Identifying Signal Molecules Involved in the Adenosine-Mediated Wound Healing and Dermal Fibrosis

MPS Paper

Bachelor's programme
"Medical and pharmaceutical biotechnology"

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Submitted on: 10.02.2009

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I.   List of Abbreviations

PDGF	Platelet-derived growth factor
IGF-1	Insulin-like growth factor 1
EGF	Epidermal growth factor
TGF-β	Transforming growth factor beta
PMNL	Polymorphonuclear leucocytes
HB-EGF	Heparin-binding EGF-like growth factor
bFGF	Basic fibroblast growth factor
IgG	Immunoglobulin G
ECM	Extracellular matrix
MMP	Matalloproteinases
TIMP	Tissue inhibitors of metalloproteinases
SPP1	Phosphoprotein 1
OPN	Osteopontin
BSP-1	Bone sialoprotein I
ETA-1	Early T-lymphocyte activation
kD	Kilo Dalton
RGD	Arginine Glycine Aspartic acid
ATP	Adenosine-5'-triphosphate
G protein	Guanine nucleotide-binding proteins
AR	Adenosine receptor
A_{1R}	Adenosine A_1 receptor
$A_{2A}R$	Adenosine A_{2A} receptor

A_{2B} R	Adenosine A_{2B} receptor
A_3R	Adenosine A_2 receptor
GTP	Guanosine-5'-triphosphate
GDP	Guanosine diphosphate
A1	Adenosine A1
A2A	Adenosine A2A
A2B	Adenosine A2B
A3	Adenosine A3
Gi	Inhibitory G-protein
cAMP	Cyclic adenosine monophosphate
Gs	Stimulatory G-protein
AA	Amino acid
GPCR	G protein-coupled receptors
O2	Oxygen
H_2O_2	Hydrogen peroxide
IL	Interleukin
TNF	Tumor necrosis factors
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
IKK	IκB Kinase
GRK	G protein- coupled receptor Kinase
IFN-γ	Interferon gamma
PKA	Protein kinase A
VEGF	Vascular endothelial growth factor

EPC   Circulating endothelial progenitor cells

EC   Endothelial cells

MTX)   (2*S*)-2-[(4-{[(2,4-diamino-7,8-dihydropteridin-6
             yl)methyl](methyl)amino}phenyl)formamido]pentanedioic acid

CGS-21680   3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-
             dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid

NECA   5'-N-ethylcarboxamido adenosine

ZM 241385   4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5] triazin-5-yl-
             amino]ethyl)phenol

Theophylline 1,3-dimethyl-7H-purine-2,6-dione

CTGF   Connective Tissue Growth Factor

Fli-1   Friend Leukemia Virus Integration 1

1 Introduction

1.1 Wound Healing

Wound healing is the process in which an organ repairs itself after injury.
It is divided into four phases which are sequential and overlapping: haemostasis, inflammation, tissue formation and tissue remodeling. [1]
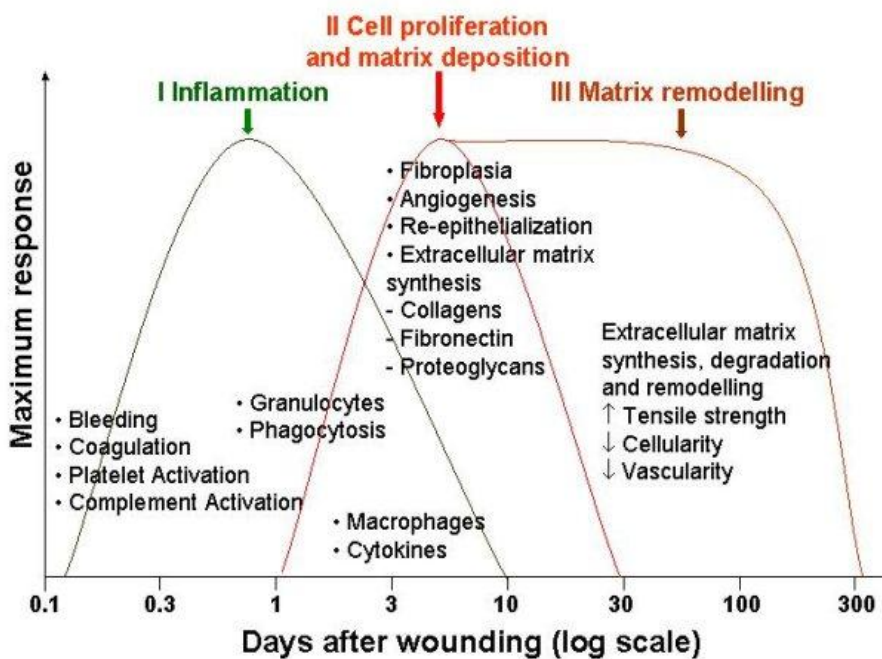

Fig.1: The wound healing process

1.1.1 Phase I: Haemostasis

When tissue is injured blood flows into the wound. To limit the blood loss a coagulation cascade is activated which drives to clot formation and platelet aggregation. The clot consists out of various proteins like fibrin, fibronectin, vitronectin, von Willebrand factor and thrombospondin which form a provisional matrix for cellular migration. Other essential growth factors for initializing the coagulation cascade are platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β) which attract and activate fibroblasts, endothelial cells and macrophages. [1]

1.1.2 Phase II: Inflammation

In the inflammation phase nucleophils, also known as polymorphonuclear leucocytes (PMNL) migrate to the wound and adhere to endothelial cells in the blood vessels. They secrete pro-inflammatory cytokines such as IL-1, IL-6 and TNF-β as well as anti-inflammatory cytokines such as IL-10 and growth factors including VEGF and TGF-β. Nucleophils also release degradation enzymes and oxygen-derived free radical species which help to kill foreign particles like bacteria. To phagocytize bacteria and damaged tissue monocytes migrate to the wound sites and mature there to macrophages. Macrophages release cytokines and growth factors into the wound, recruiting fibroblasts, keratinocytes and endothelial cells to repair the damaged blood vessels. Macrophages also release proteolytic enzymes such as collagenase which leads to depletion of tissue. Additional growth factors such as transforming growth factor-alpha (TGF-α), heparin-binding epidermal growth factor (HB-EGF), and basic fibroblast growth factor (bFGF) are secreted by the PMNLs and macrophages, which further stimulate the inflammatory response. In the last step of phase two lymphocytes are attracted by interleukin-1 (IL-1), IgG and complement products. [1]

1.1.3 Phase III: Proliferation

In the phase of proliferation the provisional matrix is replaced by new tissue by building a new extra cellular matrix (ECM). The ECM is formed by fibroblast cells which are activated by PDGF and TGF-β and migrate into the wound, proliferate and produce the matrix proteins like fibronectin, collagen type I, collagen type III and proteoglycans. Collagen accumulates in the new formed tissue. As collagen layers of the new matrix have a different architecture than the old matrix scars occur.

As another process of wound healing TGF-β and PDGF secreted by the platelets during the haemostatic phase, attract macrophages and granulocytes and promote angiogenesis. [1]

Angiogenesis is the formation of new vessels from pre-existing ones. Macrophages release angiogenic substances like TNF-α and Basic fibroblast growth factor (bFGF). Wound healing is further processed by the formation of granulation tissue which contains a loop of capillaries.

For reparation of the wound epithelialisation starts. Therefore epidermal cells migrate from the wound edges and covering the raw area exposed by the loss of epidermis. The cells migrate across the provisional matrix, start growing and differentiating which leads to the regeneration of the epithelium. [1]

1.1.4 Phase IV: Remodeling phase

The last stage of wound healing is the remodeling phase in which the ECM is remodeled.

The matrix proteins fibronectin and hyaluronan are broken down and the diameter of collagen bundles increased which leads to increased wound tensile strength. Fibroblasts, granulocytes and macrophages produce matalloproteinases (MMPs) which degrade collagen. In the remodeling phase MMPs are inhibited and the activity of tissue inhibitors of metalloproteinases (TIMPs) is increased.

The wound is closed through contractions due to the interaction of fibroblasts with the ECM. In the end of the wound healing process the outgrowth of capillaries is stopped, blood flow to the area is reduced and metabolic activity in the area declines.[1]

Dermal fibrosis is a pathological hallmark of several autoimmune disorders, such as scleroderma. The cause of this disease is the disruption in homeostasis of collagen, the principal protein of connective tissue in humans which maintains the structural integrity of many different tissues in the body. In scleroderma, excessive collagen leads to the uncontrolled proliferation of scar cells.[21] These scar cells replace healthy organ tissue and may lead to organ failure, and in some cases death. In autoimmune diseases, collagen overproduction is promoted by dermal fibroblast cells. Adenosine, a nucleoside found in many different cells, including dermal fibroblasts, causes this disruption in homeostasis when it binds to its A_{2A} receptor (A2AR). [17] The mechanisms that cause fibrosis are very complex, and involve several signaling pathways. This study investigates two signaling mechanisms by which adenosine regulates collagen expression.

2   Osteopontin

2.1 Background Information

Osteopontin (OPN), also known as Secreted phosphoprotein 1 (SPP1), bone sialoprotein I (BSP-1) and early T-lymphocyte activation (ETA-1) is an acidic protein consisting of 314 amino acids and a molecular weight of 32kD.[2] It is synthesized by fibroblasts, osteoblasts, osteoclats, macrophages, epithelial cells and endothelial cells etc. Osteopontin is a highly phosphorylated and glycosylated protein with an arginine glycine aspartic acid (RGD) binding domain as well as two heparin binding sites, one thrombin cleavage site and a putative calcium binding site. When proteases like thrombin cleave OPN separates into two functional fragments which are an RGD containing N-terminal part that binds to integrins receptors and a C terminal fragment which inter acts with CD44 receptors.[3] The protein is encoded by a single gene located on chromosome 4q13 (in humans) which consist of seven exons.[2]

OPN binds to a various receptors like the the integrins $\alpha(\beta1, \beta 3, \text{ or } \beta 5)$, ($\alpha4, \alpha5, \alpha8$, or $\alpha9$) $\beta 1$, and to CD44v6 and CD44v6 which are a variant form of CD44. As OPN is a ligand for many different receptors it mediates many different signaling pathways. [3] Osteopontin plays a role in many physiological processes like adhesion, migration, apoptosis, inflammation and wound healing.

This makes OPN to a key player in autoimmune diseases like rheumatoid arthritis as well as in cancer metastasis, fibrosis and osteoporosis.
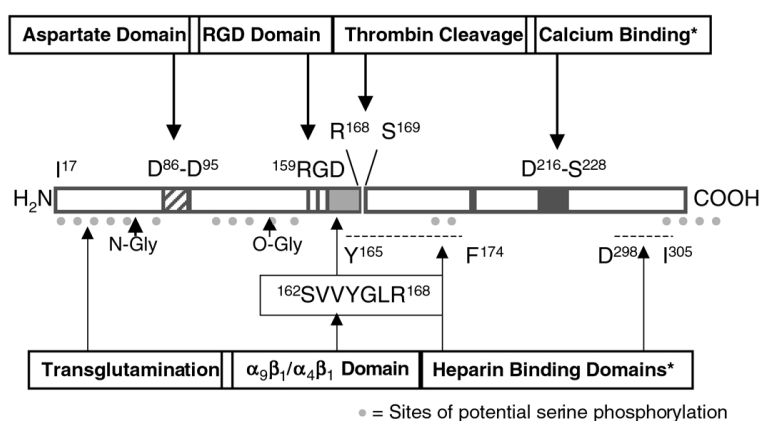

● = Sites of potential serine phosphorylation

Fig.2: Structure of OPN protein

2.2 Regulation of Wound Healing by OPN

2.2.1 OPN and Apoptosis

As mentioned the wound healing is a complicated process with four different sequential and overlapping stages. Each stage requires an increase of specific cells which have to be eliminated after accomplishing their task so that the next stage can begin by activating cells for its use. This down regulation is done by apoptosis which make the process of apoptosis vital in regulating wound healing. [4]

Research studies in prostate cancer cells have shown that OPN stimulates survival and proliferation by activation of Epidermal Growth Factor Receptor (EGFR) and thus upregulates EGFs due to its interaction with β_1 integrins on the surface of the cancer cells. Also it has been shown that OPN upregulates IL-6 in myeloma cells.[3] Both, EGF and IL-6, have crucial role in wound healing. EGF is vital for the migration of cells to the wound and stimulates the extracellular matrix remodulation.[5] IL-6 is known to regulate leukocyte infiltration, angiogenesis, and collagen deposition.[6]

2.2.2 OPN and Angiogenesis

OPN also has angiogenic properties. Studies have shown that VEGF stimulates OPN and $\alpha v \beta 3$ expression. Moreover it induces the cleavage of OPN by the protease thrombin which separates the receptor binding sites on the NH2-terminal from interacting sites on the COOH- terminal. This conformational change in OPN helps to increase blood vessel formation. OPN upregulates the expression of matrix metalloproteinases (MMP) which are required for movement through the ECM.[2] Another way how OPN regulates angiogenesis is activating Fibroblast growth factor-2 (FGF2) which is produced by mononuclear phagocytes, CD4$^+$ and CD8$^+$ T lymphocytes and endothelial cells and works pro- angiogenic by mobilization from proteoglycans of ECM. In vivo and in vitro studies have shown that FGF2 causes

OPN up-regulation in endothelial cells and hence recruits pro-angiogenic monocytes. [7]

2.2.3 OPN and Inflammation
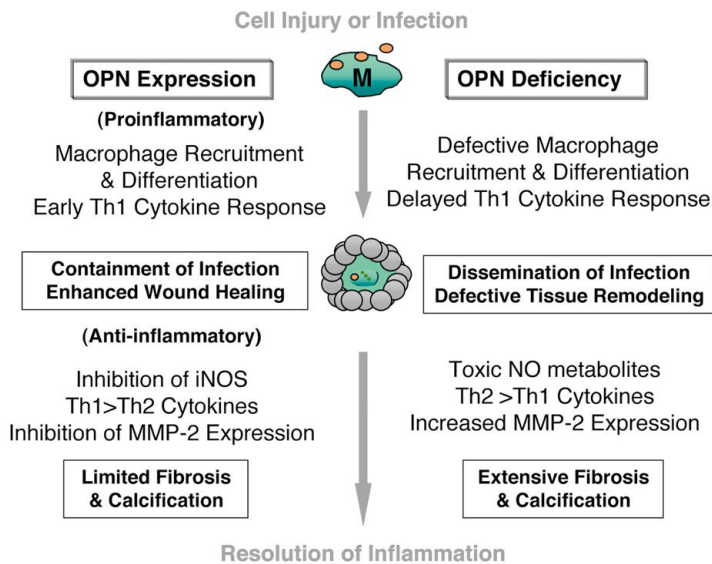
Fig.3: Pro- and anti- inflammatory effects

Researching the role of OPN in inflammation showed that OPN upregulates cytokines and growth factors which play are key regulators in the immune response, including IL-1 TNF-α and PDGF via activation of protein kinase C (PKC).

OPN has pro- inflammatory as well as anti- inflammatory effects. As a pro-inflammatory agent it is upregulated in various inflammatory cells, including T-cells, macrophages, and other NK cells. Osteopontin works as a chemotracant, facilitates adhesion of fibroblast and T-cells via CD44 variants, induces haptotaxis of T-cells and macrophages and stimulates collagenase 1 which activates the invasive behavior of macrophages and articular chondrocytes. [3]

It is also known that B-cells adhere to OPN which leads to an upregulation of various immune globulins expression, including IgM, IgG_1, IgG_{2a} and IG_{2b}. [2]

OPN also works as an inhibitor of Th2 by inhibiting the production of the Th2 cytokine IL-10 and stimulator of Th1 cytokine expression which leads to increased production of IL-12 and IFN-γ.

The anti-inflammatory effect of OPN is defined by inhibiting the expression of the pro-inflammatory mediators nitric oxide (NO) and prostaglandin E2 by downregulation of inducible NO synthase (iNOS) and thus decreasing NO production by macrophages and kidney tubule epithelial cells. This effect helps to reduce the extent of cartilage damage and help maintain tissue integrity. [3]

3 Adenosine

3.1 Introduction

Adenosine is a purine nucleoside consisting of adenine and ribose. It is continuously formed intra- and extracellularly by the breakdown of ATP due to dephosphorylation.[8] It is essential for protection of cells from stress signals and hence regulates and modulates their function. Elevated adenosine levels can be found during hypoxia, ischemia and inflammation. Increase of adenosine is also caused by cell proliferation, secretion, vasodilation, proliferation and cell death. [9]

Adenosine binds to four different receptors which belong to the family of G protein-coupled receptors called A_1, A_{2A}, A_{2B} and A_3.
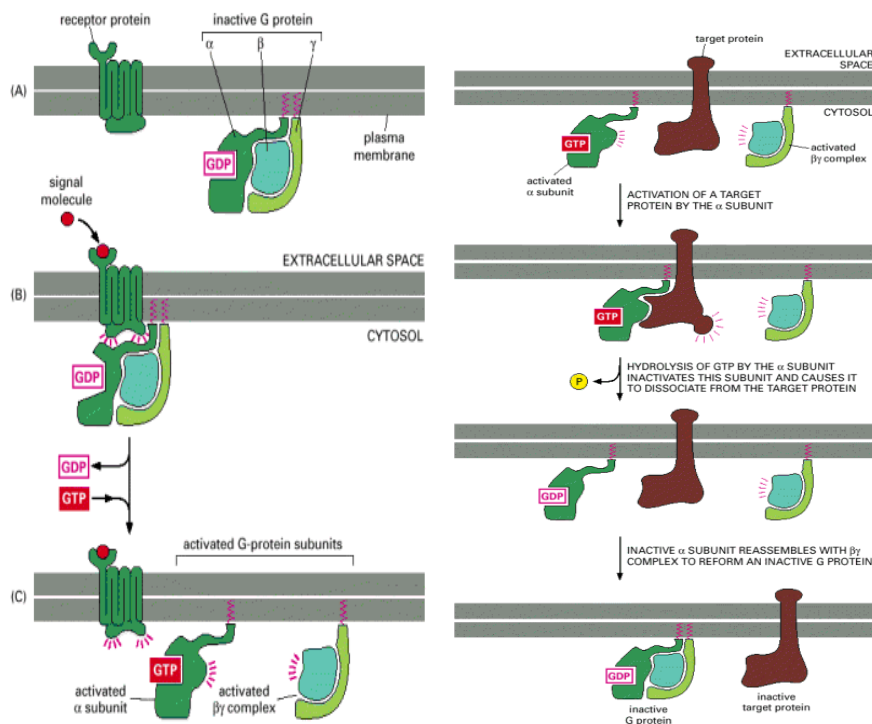
3.2 G- Proteins

Fig.4: G-protein linked Receptor Signaling

G- Protein coupled receptors are the largest family of cell-surface receptors and are found in all eucaryotes.

G- proteins consist of three protein subunits called α, β and γ. Binding of a signaling molecule to the receptors leads binding of the G-protein to its receptor. Due to conformational changes of the protein the α subunit releases GDP and binds to GTP. Thus leads to the separation of the protein into an active α complex which binds to the target protein and an active βγ complex. Signaling is switched off as soon as the signaling molecules stopped binding to the receptor. This is achieved by dephosphorylation of GTP to GDP which consequently leads to dissociation of the α subunit from the target protein and binding of the α subunit to the βγ complex. [10]

4 Adenosine Receptors

4.1 Structure

All four adenosine receptor subtypes are asparagines linked glycoproteins. The A1 and A3 receptors are coupled to Gi proteins which inhibit the production cAMP from ATP whereas A2A and A2B receptors couple to Gs proteins which activate the cAMP pathway. The structure of ARs is significant for their function and regulation. [8]

As ARs contributes to the family of G-proteins coupled receptors (GPCR) they have the typical protein structure of GPCRs. Compared to other GPCR they are quite small. Human A1AR consists of 236aa, A2AR of 128aa and A3 of 318. The A2AR is with 409aa is the biggest adenosine receptor. ARs have seven α-helical membrane spanning domains, each composed of 20–27 amino acids. The amino-terminus is oriented toward the extracellular space and the carboxyl-terminal tail to intracellular space. They are characterized by three intra- and extracellular loops with consensus sites for N-linked glycosylation as well as contain cysteine residues on the extracellular regions. [12]

4.2 **A2A Receptor Ligands**

As adenosine receptors are crucial in many different physiological processes, it is important to develop drugs targeting these receptors.

A new therapeutica approach is to find selective compounds for A2ARs which provide a class of new therapeutics. This is done by developing A2A agonists which bind to the receptor and thereby stimulate its activity and antagonists which inhibits the receptor activity.

4.2.1 A2A Agonist

Besides of being useful against inflammation A2A receptor agonists can potentially be used for the treatment of cardiovascular diseases, such as hypertension, ischemic cardiomyopathy and atherosclerosis. [13]

Some of the known A2AR agonists are (2S)-2-[(4-{[(2,4-diamino-7,8-dihydropteridin-6-yl)methyl](methyl)amino}phenyl)formamido]pentanedioic acid also known as methotrexate (MTX), 3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid (CGS-21680) and 5'-N-ethylcarboxamido adenosine (NECA). [11]

Suppressing the function of A2AR by binding to antagonists helps treating the Parkinson's disease and may also be active as cognition enhancers, neuroprotective and antiallergic agents, analgesics, and positive inotropics.[17]

Natural antagonists for A2AR are methylxanthines such as caffeine. [18] Other selective antagonists are 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5] triazin-5-yl amino]ethyl)phenol (ZM 241385) and 1,3-dimethyl-7H-purine-2,6-dione (theophylline). [11]

If the effects of CGS2180 and ZM on the downstream signaling factors of adenylyl cyclase and PKA can be understood, then researches may be able to identify novel therapeutic targets for treating wound healing.

4.1 A2A Receptors plays an important role in Wound Healing

4.1.1 A2A and Inflammation

Various studies have shown that adenosine plays an important role in inflammation by activating its receptors. The activation of A2A receptors has been proved to be most important for the anti-inflammatory effects of adenosine. [17]

A2AR works anti-inflammatory by protecting endothelial cells from damage caused by neutrophils.

Neutrophils generate reactive oxygen species like O_2^- and H_2O_2 which cause cell damage. A2AR inhibit the generation of oxygen species and prevent the adherence of neutrophils to the cell. Four families of adhesive molecules are involved in adhesion of neutrophils which are selectins, Ig-like receptors, leucine- rich glycoproteins and integrins. Two of this integrins are CD18 and CD11b. The activation of A2AR causes changes in CD11b/CD18 and thereby regulates the accumulation of neutrophils. [18]

Activation of A2AR also leads to the inhibition of inflammatory cytokine production like TNF-α, IL-1, IL-12, IL-6, IL-8 and to an enhancement of anti-inflammatory cytokine releasing like IL-10. [19]

Another way adenosine regulates inflammation is the inhibition of NF-κB activation.

NF-κB is a transcription factor which plays an important role in the regulation of the immune response and thus significant for the occurrence of inflammation. Under normal conditions NF- κB is located in the cytoplasm in its inactivated form. This inactivation is caused by inhibitory proteins called IκB which bind to NF-κB. The activation of NF- κB is achieved by various stimuli like, IL-1, okadaic acid, phorbol ester, H_2O_2 and TNF These signals induce phosphorylation, ubiquitylation, and

consequent degradation of IκB which leads to the translocation of NF-κB to the nucleus where it triggers the transcription of specific genes.

Studies have shown that the activation of A2AR suppress the activation of NF-κB by inhibiting the IκB phosphorylation and thus degradation. The suppression is done by inhibiting IκB kinase (IKK) which is activated by TNF and is responsible for the phosphorylation of IκB. This means that adenosine can regulate the NF-κB pathway by inhibiting of IKK activation, IκB phosphorylation, NF-κB binding to the DNA and NF-κB NF-B-dependent gene transcription. [20]

After the activation of TNF-α it increases the expression of A_{2A} receptors at the inflamed sites to prevent further inflammation. [21]

The enhancement of A_{2A} receptors is accomplished by inhibiting its desensitization.

G protein- coupled receptors stop stimulating intracellular signals after their activation and thereby are uncoupled from their G proteins. This process is mediated by G protein- coupled receptor Kinases (GRKs) that recognize, bind and phosphorylate the receptors when they are active. After that a protein called β-Arrestin1/2 binds to the receptor and induce receptor internalization.

TNF-α inhibits the binding of GRK2 to A2AR and suppresses β-Arrestin1/2 recruitment to the plasma membrane which leads to diminished phosphorylation and desensitization of A_{2A} receptors. [22]

Interferon gamma (IFN-γ) is another critical cytokines in acute and chronic inflammation. Studies which investigate the connection between IFN-γ and A2AR showed that IFN- γ decreased the expression of A2AR.

Another evidence that the occupancy of A2AR decrease inflammation is given by research studies with A2AR and PDGF which showed that active A2AR promote more rapid wound healing than PDGF. [23]

4.1.2 A2A and Angiogenesis

Adenosine acting at the A2 receptor promotes endothelial cell proliferation, migration and growth factor secretion. As it regulates inflammation it is also an initiator of the wound healing process and therefore stimulates angiogenesis. Activated A2ARs accelerate the wound closure by increasing cAMP levels which leads to the activation of Protein kinase A (PKA). [23] The cyclic-AMP-dependent protein kinase consists of two regulatory and two catalytic subunits. When cAMP levels are increased it binds to the regulatory subunit of PKA which leads to a conformational change of PKA and to the separation of the now active catalytic subunit from the regulatory subunit. Protein kinase A catalyzes the transfer of the terminal phosphate group from ATP to specific serines or threonines of selected target proteins, thereby regulating their activity.[24] A2ARs also regulates wound healing by promoting cell proliferation and thus angiogenesis via increase secretion of vascular endothelial growth factor VEGF locally in the wound, migration and synthesis of message for angiogenic growth factors. [25]

A_{2A} receptors increase neovascularization in healing wounds by increasing both local vessel sprouting and recruitment of endothelial progenitor cells from the bone marrow. For the process neovascularization circulating endothelial progenitor cells (EPCs) are recruited to and differentiate into endothelial cells (EC). VEGF plays an important role in the differentiation of hemangioblasts to either endothelial progenitor cells or hematopoietic stem cells. Studies have shown that VEGF increases circulating EPCs, as consequence of induced mobilization of bone marrow-derived EPCs, which resulted in increased differentiation of EPCs *and* neovascularization. [26]

5 Experimental Signaling Pathway

The possibility for cross-talk between different signaling pathways that regulate collagen expression is determined by examining members of separate, previously unrelated pathways involved in the regulation of fibrosis and wound healing.

The growth factor TNF-α is part of a separate signaling pathway, the Wnt pathway, responsible for the inhibition of collagen synthesis. [27] ß–Catenin, a subunit of a transmembrane protein involved in the Wnt pathway, is in turn involved in the mechanisim by which TNF- α inhibits collagen synthesis. [27] The Wnt pathway, and the molecule ß–Catenin are also of primary interest because they have been shown to regulate fibromatosis.[27] This makes ß–Catenin a likely candidate to be involved in A2AR-induced fibrosis. Previous studies showed that CGS-21680 induced twofold expressions of ß–Catenin in HMVEC cells and dermal fibroblasts in 4 hours and 6 hours by gene micro-array.

If ß-Catenin is involved in the signaling pathway by which Adenosine A2a receptor when occupied by agonists induces dermal fibrosis, then it may account for the incomplete downregulation of collagen found when A2AR is occupied by the antagonist ZM. [28] Moreover, identifying signaling molecules involved in cross-talk amongst different signaling pathways in the pathogenesis of fibrosis would help to identify therapeutic targets for the condition. If there is cross-talk between the Wnt pathway and the Adenosine A2a receptor, I hypothesize that A2AR occupancy by the agonist CGS will show an increase in ß–Catenin expression.

Fli-1, an ETS (E Twenty-Six) transcription factor is believed to repress collagen synthesis by down-regulating Connective Tissue Growth Factor (CTGF). An increase in CTGF is thought to stimulate collagen expression. As noted earlier, when researchers treated human fibroblast cell cultures with A2AR agonist, CGS, and antagonist ZM, results indicate that CGS can induce CTGF expression among other proteins and growth factors, as well as decrease Fli-1 expression. In contrast, blocking CTGF by the CTGF antibody showed a decrease in collagen production. [28] Building upon the known methods by which A2AR regulates collagen production and

dermal fibrosis, I determined if Fli-1 can control the levels message RNA and protein levels of CTGF. If increasing levels of nuclear Fli-1 can control the levels of CTGF, then collagen levels would be more easily regulated by decreasing the amount of dermal fibrosis in patients who are affected by scleroderma. Thus, I hypothesized that decreasing Fli-1 leads to CTGF over-expression, thereby increasing collagen production and ultimately, dermal fibrosis.

6 Materials and Methods:

6.1 Materials

Normal Human Dermal Fibroblast Cell Cultures (Lonza), Dulbecco's Modified Eagle Medium (Gibco), L-Glutamine (Gibco), Penicillin/Streptomycin (Gibco), Fetal Bovine Serum (Gibco), Tissue Culture Flask T75 (Falcon), 6-Well Plate (Falcon), β-Actin Antibody (Abcam), Collagen-1 Antibody (Southern Biotech), β-Catenin Antibody (Cell Signaling), CTGF Antibody (Santa Cruz Biotechnology), Fli-1 Antibody (Abcam), Osteopontin Antibody (RD Biosciences), Stom 860 Phosphoimager, Bio-Rad Protein Quantification Assay (Bio-Rad), Trizol Reagent (Trizol), Brilliant SYBR Green Master Mix (Stratagene).

6.2 Methods

6.2.1 Cell Culture Maintenance and Treatment

Human dermal fibroblasts obtained from Lonza were cultured for different treatments. These were grown on Falcon T75 tissue culture flasks in Dulbecco's Eagle Medium with 10% Fetal Bovine Serum,1% L-Glutamine, and 1% Penicillin/Streptomycin. These cell cultures were incubated at 37ºC in a humidified atmosphere with 5% CO_2. At 75% confluence the cell cultures were passaged, Trypsin was used to re-plate the cell on tissue culture plates. When the cell cultures reached 75% confluence in the second passage, they were treated with the following: CGS21680 (A2AR agonist) in a concentration of 10^{-6}, ZM10^{-5} (A2AR antagonist), CTGF10^{-4} antibody, and Normal IgG Rabbit Antibody10^{-4}. Cell cultures were treated for time durations of 4, 6, 8, 10, and 12 hours.

6.2.2 Effects of Adenosine Receptor Agonists and Antagonists on β-Catenin, Fli-1 and CTGF

Western Blot analysis was performed to measure message RNA levels of FLI-1 and CTGF in response to treatments of A2aR, agonist CGS, and antagonist ZM. To conduct this analysis, protein extraction was first performed. Whole cell lysates were prepared by washing cell cultures with PBS buffer solution for 3 minutes. 70μl of RIPA buffer was added to each well containing 1mL of DMEM media to detach cell from their plates. The Cells were then collected by scraping them with Falcon cell scrappers. The fibroblast cells were then immediately suspended in TRIZOL reagent for use or storage in a -80ºC environment. [18] The concentration of proteins used in gel electrophoresis was measured by a standard Lowry protein quantification assay according to BIO-RAD company instructions. Equal amounts of protein sample and loading dye were loaded onto 7.5% acrylamide gels. These gels were then transferred to nitrocellulose membranes. After transfer, the gels were blocked in a blocking buffer composed of Tris buffered saline (TBST), combined with 5% dry non-fat milk.

The blots were incubated overnight in primary antibody diluted in blocking buffer, at a temperature of 4ºC. The blots were then washed in TBST washing solution and incubated in the appropriate secondary antibody. The blots were again washed in TBST and then exposed to ECF (electro-chemifloresence) substrate. This allowed the strength of the protein bands on the blot to be measured by STORM 860 Phosphoimager. The results of the Western Blot experiments were then normalized with ß–Actin, which is highly endogenous in dermal fibroblasts.

6.2.3 Sircol Soluble Collagen Assay

To determine the collagen concetration released into cell culture medium after 24hrs treatment of dermal fibroblast cells with CGS, CGS+ZM, ZM, CGS+CTGF the Sircol Collagen Assay was performed. To measure the concentration Reagent blanks - 100μl of deionised water or 0.5M acetic acid or fresh cell culture medium or extraction buffer.

Collagen standards with known concentration of 5, 10 and 15µg/ml of the Collagen Reference Standard were used. 100 µl of Test samples, Blanks (deionised water) and the Standards were used. Afterwards 1ml of Sircol Dye Reagent was added to each sample and incubated and constantly shaken for 30 min. at room temperature. Then the samples were centrifuged at 12,000 r.p.m. for 10 minutes. Next the supernatant was discarded by inverting the tubes 750µl Washing Reagent was added to the pellet. Again the samples were centrifuged at 12,000 r.p.m. for 10 minutes and the supernatant was removed. . In the next step 250µl Alkali Reagent was added to each sample. After an incubation of 5 min the absorbance was measured at a wavelength of 540nm.

6.2.4 Effects of Adenosine Receptor Agonists and Antagonists on Osteopontin, B-Catenin, CTGF and Fli-1 RNA Message Levels

To supplement the findings via immunoblotting, Real-time Polymerase Chain Reaction (PCR) was used to measure collagen, fli-1, and CTGF RNA message levels. This was accomplished by first extracting RNA from cell cultures grown in accordance to the above procedures. Then 200µL of chloroform was added to the cells suspended in TRIZOL reagent and the tubes of cells were vortexed for 15 seconds. After centrifuging tubes of RNA and chloroform 12000 x g for 15 minutes, the clear aqueous phase containing RNA was transferred to a new tube. An amount of 100% isopropanol solution was added to the tubes and centrifuged at the same speed as described above. Following centrifugation, RNA collected on the bottom of the tube in the form of a pellet. The supernatant was removed and the pellet washed in 75% ethanol solution.

After centrifugation, the ethanol was removed by eluting it with a pipette. The pellet was suspended in 10uL of Rnase free water to create cDNA via reverse transcription. The cDNA used in Real-time PCR was reverse transcribed from 2uL of RNA in a 50 µL reaction containing the following: $MgCl_2$, 10x PCR buffer, dNTPs, Rnase inhibitors, and reverse transcriptase. In order to measure message RNA levels, the cDNA was amplified by adding Brilliant SYBR Green QPCR Master Mix to samples and primers targeted for a specific gene. Primers for Osteopontin, Fli-1 and CTGF were used to check their message levels. The amount of mRNA was measured by a

fluorescent dye reader. The results of PCR experiments were normalized by amplification of the standard gene ß–Actin.

6.2.5 Quantikine Human Osteopontin Immunoassay

To determine the osteopontin concetration released into cell culture medium after 24hrs treatment of dermal fibroblast cells with CGS, CGS+ZM, ZM, CGS+CTGF the OPN Immunoassay was done.

To measure the level of secreted osteopontin in the cell media standards of 20ng/ml, 10ng/ml, 5ng/ml, 2,5ng/ml, 1,25ng/ml, 0,625ng/ml and 0,312ng/ml were prepared. After that 100µl of Assay Diluent RD1-6 was added to each well of a 96-well plate. Next 50µl of standard, control, or sample per well was added to the wells and the plate was incubated for 2 hours at room temperature. The next step was the washing step where 400µl Washing Buffer is added to the wells and aspirated. This step was repeated 4 times. Then 200µl of OPN Conjugate was added to each well and again the plate was incubated for 2 hours at room temperature. Again the wells were washed 4 times with Washing Buffer. Next the Substrate Solution is made mixing equal amounts Color Reagent A and Color Reagent B. Then 200µL of Substrate Solution was added to each well and the plate was incubated for 30 minutes at room temperature and protect from light.

Afterwards 50µL of Stop Solution was added to each well and the absorbance was measured at 540nm.

7 Results

7.1 CTGF and fli-1 May Play Roles in the Regulation of Collagen

Preliminary results indicate that Connective Tissue Growth factor does in fact control levels of collagen. Figure 1 shows a marked decrease in collagen expression in the sample treated with the CTGF antibody, thus confirming that a increase in CTGF leads to a decrease in collagen expression in comparison to control values. However, blots tested for the presence of fli-1 also showed a significant decrease in protein levels in samples treated with ZM and the CTGF antibody. The protein expression of collagen paralleled that of CTGF and fli-1.
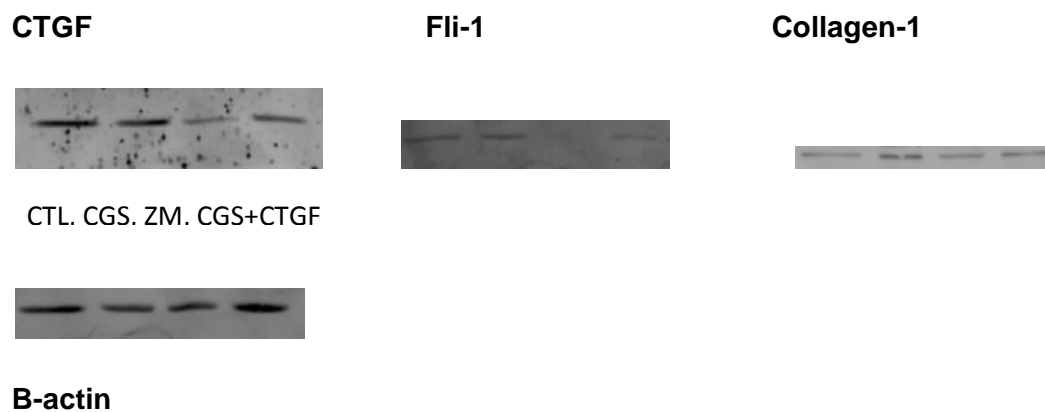
CTGF   **Fli-1**   **Collagen-1**

CTL. CGS. ZM. CGS+CTGF

B-actin

Fig.5: .Western Blot analysis for CTGF, Collagen-1 and Fli-1.
Cells were treated with A2AR agonist CGS, antagonist ZM, CTGF antibody and control cells were not treated as described in Materials and Methods. Protein expression was normalized by the concentration of β-actin in samples. (n=3)

Regulation of Collagen by CTGF was further proved by performing the Sircol Collagen Assay. The concentration of secreted collagen in the media of dermal fibroblast show a decrease in collagen production after treating with CTGF.
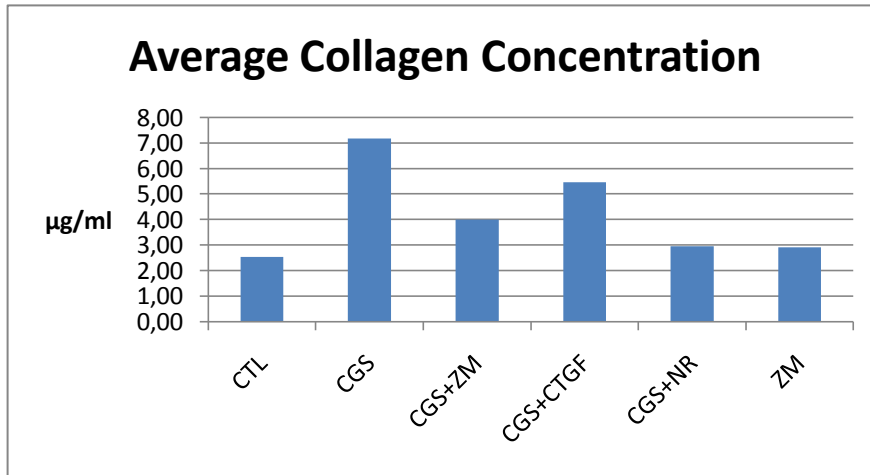

Fig.6. Average Collagen concentration measured by Sircol Soluble Collagen Assay.

The collagen assay was performed as described in Materials and methods.

Cells were treated with A2AR agonist CGS, antagonist ZM, CTGF antibody and control cells were not treated. (n=4)

7.2 A2A Receptor regulates CTGF and B-catenin gene expression

Message RNA levels show an increase in the expression of CTGF in samples treated with adenosine receptor agonist CGS. In contrast, cells treated with Zm generally showed a marked decrease in mRNA levels (Fig.7).
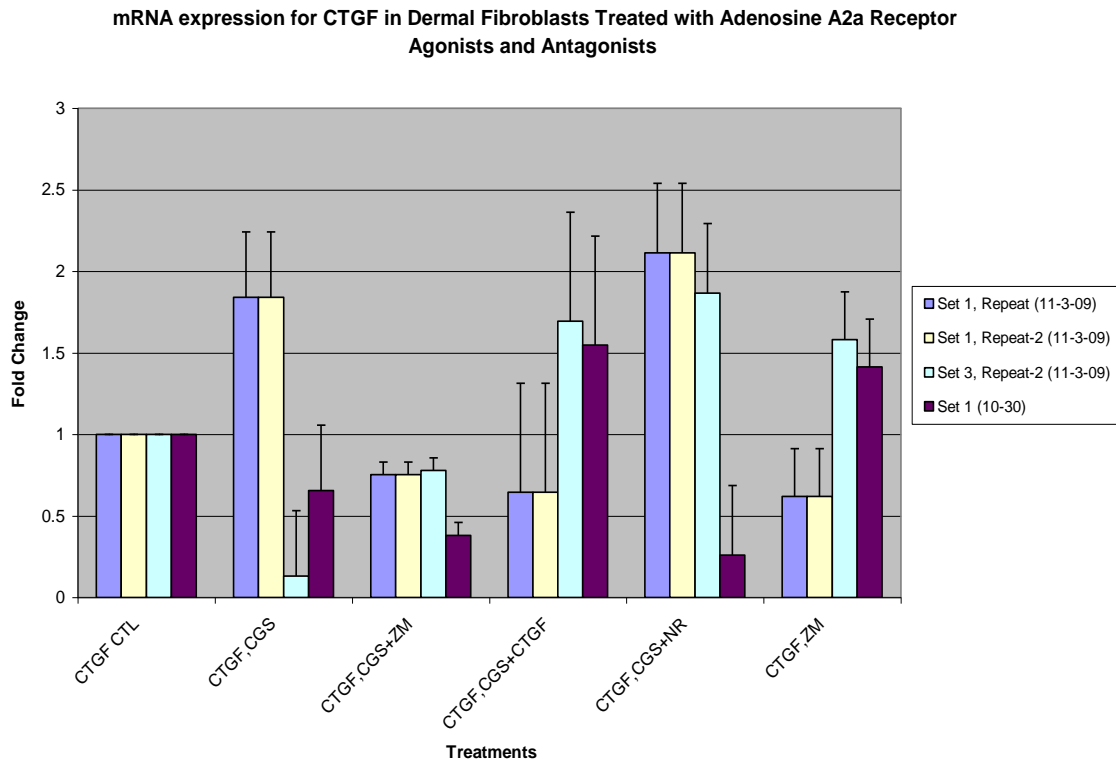

mRNA expression for CTGF in Dermal Fibroblasts Treated with Adenosine A2a Receptor Agonists and Antagonists

Fig. 7: Real- Time PCR Analysis of CTGF expression. Dermal fibroblast cells were treated and subjected to Real-time PCR analysis as described in Materials and Methods. 75% of all samples treated with CGS and the CTGF antibody showed a dramatic increase in CTGF expression levels. (n=4)

Message for mRNA Expression is Increased in Dermal Fibroblast Cells Treated with Adenosine A2a Receptor Antagonist ZM. Real time PCR analysis of dermal fibroblast cells treated with agonists and antagonists for the Adenosine A_{2A} receptor confirmed the apparent involvement of ß-Catenin in Adenosine A_{2A} receptor-mediated fibrosis. On average, cells treated with ZM 241385 showed a threefold increase in RNA expression (Fig.8). Conversely, cells treated with the Adenosine A_{2A} receptor agonist CGS showed much lower mRNA expression levels of ß-Catenin. Samples treated

with CTGF showed limited expression of ß-Catenin, averaging 1.2 times that of their expression in control samples.
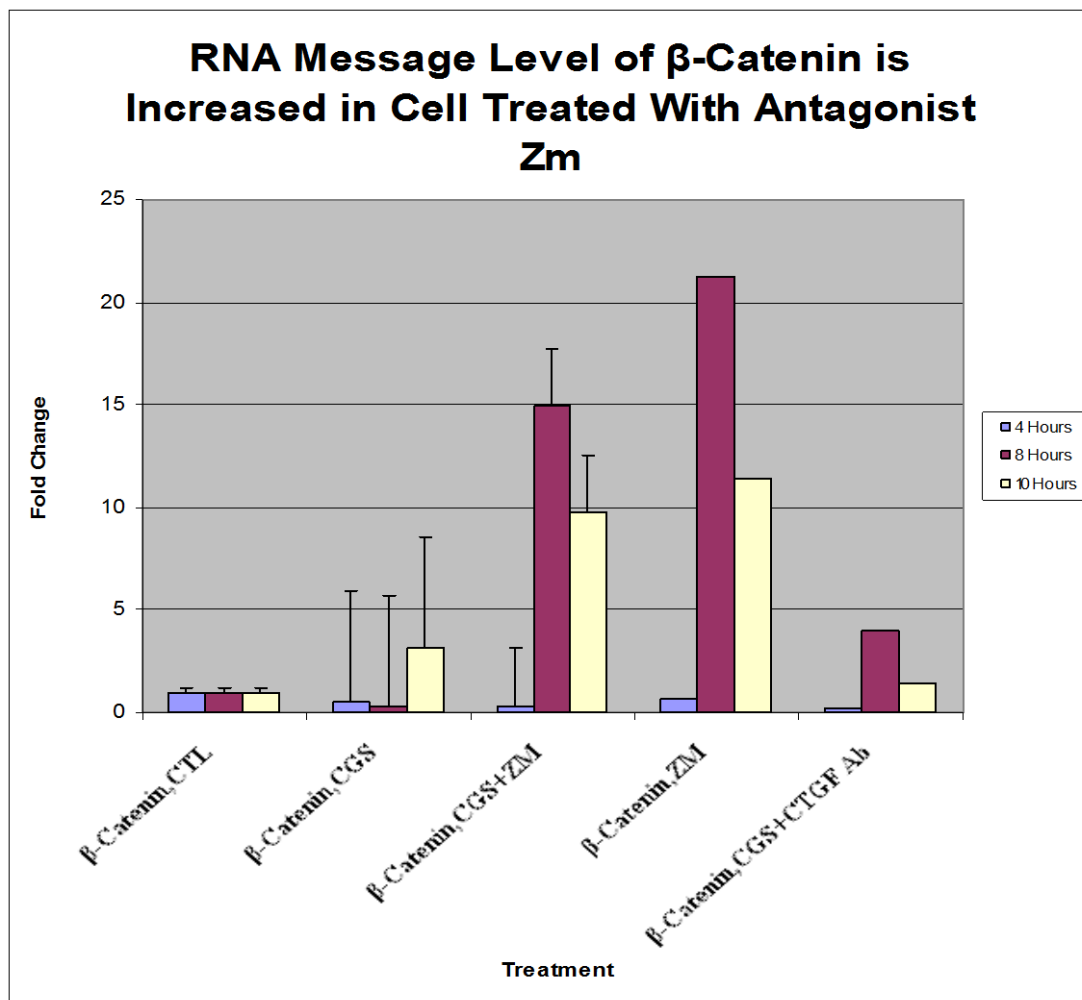

Fig. 8. Real- Time PCR Analysis of B-catenin expression
Dermal fibroblast cells were treated and subjected to Real-time PCR analysis as described in Materials and Methods. Samples treated with Zm showed a dramatic increase in ß-Catenin expression levels. (n=3)

Overall the real time PCR analysis of dermal fibroblast cells treated with agonists and antagonists for the Adenosine A_{2A} receptor confirmed the apparent involvement of ß-Catenin in Adenosine A_{2A} receptor-mediated fibrosis. On average, cells treated with ZM 241385 showed a fold increase in RNA expression (Fig. 9). Conversely, cells treated with the Adenosine A_{2A} receptor agonist CGS showed much lower mRNA expression levels of ß-Catenin. Samples treated with CTGF showed limited expression of ß-Catenin, averaging 1.5 times that of their expression in control samples.

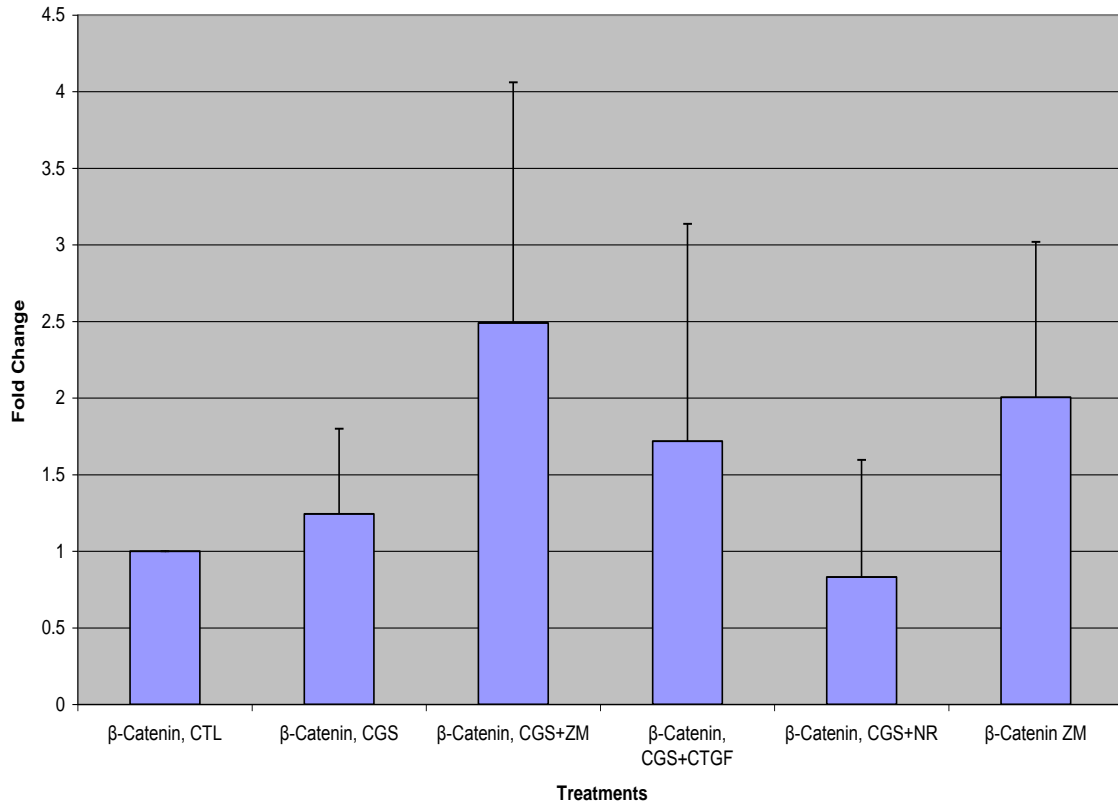RNA Message for β-Catenin is Increased by ZM in Dermal Fibroblasts Treated for 4 hours

Fig. 9. Real- Time PCR Analysis of B-catenin expression after 4hrs treatment. Dermal fibroblast cells were treated and subjected to PCR analysis as described in Materials and Methods (n=3). 100% of samples treated with ZM showed an increase in B-Catenin message RNA expression levels.

7.3 **Protein Expression of ß–Catenin**

Results concerning the protein expression of ß–Catenin are indeterminate, protein expression levels in treatments with CGS and ZM yielded similar concentrations.

ß–Catenin

Blot 1

Blot 2
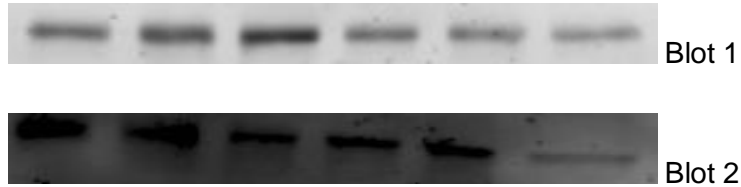
Control   CGS   CGS+ZM   ZM   CGS+ Rabbit antibody CGS+ CTGF antibody

Fig. 10. Western Blot analysis of the expression level of ß-Catenin in dermal fibroblast cells. Cells were treated with Adenosine A2AR agonist CGS, antagonist ZM, CTGF antibody and control cells were not treated as described in Materials and Methods. Both blots were subjected to the same treatments. Protein expression was normalized by that of β-actin. (n=3)

7.4 Osteopontin expression is regulated by stimulation A2A receptors

Real Time PCR, Western Blot and the Immunoassay show that the expression of Osteopontin is controlled by the A2A receptor. A2A receptor agonist CGS increases mRNA level of Osteopontin. The A2A receptor antagonist ZM reverses this effect after 4 hours treatment. However mRNA levels of Osteopontin after CGS treatment for 8 and 10 hours decrease gradually whereas ZM increase OPN expression.
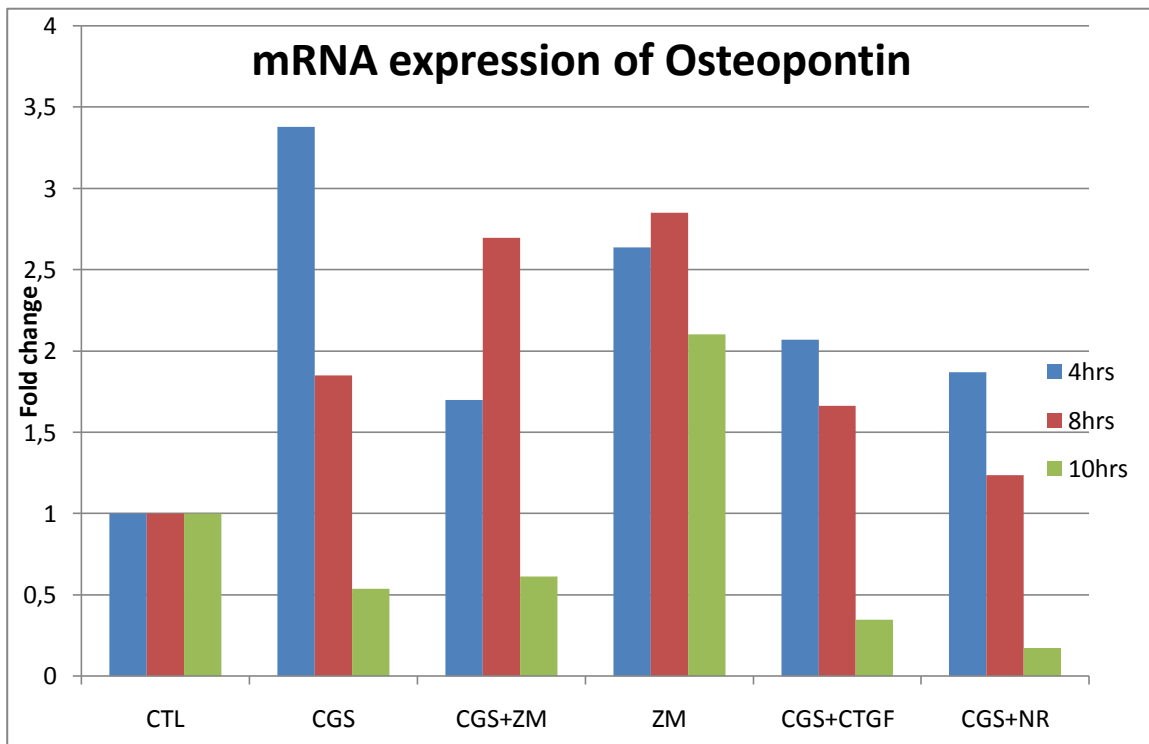

Fig. 11: Real- Time PCR Analysis OPN expression.
Dermal fibroblast cells were treated and subjected to Real-time PCR analysis as described in Materials and Methods. 100% of Samples treated with CGS showed an increase in Osteopontin expression levels.

7.5 A2A Receptors regulate Osteopontin

The regulatory effect of A2A receptors in OPN expression can be better seen in the Immunoassay and Western Blots. As hypothesized the agonist CGS increases OPN production and the antagonist ZM decreases the OPN level in dermal fibroblast cells.
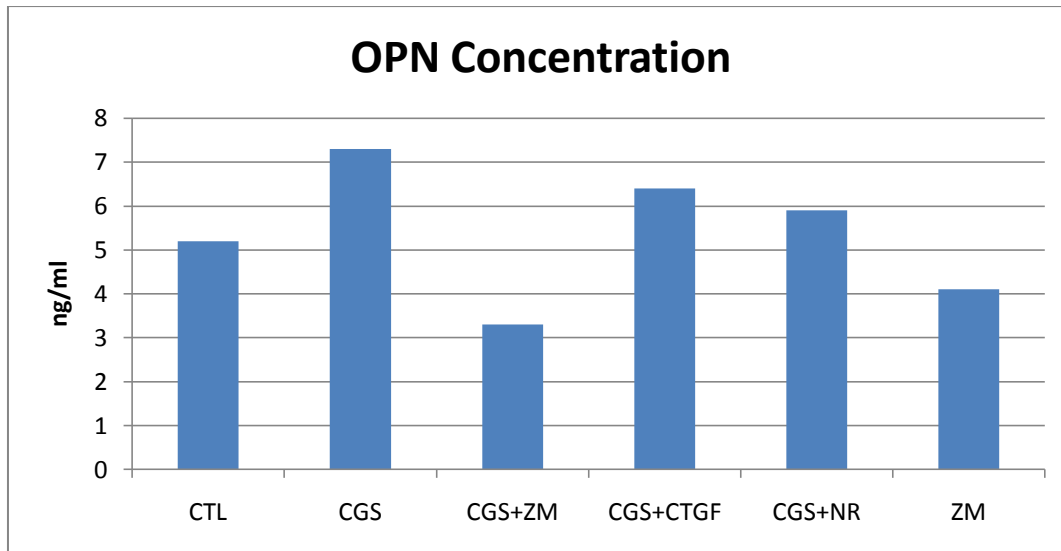

OPN Concentration

(y-axis: ng/ml, 0 to 8; x-axis categories: CTL, CGS, CGS+ZM, CGS+CTGF, CGS+NR, ZM)

Fig.12.: Average Osteopontin concentration measured by Quantikine Human Osteopontin Immunoassay.
The Immunoassay was performed as described in Materials and methods.
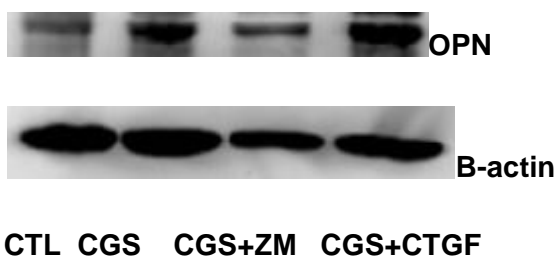Cells were treated with A2AR agonist CGS, antagonist ZM, CTGF antibody and control cells were not treated. (n=3)

OPN

B-actin

CTL CGS   CGS+ZM CGS+CTGF

Fig.13.: Western Blot analysis for Osteopontin protein expression in normal human fibroblasts. Cells were treated with A2AR agonist CGS, antagonist ZM, CTGF antibody and control cells were untreated as described in Materials and Methods. Protein expression was normalized by the concentration of β-actin in samples. (n=3)

8 Discussion

This research project addressed the mechanisms that cause fibrosis. Specifically, I examined the signaling pathways that regulated collagen expression. Understanding the pathway that regulates fibrosis and thus wound healing will help clinical pharmacologists identify therapeutic targets that can be used to regulate fibrosis, a disease for which there is currently no cure or effective treatment.

Results support the hypothesis that fli-1 decreases CTGF expression.(Figure 1) This in turn decreases collagen expression in dermal fibroblast cells as can be seen by the decrease in collagen protein expression levels in samples treated with the CTGF antibody. (Figure 1) These results corroborate observations in previous experiments. The increase in CTGF in treatments with CGS provides evidence for the mechanism by which A2AR regulates collagen expression.[16] However, results indicate that there is a negative feedback loop in the messenger RNA level between fli-1 and CTGF.   Thus, as nuclear fli-1 is repressed, levels of CTGF increase, which in turn decreases the amount of collagen, expressed in dermal fibroblast cells. Fli-1 may be a possible target for the regulating collagen expression.

Evidence for cross-talk between ß–Catenin and adenosine A2a receptors have been provided by experiments in terms of protein expression and messenger RNA expression. It is clear that RNA message is increased in samples treated with the antagonist ZM. The involvement of ß–Catenin in the signaling of fibrosis may provide researchers with another target for completely regulating collagen expression. However, these experiments did not clearly identify the function of ß–Catenin in this pathway on the protein level.

Possible cross-talk between adenosine-mediated fibrosis and B-catenin may occur during transcription in the nucleus. Adenosine-mediated fibrosis occurs when CGS binds to the A2AR. Results shown that collagen expression increases when fli-1 causes the decrease in expression of CTGF which in turn downregulates collagen expression. When the adenosine receptor is occupied by the A2AR antagonist ZM the expression level of ß–Catenin in drastically increased. Overall, this study provided evidence for the possibility for cross-talk between b-catenin which is regulated by the Wnt pathway and adenosine-mediated fibrosis. This cross-talk may explain why in previous experiments collagen expression did not completely decrease when fibroblasts were treated with treated with ZM. [16] The expression of

ß–Catenin in ZM treatments may be the cause the incomplete regulation of collagen by adenosine receptor agonists and antagonists. Another possible explanation of these results is that ß–Catenin in cells treated with ZM could stimulate fibrosis via growth factors TNF-α and TCF. The confirmation of cross talk between the Wnt Pathway and Adenosine- mediated fibrosis can help researchers better regulate this disease.

The next step in this project would be to determine what function ß–Catenin serves in the homeostasis of fibrosis. This could be done by testing the expression level of ß–Catenin in, an in vivo model of fibrosis. Keloid cells are hypertrophic scar cells and an example of in vivo fibrosis. If the presence of ß–Catenin could be confirmed in those cells, molecules downstream of ß–Catenin could be tested for their involvement in the pathogenesis of fibrosis.

The data also showed that Osteopontin is regulated by A2A receptor signaling. Western Blot results showed that OPN is better upregulated due to the treatment of CTGF than B-catenin. These results are also verified in the Real Time PCR Analysis. Comparing the mRNA level of CTGF after the treatment of B- catenin and Osteopontin it can be clearly seen that the OPN expression is much more stimulated than B- catenin. This data suggests that Osteopontin could be used as a new therapeutic approach to regulate wound healing by controlling CTGF levels which further regulates the collagen expression. Therefore osteopontin could be used to control the deposition of the collagen layers in the proliferation and remodeling process of wound healing where it is essential to control the collagen deposition to stimulate the better closure of the wound and to decrease scar formation.

9 Acknowledgements

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