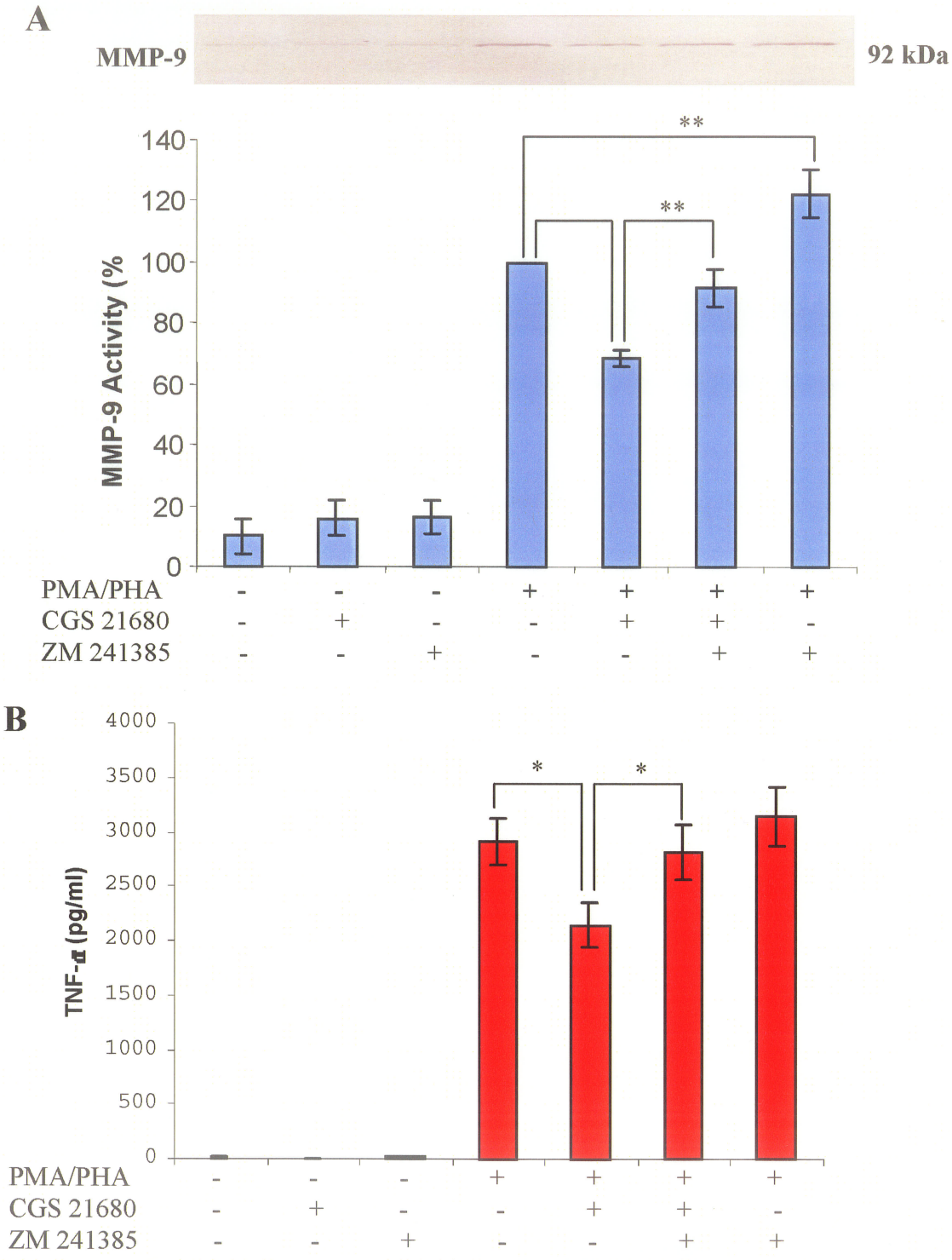

Figure 12. Adenosine A_{2A} receptor agonist CGS 21680 (100 nM) decreases MMP-9 activity and TNF–α protein levels in U937 cells stimulated with 10 ng/ml PMA and 5 μg/ml PHA for 8 hrs; these decreases were blocked by the adenosine A_{2A} receptor antagonist ZM 241385 (100 nM). (A) Densitometric analysis of MMP-9 activity as determined by gelatin zymography along with a representative zymogram. (B) TNF-α protein levels were quantified using ELISA. Results represent the mean ± standard error of three experiments (Student-Newman-Keuls, *$p < 0.05$, **$p < 0.01$).

Adenosine A_1 Receptor

The same pharmacological approach was used to examine the effects of adenosine A_1 receptor activation on MMP-9 activity. CCPA, a selective adenosine A_1 receptor agonist, co-applied to U937 cells with PMA/PHA resulted in concentration-dependent increases in MMP-9 activity; MMP-9 activity was increased significantly ($p < 0.05$) with 2 nM CCPA (116%), peaked (148% increase) with 5 nM CCPA ($p < 0.01$), and increases started to decrease at the highest CCPA concentration used (10 nM) (Figure 13A). These effects of CCPA appeared to be mediated through adenosine A_1 receptors because 30 minute pre-pretreatment with 100 nM of the A_1 receptor antagonist DPCPX decreased the CCPA (5 nM) response to that of PMA/PHA controls (Figure 13B).

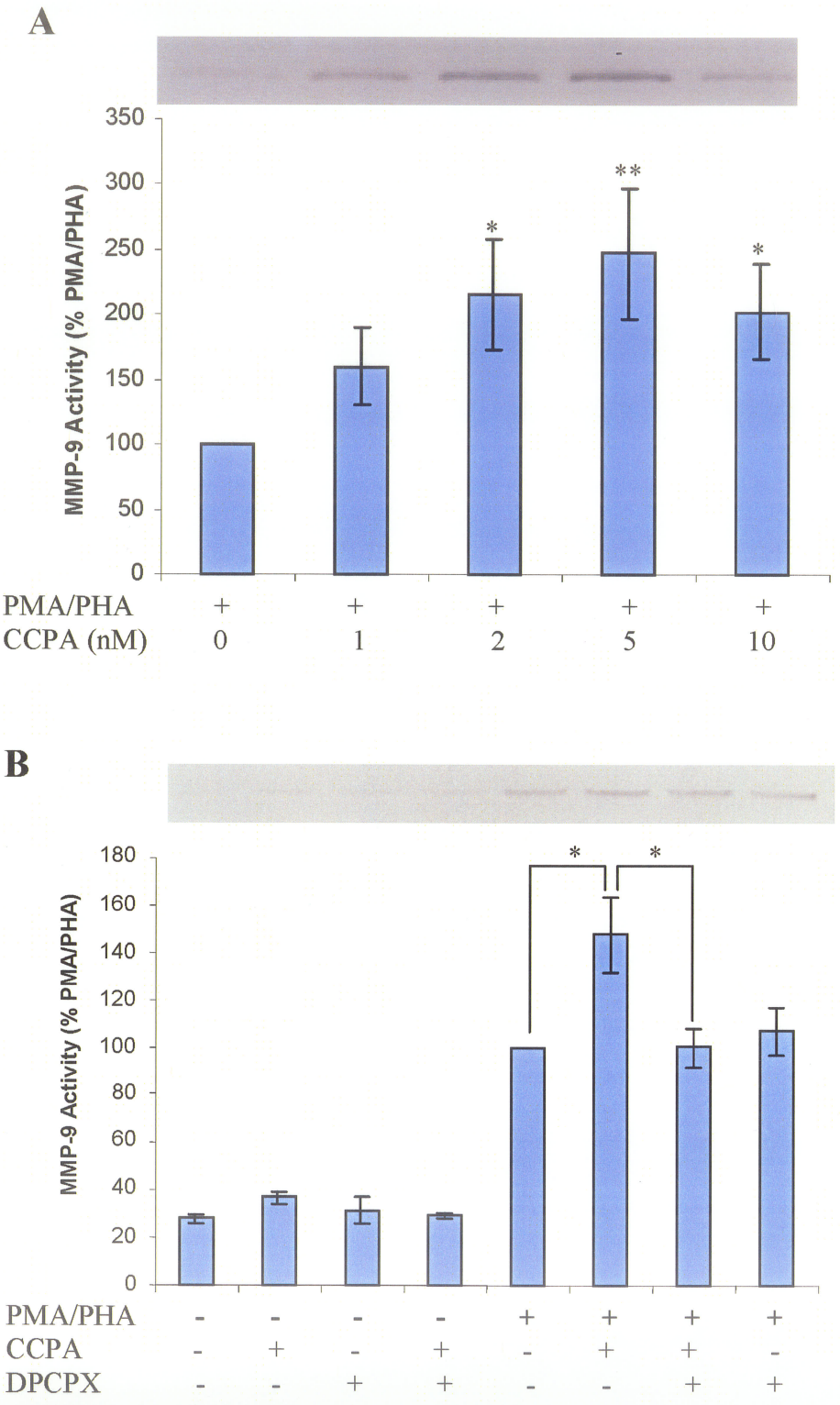

Figure 13. Activation of adenosine A_1 receptors with the A_1 specific agonist CCPA increased MMP-9 activity as measured by gelatin zymography in U937 cells stimulated with 10 ng/ml PMA and 5 μg/ml PHA for 8 hrs. (A) Effect of CCPA on MMP-9 activity was concentration dependent. (B) Treatment with the A_1 receptor antagonist DPCPX (100 nM) eliminated the CCPA (5 nM) dependent increase in MMP-9 activity. Results represent the mean ± standard error of three experiments (Student-Newman-Keuls, *$p < 0.05$, **$p < 0.01$ v. PMA/PHA control.)

Adenosine A_3 Receptor

The effect of adenosine A_3 receptor activation on MMP-9 activity was determined using the selective adenosine A_3 receptor agonist, IB-MECA. Results of concentration response experiments with IB-MECA showed a bell shaped profile of effects on MMP-9 activity (Figure 14A) as was seen with CCPA activation of A_1 receptors (Figure 13A). Although the data from 3 separate experiments were quite variable, IB-MECA caused significant increases in MMP-9 activity at concentrations of 5, 10 and 20 nM when compared to PMA/PHA control (Figure 14A). The specificity of this effect was confirmed using the adenosine A_3 receptor antagonist MRS 1220; a 30 minute pre-pretreatment with 100 nM MRS 1220 decreased the IB-MECA (10 nM) response to levels similar to PMA/PHA controls (Figure 14B).

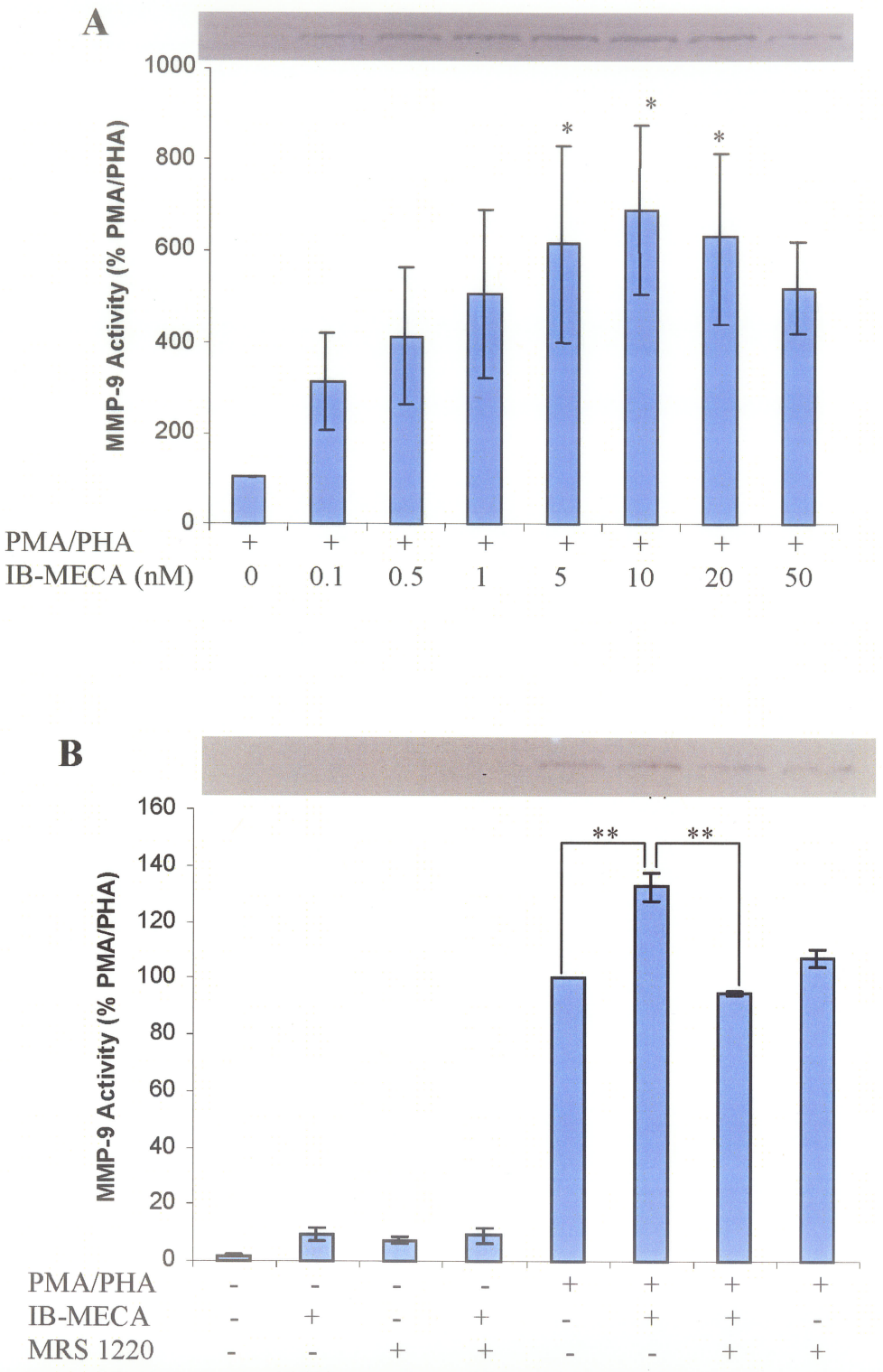

Figure 14. Activation of adenosine A_3 receptor with the A_3 specific agonist IB-MECA increases MMP-9 activity as measured by gelatin zymography in U937 cells stimulated with 10 ng/ml PMA and 5 μg/ml PHA for 8 hrs. (A) Effect of IB-MECA on MMP-9 activity was concentration dependent. (B) Treatment with the A_3 receptor antagonist MRS 1220 (100 nM) eliminated the IB-MECA (10 nM) dependent increase in MMP-9 activity. Results represent the mean ± standard error of three experiments (Student-Newman-Keuls, *$p < 0.05$, **$p < 0.01$ v. PMA/PHA control.)

Activation of the adenosine A_{2A} receptor increases MMP-9 gene expression

We next determined whether activation of adenosine A_{2A} receptor decreased not only MMP-9 activity, but also MMP-9 gene expression. The effect of adenosine A_{2A} receptor activation on MMP-9 gene expression was analyzed by RT-PCR. U937 cells were pretreated for 30 minutes with the A_{2A} receptor agonist CGS 21680 (100 nM) and then treated with 10 ng/ml PMA and 5 μg/ml PHA for 6 hrs. In PMA/PHA treated cells, activation of adenosine A_{2A} receptors with 100 nM CGS 21680 increased MMP-9 mRNA by 25% over controls (Figure 15). This effect was specific to A_{2A} receptor because 30 minute pre-pretreatment with 100 nM ZM 241385, the A_{2A} receptor specific antagonist, blocked this effect ($p<0.001$).

Treatment with 100 ng/ml TSR1 alone or in combination with 10 ng/ml PMA and 5 μg/ml PHA had no statistically significant effect on MMP-9 gene expression compared to controls.

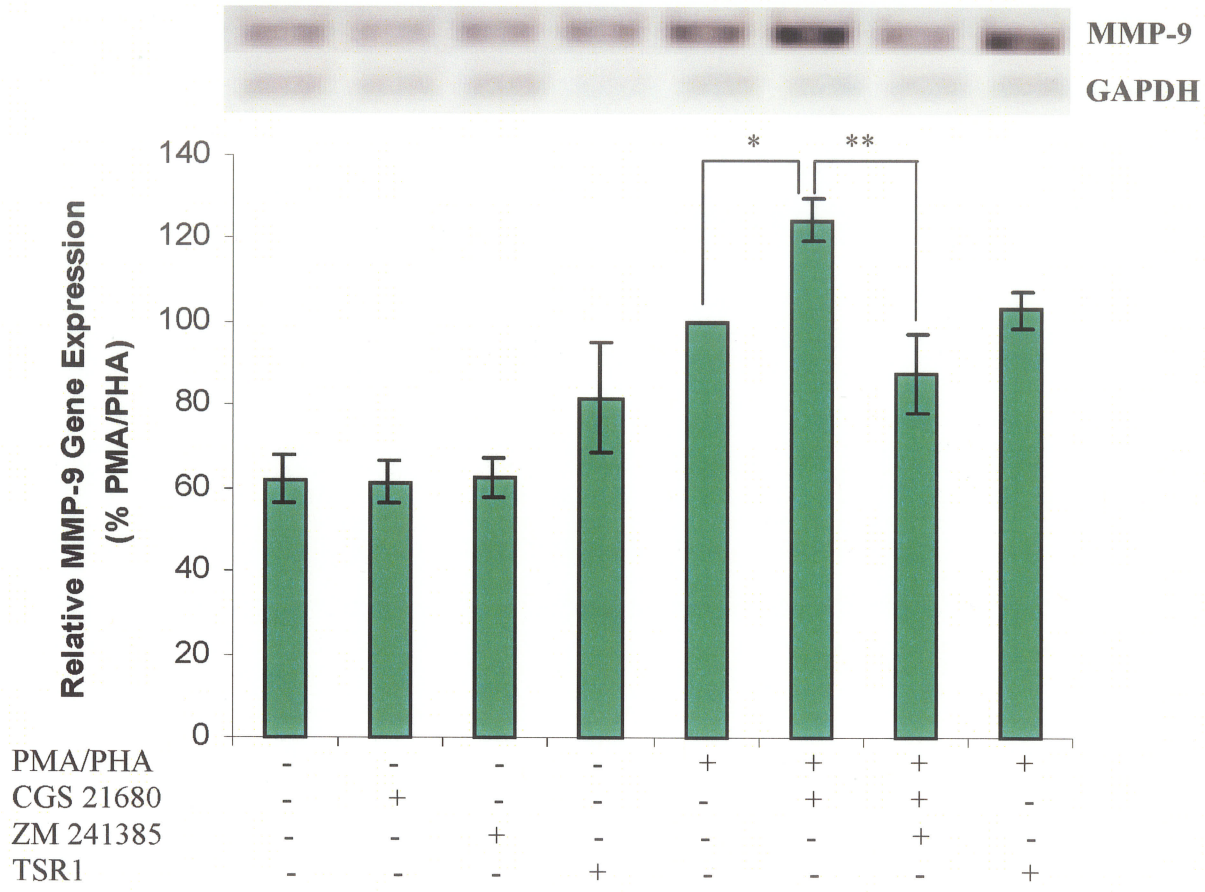

Figure 15. Adenosine A_{2A} receptor agonist CGS 21680 (100 nM) increased MMP-9 gene expression as determined by RT-PCR in U937 cells stimulated with 10 ng/ml PMA and 5 μg/ml PHA for 6 hrs. This effect was blocked by the adenosine A_{2A} receptor antagonist ZM 241385 (100 nM). No significant changes were seen using 100 ng/ml of the neutralizing TNF-α soluble receptor 1 (TSR1). MMP-9 PCR products were normalized to GAPDH. Results represent the mean ± standard error of three experiments (Student-Newman-Keuls, *$p < 0.05$, * **$p < 0.001$).

Effects of A_{2A} adenosine receptor activation on membrane bound and cytosolic MMP-9 protein levels

Activation of adenosine A_{2A} receptors decreased MMP-9 activity, but this inhibition did not appear to be occurring at the level of transcription. Therefore, the regulatory effect of adenosine A_{2A} receptor activation on MMP-9 activity might be occurring between the levels of transcription and secretion and we examined intracellular protein levels of MMP-9 to determine effects on protein translation using the techniques of immunofluorescent staining and Western blotting. Preliminary results from one experiment demonstrated that in PMA/PHA treated and untreated cells, activation of adenosine A_{2A} receptors clearly increased the fluorescent signal specific to MMP-9 as determined by immunofluorescent staining (Figure 16A). This signal was reduced upon treatment with the receptor antagonist ZM 241385 suggesting a selective role of the adenosine A_{2A} receptor. An increase in fluorescent signal was also detected in cells treated with 100 ng/ml of TSR1 in both PMA/PHA treated and untreated conditions (Figure 16A).

In addition, Western blots were performed to quantify MMP-9 protein under similar treatment conditions. Data analyzed from three separate experiments revealed no significant differences in MMP-9 protein levels with activation of the A_{2A} adenosine receptor compared to control (Figure 16B).

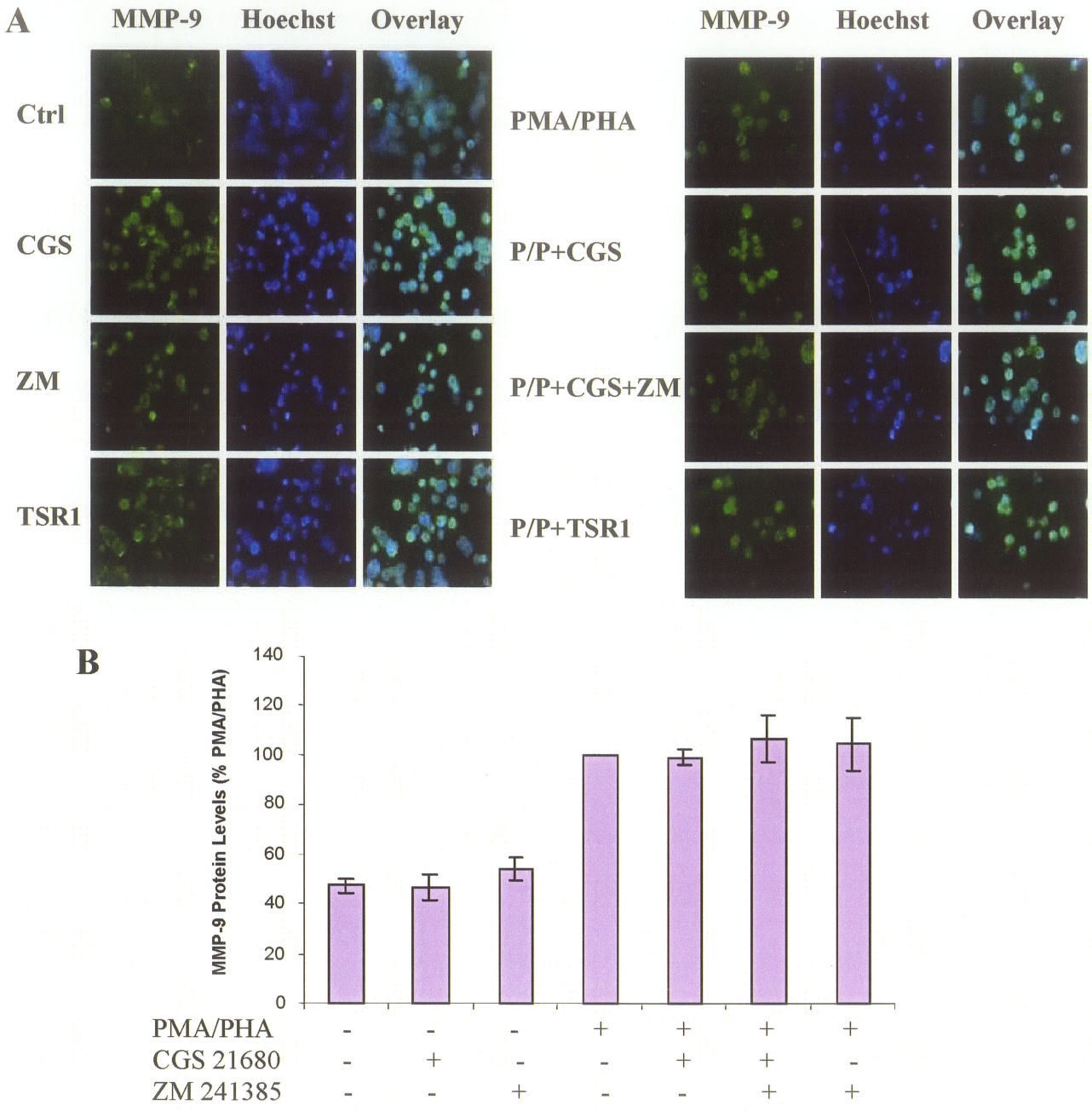

Figure 16. (A) Adenosine A_{2A} receptor agonist CGS 21680 (100 nM) and neutralizing TNF-α soluble receptor 1 (TSR1) (100 ng/ml) increased MMP-9 protein levels in U937 cells stimulated for 8 hrs with 10 ng/ml PMA and 5 μg/ml PHA; these increases were blocked by the adenosine A_{2A} receptor antagonist ZM 241385 (100 nM). Images are of immunofluorescence of MMP-9 catalytic site antibody observed at 630X magnification and Hoechst staining labels nuclei of viable cells. Experiment was done once. (B) Activation of the A_{2A} adenosine receptor did not produce a significant change in MMP-9 protein levels compared to controls as detected by Western blot. U937 cells were pretreated for 30 minutes with 100 nM ZM 241385 and/or pretreated for 30 minutes with 100 nM CGS 21680 and stimulated with 10 ng/ml PMA and 5 μg/ml PHA for 8 hrs. Results represent the mean ± standard error of three experiments (Student-Newman-Keuls)

CHAPTER 6: DISCUSSION

Optimization of experimental model

Matrix metalloproteinases produced by monocytes are involved in the migration of these cells through the basement membrane and the ensuing destruction of connective tissue in chronic inflammatory lesions. Monocytes naturally encounter a variety of cytokines at these sites and U937 promonocytic cells were chosen as our test model to study MMP-9 regulation.

With density of cells and concentration of PMA/PHA held constant, length of incubations became an important factor determining our ability to detect and analyze MMP-9 production/activity and gene expression. Zymography is the classic technique used to determine MMP-9 production and activity. By incorporating gelatin into a SDS-PAGE gel, gelatinase activity can be visually identified based on molecular mass and its ability to degrade substrate (gelatin) incorporated into the gel. Degradation of the substrate occurs during an overnight incubation step, and the change in gel density is revealed upon staining with Coomassie blue. Because the amount of substrate incorporated into a gel is finite, it is important that analysis of a gel does not occur past the point when all the substrate has been degraded. Otherwise, the opportunity to see differences in MMP activity between samples would be missed. The results of our time course experiments established 8 hrs as the treatment time that gave clear zymographic bands suitable for analysis.

We also looked at optimizing experiments for MMP-9 gene expression studies, because we did not know at what level adenosine receptor activation would affect MMP-9 regulation. Our results showed a definite trend towards an increase from hrs 2 - 4, but statistically significant increases were observed only after 6 and 8 hrs (Figure 9). This pattern of MMP-9 gene expression was similar to that reported for THP-1 cells treated with the CC chemokines CCL2 (MCP-1) and CCL5 (RANTES) (104). The up-regulation of mRNA 6 hrs after cell stimulation may reflect a delayed response needed for leukocytes to first migrate through the endothelial cell layer. The sub-endothelial layer is where MMP-9 is needed to degrade the basement membrane proteins. In fact, migration and invasion of monocytes in response to chemokines was reported to take 6 - 24 hrs *in vivo* (105). The increase in MMP-9 mRNA in this study fits within this time scale and for all subsequent experiments of MMP-9 gene expression we typically stimulated U937 cells with PMA/PHA for 6 hrs.

PMA/PHA induced TNF-α production increases MMP-9 activity

TNF-α, a proinflammatory cytokine, is involved in the etiology of numerous pathological conditions where increased MMP activity is a hallmark. In conditions such as atherosclerosis (106), tumor growth and metastasis (107), rheumatoid arthritis (108-110) and neuroinflammation (19, 53), the effects of TNF-α on MMP-9 regulation have been extensively studied.

The pathology of these diseases all involve remodeling of the extracellular matrix and increases in the biological activity of matrix metalloproteinase-9 (111). TNF-α

appears to be an important regulator of MMP because TNF-α has been shown to upregulate MMP-9 production and release in various cell types including NB4 leukemia cells (112) and monocytes/macrophages (113-115). In peripheral blood monocytes and the monocytic cell line THP-1, autocrine TNF-α was required for chemokine stimulation of MMP-9 activity (104). The addition of other cytokines in combination with TNF-α has been shown to result in synergistic increases in MMP-9 production (114).

TNF-α's role as a central mediator of inflammation has been shown convincingly for rheumatoid arthritis where treatment with neutralizing anti-TNF antibody resulted in a significant decrease in disease (116). Rheumatoid arthritis is an autoimmune disease where MMPs play a critical role in tissue disintegration (117). A correlation between TNF-α and MMP levels in serum of patients with rheumatic arthritis has been clearly demonstrated (118) supporting the idea that MMP regulation is dependent on cytokines such as TNF-α. In addition, serum TNF-α and various MMPs (MMP-1, MMP-3 and MMP-9) correlated with characteristic markers of arthritic disease progression such as erythrocyte sedimentation rate, C reactive protein level and increased number of swollen joints (118).

In our model of inflammation, we induced TNF-α production by treating U937 cells with PMA/PHA. PMA/PHA-induced TNF-α production was likely partly responsible for the increase in MMP-9 activity as TNF-α neutralizing drugs (TNF-α soluble receptor 1 and TNF-α neutralizing antibody) both blocked the MMP-9 released.

The TNF-α soluble receptor works by competitively binding to TNF-α in the supernatant and neutralizing its ability to bind to cell surface TNF-α receptors. The TNF-

α soluble receptor appeared to decreased MMP-9 activity as there appeared to be a trend towards a decrease in MMP-9 activity with increasing concentrations of TSR1.

The same samples were also analyzed by ELISA. TNF-α protein levels remained comparable to control and did not result in a concentration dependent decrease as expected (Figure 10B). These results raised questions of how effectively the soluble receptor was neutralizing TNF-α and if this method was compatible with our detection assay. It is possible that both the bound and unbound TNF-α was being detected by our ELISA method resulting in the unchanging TNF-α profile seen in Figure 10B. Another uncertainty involved the properties of the soluble receptor such as its affinity for TNF-α and differences in function between TNF-α Receptor 1 (TNF-R1) or TNF-α Receptor 2 (TNF-R2). The TNF-α soluble receptor 1 was chosen based on reports that indicated that MMP-9 induction by TNF-α occurs via signaling through p55 TNF receptor 1 (112). It was shown that blocking monoclonal antibodies against TNF-R1, but not TNF-R2, could decrease the constitutive and TPA induced expression of MMP-9 in NB4 leukemia cell line (112). Due to these complications we decided to switch strategies and use instead an anti-TNF-α neutralizing antibody.

The results obtained using the TNF-α neutralizing antibody were much more clear and so the TSR1 experiments were not pursued further. Use of the TNF-α neutralizing antibody (Anti-TNF Ab) resulted in a concentration dependent decrease in MMP-9 activity as measured by gelatin zymography (Figure 11A). Protein levels of TNF-α in cell supernatant of these samples were also measured by ELISA and results show a concentration dependent decrease with the use of the Anti-TNF Ab (Figure 11B). As the highest concentrations of Anti-TNF Ab (>250 ng/ml) decreased TNF- α to levels that

were almost undetectable, MMP-9 activity was still significantly above basal levels, suggesting the involvement of other inducers of MMP-9 in PMA/PHA stimulated cells. However, these results suggest that TNF-α is partly responsible for inducing the increase in MMP-9 in our model of inflammation.

Effects of adenosine receptor activation on MMP-9 activity.

As described in the Introduction, adenosine receptors are subdivided into A_1, A_{2A}, A_{2B} and A_3. The differences in receptor subtypes mainly involve the cell signaling mechanisms involved, as the A_1 and A_3 receptors are coupled to G_i proteins and the A_{2A} and A_{2B} are coupled to G_s proteins. Activation of G_s proteins causes a build up of intracellular cAMP whereas activation of G_i proteins decreases cAMP levels (119).

Our focus was drawn to the $A_{2A}R$ based on *in vitro* and *in vivo* evidence demonstrating anti-inflammatory properties. *In vitro* studies have implicated the A_{2A} receptor to the downregulation of numerous proinflammatory cytokines including TNF-α (83) while the development of the $A_{2A}R$ gene-deficient mice has provided convincing *in vivo* evidence that the $A_{2A}R$ is crucial for downregulation of acute inflammation (89).

Our group has shown that activation of the adenosine A_{2A} receptor can decrease TNF-α protein levels in models of inflammation, and TNF-α has been established as a potent inducer of MMP-9 activity in various models of inflammation including our own (Figure 11). Using pharmacological techniques, we demonstrated that activation of the adenosine A_{2A} receptor could decrease MMP-9 upregulation caused by PMA/PHA induced increases in TNF-α production. This response was specific to the A_{2A} receptor

because treatment with the A_{2A} specific antagonist ZM 241385 blocked this effect (Figure 12A). Interestingly cells treated with PMA/PHA and pretreated with ZM 241385 showed higher levels of MMP-9 activity than cells treated with PMA/PHA alone. This increase may reflect involvement of endogenous adenosine in MMP-9 regulation and offers the possibility that adenosine A_{2A} receptor activation may be a useful strategy against pro-inflammatory events where TNF-α and MMP-9 are involved.

Preliminary studies were done to examine the effects of adenosine A_1 and A_3 activation on MMP-9 activity. Figure 13 and Figure 14 both showed increases in MMP-9 activity as determined by zymography. As mentioned earlier, one of the main differences between the A_1 and A_3 receptors from the A_2 receptors is its effect on levels of cAMP. Activation of the G_s coupled $A_{2A}R$ increases cAMP while activation of the G_i coupled A_1 and A_3 receptor decreases cAMP. The opposite responses obtained from activation of the $A_{2A}R$ versus the A_1 and A_3 suggest that the results were not affected by cross-talk between receptors and strongly implicates cAMP to be an important mediator of MMP-9 regulation. The effects of the cell-permeable analog of cyclic AMP, dibutyryl-cAMP, or with an inhibitor of protein kinase A on MMP-9 activity have yet to be studied in our model.

Adenosine receptor agonists although specific can activate other subtypes of adenosine receptors especially at higher concentrations. Additionally, cross-talk between subtypes of adenosine receptors is known to occur (120, 121). MMP-9 activity was increased concentration dependently by A_1 and A_3 receptor agonists. However, at higher concentrations, these same agonists caused MMP-9 activity to decrease back to control levels. These results may have been due to loss of receptor selectivity or to cross-talk

between adenosine receptor subtypes at the higher concentrations. Further experiments are needed to resolve this issue.

Activation of the adenosine A_{2A} receptor increases MMP-9 gene expression.

Effects on MMP-9 transcription were examined based on reports that TNF-α increases MMP-9 messenger RNA in various cell types including mammary epithelial cells (122), bronchial fibroblasts (123) and NB4 leukemic cells (112). In monocytic cells, MMP-9 secretion is tightly controlled at the level of gene transcription and MMP-9 secretion has been shown to be increased by various cytokines including TNF-α (23). Work from our laboratory as well as from others has reported that activation of adenosine A_{2A} receptors on immune cells will suppress the production of proinflammatory mediators including TNF-α (82, 83, 100, 103). By combining these ideas, we proposed that activation of adenosine A_{2A} receptors would decrease MMP-9 gene expression by decreasing TNF-α production.

The results obtained in Figure 12A prompted us to look at MMP-9 gene expression to explain whether or not the decrease in MMP-9 secretion was due to a decrease in MMP-9 transcription. Studies of signal transduction pathways regulating MMP-9 expression have revealed that activation of the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and PI 3-kinase are crucial for upregulating MMP-9 expression in response to different stimulus (124-126). TNF-α has been shown to induce MMP-9 expression through a Ras dependent increase in the ERK signaling pathway. As a result, there is increased binding activity of the

transcription factors NF-kB and AP-1 to the MMP-9 promotor (127). Based on these reports, the results obtained in Figure 15 showing an increase in MMP-9 gene expression with activation of the adenosine A_{2A} receptor, were unexpected. Intracellular levels of cAMP would have increased with the activation of the adenosine A_{2A} receptor. MMP-9 gene expression was expected to decrease with the report that cAMP can inhibit the Ras-ERK signaling pathway (128). However, the molecular mechanism involved with cAMP-mediated modulation of inflammatory processes is complex and probably cell type specific. For instance increases in cAMP delays the apoptotic process in neutrophils (129) while inducing apoptosis in thymocytes (130) and certain leukemic cell lines (131).

Effects of A_{2A} adenosine receptor activation on membrane bound and cytosolic MMP-9 protein levels

The MMP-9 gene expression profile seen in Figure 15 did not reflect the pattern of MMP-9 activity as determined by zymography (Figure 12A), suggesting that perhaps the protein was being translated but not released into the cell supernatant. Our zymography data represent the MMP-9 protein collected from cell supernatant and reflect MMP-9 secretion which does not necessarily correspond to cytosolic or membrane bound protein levels. Post-translational regulation of proteinases is common and based on the destructive capabilities of MMPs, compartmentalization should be a significant issue concerning MMP regulation. It is important to realize that cells do not indiscriminately release proteases and studies have shown that MMPs can be regulated post-translationally by TIMPs and by binding to components of the cell membrane.

MMPs anchored to the cell membrane will restrict their proteolytic activity to specific substrates in the pericellular space (132). Reported cell-MMP interactions include MMP-2 binding to integrin $\alpha_v\beta_3$ (133), MMP-9 binding to CD44 (17) and MMP-7 attaching to surface proteoglycans (134). Recently, proMMP-9 was discovered to be a potent ligand for leukocyte β_2 integrins (particularly $\alpha_M\beta_2$, the major integrin of neutrophils) playing an important role in neutrophil migration (135). The number of MMPs and the different possible interactions with membrane proteins are enormous and determining these anchors can provide clues to MMP activation mechanisms and pericellular substrates.

Preliminary results obtained by immunofluorescent staining (Figure 16A) suggest that activation of the A_{2A} adenosine receptor increases cellular MMP-9 protein levels. Whether this was an increase in cytosolic or membrane bound MMP-9 protein has not been established and further experiments possibly using confocal microscopy may be warranted.

To obtain more quantifiable results Western blot analysis was done on cell pellets obtained under the same treatment conditions. We were expecting to obtain results to support the immunofluorescence data; however, preliminary experiments indicated no change in MMP-9 protein levels compared to control (Figure 16B).

Increased MMP-9 gene expression as a result of adenosine A_{2A} receptor activation (Figure 15) may correspond to increased levels of membrane bound MMP-9. This may be important during inflammatory events where changes occuing in the pericellular environment would allow for cell migration and binding. Signaling components influencing cell movement and adhesion overlap with the signaling pathways affected by cAMP and adenosine A_{2A} receptor activation.

A_{2A} receptor activation may increase (Figure 16A) or have no effect on (Figure 16B) MMP-9 protein levels in PMA/PHA stiumlated cells. However, A_{2A} receptor activation increases MMP-9 gene expression (Figure 15) while decreaseing MMP-9 activity (Figure 12) in activated cells. To determine how adenosine receptor activation is regulating MMP-9 activity further work needs to be done to map out the molecular mechanisms involved with gene transcription, protein translation and secretion.

CHAPTER 7: CONCLUSION

The results of our experiments have shown that activation of the adenosine A_{2A} receptor decreases MMP-9 activity in part via the TNF-α modulated pathway. There are 3 lines of evidence to support this conclusion. First, MMP-9 activity and TNF-α protein levels increases in a time dependent fashion in PMA/PHA stimulated U937 cells. This suggests a relationship between TNF-α and MMP-9. Secondly, use of TNF-α neutralizing drugs, decreases MMP-9 activity in PMA/PHA treated cells. This indicates that TNF-α is in part involved with the induction of MMP-9 in our model of inflammation. Finally, adenosine A_{2A} receptor agonist CGS 21680 decreases MMP-9 activity and TNF-α protein levels in PMA/PHA stimulated U937 cells. This effect was blocked by the adenosine A_{2A} receptor antagonist ZM 241385. This study provides further evidence to support the hypothesis that adenosine is an effective sensor and signalling molecule in inflammation.

The relationship we have elucidated between the A_{2A} adenosine receptors and MMP-9 has important implications for future therapies of inflammatory diseases. The identification of adenosine as a regulator of MMP-9 activity provides a novel pharmacological strategy in treating pathologies where MMP-9 plays a central role such as arthritis, stroke and neuroinflammation.

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