

The mechanisms involved in
intercellular adhesion molecule 1 and
hyaluronan synthase 2 induction
following monocyte - fibroblast
interaction

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DEDICATION

This thesis is dedicated to the memory of my uncle who was instrumental in helping me to become a doctor. He passed away while I was working on this research but wanted me to complete the work, till the very end of his life.

It is also dedicated to my wife and children, for whom my research and thesis became as much a part of their life as mine.

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I would like to thank my supervisors, Dr Robert Steadman and Prof Aled Philips, for their support and encouragement in not only completing my project but also bringing it to fruition by completing and submitting my thesis.

I would like to thank Prof Philips for giving me the opportunity to involve myself in research when I was at crossroads in my professional career. He trusted me to complete my work and hopefully I have lived up to the trust he put in me. I find it difficult to express my thanks to Dr Steadman for pushing me to complete my work, at all times persevering with me, despite all the deadlines that I did not meet. I simply could not have finished this piece of work without the patience of both my supervisors.

I could not have done this work without the assistance of my friends in the laboratory, especially Jason Webber, John Martin, Soma Meran, Ceri Fielding, Robert Jenkins, Girish Bommaiya and many others who I fail to name here.

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SUMMARY

An influx of leucocytes, particularly macrophages, into the glomerulus and cortical interstitium, is characteristic of most forms of progressive renal disease. Previous work in our lab has demonstrated that leucocyte adherence to primary cultures of human renal fibroblasts stimulated ICAM-1 induction (1). Induction was initiated as a result of the ICAM-1/ $\beta 2$ integrin interaction, and was mimicked by crosslinking ICAM-1 with specific antibodies. The aim of my research was to examine the effect of leucocyte/fibroblasts interaction on ICAM-1 and HAS2 expression, and to investigate the mechanisms involved. I have shown induction of HAS 2 mRNA, the principal synthase involved in generation of the extracellular matrix polysaccharide hyaluronan (HA), in parallel with ICAM-1 in the fibroblasts. There were both cell-contact and soluble factor-mediated components to this activation. In addition there was an exponential rise in this induction when monocytes were activated. This was mediated by ICAM-1 on the fibroblast surface. Two major pro-inflammatory cytokines, $\text{TNF } \alpha$ and $\text{IL } 1 \beta$, were shown to be potential soluble factor involved.

In conclusion, I was able to show two possible mechanisms through which mononuclear cells activate fibroblasts and induce ICAM 1 and HAS 2, thus perpetuating an inflammatory reaction. The first was through interaction with ICAM-1 on the fibroblast surface the second was through the release of pro-inflammatory cytokines.

PUBLISHED WORK

1. Differential control of fibroblast Hyaluronan synthase 2 and intercellular adhesion molecule 1 expression induced by monocytes: **Presented at the Renal Association Meeting, Manchester, May 17 -20th 2010.**
2. Monocytes induce Hyaluronan synthase 2 and intercellular adhesion molecule 1 in fibroblasts: **Presented at the Renal Association Meeting, Manchester, May17-20, 2010.**
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4. Monocytes induce Hyaluronan synthase 2 and intercellular adhesion molecule 1 in fibroblasts: **Presented at the International Society for Hyaluronan Sciences, Kyoto, Japan 2010**

ABBREVIATIONS

AKI	Acute Kidney Injury
APOL1	Apolipoprotein L1
BMP-7	Bone Morphogenetic Protein 7
CD44	Cluster of Differentiation-44
CKD	Chronic Kidney Disease
DCT	Distal Convoluted Tubule
ECM	Extracellular Matrix
ELISA	Enzyme linked immune sorbent assay
EMT	Epithelial Mesenchymal Transition
ERK	Extracellular Signal Regulated Kinase
ESRD	End Stage Renal Disease
FSGS	Focal Segmental Glomerulosclerosis
GN	Glomerulonephritis
HA	Hyaluronan
HAS 2	Hyaluronan Synthase 2
HMW	High Molecular weight
ICAM 1	Inter cellular adhesion molecule 1
IFN- γ	interferon γ
Ig	Immunoglobulin
IL	Interleukin
IL-1 RA	Interleukin 1 Receptor Antagonist
IL-1 β	Interleukin 1 β
LMW	Low Molecular Weight
MAP-kinase	Mitogen Activated Protein kinase

MCP-1	Monocyte Chemoattractant Protein 1
MIP-1	Monocyte Inflammatory Protein
MMP	Matrix Metalloprotease
mRNA	messenger Ribonucleic Acid
NF- κ b	Nuclear Factor kappa B
PBS	Phosphate Buffered Saline
PCT	Proximal Convoluted Tubule
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
Q-PCR	Quantitative Polymerase Chain Reaction
RRT	Renal Replacement Therapy
RT	Reverse transcription
Sp1	Specificity protein 1
Sp3	Specificity protein 3
TGF β	Transforming Growth Factor β
TNF α	Tumour Necrosis Factor A
VCAM 1	Vascular Cell Adhesion Molecule 1
WBC	White Blood Cell

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1 INTRODUCTION

1.1 KIDNEYS – STRUCTURE AND FUNCTION

Kidneys are retroperitoneal organs, normally 2 in number one on each side of the vertebral column. They are usually 11cm in length and 6 cm in width, and weigh 140g each. Despite being small in comparison to the rest of the human body, they account for receiving 20 to 25% of the cardiac output. This is about 10 times the coronary blood flow calculated per unit tissue weight (2). More than 90% of the blood supply goes to the outer cortex at a rate of 500ml/min per 100g of tissue. It then goes to the inner medulla, with a perfusion rate of approximately 100ml/min per 100g of tissue to the outer medulla and 20ml/min per 100g of tissue to the inner medulla (2).

Each kidney has about 1 million nephrons to begin with, interspersed in the cortex and medulla and bound together with connective tissue containing extra cellular matrix, blood vessels, nerves and lymphatics. These nephrons are the functional unit of the kidneys. They consist of a filtering corpuscle and a tubular part, the main function of which is secretion and reabsorption. The renal corpuscle is made of the glomerulus, which is a tuft of arteriolar vessels and a capsule, called Bowman's capsule, which surrounds the tuft. The Bowman's capsule is a cup-shaped structure at the beginning of the tubular component of the nephron into which the glomerulus sits. The glomerulus receives its blood supply from an afferent arteriole. Once the solutes and water filter through the vessels into the Bowman's space, the remaining blood leaves via the efferent arteriole. The segment of the Bowman's capsule towards the glomerular tuft provides the filtering component and has 3 layers. The first is the endothelial layer of the blood vessels, second is the glomerular basement membrane that is made of collagen and glycoproteins. This layer provides the main barrier to larger molecules such as albumin because of the smaller pores and negative charge it carries from high concentration of sialic acid and heparan sulphate. The third layer is formed of podocytes.

The tubular part of the nephron is divided into a proximal convoluted tubule (PCT), loop of Henle, Distal Convoluted Tubule (DCT) and a collecting system. The function of the proximal tubule is essentially reabsorption of filtrate in accordance with the needs of homeostasis, whereas the distal part of the nephron and collecting duct are mainly concerned with the detailed regulation of water, electrolyte, and hydrogen-ion balance. The loop of Henle is principally involved in maintaining the concentration gradient in the medulla, which is important for concentrating the urine.

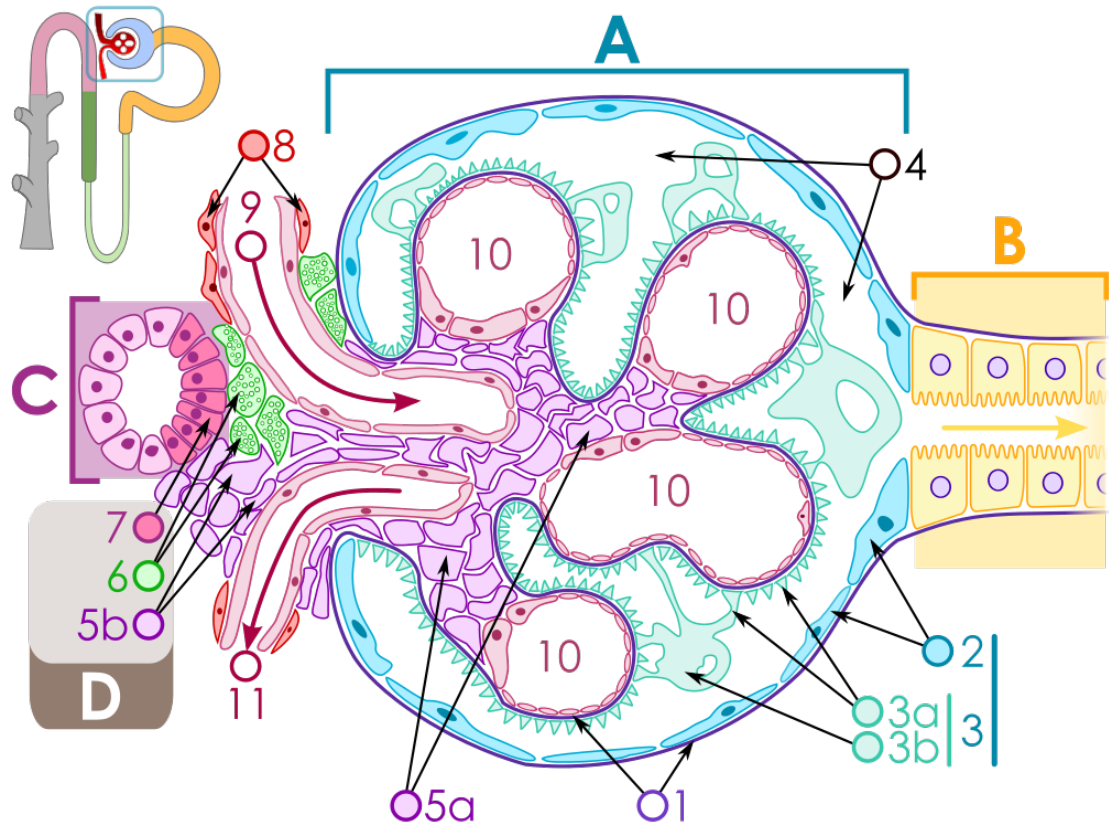


Figure 1-1 Diagram of renal corpuscle structure

A – Renal corpuscle B – Proximal tubule C – Distal convoluted tubule D – Juxtaglomerular apparatus 1. Basement membrane (Basal lamina) 2. Bowman's capsule – parietal layer 3. Bowman's capsule – visceral layer 3a. Pedicels (Foot processes from podocytes) 3b. Podocyte 4. Bowman's space (urinary space) 5a. Mesangium – Intraglomerular cell 5b. Mesangium – Extraglomerular cell 6. Granular cells (Juxtaglomerular cells) 7. Macula densa 8. Myocytes (smooth muscle) 9. Afferent arteriole 10. Glomerulus Capillaries 11. Efferent arteriole

From

[https://en.wikipedia.org/wiki/Glomerulus_\(kidney\)#/media/File:Renal_corpuscle.svg](https://en.wikipedia.org/wiki/Glomerulus_(kidney)#/media/File:Renal_corpuscle.svg)

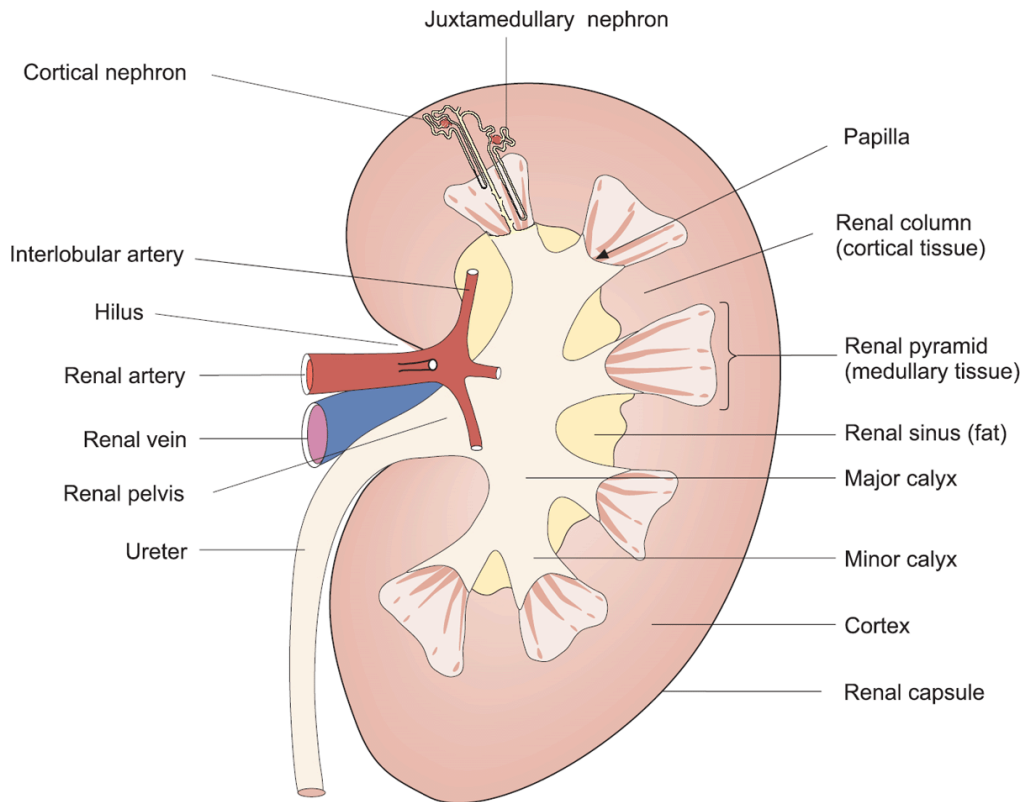


Figure 1-2 Cross section of a Human Kidney

From: Human Physiology, 3rd edition, Pocock, Gillian; Richards, Christopher D.

Figure 1.2 shows a cross section of one of the human kidneys. As mentioned before, the kidney has an outer cortex and inner medulla. Cone-shaped renal pyramids form the renal medulla deep to the renal cortex. The renal pyramids are aligned with their bases facing outward toward the renal cortex and their apices point inward toward the centre of the kidney. Each apex connects to a minor calyx, a small hollow tube that collects urine. The minor calyces merge to form larger major calyces, which further merge to form the hollow renal pelvis at the centre of the kidney. The renal pelvis exits the kidney at the renal hilum, where urine drains into the ureter. The hilum is the indentation in the concave side of the kidney that provides a space for the renal artery, renal vein, and ureter to enter the kidney.

The kidneys make on an average 1 to 1.5L urine every 24 hours, filtering up to 200L of blood in this time. They manage multiple functions, including fluid and electrolyte balance, excretion of harmful metabolites and by-products, endocrine functions including production of renin and erythropoietin, and metabolic functions such as activation of vitamin D, catabolism of low molecular weight proteins such as insulin, parathyroid hormone etc.

1.2 KIDNEY DISEASE

Injury to the kidneys can be acute, from which recovery is possible, or chronic and progressive. There is an increasing focus on targeting early detection and prevention of progressive conditions such as chronic kidney disease. This is especially important, given the rise in multiple risk factors including obesity, hypertension and diabetes promoting chronic kidney disease.

1.2.1 ACUTE KIDNEY INJURY (AKI)

Acute Kidney Injury (AKI) is the term that has replaced the previous term acute renal failure. This is because insults to kidneys lead to a spectrum of injury rather than being an all-or-none phenomenon. It is characterised by a rapid reduction in kidney function. This is assessed clinically in terms of serum creatinine and urine output.

Acute kidney injury is common in hospitalised patients and also has a poor prognosis with mortality ranging from 10%-80%, dependent upon the patient population studied. Patients who present with uncomplicated AKI have a mortality rate of up to 10%. In contrast, patients presenting with AKI and multi-organ failure have been reported to have mortality rates of over 50%. If renal replacement therapy is required, the mortality rate rises further to as high as 80% (3-5). AKI has been demonstrated to be an independent risk factor for mortality (6).

Acute kidney injury is most frequently caused by ischaemia, sepsis or nephrotoxic insults like contrast dye exposure to the kidney for various imaging modalities. Depending on the cause and severity of the insult to the kidney, patients can recover from the injury with renal functions returning to baseline, have partial recovery with scarring or fibrosis or develop progressive chronic kidney disease. In some patients AKI can be fatal.

1.2.2 CHRONIC KIDNEY DISEASE (CKD)

Chronic kidney disease is the reduced ability of the kidney to carry out the functions mentioned above in the long-term. A UK population study estimated that in people with CKD there was a 4% risk of progression to end stage renal disease (ESRD) over a 5.5 year follow-up period (7).

Many different types of insults to the kidney can cause CKD. These include toxic, ischaemic, infectious, paraneoplastic, congenital, genetic, endocrine, and immunological diseases. CKD is characterised by glomerulosclerosis, interstitial fibrosis, tubular atrophy and inflammation.

Most cases of CKD are acquired, but congenital syndromes like Alport's syndrome and autosomal dominant polycystic kidney disease are well known. More recently, research has started to identify genetic risk factors that put individuals at risk of developing CKD. A study revealed that African-American black people with genetic variants in both copies of apolipoprotein L1 (*APOLI*) are at higher risk for hypertension-attributable ESRD and FSGS (Focal segmental glomerulosclerosis). In contrast, black individuals without the risk genotype and European Americans appear to have similar risk for developing non-diabetic CKD(8).

An episode of AKI puts a person at risk of developing CKD as discussed above. This has especially been studied in patients with diabetes, where it has been shown that each episode of AKI led to a doubling of risk of developing CKD (9).

ESRD (End Stage Renal Disease) is a long-term irreversible decline in kidney function, for which renal replacement therapy (RRT) is required if the individual is to survive. RRT can take a number of forms; kidney transplantation, haemodialysis and peritoneal dialysis.

1.3 INFLAMMATION AND FIBROSIS

Inflammation is a protective response to eliminate the initial cause of cell injury, the necrotic cells and tissues resulting from the initial injury, and to initiate the process of repair (10). Although the aim of the inflammatory response is to get rid of the offending organism or stimuli, sometimes the response itself can cause considerable harm.

Inflammation can be acute or chronic. Acute inflammation has a rapid onset, is of short duration and is characterised by a neutrophilic leucocyte accumulation and fluid and protein exudation at the site of injury. Acute inflammation has 2 main components, involving vascular and/or cellular changes. Vascular changes involve vasodilatation and increased permeability. Vasodilatation takes place to increase blood flow to the site of inflammation, in order to carry participants of the inflammatory process to the site of injury. These include circulating antibodies, cells, oxygen, nutrients and cytokines. Increased vascular permeability involves leakage of circulating protein into the extravascular tissue through formation of endothelial gaps in venules, direct endothelial damage, necrosis or detachment, and leucocyte-mediated endothelial injury that ultimately results in the loss of circulating protein into the extravascular tissue (11).

Cellular changes involve emigration of leucocytes (mainly neutrophils) to site of injury and activation of leucocytes. Leucocytes, including monocytes and macrophages, are needed at the site of injury and inflammation. For this purpose, leucocytes adhere to the endothelial lining of the blood vessels, transmigrate across the endothelium (a process called as diapedesis), and migrate in interstitial tissues toward chemotactic stimuli to reach the site of inflammation or injury. For this extravasation to occur and for the leucocytes to adhere and transmigrate from the blood into tissues, both leucocytes and endothelial cells express complementary adhesion molecules, whose expression, in turn, is regulated largely by cytokines. The adhesion receptors involved in this process belong to four major molecular families, which are discussed further below. The multi-

step process of leucocyte migration through blood vessels involves: leucocyte rolling, activation and adhesion of leucocytes to endothelium, transmigration of leucocytes across the endothelium whilst piercing the basement membrane, and finally migration towards chemo-attractants emanating from the site of injury or inflammation.

Acute inflammation usually has one of the following outcomes:

1. Resolution: If the injury is short lived and there has been no or limited tissue damage, this is the usual outcome. The aim is restoration of normal structure as well as function.
2. Chronic inflammation: This may be present from the outset or follow an acute inflammatory process. This may again, over time, lead to resolution or proceed to scarring.
3. Scarring: This occurs when either the injury to tissue is substantial enough to outweigh the regenerative potential of the tissue, or the tissue involved does not regenerate and is filled with connective (scar) tissue.

Progressive, uncontrolled deposition of extracellular matrix proteins leading to scar formation and organ failure represents a final common pathway of tissue response to chronic injury (12).

In organs where considerable connective tissue deposition takes place to heal the damage, the function of the organ can be significantly compromised. This is called Fibrosis (10). Renal fibrosis is the inevitable consequence of an excessive accumulation of extracellular matrix that occurs in virtually every type of chronic kidney disease. The pathogenesis of renal fibrosis is a progressive process that ultimately leads to end-stage renal failure, a devastating disorder that requires dialysis or kidney transplantation(13).

1.4 INFLAMMATION IN KIDNEY DISEASE:

Inflammation contributes to progression of chronic kidney disease by inducing release of various pro-inflammatory cytokines. It also increases the production and activity of adhesion molecules that promote adhesion and migration of inflammatory cells into the interstitium.

There are multiple mediators of inflammation in CKD and ESRD such as hypoalbuminaemia, atherosclerosis, LPS, β -glucans etc (14).

It has also been shown that the pro-inflammatory cytokines TNF α (Tumor Necrosis Factor – α) and IL-1 β (Interleukin-1 β) are elevated in ESRD and also once dialysis is initiated (15, 16). There is a direct correlation between serum albumin and circulating pro-inflammatory cytokines such as TNF α . Studies in cancer patients have shown this link where TNF has also been related with cachexia(17). A significant correlation was seen between degree of renal impairment, serum albumin levels and serum TNF α levels in the CRIC study, a prospective observational study with almost 4000 participants(18). This study showed the levels of TNF were higher and serum albumin lower in patients with lower eGFR, a measure of kidney function.

Pro-inflammatory cytokines take part in pathways that promote inflammation that in turn can promote renal fibrosis. These induce the production and activity of adhesion molecules in capillary endothelial cells. The adhesion molecules bind to receptors on activated T cells causing T cell adhesion and migration in to the interstitium (19).

Further evidence of a role of inflammation in promoting progression of CKD is seen in the various treatments used in CKD. Use of atorvastatin to treat hyperlipidaemia in patients with CKD stage 2-4 resulted in reduction of lipid levels but also of inflammation as measured by CRP, TNF α and IL-1 β . No changes were observed in untreated patients (20). Corticosteroids and calcineurin inhibitors have also been used for their anti-inflammatory roles for a long time in treating glomerular diseases.

In summary, inflammation is an important safety mechanism to prevent uninterrupted harm to organs and tissues. There is also a strong possibility that this in itself can become harmful. There is extensive evidence that a pro-inflammatory state exists in CKD and this is heightened as CKD progresses and promotes further impairment. There is also extensive evidence for the role of leucocytes, adhesion molecules and cytokines in inflammation and progression of CKD, as detailed above. It has also been shown that the extracellular matrix (ECM) has an important role in influencing immune cell behaviour in inflamed tissues. The individual components of the ECM and its three-dimensional ultrastructure and biophysical properties can signal specific information to cells and modulate essential immune functions, such as immune cell migration into and within inflamed tissues, immune cell activation and proliferation, and cell differentiation processes, such as T cell polarization (21).

In inflamed tissues, inflammatory cytokines that are released by extravasating cells or by activated tissue-resident cells can modify the ECM. This results in the generation of 'bioactive' fragments of the ECM that may influence the activity and/or function of both infiltrating and resident cells. Improved understanding of mechanisms involved in mediating inflammation may lead to novel therapies to combat the devastating outcome of inflammation in CKD.

Inflammation leading to fibrosis and scarring involves a complex multistage process with inflammatory cell infiltration, mesangial and fibroblast activation, tubular epithelial to mesenchymal transition, endothelial to mesenchymal transition, cell apoptosis, and extracellular matrix expansion that is orchestrated by a network of cytokines/ chemokines, growth factors, adhesion molecules, and signalling processes.

While the source of injury could be of any form such as autoimmune, metabolic, haemodynamic instability, trauma etc., the response initially is inflammation in most cases. Beyond the embryonic stage, any insult is always followed by repair by

inflammation, followed by resolution or scarring (4, 5). Inflammation is closely related to tissue repair with regeneration of parenchymal cells and the filling of tissue defects with fibrous tissue, namely, scar formation. The inflammatory response therefore represents a two-sided sword: beneficial in terms of the repair process to injury; detrimental when proceeding in an uncontrolled manner, which then leads to progressive fibrosis with a loss of function (6).

1.5 ANTI INFLAMMATORY THERAPY IN RENAL DISEASE

A number of therapeutic options exist to treat or control renal disease using anti-inflammatory and immunomodulatory actions, suggesting the significant role played by inflammation. This is true, not only for conditions that are primarily inflammatory in nature such as glomerulonephritis or vasculitis but also conditions such as diabetic nephropathy and hypertension related CKD. Progression of diabetic nephropathy consists of three steps: (i) glomerular hypertrophy and hyperfiltration; (ii) inflammation of glomeruli and tubulointerstitial regions; and (iii) reduction of cell number by apoptosis and accumulation of ECM(22). Proinflammatory cytokines have been implicated in progression of diabetic nephropathy. Studies have shown elevation of serum TNF levels and structural changes in kidney tissues in diabetes (23).

Activation of the renin–angiotensin system (RAS) and an increase in the local production of angiotensin II (AngII) is one of the main mechanisms involved in hypertension- induced tissue damage in kidneys, heart and brain. Angiotensin II acts as a pro inflammatory cytokine by activating circulating immune cells and regulating many of their functions, including chemotaxis, proliferation, differentiation and phagocytosis. It is known to play a role in expression of adhesion molecules as well as cytokines and chemokines (24)

ACE inhibitors (Acetylcholine esterase inhibitors) and Angiotensin 1 blockers are the most common drugs used in preventing worsening of renal disease especially in hypertension and diabetes. Given the presence of angiotensin receptors on the surface of monocytes, a role of immune system regulation by these drugs cannot be ruled out (25). The direct renin inhibitor, aliskiren has been shown to reduce TGF β related renal fibrosis and albuminuria in mouse models (26).

The oral hypoglycaemic agents, thiazolidinediones such as pioglitazone and rosiglitazone also have anti-inflammatory properties. They reduce pro-fibrotic cytokines such as TGF β and PAI-1(Plasminogen Activator Inhibitor-1) as well as pro-inflammatory cytokines such as IL-1, IL-6 and TNF – α (27, 28).

Anti-cytokine therapy is well established in chronic diseases such as rheumatoid arthritis and inflammatory bowel disease. There is increasing evidence for the role of similar therapy in renal diseases. Tocilizumab, a monoclonal antibody against IL-6 receptor, has been shown to have some benefit in lupus nephritis and crescentic GN (29).

1.6 CELLULAR MEDIATORS OF INFLAMMATION

1.6.1 LEUCOCYTES

The term leucocyte comes from the Greek word leuko- meaning "white" and cyte translated as "cell". White blood cells (WBCs) as they are commonly known are derived from the pluripotent stem cells in the bone marrow. These comprise only 0.01% of the total marrow cells and produce a hierarchy of lineage committed stem cells. There are 5

types of leucocytes found in blood: granulocytes (neutrophils, eosinophils, basophils), monocytes and lymphocytes. This is based on morphology and staining characteristics.

1.6.1.1 Neutrophils

These are the most common type of leucocyte in the blood of adults. They are 10 - 14 microns in diameter with a multilobular nucleus containing 2 to 5 segments and granules in their cytoplasm, hence the name granulocytes. They get their name from the characteristic neutral pink staining with haematoxylin and eosin (H&E).

In addition to activating other cells at the site of infection or inflammation, neutrophils play a very direct and active role in fighting foreign antigens such as microbes. They do so in 4 ways:

- a. Phagocytosis: Neutrophils have the capacity to ingest and internalise microbes into phagosomes. There is release of hydrolytic enzymes and free oxygen radicles into these phagosomes to digest the microbes (30).
- b. Degranulation: Neutrophils contain 3 types of granules (31)-
 - i) Primary granules or azurophilic granules: myeloperoxidase, bactericidal/permeability-increasing protein (BPI), defensins, and the serine proteases neutrophil elastase and cathepsin G.
 - ii) Secondary or specific granules: alkaline phosphatase, lysozyme, NADPH oxidase (Nicotinamide adenine dinucleotide phosphate-oxidase), collagenase, lactoferrin and cathelicidin.
 - iii) Tertiary granules: cathepsin and gelatinase.
- c. Neutrophil Extracellular Traps (NET): These comprise a web of fibres composed of chromatin and serine proteases that trap and kill microbes extracellularly (32).

d. Oxygen-free radicals

1.6.1.2 Eosinophils

Eosinophils represent 1 to 6 % of the circulating WBCs. They have 2 lobes in the nucleus and prominent granules on staining. These cells express cytoplasmic granules containing enzymes that are harmful to the cell walls of parasites but can also damage host tissues. The granules of eosinophils contain basic proteins that bind acidic dyes such as eosin. This gives the characteristic red staining with H&E stains.

Their granules contain peroxidase involved in intracellular killing of protozoa and helminths. These are also involved in allergic reactions, atopic diseases, asthma and interstitial nephritis.

1.6.1.3 Basophils

These represent less than 1% of circulating WBCs. Basophils contain granules that bind basic dyes and they are capable of synthesizing many of the same mediators as mast cells. Basophils express IgG and IgE receptors, bind IgE, and can be triggered by antigen binding to the IgE. They bind to IgE antibody on their surface and release heparin, leucotrienes and histamines. Basophils are involved in hypersensitivity reactions.

1.6.1.4 Lymphocytes

These are the most abundant of the white cells till the age of 7, after which neutrophils become more abundant. They are quite heterogeneous in size. There are 2 types of lymphocytes:

- a. T cells, which represent about 80% of the lymphocyte population and mediate cellular immunity. There are 2 subtypes of T cells: CD4 positive helper cells and CD8 positive suppressor cells.
- b. B cells, which mediate humoral immunity.

1.6.1.5 Monocytes

These are the largest of the white cells with a diameter of 12 - 20 microns and an irregular nucleus in abundant pale blue cytoplasm when stained with Giemsa stain. Once they enter tissues, these monocytes mature and become macrophages (33). Macrophages in different tissues have been given special names to designate specific locations. For instance, in the central nervous system, they are called microglial cells; when lining the vascular sinusoids of the liver, they are called Kupffer cells; in pulmonary airways, they are called alveolar macrophages; and multinucleate phagocytes in bone are called osteoclasts. These produce a variety of cytokines including Interleukin 1, Tumour necrosis factor- α and GM-CSF (Granulocyte monocyte colony stimulating factor) (34-36). Monocytes, once activated, also play an important role in phagocytosis of microbes, dead neutrophils and apoptotic cells thereby preventing unwanted release of pro-inflammatory mediators from neutrophils(37). Another important function of macrophages is to promote repair of damaged tissues by stimulating new blood vessel growth (angiogenesis) and synthesis of collagen-rich extracellular matrix (fibrosis). This function is mediated by specific cytokines secreted by the macrophages that act on various tissue cells.

While there are small numbers of the various leucocyte lineages present in resting tissues, these numbers are massively augmented by recruitment from the circulation in response to inflammatory cues. Figure 1.3 shows the approximate time course of influx of neutrophils, macrophages, T lymphocytes and mast cells in a murine wound response superimposed on the three classically considered and overlapping phases of tissue repair, namely inflammation, proliferation/ migration and maturation/remodeling. Amongst the WBCs, neutrophils are the first cells to arrive at the site of inflammation or injury. They have a short life span. The short lifetime of neutrophils reduces spread of those pathogens that parasitize phagocytes because the more time such parasites

spend outside a host cell, the more likely they will be destroyed by some component of the body's defenses. Also, because neutrophil antimicrobial products can also damage host tissues, their short life limits damage to the host during inflammation (38).

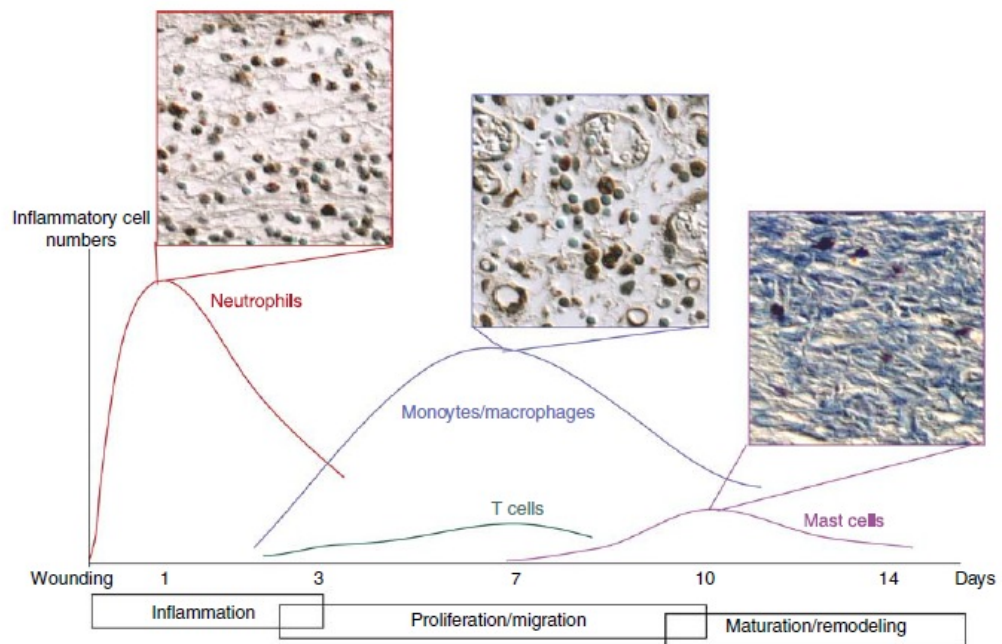


Figure 1-3 Relative Time course of inflammatory cell recruitment to sites of tissue damage.

From The Inflammation–Fibrosis Link? A Jekyll and Hyde Role for Blood Cells during Wound Repair; Brian M. Stramer, Ryoichi Mori and Paul Martin; Journal of Investigative Dermatology (2007) 127, 1009–1017. Doi: 10.1038/sj.jid.5700811

1.7 ADHESION MOLECULES

Adherence, whether it is cell to cell or cell to proteins in the extra cellular matrix, plays a critical role in various biological processes such as immune responses, embryogenesis, haemostasis and inflammation (39-41).

The ability to characterize cellular adhesion molecules (CAMs) at the molecular level has enabled us to classify molecules into several discrete groups that include integrins, cadherins, members of the immunoglobulin superfamily, and selectins (42).

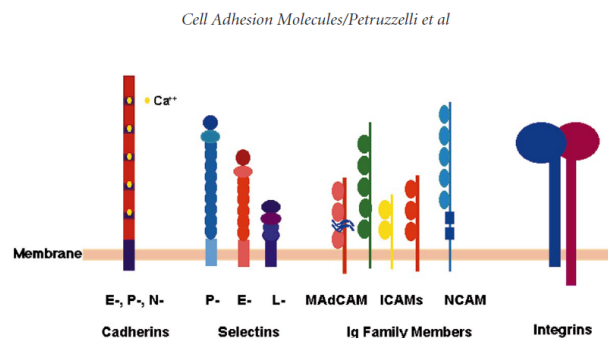


Figure 1-4 Schematic representation of four classes of adhesion molecules; cadherins, selectins, Ig family members and integrins.

From Structure and Function of Cell Adhesion Molecules: Lilli Petruzzelli, MD, PhD, Mimi Takami, MD, H. David Humes, MD, Am J Med. 1999; 106:467– 476.

1.7.1 CLASSIFICATION OF ADHESION MOLECULES

1.7.1.1 CADHERINS

The classic cadherins were among the earliest identified in this family and are comprised of the N, P, R, B, and E cadherins (40). Cadherins mediate homotypic cellular interactions by binding to their homologues on an adjacent cell (43). The brain

expresses the largest number of different cadherins, presumably due to the necessity of forming very specific cell-cell contacts.

Calcium is critical to their function and serves to maintain the structural integrity of the protein. During differentiation and in some diseases, the amount or nature of the cell-surface cadherins changes, affecting many aspects of cell-to-cell adhesion and cell migration. For example, the metastasis of tumour cells is correlated with the loss of cadherins on their cell surface (44).

The cadherins function not only to maintain the integrity of the epithelial layer but also to organize the formation of the correct architecture.

1.7.1.2 SELECTINS

Movement of leucocytes to a site of inflammation requires extravasation of leucocytes from circulation. This requires successive formation and breakage of cell-to-cell contacts between leucocytes in the blood and endothelial cells lining the vessels. These contacts are mediated by selectins, a class of cell-adhesion molecules that are specific for leucocyte – vascular cell interactions. Each type of selectin binds to specific oligosaccharide sequences in glycoproteins or glycolipids. As with cadherins, the binding of selectins to their ligands is Ca^{2+} dependent. There are 3 classes of selectins: E-, L- and P-Selectins (45). P-selectin is localized to the blood-facing surface of endothelial cells in Weibel-Palade bodies and α granules of platelets. In normal endothelial cells, P-selectin is localized to intracellular vesicles and is not present on the plasma membrane. L-selectin is expressed on all granulocytes and monocytes and on most lymphocytes. E-selectin is not expressed under baseline conditions but is induced by inflammatory cytokines (46).

Activation allows exocytosis of the selectins onto the surface of the plasma membrane. As a consequence, passing leucocytes adhere weakly to the endothelium; because of the

force of the blood flow, these “trapped” leucocytes are slowed but not stopped and roll along the surface of the endothelium.

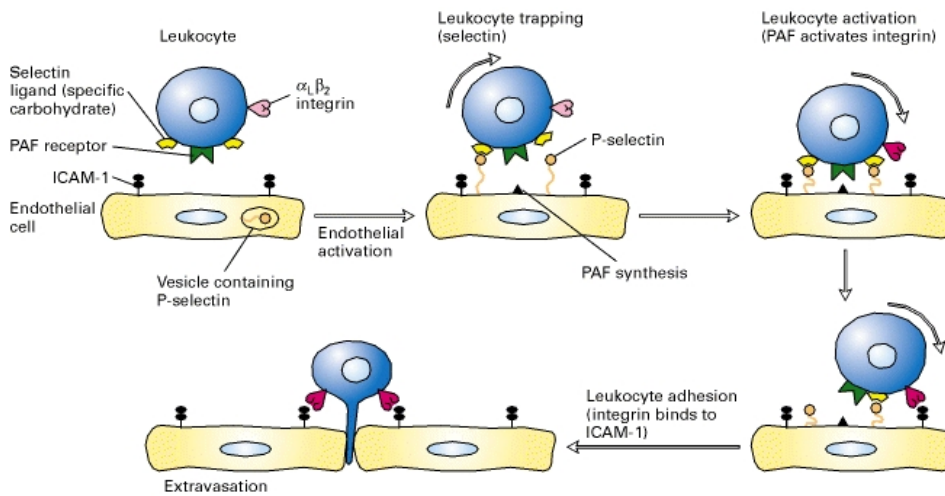


Figure 1-5 Interactions between cell-adhesion molecules during the initial binding of leucocytes to activation of endothelial cells

From R. O. Hynes and A. Lander, 1992, *Cell* 68:303

1.7.1.3 INTEGRINS

The integrins are a set of cell surface adhesion molecules that regulate cell to cell and cell to extracellular matrix protein interactions. The integrin family is composed of two subunits, α and β , which traverse the cell membrane and are characterized by non-covalent interactions. The integrins, now numbering more than 20, are further divided into subgroups based on their β subunit. These play multiple roles such as tissue organization by binding to molecules both in the extracellular matrix (ECM) within many tissue and in the basement membranes found in muscle, the nervous system, epithelial tissue, and endothelium (47), lymphocyte homing and leucocyte migration (48), platelet activation and thrombosis (49).

The major ligands for the integrins fall into two categories:

1. Cell surface molecules that are members of the immunoglobulin superfamily [such as intracellular adhesion molecules (ICAM-1, ICAM-2), vascular cell

adhesion molecule (VCAM-1), or mucosal addressin cell adhesion molecule (MAdCAM-1)], and

2. A variety of large extracellular matrix proteins (such as fibronectin, vitronectin, fibrinogen) (42, 48).

Some integrins can bind to ligand in the absence of cell stimulation. The recruitment of either leucocytes or platelets from the circulation requires the participation of several different adhesion molecules as well as the activation of inside-out signalling pathways (48-50). Usual binding processes involve activation, ligand binding, reorganisation of the cytoskeleton and finally binding (51).

1.7.1.4 IMMUNOGLOBULIN SUPERFAMILY

These proteins are classified together, because they contain one or more of a common immunoglobulin-like repeat that is characterized by two cysteines separated by 55 to 75 amino acids. The members of this group play a critical role in the development of the nervous system, in immune and inflammatory responses, and in embryonic development (42, 52).

In the immune system, immunoglobulin superfamily members play a critical role in cellular adhesion. These members include ICAMs, VCAMs and the peripheral addressin, MAdCAM-1. These proteins serve as ligands for the integrins, and their adhesive interactions depend on the endogenous cell (for example, endothelial, epithelial or fibroblast cells) and the individual leucocyte.

Structurally, the ICAMs include three family members, ICAM-1, ICAM-2, and ICAM-3, which contain two to five extracellular immunoglobulin domains.

1.8 EXTRACELLULAR MATRIX

The extracellular matrix (ECM) is the non-cellular component present within all tissues and organs, and provides not only essential physical scaffolding for the cellular constituents but also initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis.

The composition of ECM varies between different tissues. It is a complex assembly of many proteins and polysaccharides forming an elaborate meshwork within tissues. The main fibrous ECM proteins are collagens, elastins, fibronectins and laminins (53). Collagens, which constitute the main structural element of the ECM, provide tensile strength, regulate cell adhesion, support chemotaxis and migration, and direct tissue development (54). These are mainly secreted by fibroblasts that are present in the matrix or recruited from surrounding tissues(55).

Adhesion of mesenchymal cells such as fibroblasts mediates cytoskeletal coupling to the ECM and is involved in cell migration through the ECM (56). The ECM is a highly dynamic structure that is constantly being remodelled; it generates the biochemical and mechanical properties of each organ, such as its tensile and compressive strength and elasticity, and also mediates protection by a buffering action that maintains extracellular homeostasis and water retention. The ECM also allows binding to growth factors and other cell surface receptors to regulate gene transcription. The structure and composition of ECM can vary from time to time, depending on the physiological state such as normal to cancerous or post-inflammation.

There are multiple growth factors that are bound to the ECM which modulate cell growth and cell migration and are essential for normal homeostasis (57, 58). Any insult, acute or chronic, activates the fibrogenic machinery and induces wound healing. One of the first events that characterises a wound response is vascular damage and the

formation of a fibrin clot, which stimulates monocyte infiltration to the damaged ECM. These differentiate into macrophages under the influence of various cytokines (33). The activated macrophages, in turn, secrete and release multiple Growth Factors, MMPs (Matrix Metalloproteinases) and cytokines that promote angiogenesis and stimulate fibroblast migration and proliferation (59). Thereafter, recruited fibroblasts begin to synthesize and deposit large quantities of ECM proteins, including collagen type I and III, fibronectin and hyaluronic acid. The elevated mechanical stress associated with this profound ECM deposition can induce the differentiation of fibroblasts and other tissue-resident cells – i.e. epithelial-to mesenchymal transition (EMT) of epithelial cells – or of circulating bone marrow-derived mesenchymal stem cells into myofibroblasts (59, 60)

1.8.1 ROLE OF EXTRACELLULAR MATRIX IN KIDNEYS

ECM constituents and their receptors play a significant role in development. Matrix molecules and matrix receptors act at multiple steps during kidney development, from the onset of ureteric bud development, during its branching morphogenesis and during the formation of epithelial tubules from condensing mesenchymal cells (61).

In the adult kidneys, ECM is present in distinct areas and its function depends on the specific molecular components

1. In the glomeruli

- a. Glomerular basement membrane
- b. Bowman's capsule
- c. Mesangial ECM

2. In the tubulointerstitium

- a. Tubular basement membrane (in part segment specific)
- b. Peritubular capillary basement membrane
- c. Interstitial ECM

3. In larger vessels

- a. Within the vessels (lamina elastica interna and externa)
- b. Around the vessels (adventitia of arteries and veins)

Medullary interstitial ECM is physiologically more prominent compared to the cortical interstitial ECM. It steadily increases in quantity in the direction from outer to inner medulla/papilla (62).

The glomerular basement membrane (GBM) is thicker compared to most other basement membranes. It contains four main macromolecules: laminin, collagen type IV, nidogen and heparan sulphate proteoglycans. The main function of the GBM is to act as a charge- and size- selective filtration barrier between the vascular system and the urinary space (62). It therefore acts as a barrier to bigger molecules and proteins being lost in urine.

The mesangial ECM provides structural support for the glomerular capillaries and has a role in cell-matrix signalling. Its major components are fibronectin, collagen type IV, collagen type V, laminin A, B1 and B2, chondroitin sulphate and heparan sulphate proteoglycans (63).

The interstitial extracellular matrix is normally composed of collagen type I, III, V, VI, VII and XV, both sulphated and non-sulphated glycosaminoglycans, glycoproteins and polysaccharides (62). Collagens provide the tensile strength, regulating cell adhesion, support, chemotaxis, cell migration and tissue development (54). Among the glycoproteins, fibronectin is the most important one and its accumulation is one of the first events during renal fibrosis (64). Proteoglycans are a subgroup of glycoproteins with a high content of carbohydrates, which fill renal extracellular interstitial space. They have a wide variety of functions, such as hydration, force-resistance and growth factor binding (65).

The ECM plays an important role in several renal disorders. The accumulation and dysregulated remodelling of ECM can affect all major compartments of the kidney, and

is called glomerulosclerosis in the glomeruli, tubulo-interstitial fibrosis in the tubulointerstitium and arteriolosclerosis in the vasculature. Mutations in the gene for the $\alpha 5$ chain of collagen type IV causes the X-linked Alport's syndrome in humans, a rare genetic disease characterized by progressive glomerular injury. Collagen type IV is also the target of two autoimmune diseases affecting the kidney: Goodpasture's syndrome and Alport's post-transplantation disease. Both diseases are characterized by autoantibodies attacking the GBM and causing rapidly progressive glomerulonephritis.(66) Expression of versican, an ECM proteoglycan, was found to be increased in areas with marked tubulointerstitial fibrosis in patients with proteinuric CKD, suggesting a role for it in progression of CKD.(67)

1.9 HYALURONAN

Hyaluronan (HA) is a glycosaminoglycan of alternating N-acetyl glucosamine (GlcNAc) and glucuronic acid (GlcA) residues synthesized by essentially all organisms from bacteria to mammals (68). Hyaluronan is synthesised at the inner face of the plasma membrane by one of three distinct hyaluronan synthases (HAS) (69, 70). It is synthesised as a linear polymer on the inside of the plasma membrane and then extruded to the outside (68, 70). The synthases HAS1, HAS2, and HAS3 are encoded on separate chromosomes but possess amino acid and structural similarities (71, 72). HAS 1 gene is localised to chromosome 19, HAS 2 gene to chromosome 8 and HAS 3 gene to chromosome 6 (72). In the normal kidney, all three HAS are expressed in larger amounts in the medulla than in the cortex. Furthermore, the relative expressions are such that $HAS2 > HAS1 > HAS3$ (73).

HAS 2 is considered to be the major source of HA production during embryo development and tissue regeneration (74, 75). Hyaluronan chains, being negatively charged, attract water and salt. HA also binds to other extracellular molecules resulting

in a strong structural meshwork which is resistant to biomechanical pressure (76). The key capabilities of HA are its water-attracting properties (1 g HA attracts one litre of water) and its ability to form gels in higher concentrations (>0.2 mg/ml) (77).

Various biological and physiological roles of hyaluronan are related to its molecular weight. This is governed by the isoform of HAS from which it is synthesized. HAS 1 seems to be least active and is responsible for high molecular weight HA (HMW HA), up to 2×10^6 Da. HAS 2 is responsible for high molecular weight HA as well (more than 2×10^6 Da). HAS 3 is most active and makes large amounts of low molecular weight HA (LMW HA) (69, 71, 72).

Hyaluronan also plays a key role in embryogenesis. The HA contribution to kidney development is mostly accomplished by inducing morphogenic branching and differentiation. The mechanism by which HA is reduced in the kidney during maturation involves reduced HAS2 and increased Hyal1 expression (78).

HMW HA possesses anti-inflammatory and anti-angiogenic properties and can promote cell quiescence, whereas LMW HA is pro-inflammatory and can induce cytokine and chemokine secretion, activation of signalling pathways, cell proliferation, and angiogenesis (79).

Hyaluronan plays an important role in the pathogenesis and progression of chronic inflammatory conditions such as atherosclerosis, chronic wounds and inflammatory bowel diseases like Crohn's disease. Elevated Hyaluronan levels in inflammation highlight this. It is also involved in tumour genesis and normal development (68, 80).

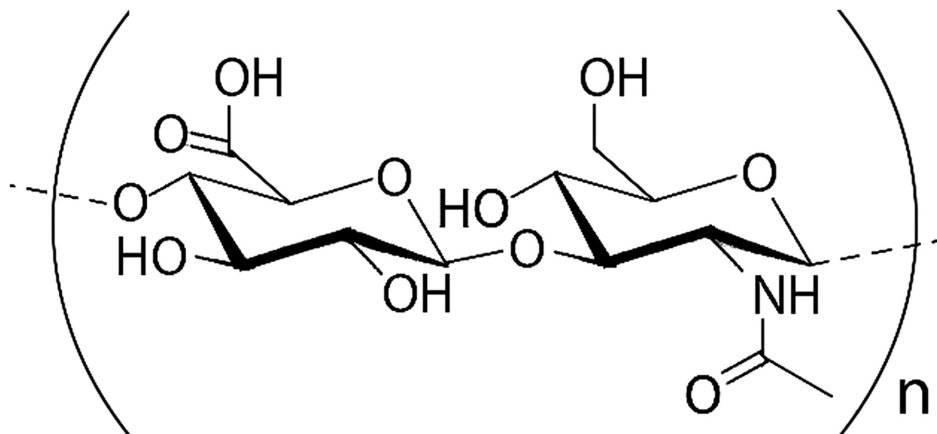


Figure 1-6 Structure of hyaluronan (HA) demonstrating the repeated D-glucuronic acid and N-acetyl-D-glucosamine moieties.

From Renal interstitial hyaluronan: functional aspects during normal and pathological conditions. Sara Stridh , Fredrik Palm , Peter Hansell. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology Published 1 June 2012 Vol. 302 no. R1235-R1249

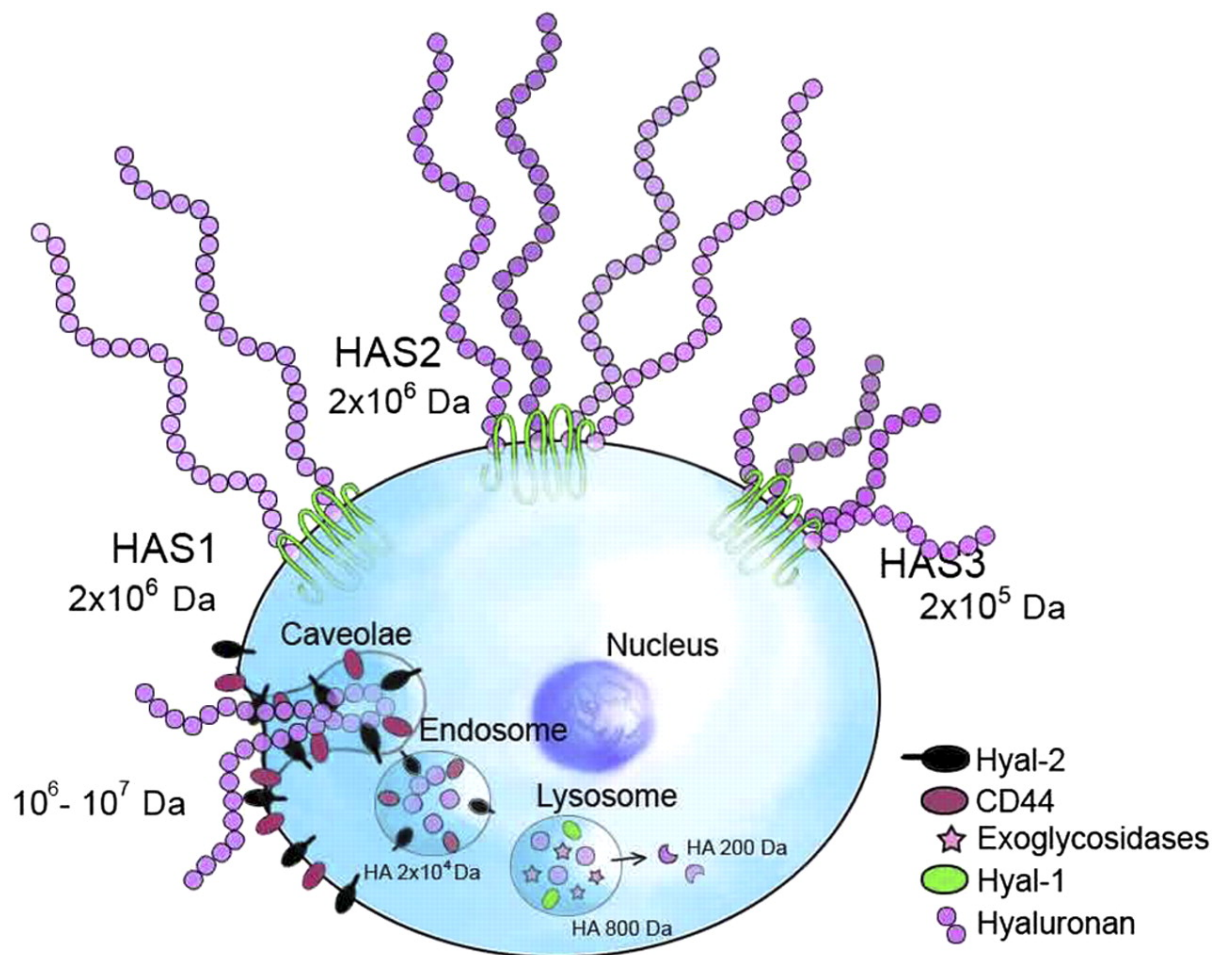


Figure 1-7 Synthesis and turnover of HA

The HA synthases (HAS) 1–3 produce HA in the plasma membrane of different sizes and at different rates. The hyaluronidases (Hyal) hydrolyze HA. This may begin already 1) on the plasma membrane by Hyal2, followed by 2) binding to CD44, which is a scavenger receptor for HA. After 3) internalization and degradation in endosomes by Hyal2, HA is 4) further degraded by Hyal1 in lysosomes

From: Renal interstitial hyaluronan: functional aspects during normal and pathological conditions
Sara Stridh , Fredrik Palm , Peter Hansell, American Journal of Physiology - Regulatory, Integrative and Comparative Physiology Published 1 June 2012 Vol. 302 no. R1235-R1249

1.9.1 ROLE OF HYALURONAN IN KIDNEY DISEASE

In the kidneys, hyaluronan is primarily expressed in the medullary interstitium (81). The main purpose here is to provide structural stability to the tubules and blood vessels. It also helps in urine concentration. The amount of renal papillary HA changes in response to water balance. HA plays a role in renal water handling by affecting physicochemical characteristics of the papillary interstitial matrix and influencing the interstitial hydrostatic pressure, thereby determining interstitial water diffusion (82).

HA can also be secreted by cortical interstitial cells, fibroblasts, and apical membrane of tubular cells (83-85).

Hyperglycaemia can stimulate vascular smooth muscle cells and mesangial cells to produce hyaluronan (86, 87). There is evidence for the role of hyaluronan in various forms of renal injury. This ranges from acute flare up of autoimmune conditions such as lupus nephritis, to renal impairment in transplant recipients. Serum levels of IL-6, TNF- α , IFN- α , and hyaluronan (HA) are increased in patients with lupus nephritis. It has been shown that their expression is increased in the renal parenchyma of patients and mice with active lupus nephritis, mediated in part through stimulation of resident renal cells with anti-dsDNA antibodies, which contribute to the development and progression of disease (88).

There is also evidence to suggest that during chronic kidney inflammation, mesangial cells and proximal renal tubular epithelial cells synthesize HA that forms long cable-like structures which function as an adhesive matrix, binding leucocytes and macrophages and preventing them from interacting with adhesion molecules, thereby limiting glomerular and tubulointerstitial inflammation (86, 89, 90).

Up-regulation of HA and its binding receptors is involved in interstitial fibrosis in chronic cyclosporin-induced renal injury (91). Experimental evidence is also available that suggests a role of hyaluronan in ischaemic reperfusion injury (92, 93). Kidney

transplant increases the chances of ischaemic reperfusion injury. There is also risk of rejection which is an immune reaction leading to inflammation with release of several cytokines. These play a role in modifying the extra-cellular matrix. HA is seen early in transplant rejection and is especially localised to cortex and sclerotic vessels (94). Hyaluronan has also been implicated in formation of renal stones (95).

Diabetic nephropathy is the leading cause of end stage renal failure across the world and 25% of all type 1 Diabetes patients are likely to develop nephropathy (96). As mentioned above, several cells in the kidney produce hyaluronan at an increased rate. There is also accumulation of all components of the extra-cellular matrix as diabetic nephropathy progresses. HAS2 mRNA activity is elevated and coincides with proteinuria, overt diuresis, and depressed kidney function (97).

1.10 FIBROBLASTS

Fibroblasts are elongated cells with extended cell processes that show a fusiform or spindle-like shape in profile (98). Fibroblasts are morphologically heterogeneous with diverse appearances, depending on their location and activity. They are the most abundant cell type in connective tissue, and play a central role in extracellular matrix (ECM) remodelling and wound contraction during tissue repair. Characteristic features include expression of vimentin in the absence of desmin and α -smooth muscle actin.

Fibroblasts synthesize many of the constituents of the fibrillar ECM such as type I, type III and type V collagen, and fibronectin (99). They also contribute to the formation of basement membranes by secreting type IV collagen and laminin (100). Fibroblasts are an important source of ECM-degrading proteases such as matrix metalloproteinases (MMPs), which highlights their crucial role in maintaining an ECM homeostasis by regulating ECM turnover (100). Fibroblasts continually synthesise ECM proteins and it has been estimated that each cell can synthesise approximately 3.5 million procollagen

molecules/cell/day (101). However, this is regulated by lysosomal enzymes, such as cathepsins B, D and L, with between 10% and 90% of all procollagen molecules being degraded. Regulation of this process appears to provide a mechanism for rapid adaptation of the amount of collagen secreted following injury (102).

1.10.1 ACTIVATION OF FIBROBLASTS

Fibroblasts that are isolated from the site of a healing wound or from fibrotic tissue secrete higher levels of normal ECM constituents and proliferate more than their normal counterparts, isolated from healthy organs (99, 103). Thus a normal fibroblast can become 'activated'. The stimulus for the activation comes from growth factors such as Platelet-derived growth factors, TGF β , Fibroblast growth factor 2 (FGF 2), and Epidermal Growth Factor (EGF) released from monocytes, macrophages and injured cells (104-106). Activation also takes place with interaction of fibroblasts with leucocytes via adhesion molecules (1).

To synthesize large amounts of ECM constituents, activated fibroblasts typically contain a large oval euchromatic nucleus with one or two nucleoli, rough endoplasmic reticulum, and a prominent Golgi apparatus. In inactive adult fibroblasts, the endoplasmic reticulum is smaller and the nucleus is flattened and heterochromatic (98). Activated fibroblasts express α -smooth-muscle actin, leading to the term 'myofibroblasts'. These secrete matrix metalloproteases such as MMP2, MMP3 and MMP 9. These are ECM degrading proteases, suggesting increased ECM turnover and remodelling (99). Activated fibroblasts are involved in secretion of growth factors such as hepatocyte growth factor (HGF), insulin-like growth factor (IGF), nerve growth factor (NGF), Wnt1, EGF and FGF2, secretion of cytokines such as interleukin-1 and chemokines such as monocyte chemotactic protein 1 (MCP1) (107, 108).

The difference between activated fibroblasts (myofibroblasts) from a wound that is healing and from fibrosing tissue is that the myofibroblasts in a healing wound revert to inactive form whereas the myofibroblasts from a fibrosing organ/tissue remain activated. These continue to secrete growth factors, cytokines etc. and thereby activate further fibroblasts in the vicinity (109).

Hyaluronan appears to play a significant role in myofibroblast differentiation and maintenance of myofibroblast phenotype (110). This has been well described in studies from our laboratory using dermal and oral fibroblasts as representative of scarring and non- scarring fibroblast phenotypes. The inability of the oral fibroblast to differentiate into myofibroblasts in response to the profibrotic cytokine, TGF β 1 was associated with the inability to induce the HAS 2 enzyme or assemble a pericellular HA coat. The converse was true for the scarring dermal fibroblasts. These, when stimulated with TGF β 1, expressed HAS 1 and HAS 2, showed development of an HA coat and myfibroblast differentiation. In the same study HAS2 activity and HA synthesis were shown to be essential for differentiation as siRNA to HAS2 or inhibiting HA synthesis with 4-methyl umbelliferone in dermal fibroblasts altered TGF β 1-dependent responses in these cells preventing fibroblast to myofibroblast differentiation(111).

Aged cells also show dysregulated responses to TGF β 1 and this is also due to reduced HAS2 and HA pericellular coat induction in these cells. (112). These studies highlight the importance of the induction of HAS 2, the synthesis of HA and the assembly of the HA coat in fibroblast to myofibroblast differentiation.

It has been shown that expression of Bone morphogenetic protein-7(BMP-7) is reduced in renal fibrosis. BMP-7, a member of TGF β family, may work by antagonizing the effects of TGF β 1, its profibrotic counterpart (113, 114). One of the possible mechanisms is likely to be the internalization of the HA coat into hyaluronidase

containing endosomes (115). This again highlights the importance of HAS2 and HA in fibroblast differentiation and also maintenance of the myofibroblast phenotype.
(111-115)

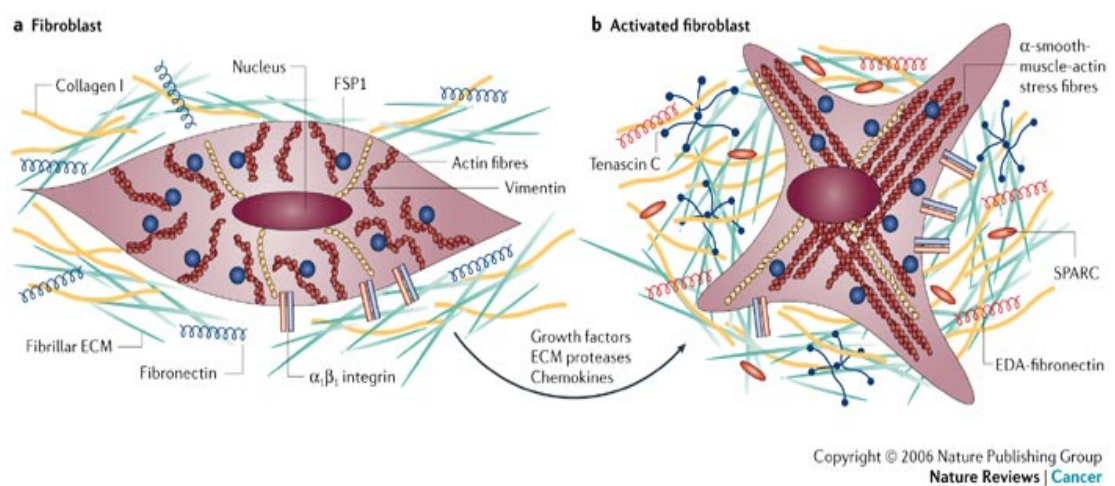


Figure 1-8 Fibroblasts and myofibroblasts

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| doi:10.1038/nrc1877

1.10.2 EPITHELIAL MESENCHYMAL TRANSITION (EMT)

Fibroblasts display the highly activated phenotype characteristic of myofibroblasts, at sites of inflammation and fibrosis. These fibroblasts could come from the proliferation of pre-existing stromal fibroblasts, recruited from the bone marrow. Increasingly, it is becoming evident that transition of epithelial cells to mesenchymal phenotype plays an important role in tissue repair and even fibrosis. Epithelial Mesenchymal Transition (EMT) refers to this orchestrated transition of epithelial cells to migratory mesenchymal cells, which develop the capacity to generate extracellular matrix.

EMT was first identified in kidneys while investigating features of tubular atrophy in end-stage kidney disease. It identified cytokeratin- expressing single cells or loosely associated cell clusters dispersed within the fibrotic interstitium(116). In an experimental study, 36 % of interstitial FSP1/S100A4-immunoreactive fibroblasts within the fibrosing kidney were found to have come from EMT of the labelled tubular epithelium(117). Markers of myofibroblasts transition such as Vimentin, HSP-47 and α SMA have been identified in epithelial cells in renal biopsies from patients with diabetes, chronic allograft nephropathy and various glomerulonephritis. On several occasions, these are seen even before histological evidence of progression of disease and might be used as prognostic markers (118).

Fibroblasts therefore play a significant role in promoting renal fibrosis. Once activated, they are the primary source of extra cellular matrix deposition and therefore increase the loss of architecture in the kidney.

1.11 AIMS

In previous studies, the ligation of ICAM-1 on fibroblasts by leucocytes or specific cross-linking with anti-ICAM-1 antibody has been shown to activate the cells, which then adopt a pro-inflammatory phenotype (1, 119). TGF β 1 transcription is also induced in renal epithelial cells by leucocyte binding and is down-regulated by HAS 2 dependent HA generation (120). The aim of the work described in this thesis was to study the interaction between fibroblasts and monocytes and focus on the role played by the adhesion molecule ICAM 1 in this interaction. I investigated how this interaction led to further pro-fibrotic events by impacting on production of ICAM 1 and HAS 2, the chief synthase involved in the production of HA.

2 METHODS

2.1 TISSUE CULTURE:

2.1.1 LUNG FIBROBLASTS MONOLAYER CULTURE

Human Lung Fibroblasts (AG02262) used in this work were primary human fibroblasts obtained from Coriell Cell Repositories. These were cultured in Dulbecco's Modified Eagle's / HAMS F-12 medium (Sigma-Aldrich), containing 100 units/ml Penicillin and 0.1 mg/ml Streptomycin (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and 10% Foetal calf serum (FCS) (Biosera). They were maintained at 37°C in an atmosphere of 5% CO₂ in a humidified incubator (Cell House 170, Heto Holten, Derby, UK) and medium was replenished every 3 days.

Confluent cell layers were sub-cultured (1:3 ratio) using trypsin solution diluted 1:1 with sterile phosphate buffered saline pH 7.3 (PBS). The protease activity in the solution was then neutralized with an equal volume of FCS. Cells were pelleted at 1500 rpm for 7 minutes at room temperature. The pellet was resuspended in three times the original volume of the medium and cells seeded in appropriate culture flasks (T25 or T75) or plates (BD FalconTH Bioscience, Bedford, USA).

Cells were growth arrested in serum-free medium for 72 hours before experiments to allow cell cycle synchronisation. Only Cells having less than 10 passages were used for experiments.

2.1.2 U937 CELL CULTURE

U937 cells are a human cell line extracted from a 37 year old with diffuse histiocytic lymphoma. It is one of only a few human lines still expressing many of the monocytic like characteristics exhibited by cells of histiocytic origin. The cells are committed to the macrophage branch of the myeloid lineage and can be induced by a variety of agents to mature into a monocytic stage of development.

Cells were grown and subcultured in RPMI 1640 medium. Each 500ml of medium was supplemented with 10 ml of HEPES Buffer 10.25 ml of L Glutamine. FCS was added to a final concentration of 5%.

U 937 cells are stored frozen in liquid nitrogen. The tubes are thawed at room temperature. 10 % FCS was used in the first instance when freshly thawed cells are suspended and incubated for 24 hours at 37° C in a humidified atmosphere and 5% CO₂.

Medium was then changed every 3 days. The cell suspension was harvested and centrifuged at 1600 rpm for 6 minutes. The pellets were then resuspended in 5% medium and transferred to fresh flasks or plates as required.

2.1.3 CO-CULTURE EXPERIMENTS

Lung Fibroblasts were cultured in 6 well plates as described above. Once confluent the cells were growth arrested for 48 hours in serum free medium.

U937 cells that have been grown in RPMI 1640 medium were pelleted by centrifugation at 1600 rpm for 6 minutes. The supernatant was discarded and the cells re-suspended in DMEM F12 HAM medium. Cells were counted using a Beckman coulter counter.

5×10^5 cells/ml were added to the lung fibroblasts in a final volume of 2ml. The co-cultures were incubated for varying time periods. Cells were washed with sterile PBS three times at specific time points and RNA extracted as detailed above.

The above experiments were also conducted with the fibroblasts stimulated with 1×10^{-12} M TNF α for 24 hours before co-incubation with U 937 cells.

2.2 ACTIVATION OF U937 CELLS

U 937 cells were grown in 5% FCS in RPMI medium in T75 flasks as described. The cells were then centrifuged and the pellet re-suspended in 5% FCS with 160 nM PMA for 48 hrs. Most of the cells adhered to the flask after this time and these were the activated cells. The medium containing the remaining cells in suspension was aspirated and discarded.

Two methods were employed to harvest the activated cells from the flask.

1. 0.1% EDTA: 5 ml of cold 0.1% EDTA was added to the flask and it was left on ice for 3 to 5 min. It was then agitated and most of the cells came off by this time. The cells were aspirated and centrifuged at 1600 rpm for 6 min. The supernatant was discarded and the activated U937 cells were suspended in the DMEM F12 HAM medium and used for the experiment.

2. 10% TRYPSIN: Once the medium with the remaining suspended cells was removed, 5 ml of 10% trypsin was added and incubated for 5 min. The trypsin was then neutralized with 5 ml FCS. The cells were then pelleted by centrifugation and re-suspended in serum free DMEM F12HAM medium for use in the experiment.

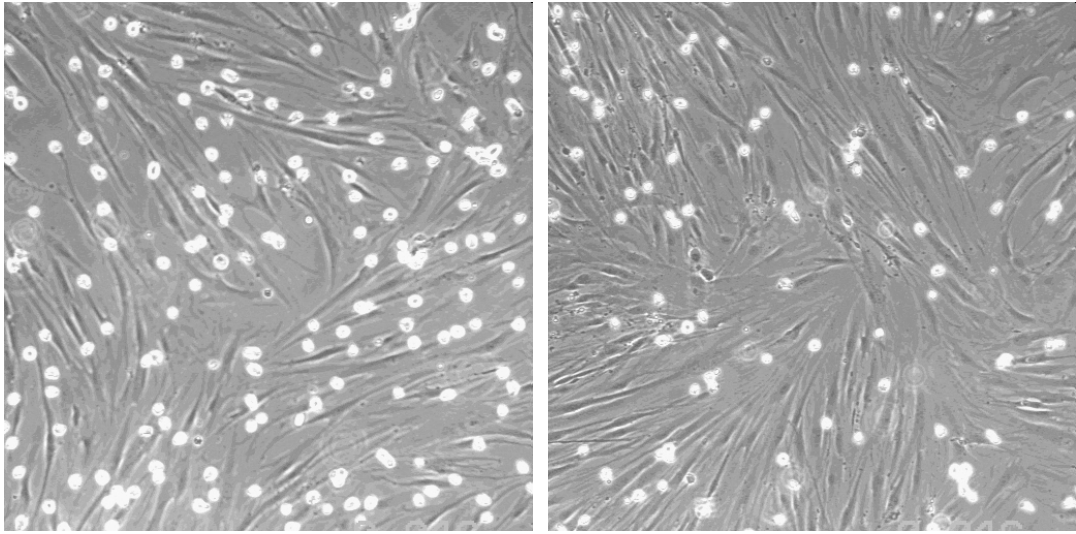
The “Alamar Blue” assay was carried out to determine the viability of cells extracted by both methods and cell counts performed using a Coulter counter.

2.3 CO CULTURE OF FIBROBLASTS AND ACTIVATED U937 CELLS

Fibroblasts were grown as described previously in 6 well plates. Once confluent, medium was changed to serum free medium to growth arrest and synchronise cell cycles. U 937 cells were activated and extracted as described above. Cells were then resuspended in DMEM F12 HAM medium. Cell number was determined using a Coulter counter and 5×10^5 cells/ml in 2 ml medium were added to each well and co

incubated for a set period of time. At relevant time points the medium was aspirated and cells washed to remove any non-adherent cells. Adherent U937 cells were removed from the fibroblasts monolayers again using EDTA treatment as described above. RNA extraction and q PCR was then carried as described above.

The possibility of contamination of fibroblasts with adherent U937 cells was considered. Adherent U937 cells were disassociated from fibroblasts using EDTA treatment after washing cells with PBS 3 times. Experiments relied on the ability of EDTA to remove the adherent U 937 cells from the surface of adherent fibroblasts (121) . The figures (2-1 And 2-2 illustrate successfully removal of U937 cells from fibroblasts in co-culture experiments.



a

b

Figure 2-1 Fibroblasts and U937 cells co-culture (a) pre and (b) post PBS wash

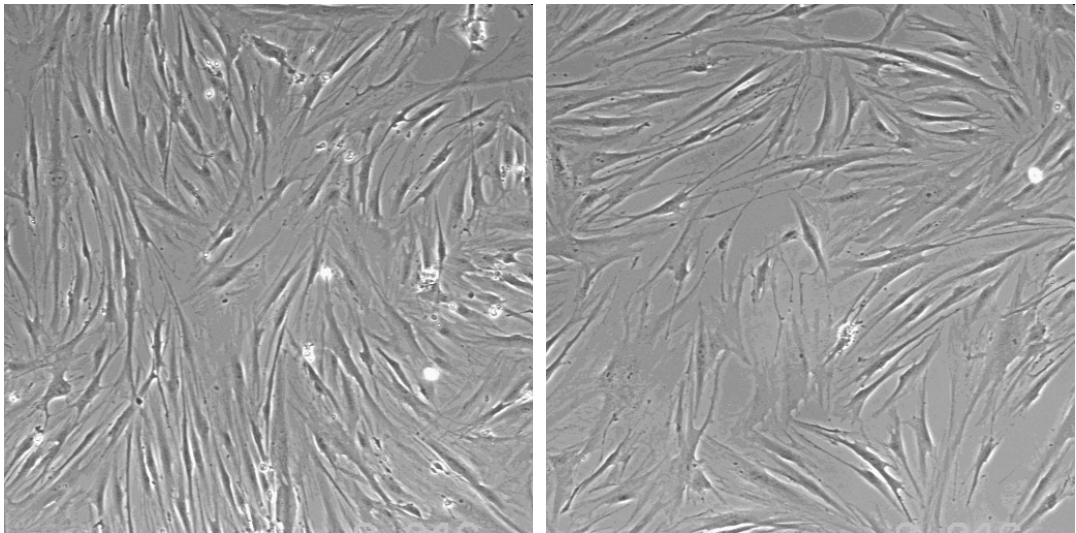


Figure 2-2 Fibroblasts and U937 cells co-culture post PBS wash and EDTA treatment

2.4 CROSS LINKAGE EXPERIMENTS

Lung fibroblasts (AG02262) were grown in 6 well plates as described above. Once confluent they were growth arrested for 48 hours for cell cycle synchronisation by growing in serum free medium. Cells were then incubated for 24 hours in serum free medium with 1×10^{-12} M TNF α . This allows optimal protein expression on the surface of the cells for cross linkage to take place. Cells were washed with sterile PBS and incubated with anti – ICAM 1 monoclonal IgG (Catalogue number: BBA4, R&D Systems) in serum free DMEM F12 HAM for 1 hour. The anti ICAM 1 was used at a concentration of 10 μ g/ml as employed in previous experiments in the lab.

Cells were then washed with warm sterile PBS. The primary antibody was cross-linked using goat anti mouse IgG antibody (Catalogue number M8642, Sigma) at a concentration of 10 μ g/ml for varying time periods. RNA was extracted as described above at 1 hour, 2 hours, 4 hours, 8 hours, 12 hours and 24 hours. Reverse Transcription and quantitative PCR was carried out to examine the expression of ICAM 1, and HAS 2 mRNA.

2.5 CELL LYSIS, RNA EXTRACTION AND ANALYSIS

1.1.1 CELL LYSIS AND RNA ISOLATION

Medium was aspirated off the plates. The majority of the experiments described were done using 10 cm² 6 well plates. Cells were washed with sterile PBS x 3 times. 0.5 ml of TRI reagent (Sigma) was added and the plates left for 1 minute. The lysate was pipetted up and down several times to break up any complexes, and samples were collected in clear tubes. Another 0.5 ml of TRI reagent was added to the well and the above procedure repeated. The homogenate was added to the first tube.

This could then be stored at -70°C for future use. TRI reagent is a mixture of guanidine thiocyanate and phenol in a monophasic solution which effectively dissolves DNA, RNA, and protein on homogenization or lysis of tissue sample. After adding chloroform and centrifugation, the mixture separates into 3 phases: an aqueous phase containing the RNA, the interphase containing DNA, and an organic phase containing proteins. Each component can then be isolated after separating the phases. One ml of TRI Reagent is sufficient to isolate RNA, DNA, and protein from 50-100 mg of tissue, $5-10 \times 10^6$ cells, or 10 cm^2 of culture dish surface for cells grown in monolayer; according to manufacturer's protocol.

Sample was thawed when required and 200 μl of chloroform was added to each tube. This was then mixed by shaking and incubated at room temperature for 5 to 10 minutes. It was then centrifuged at $12000\times g$ for 20 minutes at 4°C and the aqueous phase transferred to a fresh tube.

500 μl of isopropanol (Sigma-Aldrich) was added to each tube, mixed and RNA allowed to precipitate overnight at 4°C . Following centrifugation as before the supernatant was discarded and two washes were performed with 1ml of 70% ethanol and repeat centrifugation performed at $12000\times g$ for 20 minutes. After the final wash, the supernatant was removed and the pellets were air-dried for 10 minutes before dissolving in 21 μl of sterile water.

1.1.2 RNA QUANTIFICATION

1 μl of each sample was diluted with 49 μl of nuclease free water.

The spectrophotometer (Beckman UV -DU64 - Beckman Instruments Ltd, High Wycombe, UK) was blanked using nuclease free water and the first reading taken was of the blank as a control. Optical density at 260 and 280 was measured. The amount of RNA in each sample was quantified using the following formula:

$\text{Abs}_{260} \times \text{dilution factor (50)} \times \text{RNA Coefficient (40)} = \text{RNA in } \mu\text{g/ ml}$

The A260/A280 ratio was also used as an indicator of RNA purity.

1.1.3 REVERSE TRANSCRIPTION

Quantification of specific mRNA was carried out by quantitative Polymerase Chain Reaction (qPCR) and the first step for this was generating cDNA by Reverse Transcription. Accurate quantification of RNA targets depends upon the performance of this step.

The method employed uses the random primer scheme to initiate cDNA synthesis. The total volume for the reaction was 20 μl . (High Capacity cDNA Reverse Transcription Kit Applied Biosystems)

1.1.3.1 PREPARATION OF 2X RT MASTER MIX:

2 μl of RT (Reverse Transcription) Buffer, 0.8 μl of 25X dNTP Mix, 2 μl of 10XRT Random Primers, 1 μl of multiscribe Reverse Transcriptase, 1 μl of RNase inhibitor and 3.2 μl of nuclease free water were combined making a total of 10 μl per reaction.

1.1.3.2 PREPARATION OF cDNA REVERSE TRANSCRIPTION REACTION:

10 μl of the master mix was added to each well of an 8 well strip and 1 μg of RNA sample was added to each corresponding well, together with nuclease free water to give a final volume of 20 μl , and the contents mixed. As negative control 1 tube was loaded with nuclease free water instead of the RNA sample. The strips were then sealed and centrifuged. All the above steps are done on ice.

The strips were then loaded on the thermal cycler (Applied Biosystems Gene Amp PCR System 7000 thermocycler) and the following programme was followed as per the kit protocol

	Step 1	Step 2	Step3	Step4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 sec	∞

Once the cycle had ended the strips containing the cDNA could stored at 4°C or -20°C until required.

1.1.4 QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

A relative quantitation assay was used to analyse changes in gene expression in a given sample relative to a reference sample. The comparative CT method was used for this. CT refers to threshold cycle where amplification is in linear range of the amplification curve. It is also defined as the fractional cycle number at which the fluorescence passes the threshold. To use this method a validation experiment was run to show that the efficiencies of the target and endogenous control amplifications were approximately equal. The advantage of using the comparative CT method is that the need for a standard curve is eliminated. This eliminates the adverse effect of any dilution errors made in creating the standard curve samples. Ribosomal RNA (rRNA) or rRNA was used as the internal control for all samples, and the results presented as the RQ calculated by the delta delta CT method using the equation: $2^{-[\Delta CT(1) - \Delta CT(2)]}$ (where $\Delta CT(1)$ is the mean ΔCT calculated for the experimental samples and $\Delta CT(2)$ is the mean ΔCT calculated for the control samples).

PCR was carried out using 7900 Fast Real-Time PCR system from Applied Biosystems.

TaqMan reagent-based chemistry uses a fluorogenic probe to enable detection of a specific PCR product as it accumulates during PCR cycles. A single primer pair was present in the reaction well. Only one target sequence or endogenous control was amplified per reaction. Three replicate reactions per sample and endogenous control were carried out to ensure statistical-accuracy.

Figure : Taqman gene expression assays (Applied Biosystems).

PRIMER	CATALOGUE NUMBER (Applied Biosystem)
ICAM 1	HS00164932_m1
HAS 2	Hs 00193435_m1
rRNA	4310893E
TNF α	HS00174128_m1
IL 1 β	HS01555410_m1
CD45	Hs00236304_m1

2.6 IMMUNOHISTOCHEMISTRY

Immunohistochemistry experiments were done to review sub-maximal stimulation of surface ICAM 1 on lung fibroblasts stimulated with varying concentration of TNF α .

Cells were grown in 8 well chamber slides. Cells were growth arrested for 48 hours and then stimulated with medium containing varying concentrations from 1×10^{-9} to 1×10^{-14} M of TNF α for 24 hours. Wells used as controls contained only serum free medium.

After 24 hours the medium was aspirated and cells washed with sterile PBS. Cells were fixed with 3.5% (w/v) paraformaldehyde for 15 minutes at room temperature, after

which the paraformaldehyde was removed and the cells washed with sterile PBS. Non specific binding was prevented by blocking with 1% BSA (wt/vol) in PBS for 1 hour.

Cells were then washed x2 with PBS 0.1% BSA and incubated with primary monoclonal antibody (Anti ICAM 1 antibody 10 µg/ml) for 2 hours at room temperature. Positive control staining was carried out using Vimentin and negative control staining was Cytokeratin (both at 1 in 50 dilutions)

Unbound primary antibody was removed after 2 hours with repeated washes with 1% BSA in PBS. Secondary antibody, Fluorescein Isothiocyanate (FITC) conjugated anti-mouse IgG (Dako) was then added and incubated for 1 hour. Cells were again washed as above to remove unbound secondary antibodies.

The cells were then mounted in Vectashield fluorescent mountant (Vecta Laboratories, Peterborough, UK) and examined under UV-light on a Leica Dialux 20 fluorescent microscope (Leica Microsystems Ltd, Milton Keynes, UK).

2.7 RNA INTERFERENCE

RNA interference offers a valuable tool to study the effect of selective gene knockdown with in the cells. This was done using transient transfection with specific siRNAs (short interfering RNA).

4×10^4 cells/ml were plated on 6 well plates and grown to 70 to 80% confluence The medium was changed to antibiotic and serum free medium for 4 hours prior to transfection. Transient transfection of fibroblasts was done using Lipofectamine 2000 transfection reagent (Invitrogen, Catalogue number 11668 -027). The transfection was done as per manufacturer's protocol.

Two master mixes were made. First mix was for the siRNA oligomers and second for the lipofectamine 2000. Specific siRNA oligonucleotides (sp1 and p3) were diluted in the Opti-MEM®I medium such that the final concentration of the siRNA when added to

the cells would be 33nM. 250 µl of the diluted complex is prepared for each well of a 6 well plate. 5 µl of lipofectamine was diluted in 250 µl of Opti-MEM® I. This was mixed gently and incubated for 5 minutes at room temperature.

The two master mixes were then mixed and incubated for 20 minutes at room temperature. At the end of this incubation 500 µL of the oligomer-Lipofectamine TM 2000 complexes was added to 500 µl antibiotic and serum free medium per well of the 6 well plate. The plates were gently rocked to allow the complexes to mix. They were then incubated for 24 hours at 37 C with 5% CO² for 24 hours. After incubation the medium containing the complexes was removed by aspiration and substituted with fresh serum and antibiotic free medium. RNA was then extracted at various time points as described before. Target gene expression was compared against that in cells transfected with scrambled control siRNA.

The transfection time was optimized prior to performing the experiments with siRNA transfection. Optimum time was considered to be the time point with maximal knockdown of the target gene with no loss of cell viability as assessed by the Alamar Blue method.

2.8 PROTEIN ESTIMATION: ELISA

Enzyme linked immunosorbent assay (ELISA) was used to analyse IL 1β and TNFα concentrations in cell culture. This was assessed using a commercial available kit (Human IL 1β BD OptEIA, BD Biosciences) and anti-human TNFα paired antibodies and recombinant standard, (R&D Systems).

A 96 well high-binding ELISA plate was initially coated with 100µL of capture antibody, 1 in 250 dilution in coating buffer, 0.1 molar sodium carbonate at 9.5 pH and

incubated overnight at 4degree C. They were then washed with wash buffer (PBS-0.05% Tween20) ad then blocked with 3% BSA PBS. Serially diluted assay standards (1000 pg/ml, 500pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml) and samples were added in duplicate and incubated for 2 hours at room temperature. Plates were washed again with the wash buffer 3 times. Biotinylated detection antibody was then added to each well and incubated for 1 hour for TNF and 2 hours for IL 1 β as per manufacturer's instructions. The plates were again washed with wash buffer 3 times.100 μ L of diluted (1 in 250 dilution) streptavidin-Horse radish Peroxidase conjugate was added and incubated for 30 minutes at room temperature. The plates were again washed with wash buffer 3 times. 100 μ L of TMB (Tetramethylbenzidine) substrate (SureBlue check manufacturer, KPL Inc, Maryland, USA) was added and incubated for 20 minutes. A blue colour develops in the standards wells. 50 μ L 2N sulphuric acid stop solution was added to each well and absorbance read on the plate reader at 450nm (with reference wavelength at 570nm).

The standard curve was calculated by plotting mean optical density vs standard concentration. R² value and equation of the trend line was generated. This equation was used to calculate the concentration of the proteins in the experimental samples.

2.9 ASSESSMENT OF CELL PROLIFERATION AND VIABILITY

Cell proliferation and viability was measured using the alamarBlue (Invitrogen) assay as per the manufacturers recommendations. Alamar Blue is a non toxte growth indicator that detects metabolic activity. The metabolic activity of growing cells causes a reduction of the alamarBlue molecule which is detected by an increase in the fluorescence of the molecule.

Briefly, the alamarBlue was added to a final of 10% (v/v) in fresh culture medium on the cells under investigation. Typically this was in a final volume of 250 μ l per well of a

24 well plate and 500 µl per well of a 6 well plate. The incubation was then continued for 1 hour, at which point 100 µl medium samples were taken and the fluorescence intensity read (Excitation 540 and Emission 590). Experimental cells were compared to untreated control cells in order to detect any effect of the various treatments on cell viability or proliferation.

2.10 STATISITCAL ANALYSIS

Statistical analysis was carried our using the ANOVA test and t - test.

3 ICAM 1 AND HAS 2 REGULATION IN A MODEL SIMULATING INTERACTION BETWEEN FIBROBLASTS AND LEUCOCYTES

3.1 INTRODUCTION

The role of adherence in various stages of life, in development and pathological, has already been discussed previously. One of the most important adhesion molecules and the primary Cell Adhesion Molecule in our study is Intercellular adhesion molecule-1 (ICAM-1).

3.1.1 INTERCELLULAR ADHESION MOLECULE-1

The human ICAM-1 gene is located on chromosome 19 and consists of seven exons and six introns, with each of the five immunoglobulin-like domains encoded by a separate exon. Intercellular adhesion molecule-1 (ICAM-1, CD54) is a transmembrane glycoprotein of 505 amino acids. It is a member of the immunoglobulin family. In contrast to other cell adhesion molecules, ICAM-1 mediates adhesive interactions by binding to two integrins belonging to the $\beta 2$ subfamily i.e., CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1) (42).

It is present on the cell surface of a wide variety of cell types including fibroblasts, leucocytes, keratinocytes, endothelial cells and epithelial cells. It is up-regulated by various pro-inflammatory signals including cytokines (IL 1β , TNF α and IFN γ), viruses, oxidative stress etc. (122, 123). This takes place through various signalling pathways involving MAP Kinases and transcription factors such as NF κ B and AP-1 (124-127). Many of these triggers act synergistically when present together rather than independently. For instance, TNF α and IFN γ when present together, up-regulate ICAM 1 expression much more than when they are present alone. The level of ICAM-1 expression on the surface of any given cell type depends on the concentrations of pro- and anti-inflammatory mediators and on the availability of specific receptor-mediated signal transduction pathways and their nuclear transcription factor targets on the ICAM-1 promoter. A number of signalling pathways and transcription factors are involved in

ICAM-1 transcription. This reflects the complex cell type-specific and stimulus-specific regulation of the ICAM-1 gene. The induction of ICAM-1 transcription occurs rapidly, being detected by nuclear run-on analysis as early as 30 min after treatment with TNF α (128).

ICAM 1 is the major adhesion molecule involved in cell-to-cell adhesion when mononuclear cells infiltrate the injury site. Cell adhesion mediated by ICAM-1 is critical for the trans-endothelial migration of leucocytes and the activation of T cells, where ICAM-1 binding functions as a co-activation signal as well (129).

3.1.2 ROLE OF MONOCYTES AND MACROPHAGES IN THE KIDNEY

Monocytes play an important role in all types of renal injury, be it in native kidneys or in renal transplants (34, 130, 131). Macrophages have been recorded in large numbers in acute diseases such as post-streptococcal glomerulonephritis (GN), ANCA associated GN and in chronic diseases such as IgA nephropathy or Systemic Lupus Erythematosis (SLE) (34, 130, 132).

Macrophages have an associative or causative role inflammation in the kidneys in various disorders, and also carry prognostic implications. In several renal diseases, the number of macrophages seen in renal biopsies is related to the outcome of the disease (133). Once activated, these macrophages secrete a broad range of cytokines through intracellular signalling pathways including NF κ B and MAP Kinases. These can be pro-inflammatory cytokines like TNF α , IL-1 β , IL-12, IL-18, IL-23, IL-6. They can also be pro-inflammatory chemokines like MIP-1, MIP-2, MCP, and generate reactive nitrogen and oxygen species (134, 135). Macrophages also secrete cytokines that can be beneficial to the kidneys; IL-10, HGF, FGF2, VEGF among others. One of the most important functions of macrophages is their ability to phagocytose. They can do this even in an inactive form in the absence of any of the above cytokines (136, 137). This

is important for a normal kidney to scavenge particles such as dying erythrocytes, immune complexes, dying leucocytes or cellular debris, which exist even in a non pathological state.(138, 139).

One interpretation would be that macrophages respond initially helping with repair of any injury, but in chronic injury or repetitive injury states they get activated with harmful effects (140, 141).

3.1.3 ROLE OF MONOCYTE AND ICAM 1 INTERACTION IN INFLAMMATION

Interactions between monocytes and adhesion molecules play a significant and varied role in inflammation and progression of various disorders. Monocyte and endothelial cell interaction, through ICAM-1 binding, plays a significant role in atherosclerotic disorders as well as vasculitis. Increased ICAM-1 expression has been correlated with increased infiltration of monocytes in cell culture experiments and in animal models (142, 143). Inflammatory mediators associated with endothelial cell activation, such as oxidized lipids and cytokines, can significantly increase ICAM-1 expression on cultured endothelial cell lines (144). Loss or inhibition of ICAM-1 expression has been reported to decrease both atherosclerotic and vasculitic lesion formation in animal models (145). Accumulation of LFA -1(CD-11+ and CD18+) cells in areas of tubulointerstitial damage has been noted in experimental models showing significant up-regulation of ICAM-1. In this model IL-1RA (IL-1 receptor antagonist) treatment partially reduced glomerular ICAM-1 expression and leucocyte infiltration. However, IL-1RA treatment resulted in a dramatic inhibition of interstitial ICAM-1 expression, interstitial leucocyte infiltration, and tubulointerstitial damage. This again highlights the importance of ICAM-1 in renal disease (146). Again in rat models it has been shown that up-regulation of periglomerular /peritubular capillary ICAM-1 expression is important for mononuclear cell entry into the interstitium, while interaction with fibroblast-like cells

may facilitate movement and subsequent focal accumulation of mononuclear cells at sites within the interstitium (19).

3.2 *AIMS*

The aim of the experiments in this chapter was to investigate the interaction between fibroblasts and monocytes with respect to the potential pro-inflammatory effects on ICAM-1 and HAS2 induction.

3.3 RESULTS

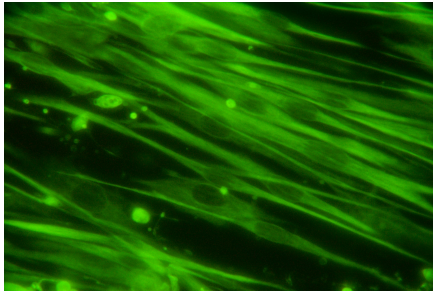
3.3.1 TNF α DEPENDENT ICAM 1 RNA INDUCTION ON THE SURFACE OF FIBROBLASTS IS DOSE DEPENDENT.

The aim of this experiment was to decide the concentration of TNF α that induced sub-optimal expression of ICAM 1 mRNA and protein on the surface of fibroblast for further experiments.

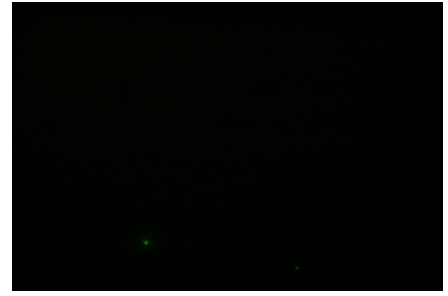
Fibroblasts were grown in 8 well plates as described in the Methods, and stimulated with various concentrations of TNF α for 24 hours. Surface expression of ICAM 1 was studied by immunohistochemistry. Vimentin was used as a positive control. Vimentin is the major structural component of intermediate filaments in cells of mesenchymal origin, e.g. fibroblasts and endothelial cells (147). Cytokeratin was used as a negative control, given that keratin polypeptides are constituents of tonofilaments found exclusively in epithelial cells and therefore are used as markers for epithelial cells (148).

ICAM 1 surface expression was induced by stimulation of fibroblasts with TNF α and was dose dependent. Strong expression was seen when fibroblasts were stimulated with 1×10^{-9} M TNF α . The expression decreased as the concentration of TNF α reduced and expression was barely visible at 1×10^{-13} M TNF α (*Figure 3-1*)

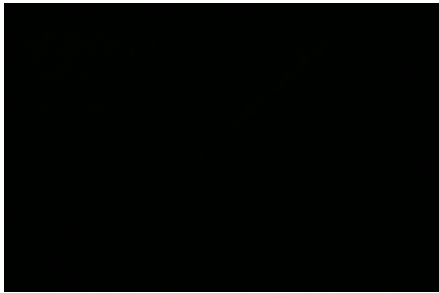
Figure 3-1 *Expression of ICAM 1 on surface of Lung Fibroblast after stimulation of Fibroblasts with TNF α : A dose response review*



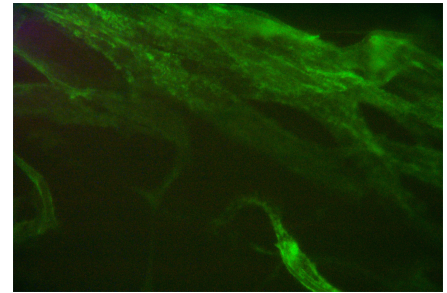
A. Positive control: Vimentin



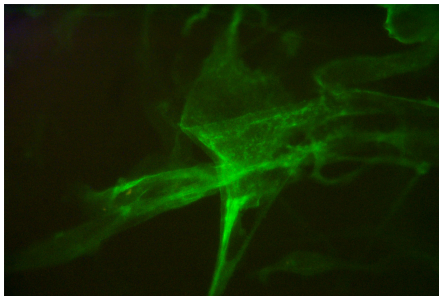
B. Negative Control: Cytokeratin



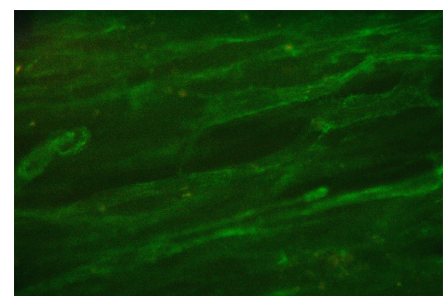
C. Unstimulated Lung fibroblasts



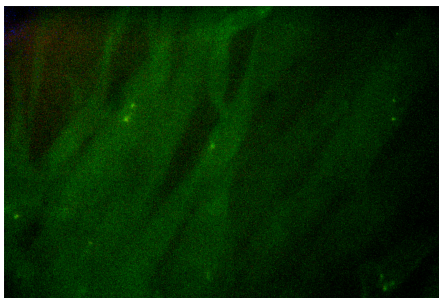
D. Stimulated with 1×10^{-9} M TNF



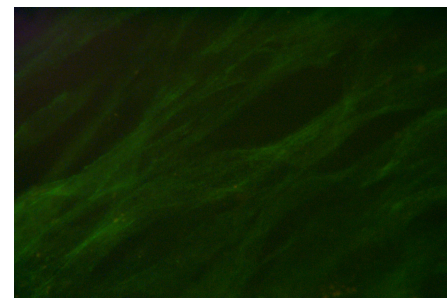
E. Stimulated with 1×10^{-10} M TNF α



F. Stimulated with 1×10^{-11} M TNF α



G. Stimulated with 1×10^{-12} M TNF α



H. Stimulated with 1×10^{-13} M TNF α

Experiments were then designed to quantify the up-regulation of ICAM 1 mRNA in response to stimulation with different concentrations of TNF α . Fibroblasts were plated in 6 well plates. Growth arrested fibroblasts were then treated with different concentrations of TNF α for 24 hours. Cells were washed with PBS 3 times and RNA extracted as discussed in methods. Reverse transcription and QPCR analysis for ICAM 1 showed a stable up-regulation of ICAM1 at a basal level with 1×10^{-12} M of TNF α . At 10×10^{-12} M TNF expression began to increase from basal levels and there was strong induction at 100×10^{-12} M (Figure 3-1). A TNF concentration of 1×10^{-12} M was used for sub-optimal induction of ICAM-1 in subsequent experiments.

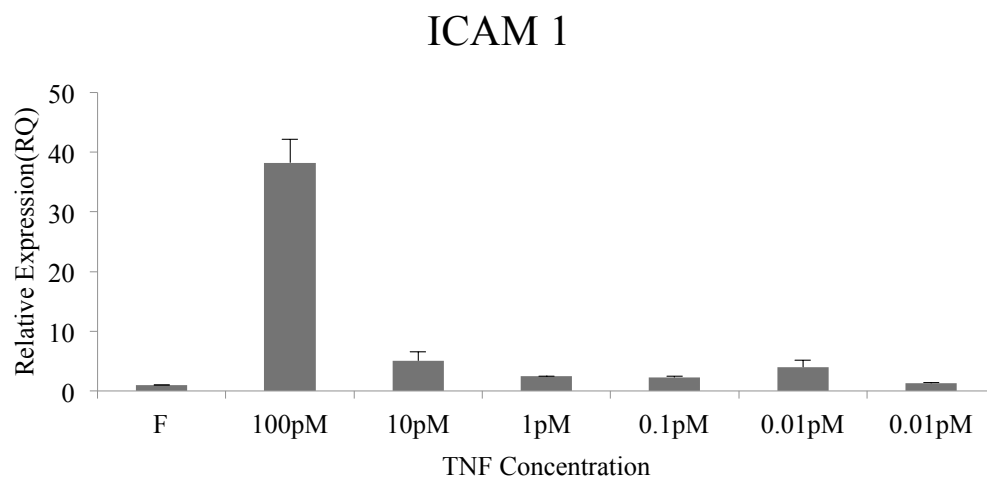


Figure 3-2 *Stimulation of Fibroblasts with different concentrations of TNF α*

Fibroblasts were incubated with different concentrations of TNF α from 10^{-14} M TNF α to 1×10^{-10} M TNF α . After 24 hours medium was aspirated and cells washed with PBS x 3 times. mRNA was extracted as described before. Q PCR results for ICAM 1 are represented as mean + SE of mean(n = 3)

3.3.2 TNF α CAUSES UP REGULATION OF ICAM 1 BUT NOT HAS 2 RNA

TNF α is known to stimulate expression of ICAM 1 on the surface of fibroblasts. It was important to investigate whether 1pM TNF α , which caused submaximal expression of surface ICAM 1 would also up-regulate HAS 2. If proven this could prove a synergetic effect of TNF α in promoting pro-fibrotic process by inducing expression of both ICAM 1 and HAS 2. On the other hand if it showed a differential up-regulation, it would suggest a secondary process occurring simultaneously when fibroblasts interact with monocytes.

Fibroblasts were grown in 6 well plates. Cells were growth arrested for 48 hours once confluent and stimulated with 1×10^{-12} M TNF α for a time course. RNA was extracted at times up to 24 hours. RT and QPCR were done as described above.

There was significant up-regulation of ICAM 1 mRNA peaking at 4 hour that then came down to baseline levels within 24 hours. In contrast there was no significant up-regulation of HAS 2 at mRNA level (*Figure 3-3*)

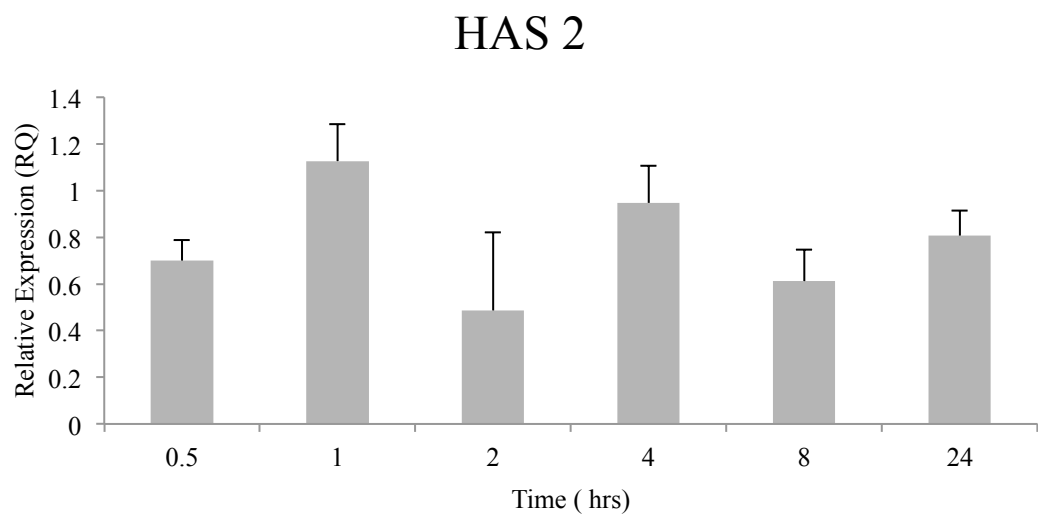
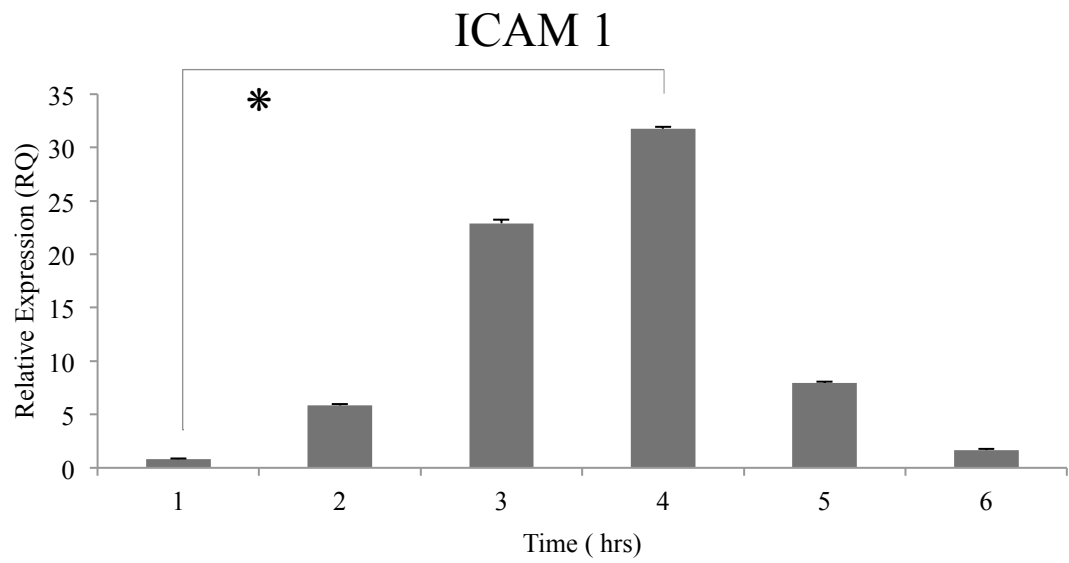


Figure 3-3 *Stimulation of Lung Fibroblasts with 1×10^{-12} M $TNF \alpha$*

Fibroblasts were incubated with 1×10^{-12} M $TNF \alpha$. At set time points cells were washed with PBS x 3 times. mRNA was extracted as described before.

Q PCR results for ICAM 1 and HAS 2 expression are represented as mean + SE of mean normalised to 0.5hrs time point (n = 3). P value for ICAM1 comparing time 0 and 4 hours (p value: * <0.001)

3.3.3 CROSS LINKING OF SURFACE ICAM 1 ON FIBROBLASTS LEADS TO UP-REGULATION OF ICAM 1

Leucocyte binding to fibroblasts (isolated from both the human renal cortex and lung) and to endothelial cells induced the de novo synthesis of ICAM-1 mRNA and protein through the ICAM-1- dependent activation of the cultured cells. This has been mimicked by cross-linking ICAM 1 on the surface of different cell lines using anti-ICAM 1 antibodies. Cross-linking of cell surface ICAM 1 on TNF α -stimulated lung fibroblasts in this study also led to an increase in the ICAM 1 mRNA which peaked at 1 hour (1, 119) (*Figure 3-4*). In addition, cross-linking cell surface ICAM-1 also up-regulated HAS 2mRNA, which peaked at 4 hours. Given the cross-linking model was designed to imitate the ICAM-1-dependent interaction of fibroblasts with leucocytes, it would suggest this interaction may also lead to induction of HAS 2 with ICAM 1-dependent binding playing an important role.

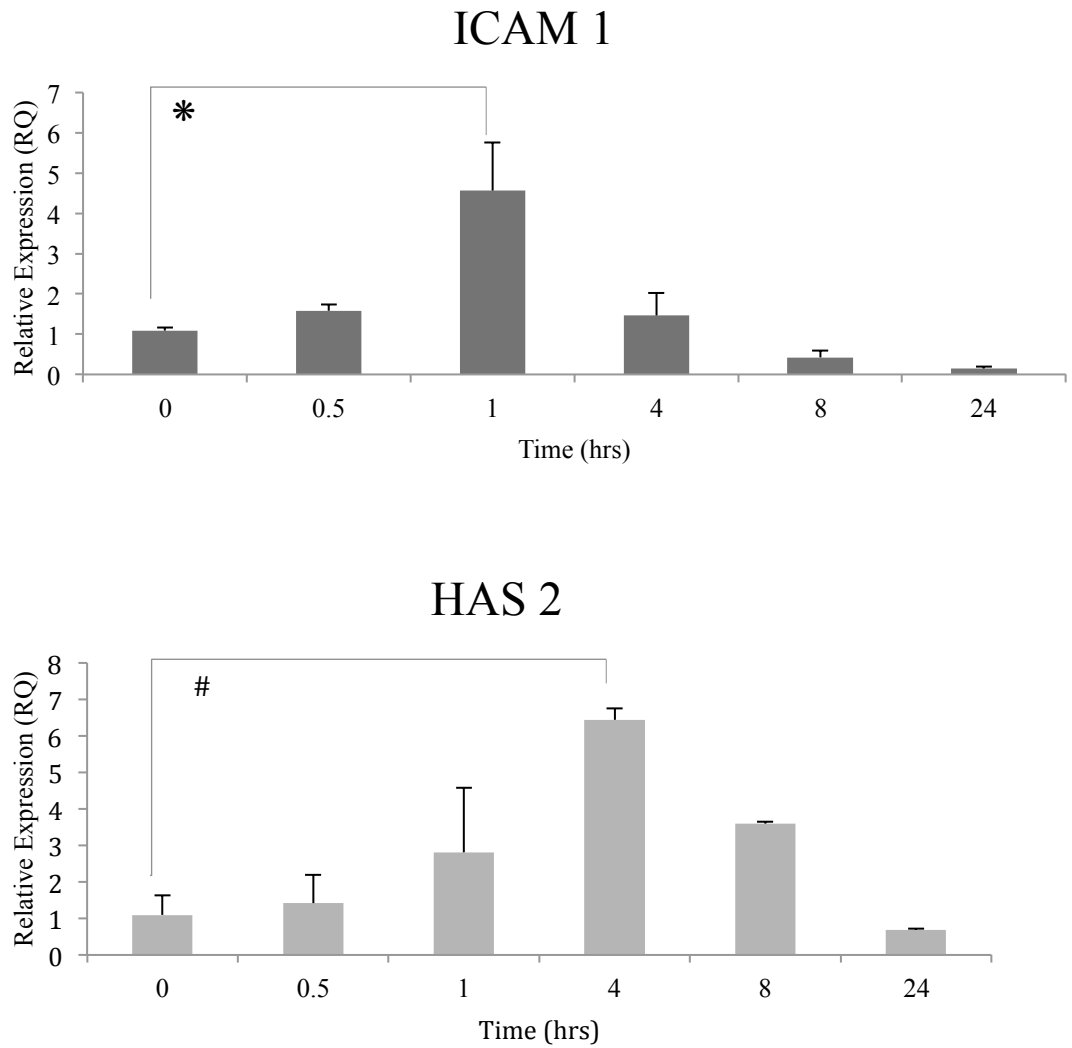


Figure 3-4 *ICAM 1 and HAS 2 mRNA expression following ICAM-1 cross-linking*

Fibroblasts were incubated with 10^{-12} M $\text{TNF}\alpha$ for 24 hours after growth arresting. Cells were washed with sterile PBS and incubated with anti – ICAM 1 monoclonal IgG in serum free DMEM F12 HAM for 1 hour. Cells were then washed with PBS (\times 3 times) and primary antibody was cross linked using goat anti mouse IgG antibody at a concentration of $10\mu\text{g/ml}$. mRNA was extracted as discussed in methods. RT and qPCR for ICAM-1 and HAS 2 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=6) , p value *=0.020,#<0.001

3.3.4 CO-CULTURE OF FIBROBLASTS WITH U937 CELLS LEADS TO UP-REGULATION OF ICAM 1 AND HAS 2

Lung Fibroblasts were plated in 6 well plates and growth arrested for 48 hours when near confluence. Co-culture with 5×10^5 U937 cells/ml was then carried out using the method described before. There was significant up-regulation of ICAM 1 mRNA. This peaked at 4 hours. There was also up-regulation of HAS 2 mRNA, which peaked at 4 to 8 hours (*Figure 3-5*).

Co-culture with U937 cells was carried out with lung fibroblasts stimulated with 1×10^{-12} M TNF α after cell cycle synchronisation as well as unstimulated fibroblasts. No significant difference was seen in the peak up-regulation of either ICAM 1 or HAS 2 between the two co-culture models (TNF α stimulated fibroblasts or unstimulated fibroblasts with U937 cells). (*Figure 3-6*). The increase, however, was maintained for longer. This is important, as it would suggest that even unstimulated fibroblast have the capacity to up-regulate ICAM 1 and HAS 2 and therefore may have a role to play in propagating inflammation. Under the influence of an inflammatory cytokine, however, this response would be prolonged.

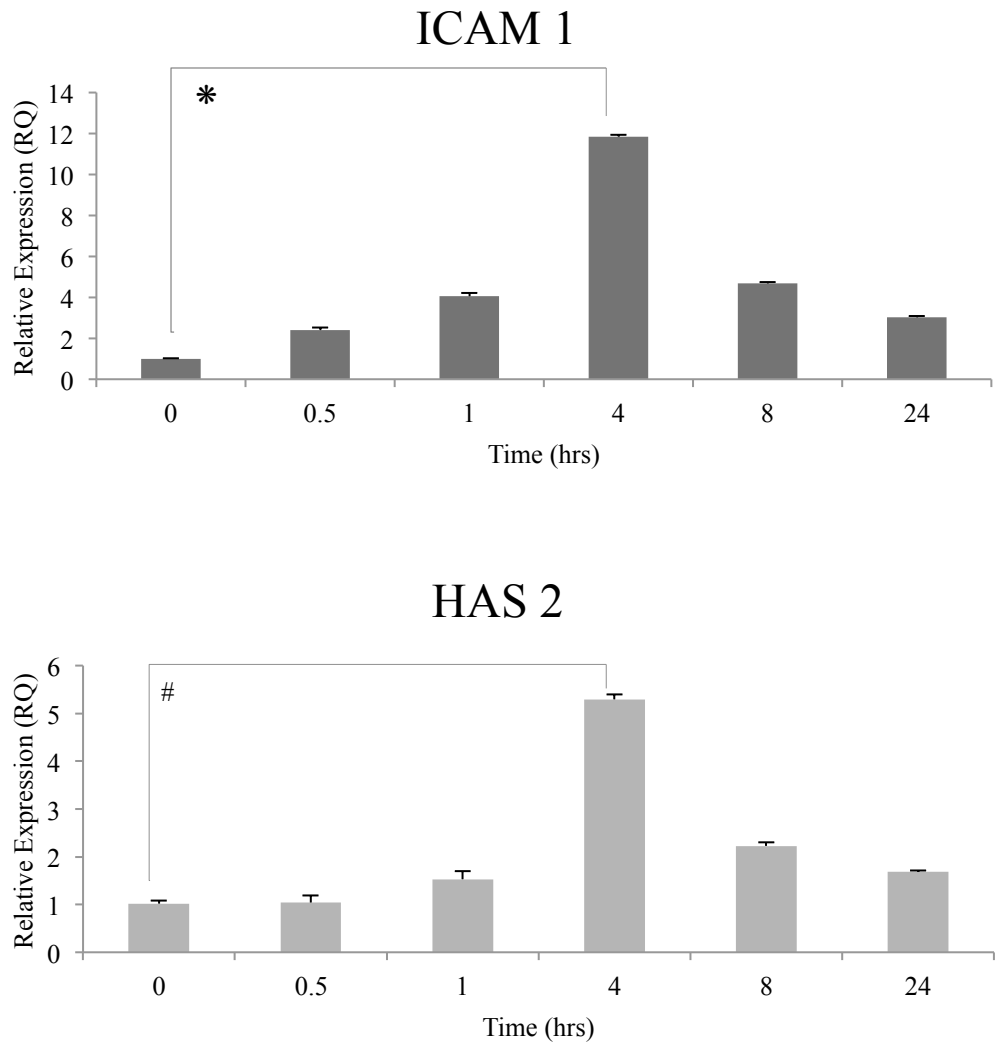
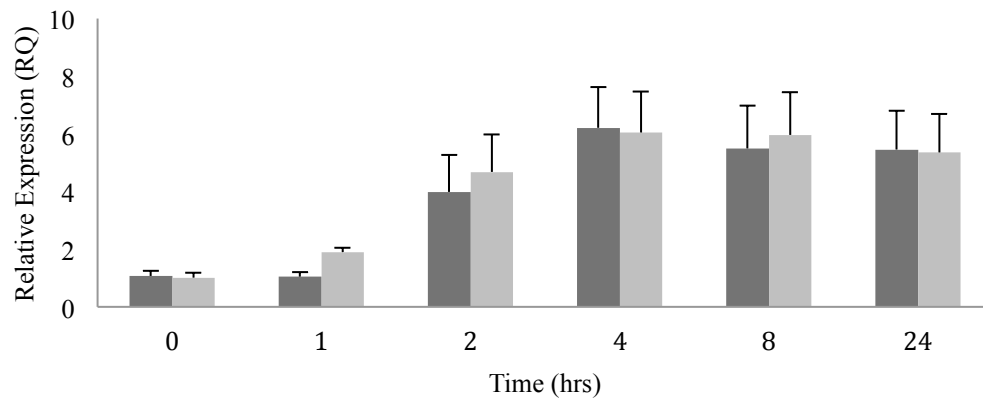


Figure 3-5 *ICAM 1 and HAS 2 mRNA expression following Lung Fibroblasts and U937 cell co-culture*

Fibroblasts were incubated with 5×10^5 U937 cells for times up to 24 hours. Cells were washed with PBS ($\times 3$ times) to wash the U937 cells off and mRNA extracted as described. RT and q-PCR for ICAM-1 and HAS 2 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=6), p value: * = 0.006, # = 0.022

ICAM 1



HAS 2

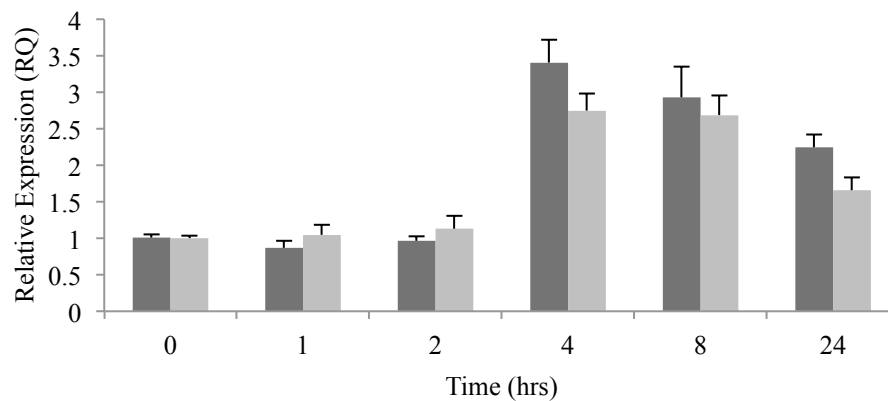


Figure 3-6 Comparison of ICAM 1 and HAS 2 mRNA expression following co-culture of TNF α stimulated Fibroblasts and U937 cells with co culture of unstimulated Lung Fibroblasts and U937 cells.

■ Fibroblasts were incubated with 10^{-12} M TNF α for 24 hours. Medium was aspirated and cells washed with PBS $\times 3$ times. These were then incubated with 5×10^5 U937 cells for times up to 24 hours. Cells were washed with PBS ($\times 3$ times) to wash the U937 cells off and mRNA extracted as described before. RT and qPCR for ICAM-1 and HAS 2 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=6).

□ At the same time Fibroblasts were incubated with 5×10^5 U937 cells for times up to 24 hours. Cells were washed with PBS ($\times 3$ times) to wash the U937 cells off and mRNA extracted as described before. RT and qPCR for ICAM-1 and HAS 2 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=6).

3.3.5 CO-CULTURE OF FIBROBLASTS AND ACTIVATED U937 CELLS LEADS TO EXPONENTIAL UP-REGULATION OF ICAM 1 AND HAS 2 mRNA

In an on-going inflammatory process the monocytes recruited to the site of inflammation get activated into a more macrophage-like state. To assess whether the interaction between fibroblast and U937 cells was altered if the U937 cells were activated, we repeated the experiment above with PMA-activated U937 cells. PMA (phorbol myristate acetate) leads to clustering of CD11a/CD18 integrin molecules at the cell surface, without any increase in integrin affinity (149). It therefore offers greater possibility of cell-to-cell binding. This is important as we have already shown that even in an unstimulated state fibroblasts and U937 cells up-regulate ICAM 1 and HAS 2, thus propagating pro-inflammatory process.

Co-culture experiments were carried out with lung fibroblasts and PMA activated U 937 cells, as described earlier. There was an exponential increase in both ICAM 1 and HAS 2 mRNA that was statistically significant (*Figure 3-7*).

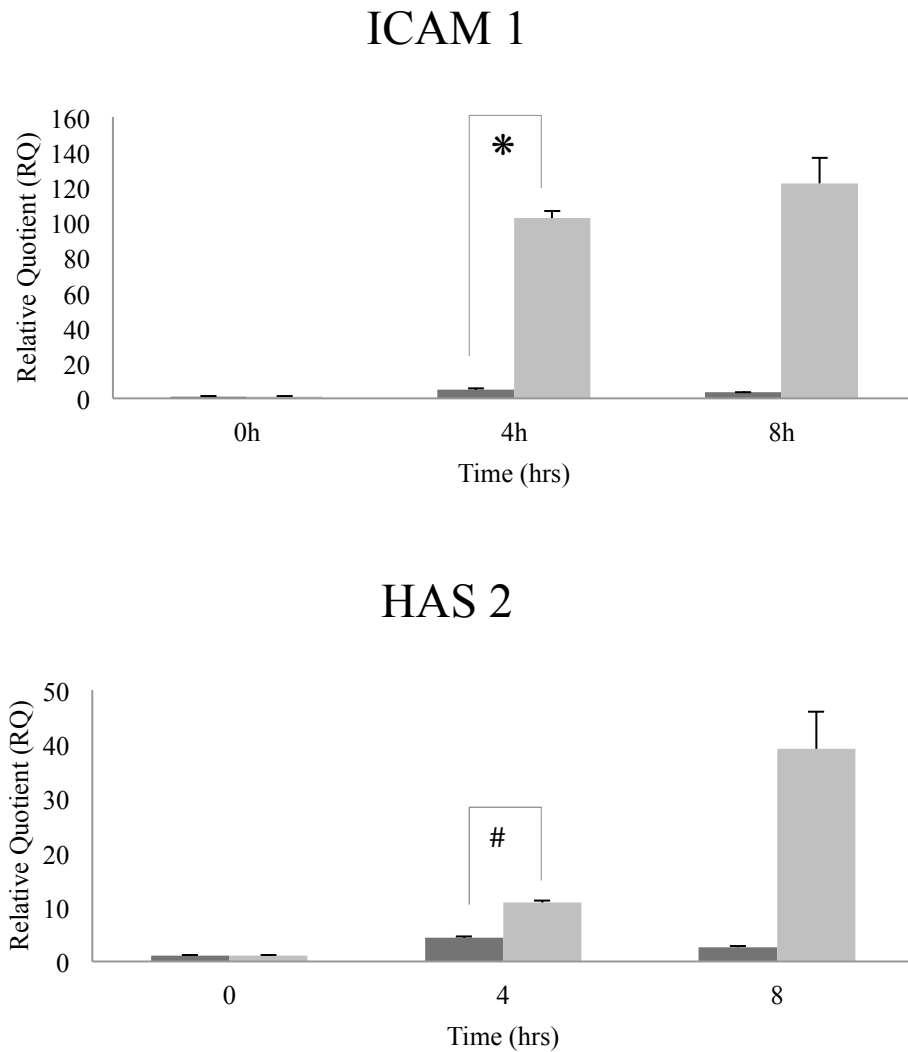


Figure 3-7 Comparison of ICAM 1 and HAS 2 mRNA expression following co culture of Lung Fibroblasts and Activated U937 cells and Lung Fibroblasts and U937 cells.

U937 were activated by treating them with 160nM Phorbol Myristate Acetate (PMA) for 48hours. Majority of the cells were adherent to the plastic flasks. Cells were taken off the flask using 0.1% EDTA and then centrifuged and re-suspended in DMEM F12 HAM medium.

■ Fibroblasts were incubated with 5×10^5 U937 cells for times up to 24 hours. Cells were washed with PBS ($\times 3$ times) to wash the U937 cells off and mRNA extracted as described before. RT and qPCR for ICAM-1 and HAS 2 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=6).

□ Fibroblasts were incubated with 5×10^5 activated U937 cells for times up to 24 hours. U937 cells were taken off the flask using 0.1% EDTA and Cells were washed with PBS ($\times 3$ times) to wash the U937 cells off and mRNA extracted as described before. RT and qPCR for ICAM-1 and HAS 2 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=6)

P value: * <0.001 , # <0.001

It was important to confirm that activated U 937 cells were not the source of the exponential rise of HAS 2 mRNA . For this purpose U 937 cells and activated U 937 cells were plated in 6 well plates and RNA extracted as explained before at times up to 24 hours. HAS 2 expression was reviewed using reverse transcription and Q-PCR as before. There was no rise in HAS 2 expression by U 937 cells over 24 hrs as shown by lack of amplification of the HAS 2 sequence by Q-PCR (*Figure 3-8*). This would therefore confirm that the HAS 2 mRNA was expressed by the fibroblasts in the fibroblast and activated U937 cells interaction, as there was no detectable HAS 2 expression in the U937 cells and activated U937 cells. I have shown that U937 cells and act U937 cells, in isolation do not express HAS 2 using qPCR. Another way of confirming this would be to separate the fibroblasts and U937 cells from each other using cell separation technique such as FACS and then performing RT qPCR for HAS 2. Newer techniques are also available which rely on mechanical separation of monocytes off the fibroblasts using micromanipulators such as CellclectorTM. I have shown upregulation of ICAM 1 and HAS2 in experiments where ICAM 1 has been cross-linked on the surface of the fibroblasts. Additional experiment using U937 cells and cross linking surface receptors such as CD18 can be attempted to simulate activation of U937 cells and investigated as possible source of ICAM 1 and HAS2 but were not done in the present thesis.

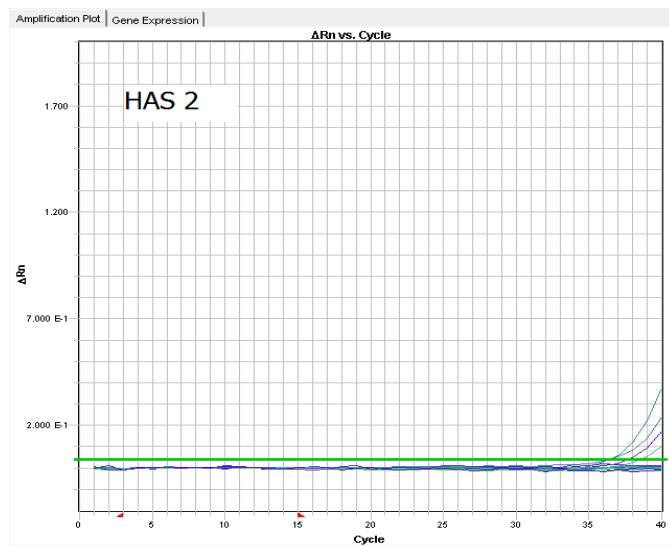


Figure 3-8 *HAS 2* expression in plated U937 cells and PMA activated U937 cells over a time course: This showed the lack of any amplification after 40 cycles

DISCUSSION

In this chapter, I have shown that co-culture of fibroblasts and U937 cells resulted in up-regulation of ICAM 1 and HAS 2 mRNA. This was mimicked in experiments where ICAM-1 on the cell surface was cross-linked using primary and secondary antibodies (119).

Fibroblast and U937 cell interaction has been studied previously in various models. In systemic sclerosis it has been shown that there is an over expression of ICAM-1 on dermal fibroblasts. In these experiments, the use of neutralising anti-ICAM 1 antibody greatly reduced U937 cell binding to these fibroblasts (150). It has also been shown in the alveolar epithelial carcinoma cell line, A549, that binding to U937 cells is ICAM-1 dependent (126). ICAM-1 up-regulation in this co-culture was found to be TNF α mediated and involved MAP kinases and NF κ B and AP-1 activation (151). U937 cells can also induce ICAM 1 mRNA in other cell lines. Interaction between Human Umbilical Vein endothelial cells and U937 cells led to up-regulation of adhesion molecules ICAM 1 and VCAM-1. This was found to be IL 1 β and TNF α mediated(152).

The interaction in fibroblast-U937 cell co-culture is therefore possibly mediated by ICAM 1 linked binding. Up-regulation of ICAM 1 would promote leucocyte infiltration and inflammation at site of any form of injury. Cross-linkage experiments have shown that up-regulation of ICAM 1 causes a positive feedback loop which would further up-regulate induction of ICAM-1.

In addition to up-regulation of ICAM-1, I have also shown up-regulation of HAS 2 mRNA in fibroblast-U937 cell co-culture. It has also been shown that intermediate molecular weight products of hyaluronan markedly stimulate the expression of the adhesion molecules ICAM-1 and VCAM-1 in cultured kidney tubular cells (153). This response was seen in some cell lines but not in others such as the 3T3 cell line (mouse

fibroblasts). In the cells that responded, it was seen that NF- κ B and AP-1 activities were induced in response to HA and the authors suggested that these transcription factors could participate in the regulation of adhesion molecule expression in MCT cells. Given hyaluronan can also stimulate ICAM 1 induction, the above process could work synergistically in promoting fibrosis and offer two different targets to alter the fibrosis promoting events.

ICAM 1 mRNA up-regulation, is exponentially increased when U937 cells are activated with phorbol esters. Phorbol esters are strong activators of leucocyte adhesion. Activation of adhesion by these agents takes place by clustering of CD11a/CD18 integrin molecules at the cell surface, allowing more receptors to be available for binding (154). Phorbol esters such as phorbol-12-myristate 13-acetate (PMA) have pleiotropic effects on cells. Phorbol exerts its biologic effects by altering gene expression through the activation of PKC and modulating the activity of transcriptional factors such as AP-1, NF κ B and PU-1 (155, 156). Increased leucocyte binding after activation with PMA has been seen in mesangial cells and has been shown to be ICAM 1 dependent (157).

I have shown that ICAM 1 and HAS 2 up-regulation takes place in the fibroblast and U 937 cell interaction and this is also confirmed in ICAM 1 cross linkage experiments which mimic ICAM 1 binding. It would therefore be logical to hypothesise that ICAM 1 binding plays a significant role in the fibroblast and U937 interaction. A significant increase in the up-regulation of ICAM 1 and HAS2 mRNA in the presence of PMA-activated U937 cells also adds evidence for the role of ICAM 1 binding based on mechanisms involving increased interaction of fibroblast ICAM-1 with the up-regulated CD18 integrins on the activated U937 cell surface. This will be further investigated in subsequent chapters.

**4 MECHANISM BEHIND UP-REGULATION OF ICAM 1 AND
HAS 2: COMMON OR DIFFERENT PATHWAY?**

4.1 INTRODUCTION

In the previous chapter I have shown that there is a significant role played by cell interaction (probably through ICAM 1 binding) in the up-regulation of ICAM 1 and HAS2. In previous studies, when U937 cells were co-cultured with synovial fibroblasts they created an environment very similar to osteoarthritic synovial fluid. Expression of CD80, a macrophage activation marker, suggested that U937 cells became activated when cultured with the synovial fibroblasts. This model also showed the importance of cell-to-cell contact between the synovial fibroblasts and U937 cells (158). Cell-to-cell contact is also deemed important in studies with lung fibroblasts to assess the role of mononuclear cell and fibroblast contact in promoting alveolar inflammation and fibrosis (159).

Conversely, there is also work that shows the cell-to-cell contact may not be essential in other fibroblast-U937 cell co-cultures. Fibroblasts, obtained from either rheumatoid arthritis or osteoarthritis synovial tissue, could mediate cartilage degradation if co-cultured with U937 macrophages. Fibroblast-macrophage contact was not required for cartilage degradation. Macrophage-conditioned media was sufficient to cause the degradation. Soluble factors released by the macrophage appear to be, at least in part, responsible for the fibroblast activation. Cartilage degradation by synovial fibroblasts was inhibited by antibodies to TNF α , IL-1 β and IL-6. The degradation process was almost completely inhibited by a combination of antibodies to TNF α and IL-1 β (160).

Co-culture experiments between fibroblast cell lines (16Lu) and mononuclear cells isolated from blood have shown differential up-regulation of different chemokines. It showed that even though the two chemokines are produced during the adhesion event, they are differentially regulated. MIP-1 α appears to be dependent upon adhesion-mediated pathways, where as MCP-1 appears to require cytokine signalling pathways for its production. They appear to be differentially induced during the cell-to-cell

interactions (135). It has also been shown that in T cell-synovial fibroblast interactions $\text{TNF}\alpha$, ICAM 1 and VCAM 1 get up-regulated. There was, however, reduced ICAM 1 and $\text{TNF}\alpha$ expression when cells were co-incubated in the presence of inserts preventing cell-to-cell contact (161).

4.2 *AIM*

My observations and the evidence discussed above raised important questions about the role of cell-to-cell contact in causing up-regulation of ICAM 1 and HAS 2 and the role of ICAM 1 binding. Cross-linking experiments had shown the potential role of cell contact with ICAM 1 cross-linking causing up-regulation of ICAM 1 and HAS 2 mRNA.

This led to the hypothesis that if the cell-to-cell contact between adhesion molecules on fibroblasts and U937 cells in co-culture experiments was interrupted, the up-regulation of ICAM 1 and HAS2 mRNA should also be inhibited. In this chapter I also studied the possible mechanisms behind ICAM-1 and HAS 2 mRNA up-regulation and assessed if these were along common or different pathways.

4.3 RESULTS

4.3.1 ROLE OF CELL TO CELL CONTACT IN FIBROBLAST-U937 CELLS CO-CULTURE

Co-culture experiments were designed using 0.4-micron membrane inserts to eliminate the cell-to-cell contact and binding. The inserts were chosen on the basis that the U937 cell size is 13microns. To investigate the degree to which cell-to-cell contact was involved in the activation of fibroblasts, cells (AG02262) were plated in 6 well plates and cultured to near confluence. They were then growth arrested by growing them in serum free culture media for 48 hours. Fibroblasts and 5×10^5 U937 cells/ml were incubated in separate plates as well allowing cell to cell contact for 4 and 8 hours as control. In the experimental plates 0.4 micron membrane inserts were suspended over growth-arrested fibroblast monolayers and 5×10^5 U937 cells/ml were added into the insert to interrupt the cell to cell contact but still allow liquid phase contact with the medium. Medium was added outside the insert to allow contact between the 2 phases to allow movement of solutes. Cells were washed with PBS ($\times 3$ times) to wash the U937 cells off and mRNA extracted as described before. RT and Q-PCR for ICAM-1 and HAS 2 were performed.

Preventing cell-to-cell contact using the membrane inserts resulted in a significant reduction in ICAM-1 mRNA induction, p value < 0.001 . In contrast, HAS 2 up-regulation was unaffected in the presence of inserts (figure 4-1). This would suggest that there might be additional mechanisms involved in addition to cell-to-cell contact in the regulation of HAS 2 in comparison to ICAM 1, which appears to be regulated mainly by cell adhesion.

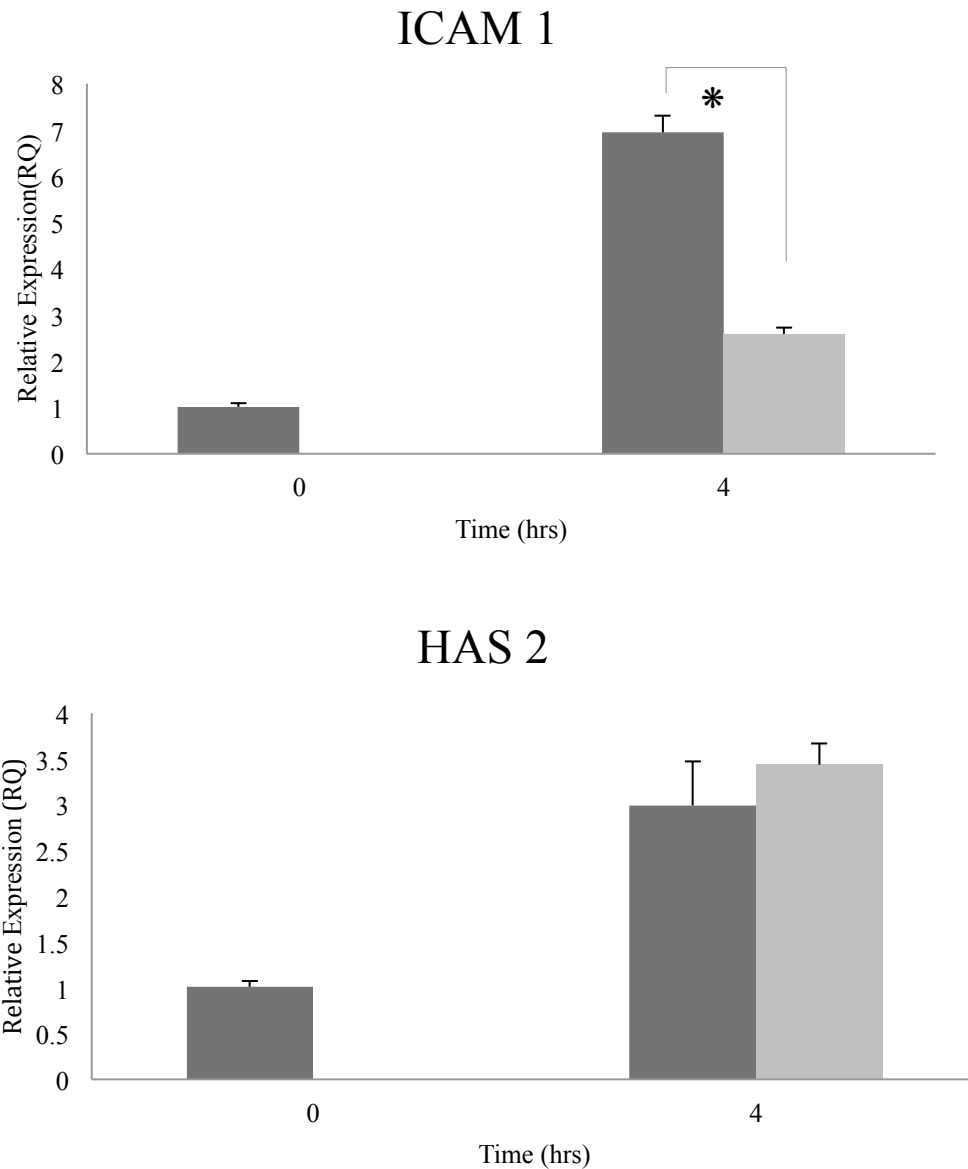


Figure 4-1 Role of Cell-to-Cell Contact: ICAM-1 and HAS-2 mRNA expression following Fibroblasts and U937 cells incubation in the presence/ absence of 0.4 μ membrane inserts.

■ Fibroblasts and 5×10^5 U937 cells/ml were incubated for 4 and 8 hours as control. □ 0.4 μ membrane inserts were suspended over Fibroblasts monolayer and 5×10^5 U937 cells/ml were added into the insert. Medium was added outside the insert to allow contact between the 2 phases to allow movement of solutes. Cells were washed with PBS ($\times 3$ times) to wash the U937 cells off and mRNA extracted as described before. RT and qPCR for ICAM-1 and HAS 2 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=6), p value * < 0.001

4.3.2 INHIBITION OF FIBROBLAST-U937 CELLS INTERACTION WITH SOLUBLE ICAM

To confirm whether the interaction between fibroblasts and U937 cells involved ICAM-1 dependent binding to U937 CD18 receptors, fibroblasts were incubated with U937 cells in the presence of soluble ICAM-1. Fibroblasts were grown to near confluence in 6 well plates, growth arrested and incubated with varying concentrations of soluble ICAM 1 for 1 hour. After 1 hour the medium was aspirated and cells washed with PBS x 3 times. Co-culture experiments using 5×10^5 U937 cells were then conducted as before. There was a dose dependent reduction in the up-regulation of ICAM 1 (p value 0.045) and HAS 2 (p value 0.020) in the presence of increasing concentrations of soluble ICAM-1 (figure 4-2a). This again showed that there was a role for cell binding in the up regulation of ICAM 1 and HAS 2. The binding was assessed directly using CD45 as a marker for U937 cells binding. There was a dose-dependent reduction in the expression of CD45 mRNA in the presence of soluble ICAM, though not statistically significant (p value 0.080), thereby supporting the role of ICAM dependent binding interaction (figure 4-2b).

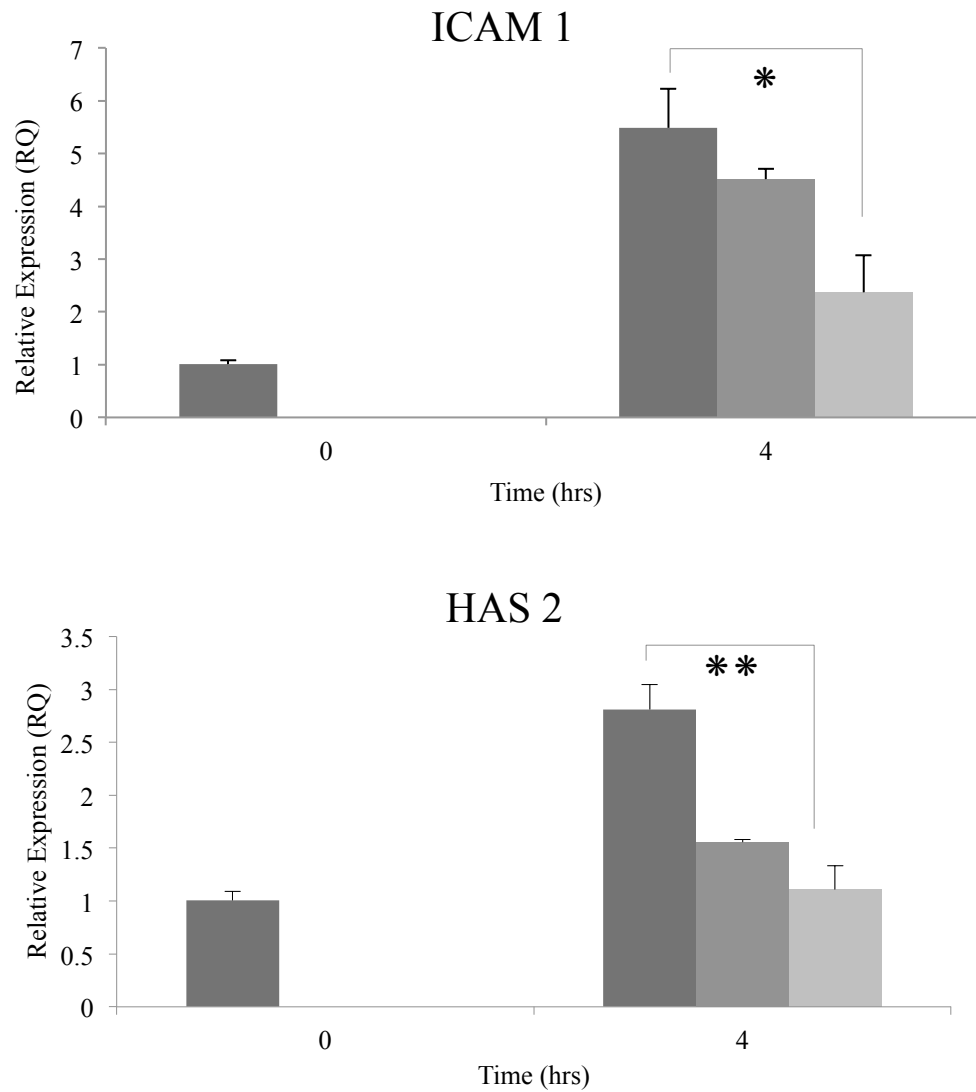


Figure 4-2a: Fibroblast and U937 interaction in the presence of Soluble ICAM 1

■ Fibroblasts were incubated with 5×10^5 U937 cells/ml in the presence/ absence of soluble ICAM in varying concentration to assess dose response,
 □ 800ng/mL S ICAM, ■ 400ng/mL S ICAM
 Cells were washed with PBS (x3 times) to wash the U937 cells off and mRNA extracted as described before. RT and qPCR for ICAM-1 and HAS 2 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=3), p value: *=0.045,**=0.020

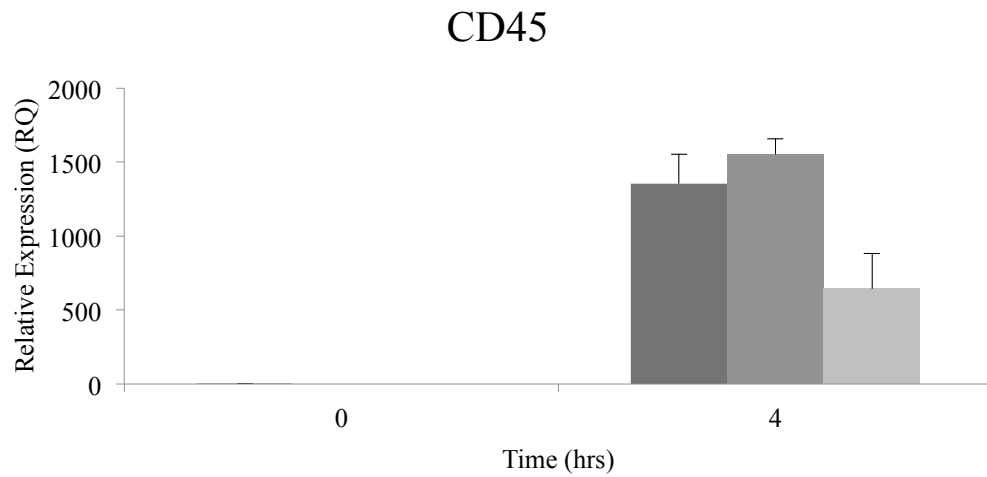


Figure 4-2b: *Fibroblast and U937 interaction in the presence of Soluble ICAM 1*

■ Fibroblasts were incubated with 5×10^5 U937 cells/ml in the presence/ absence of soluble ICAM in varying concentration to assess dose response,

□ 800ng/mL S ICAM

■ 400ng/mL S ICAM

Cells were washed with PBS (×3 times) to wash the U937 cells off and mRNA extracted as described before. RT and qPCR for CD45 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=3)

4.3.3 FIBROBLAST AND U937 CELLS CO-CULTURE IN PRESENCE OF β 2 INTEGRIN ANTIBODY

Another way to confirm that ICAM 1 - CD18 interaction was involved in the response of fibroblast to U937 cells was to block the β 2 integrins on the U937 cell. Fibroblasts were again grown to near confluence and then growth arrested for 48 hours before the experiment. U937 cells were incubated with 20mcg/ml of β 2 integrin antibody (Integrin β 2 (TS1/18), Santa Cruz Biotechnologies Inc.) for an hour before the co-culture. Co-culture was then carried out as in previous experiments. The experiment was conducted as a 4-hour timed course based on previous experiments and mRNA extracted for analysis by reverse transcription and qPCR.

The experiment showed down-regulation in the expression of both HAS 2 and ICAM 1 mRNA in the presence of β 2 integrin antibodies, highlighting the importance of cell-to-cell contact in the up-regulation of ICAM 1 and HAS 2 in fibroblast and U 937 cell co-culture using CD18-mediated ICAM-1 binding. The reduction in ICAM 1 mRNA induction was significant with a p-value of 0.038. Despite this reduction, there was still statistically significant induction of ICAM 1 mRNA in comparison to plated fibroblasts with a p-value of 0.013. This would suggest that there are other mechanisms also involved in this process, though ICAM 1 binding may be the more dominant one.

The reduction in HAS 2 mRNA up-regulation was present but not significant statistically with a p value of 0.394 (*Figure 4-3*). This would suggest a greater role of cell-to-cell contact with ICAM 1 binding playing a more significant role in ICAM 1 mRNA up-regulation in comparison to HAS2 mRNA induction.

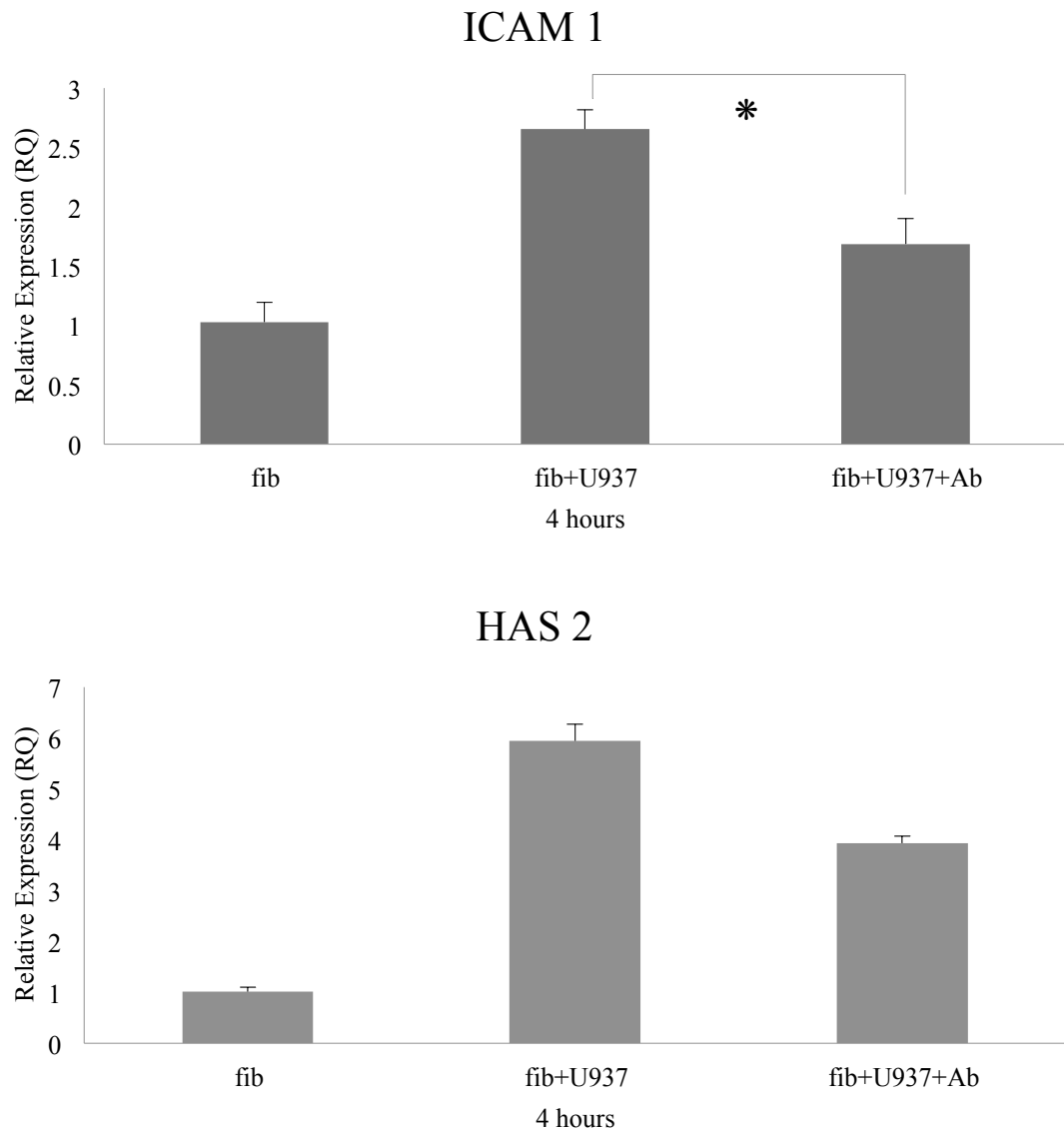


Figure 4-3: Fibroblast and U937 cells co-culture in the presence of $\beta 2$ Integrin Antibody

Fibroblast monolayer were incubated with 5×10^5 U937 cells in the presence/absence of $20 \mu\text{g/ml}$ of $\beta 2$ Integrin Antibody, (TS1/18), sc-53712. Cells were washed with PBS (3 times) to wash the U937 cells off and mRNA extracted after 4 hours. RT and qPCR for ICAM-1 and HAS 2 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=3).p value: *=0.038

4.3.4 FIBROBLAST RESPONSE TO THE PRESENCE OF CONDITIONED MEDIUM FROM FIBROBLAST AND U937 CELL INTERACTION

Experiments with the use of medium conditioned by the contact of fibroblast and U937 cells were designed to study other mechanisms that may be involved in the up-regulation of ICAM 1 and HAS2 mRNA. This medium would contain any soluble factors released from the cells that may play a role in the up-regulation of ICAM 1 and HAS 2.

Lung fibroblast monolayers were growth arrested when nearly confluent. After 48 hours 5×10^5 U 937 cells/ml were added to the 6 well plates. Medium was then aspirated after 15 minutes, 30 minutes, 1 hour, 2 hour and 4 hours of co-incubation. This was then centrifuged at 7000 rpm for 7 min. The supernatant was then used as conditioned medium. This initial experiment was designed to decide the duration of time the fibroblasts need in which to interact with the U 937 cells (*Figure 4-4*).

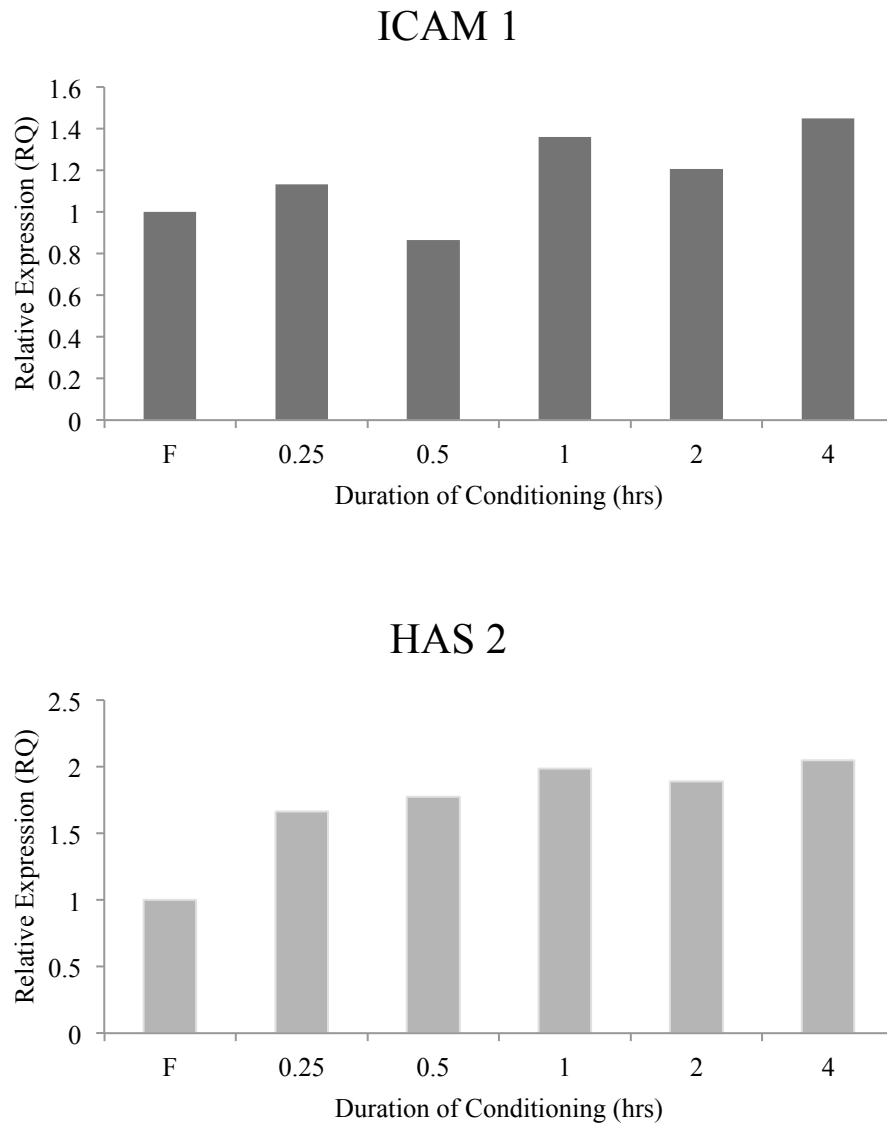


Figure 4-4: Fibroblast response to incubation with conditioned medium from fibroblast-U937 cell co-culture

Fibroblast monolayer was plated in 6 well plates. After 48 hours of growth arrest period in serum free medium 5×10^5 U 937 cells were added in serum free medium. The supernatants were aspirated from respective wells at 15 minutes, 30 minutes, 1 hour, 2 hour and 4 hour of co-incubation. Any cells in the aspirate were pelleted by centrifugation. Fibroblasts were then exposed to this medium for 4 hrs. RNA was extracted and analysed as described before.

Based on the results of the initial experiment, I decided to use the 2-hour conditioned medium, given that in the fibroblast and U937 co-culture model, the maximum up-regulation in ICAM 1 and HAS 2 mRNA was noted at 4 hours. Fibroblasts were plated in 6 well plates and growth arrested in serum free medium when near confluence. They were then incubated with 5×10^5 U 937 cells for 2 hours. After 2 hours the medium was aspirated and centrifuged at 7000 rpm for 7 minutes. The supernatant was used as conditioned medium. This conditioned medium was added to growth arrested fibroblasts plated on separate 6 well plates. They were allowed to incubate for 4 hours. After 4 hours medium was aspirated, cells washed with PBS x3 and RNA extracted as detailed before.

These conditioned medium experiments confirmed that though cell-to-cell contact plays an important role in up-regulation of ICAM 1 and has some role in HAS 2 up-regulation, there are other mechanisms involved which lead to up-regulation of ICAM 1 and HAS 2 mRNA even in the absence of the U937 cells contact. ICAM 1 mRNA up-regulation in the presence of conditioned medium was significant with a p value of <0.001 . This is despite the fact that the induction was significantly reduced when compared to induction in the presence of cell-to-cell contact (p value=0.001). HAS 2 mRNA up-regulation from fibroblasts was also significant in the presence of conditioned medium (p value= 0.004) but there was no significant difference in this up-regulation in comparison to fibroblast and U 937 cells interaction (p value=0.478). The results show that cell-to-cell contact plays a significant role in the up-regulation of ICAM 1 but possibly a contributory role to the up-regulation of HAS 2 (*Figure 4-5*).

Rather, some factor may be present in the medium generated either by the interaction of fibroblasts with U 937 cells or secreted by the U 937 cells themselves.

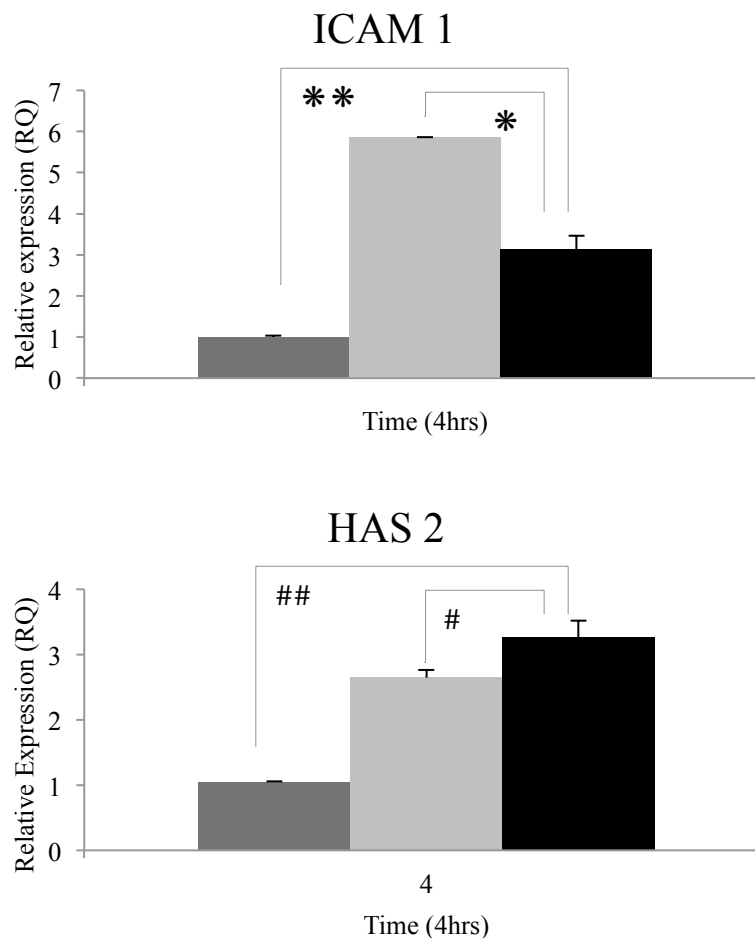





Figure 4-5: ICAM 1 and HAS 2 expression in fibroblasts in response to incubation with conditioned medium from fibroblast and U937 cell co-culture

Growth arrested fibroblast  were incubated with 5×10^5 U 937 cells in 6 well plates . At the same time fibroblasts were also incubated with conditioned medium from Fibroblast and U 937 co-culture taken after 2 hours of co-incubation. . Cells were washed with PBS (3 times) to wash the U937 cells off and mRNA extracted after 4 hours. RT and qPCR for ICAM-1 and HAS 2 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=3).

ICAM 1 mRNA up-regulation in the presence of conditioned medium was significant with a p value of <0.001(*). The reduction in the ICAM 1 up-regulation in the presence of conditioned medium in comparison to fibroblast and U937 cell interaction was also significant, p value=0.001(**)

HAS 2 mRNA up-regulation from fibroblasts was also significant in the presence of conditioned medium, p value= 0.004(#) but there was no significant difference in this up-regulation in comparison to fibroblast and U 937 cells interaction, p value=0.478 (##).

4.3.5 ROLE OF CELL TO CELL CONTACT WITH ACTIVATED U937 CELLS

The interaction of PMA activated U937 cells with fibroblasts leads to an exponential increase in ICAM 1 and HAS2 mRNA in comparison to interaction with unstimulated U937 cells. I have shown that there are different mechanisms involved in ICAM 1 and HAS 2 mRNA induction, with cell-to-cell contact playing a more dominant role in ICAM 1 induction. To investigate the role of cell-to-cell contact in this interaction, fibroblasts were incubated with PMA-activated U937 cells separated from each other by membrane inserts.

In contrast to co-incubation with untreated U937 cells, co-incubation with activated U937 cells induced an approximate 200 fold greater induction of ICAM-1 and 10 fold greater induction of HAS 2 mRNA. Both increases were inhibited in the presence of cell culture membrane inserts but still present to a significant level in comparison to fibroblasts alone (*Figure 4-6*). These results suggest that there was a binding interaction triggered once the monocytes were activated, which was a potent mechanism for fibroblast activation.

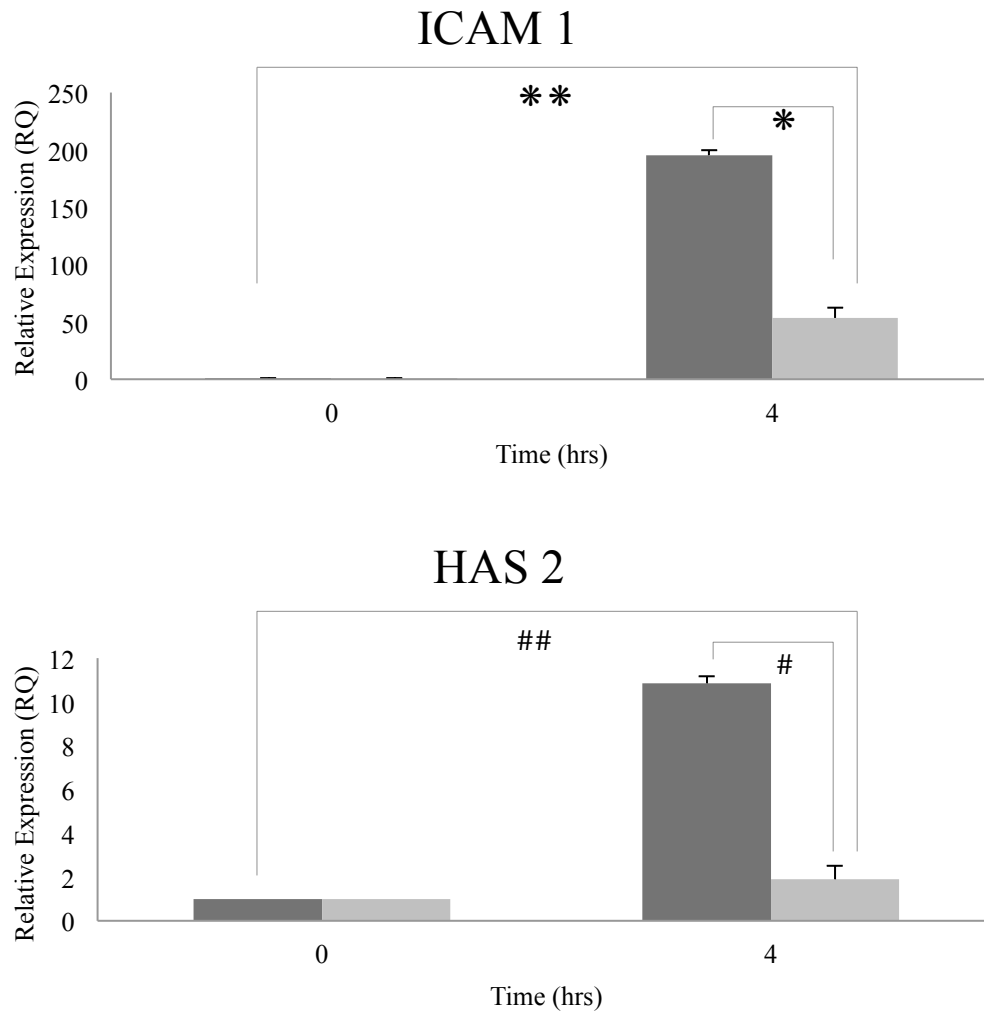


Figure 4-6: ICAM-1 and HAS-2 mRNA expression following Fibroblasts and PMA activated U937 cells incubation in the presence/ absence of 0.4μ membrane inserts.

■ Fibroblasts and 5×10^5 activated U937 cells/ml were incubated for 4 hours Similarly fibroblasts and 5×10^5 activated U937 cells were plated in the presence of 0.4μ membrane inserts suspended over Fibroblasts monolayer □. Medium was added outside the insert to allow contact between the 2 phases to allow movement of solutes but to prevent cell-to-cell contact. Cells were washed with PBS ($\times 3$ times) and mRNA extracted as described before. RT and qPCR for ICAM-1 and HAS 2 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=6)
p-value:*=0.001,**=0.010,#<0.001,##=0.026

4.3.6 FIBROBLAST AND PMA ACTIVATED U937 CO-CULTURE IN PRESENCE OF SOLUBLE ICAM

The activation of fibroblasts was many fold greater in the presence of activated U937 cells. To investigate if this was due to increased ICAM-1 dependent binding, the interaction was repeated in the presence of soluble ICAM-1. A small non-significant effect was seen in the induction of ICAM 1 and HAS 2. The effect was seen most with ICAM 1 induction in the presence of 800ng/ml of sICAM1 with a p value of 0.062. These results would suggest that either the concentrations of soluble ICAM used were too low for the binding interaction induced by activated U937 cells or that there were other mechanisms in which U937 cells bind or interact with the fibroblasts (*Figure 4-7*).

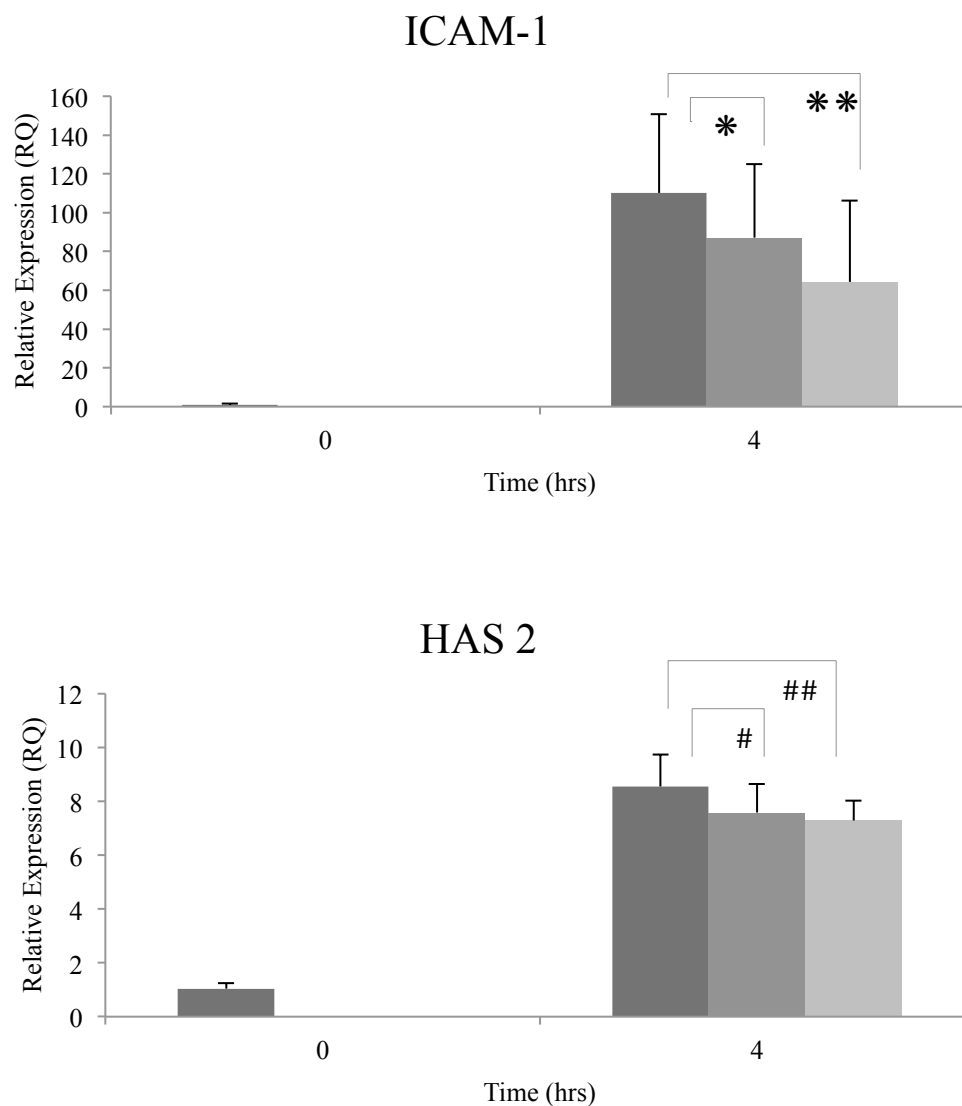


Figure 4-7: *ICAM-1 and HAS-2 mRNA expression following Fibroblasts and PMA activated U937 cells incubation in the presence/ absence soluble ICAM 1*

■ Fibroblasts were incubated with 5×10^5 PMA activated U937 cells/ml in the presence/ absence of soluble ICAM in varying concentration to assess dose response.

■ 800ng/ml soluble ICAM ■ 400ng/ml soluble ICAM

Cells were washed with PBS (3 times) to wash the U937 cells off and mRNA extracted as described before. RT and qPCR for ICAM-1 and HAS 2 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=3).

P value: *=0.276, **=0.062, #=0.576, ##=0.415

4.3.7 FIBROBLASTS AND ACTIVATED U937 CO-CULTURE IN PRESENCE OF β 2 INTEGRIN ANTIBODY

PMA activated U937 cells were pre-incubated for one hour with 20 μ g/ml of blocking anti-CD18 antibodies, to clarify whether there were other binding interactions occurring when activated U937 cells were incubated with fibroblasts. The cells were then co-incubated with growth arrested fibroblasts for 4 and 8 hours. There was no significant effect on the levels of ICAM 1 and HAS 2 mRNA. ICAM 1 mRNA up-regulation was reduced by 18% (p value 0.166), HAS 2 mRNA up-regulation was reduced by 12% (p value 0.453) and CD45 by 8% (p value 0.654) (*Figure 4-8*). These results suggest that other binding interactions were also involved in fibroblast activation with PMA stimulated U937 cells.

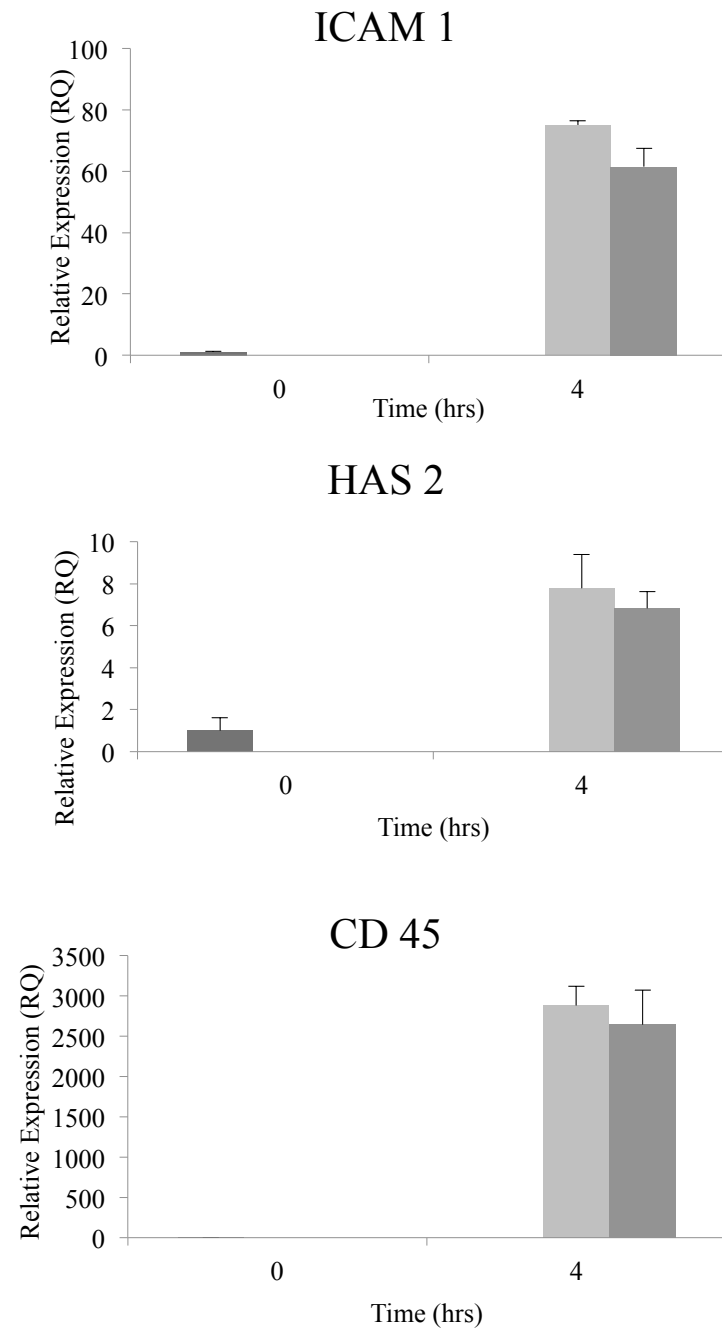


Figure 4-8: ICAM 1 and HAS 2 expression following fibroblast and PMA activated U937 cells co-culture in the presence of β 2 Integrin antibodies.

- Fibroblast monolayers were incubated with 5×10^5 activated U937 cells as control.
- Fibroblast monolayers were also incubated with 5×10^5 PMA activated U937 cells in the presence/absence of 20μ g/ml of β 2 Integrin Antibody. Activated U937 cells were pre-incubated with the β 2 integrin Antibody for 1-hour prior to co-culture with Fibroblast monolayers. Cells were washed with PBS (x 3 times) to wash the U937 cells off and mRNA extracted. RT and qPCR for ICAM-1, HAS 2 and CD45 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=3)

4.3.8 FIBROBLASTS RESPONSE TO THE PRESENCE OF CONDITIONED MEDIUM FROM FIBROBLAST AND ACTIVATED U937 CELL INTERACTION

Similar to the conditioned medium experiments with unstimulated U937 cells, conditioned medium experiments were designed for U937 cells stimulated with PMA. Growth arrested fibroblasts were co-cultured with 5×10^5 PMA activated U 937 cells for 2 hours and the conditioned medium generated as before. This medium was added to separate growth arrested lung fibroblasts in 6 well plates and incubated for 4 hours. Cells were washed at the end of 4 hours with PBS and RNA extracted as before. ICAM 1 mRNA induction was significantly increased in the presence of the conditioned medium (p value < 0.001). This up-regulation, however, was significantly reduced in comparison to the up-regulation seen when the fibroblasts interacted directly with the activated U937 cells (p value < 0.001). This would again support the significant role played by cell-to-cell binding in the interaction of fibroblasts with U937 cells and of the existence of other potential mechanisms in the process.

Similar results were seen with HAS 2 mRNA being up regulated significantly with a p value of 0.013 when compared to resting fibroblasts. Interaction of fibroblasts with conditioned medium from activated U 937 cells led to a reduced up-regulation of HAS 2 in comparison to the interaction with activated U937 cells directly (p value <0.001). This would suggest a greater role played by cell-to-cell contact once the U937 cells are activated (*Figure 4-9*).

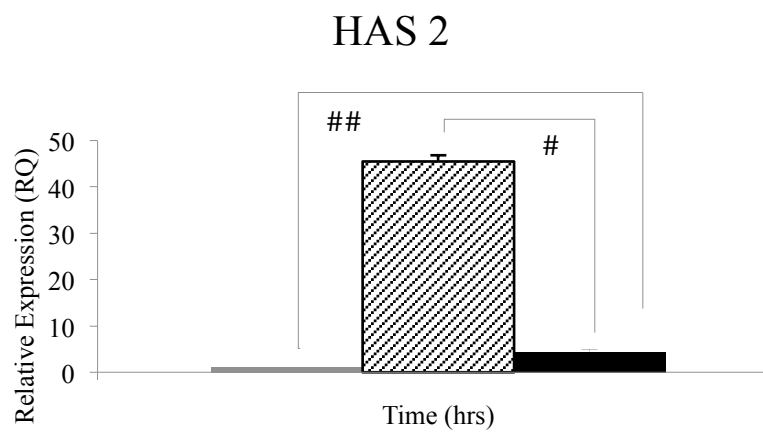
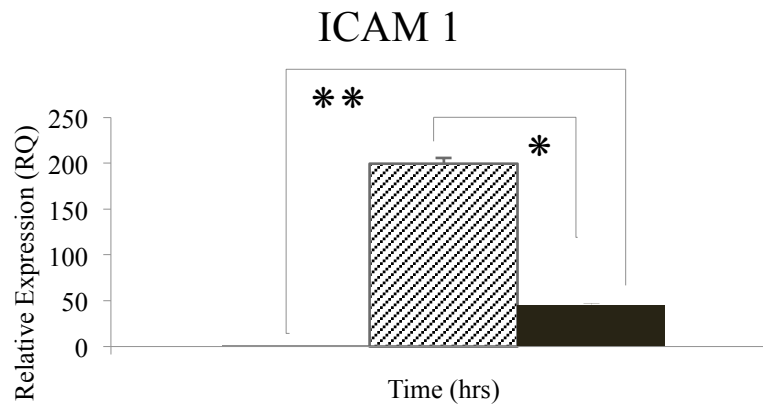


Figure 4-9: *ICAM 1 and HAS 2 expression in fibroblasts in response to incubation with conditioned medium from fibroblast and PMA activated U937 cell co-culture*

Growth arrested fibroblast were incubated with 5×10^5 PMA activated U 937 cells in 6 well plates .

At the same time fibroblasts were also incubated with conditioned medium from Fibroblast and activated U 937 co-culture taken after 2 hours of co-incubation . Cells were washed with PBS (x3 times) to wash the U937 cells off and mRNA extracted after 4 hours. RT and qPCR for ICAM-1 and HAS 2 were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=3).

4.4 DISCUSSION

The co-culture of fibroblasts and U 937 cells led to up-regulation of both ICAM 1 and HAS 2 mRNA. The up-regulation was highly statistically significant when the U937 cells were activated, suggesting a greater role of cell binding once monocytes were activated. The role of cell-to-cell contact was investigated by interrupting the contact between growth-arrested fibroblasts and U937 cells using membrane inserts in co-culture experiments. This prevented direct contact between the U937 cells and the fibroblasts, leaving communication only via the medium in which the cells were incubated.

These experiments showed that although there was up-regulation of ICAM 1 mRNA in the insert experiments, this was reduced in comparison with the co-culture setting where the U 937 cells came into contact with the fibroblasts. The up-regulation was also significantly reduced in the presence of activated monocytes, when fibroblasts were incubated with PMA-activated U 937 cells in inserts. This showed that the cell-to-cell binding had a greater role in up-regulation of ICAM 1 mRNA, especially in the presence of activated U937 cells. At the same time, it also confirmed that there were other mechanisms at play responsible for the up-regulation of ICAM 1 mRNA, as there was still some up-regulation despite the barrier between the 2 cell lines. It would suggest a role of soluble factors in the medium secreted either by the U937 cells or fibroblasts.

In the same experiments we saw that HAS 2 mRNA up-regulation was unaffected when fibroblasts were incubated with inactive U 937 cells in inserts but more suppressed when fibroblasts were incubated with PMA activated U 937 cells in inserts. This again highlighted a more significant role of cell-to-cell contact and binding in the presence of

activated U937 cells but suggests that a different mechanism may be involved in the up-regulation of HAS 2 when inactive monocytes interact with fibroblasts.

It is important to understand that for any cell to fulfill its function, it has to interact with other cells and the extracellular matrix. It is therefore not surprising that adhesion molecules play a central role in multiple aspects of pathology and inflammation.. Adhesion molecules are not merely passive linkage between cells but play an important role in various molecular events by communicating with various signalling pathways.

The dynamic role of adhesion molecules is highlighted in cell migration that involves constant binding and release by the cells. Once inflammatory cells have moved to the site of inflammation, the monocyte and macrophages continue to interact with the resident cells by cell to cell contact and produce cytokines and growth factors that are responsible for the persistence of leucocytes at the site of inflammation and chronic progression.

These results highlight important findings. Firstly, it would suggest that pathways involved in ICAM 1 and HAS 2 mRNA up-regulation may be different as ICAM 1 mRNA up-regulation is more suppressed than HAS 2 mRNA up-regulation when the cell-to-cell contact is interrupted in the fibroblasts and inactive U937 cells co-culture. Secondly, PMA activated U937 cells, when incubated with fibroblasts separated by inserts, show greater suppression in up-regulation of both ICAM 1 and HAS 2 mRNA, highlighting that the up-regulation may be taking place via different mechanisms depending on activation of U937 cells/ mononuclear cells. Finally, one or more soluble factors may be secreted by either monocytes or fibroblasts or both, causing differential up-regulation of ICAM 1 and HAS 2.

The role of cell-to-cell contact was further analysed using soluble ICAM (sICAM) to saturate the surface ICAM and therefore reducing binding. This showed that although

there was some up-regulation in both ICAM 1 and HAS 2 mRNA, this was suppressed in the presence of sICAM. Results were similar in co-culture experiments in the presence of β 2 integrin antibodies. These experiments highlighted that ICAM 1 binding or cell-to-cell contact does play a role in the up-regulation of ICAM 1 and HAS 2, but there are other mechanisms also involved or contributing to this pathway, given the persistence of the up-regulation despite interrupting the ICAM 1 LFA binding.

This led us to hypothesize that there might be a role for some soluble factor that may be produced by either the fibroblasts or U 937 cells, which may play a role in this process. This was investigated with experiments using medium conditioned from fibroblasts and U 937 cells co-culture or activated U937 cells. The consistent up-regulation of ICAM 1 and HAS 2 mRNA in the presence of conditioned medium confirmed the role of soluble factors in the process. Work from others has also shown a role of soluble factors in the increase in various chemokines and cytokines (135). Differential up-regulation of chemokines macrophage inflammatory protein-1 α (MIP-1 α) and monocyte chemoattractant protein-1 (MCP-1), during fibroblast- monocyte interactions, was seen in the presence of anti-TNF antibodies and blocking β 3-integrins (135).

Interaction of gingival fibroblasts and monocytes led to up-regulation of Matrix metalloproteinase-1 (MMP-1). Treatment of fibroblasts with conditioned medium from monocytes also stimulated the production of MMP-1 in the fibroblasts (162).

Production of several growth factors and cytokines are affected when monocytes interact with different cells. Some of these are profibrotic and pro-inflammatory while others are anti-inflammatory. It depends on the type of cell interacting with the monocytes, stimulus involved and whether the monocytes are activated. For example macrophages in the atherosclerotic plaque are highly activated and are known to produce interferon- gamma (IFN- γ) and tumor necrosis factor -alpha and -beta (TNF α

and TNF β) and interleukin-12 (IL-12) (163). Stimulation of monocytes with bacteria causing meningitis, i.e., H. influenza or S. pneumonia showed that the amount of TNF α and IL10 produced was dependent on the concentration of bacteria used to stimulate monocytes and the time of stimulation in vitro (164).

Conversely, the interaction between monocytes and different cell types is itself modified in the presence of various cytokines. For example, IL 1 β promotes ICAM-1 dependent binding of renal proximal tubular cell line HK 2 cells to monocytes(165). Expression of adhesion molecules can also be induced by proinflammatory cytokines on the surface of different cell types(1, 166, 167).

Work done in our lab has shown the importance of cytokines TNF α and IL 1 β in the up-regulation of ICAM 1 and HAS 2 in the past. We have shown that TNF α stimulates ICAM 1 expression at mRNA level and protein level in lung fibroblasts in the past and in my experiments(1). It has also been shown that ICAM-1 cross-linking on the surface of E11 human synovial cells induced the transcription of IL-1 β (168).

Both IL-1 β and TNF- α act in an autocrine manner on macrophages to stimulate their own transcription, as well as the production of each other(169).The combination of TNF α and IL-1 β produces a greater inflammatory response than is observed with either cytokine alone(170).

TNF α and IL 1 β also play a role in modulating HAS transcription in cell cultures(171).This also depends on the cell type(172). The treatment of normal fibroblasts with TNF- α , IFN- γ , and IL-1 β significantly increased the expression of all HAS isoforms. IL-1 β was more effective than TNF- α and IFN- γ and all cytokines were able to induce HAS mRNA in a dose dependent manner(173). Previous work in our lab has also shown IL 1 β mediated HAS 2 up-regulation is transcriptionally controlled by

Sp1 and Sp3. Knocking out Sp1 and Sp3 leads to reduction in HAS 2 expression in proximal tubular cells(174). This led us to investigate the role of TNF α and IL 1 β as soluble factors contributing to up regulation of HAS 2 and ICAM 1 in fibroblast /U 937 co culture.

5 SOLUBLE FACTOR: ROLE OF TNF α AND INTERLEUKIN 1 β

5.1 INTRODUCTION

Cytokines are proteins secreted by immune cells that act on other cells to coordinate appropriate immune responses. Cytokines include a diverse assortment of interleukins, interferons, and growth factors. These are usually extracellular signalling proteins less than 80kD in size (175). Their interaction could be on the cells they originate from (autocrine), on adjacent cells (paracrine) or distant cells (endocrine). The term lymphokine was coined to describe cytokines derived from lymphocytes, while monokines were produced by monocytes or macrophages. These distinctions were quite artificial and rapidly broke down.

Cytokines act by binding to cell surface receptors. These are usually low in number but get up-regulated when cells are activated. Cytokines can have an effect on their own receptor expression on the surface, or affect the expression of receptors of other cytokines (176). Two examples are the actions of interferon γ (IFN- γ) in decreasing the effect of tumour necrosis factor α (TNF- α) receptors on macrophages and that of interleukin 1 β (IL-1 β) in increasing the expression of the same receptors (176).

Many cell populations produce cytokines, but predominant among them are helper T cells and macrophages.

5.1.1.1 PROINFLAMMATORY CYTOKINES

These are mainly produced by activated macrophages and cause up-regulation of inflammatory reactions. Chief among these are IL 1 β , IL 6 and TNF α . There is further discussion on IL-1 β and TNF α in the following section.

A number of cytokines are also responsible for enhancing chemotaxis. These are called CHEMO tactic cytoKINES or chemokines. Their main role is in promoting activation and migration of leucocytes to site of inflammation. They are of low molecular weight and have conserved cysteine residues that allow them to be assigned to four groups: C-

C chemokines (RANTES, monocyte chemo attractant protein or MCP-1, monocyte inflammatory protein or MIP-1 α , and MIP-1 β), C-X-C chemokines (IL-8 also called growth related oncogene or GRO/KC), C chemokines (lymphotactin), and CXXXC chemokines (fractalkine) (177).

5.1.1.2 ANTI INFLAMMATORY CYTOKINES

Anti-inflammatory cytokines include interleukin (IL)-1 receptor antagonist, IL-4, IL-10, IL-11, and IL-13. These act by controlling the response of some of the proinflammatory cytokines in concert with soluble cytokine receptors and cytokine inhibitors (177). These are mainly produced by lymphocytes.

Some cytokines like TGF β , Interferon α and Leukaemia Inhibitory factor can act as both pro-inflammatory and anti-inflammatory factors, depending on the circumstances and situation. Specific receptors to TNF α and IL 1 β also act to down-regulate the pro-inflammatory cytokines.

Of the anti-inflammatory cytokines, IL-10 is supposed to be the most potent, down-regulating the expression of TNF α , IL-1 and IL-6 from activated macrophages. IL-10 can also up-regulate endogenous anti-cytokines and down-regulate pro-inflammatory cytokine receptors. There are studies that show low levels of anti-inflammatory cytokines such as IL-10 and IL-4 in chronic pain conditions (178).

Secretion of IL-1 β is counterbalanced by secretion of IL-1RA and TNF α secretion is counterbalanced by soluble TNF receptors (179, 180). The balance between pro- and anti-inflammatory mediators dictates the overall effect of an inflammatory response.

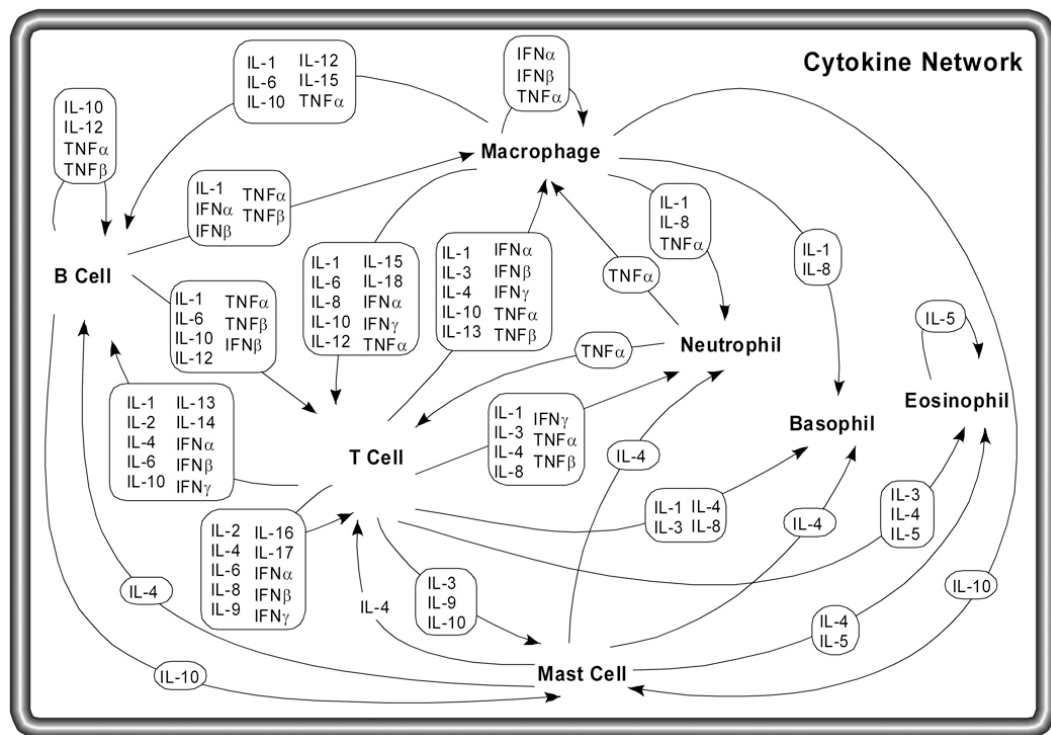


Figure 5-1 Cytokine network

From: Int Anesthesiol Clin. 2007 Spring; 45(2): 27–37. doi:10.1097/AIA.0b013e318034194e

CYTOKINE NETWORK: Several different cell types coordinate their efforts as part of the immune system, including B cells, T cells, macrophages, mast cells, neutrophils, basophils and eosinophils. Each of these cell types has a distinct role in the immune system, and communicates with other immune cells using secreted cytokines. Macrophages phagocytose foreign bodies and are antigen-presenting cells, using cytokines to stimulate specific antigen dependent responses by B and T cells and non-specific responses by other cell types. T cells secrete a variety of factors to coordinate and stimulate immune responses to specific antigen, such as the role of helper T cells in B cell activation in response to antigen. The proliferation and activation of eosinophils, neutrophils and basophils respond to cytokines as well.

5.1.1.3 ROLE OF CYTOKINES IN RENAL DISEASE

Of all organ systems, the kidneys play a leading role in the clearance of circulating cytokines (181-183). Cytokines play an important role in both acute kidney injury (AKI) as well as chronic kidney disease (184, 185). They can act by up-regulating the endothelial adhesion molecules and chemokines that can further stimulate infiltration of immune cells (186, 187). They also activate various signalling pathways that activate transcription factors such as NF kappa B and MAP kinases. NF kB activation is seen in patients with diabetic nephropathy (188), acute kidney injury (189) and glomerulonephritis (190). TNF α , TGF β and interleukins can also influence sodium excretion and renal blood flow (191, 192).

Hypertensive patients are known to have elevated levels of TNF α , IL-6, MCP-1 and adhesion molecules (193, 194). Etanercept, a TNF α antagonist, has been shown to reduce blood pressure in autoimmune associated hypertension and angiotensin II related hypertension (195, 196). Experimental work also shows role of cytokines in nephrotic syndrome (197). Mononuclear cells from patients with nephrotic syndrome cause TNF levels to go up in plasma and urine (198). There is evidence to suggest a positive role for IL-1 receptor antagonists in management of anti-GBM antibody associated glomerulonephritis(199). They have also been shown to suppress experimental crescentic glomerulonephritis (200). Cytokines also play an important role in IgA nephropathy. TNF- α and IL-6 levels increase in IgA nephropathy and are reduced by immunoglobulin therapy (201).

INTERLEUKIN 1

The IL – 1 family has 11 different member proteins, coded by 11 genes (178, 202). Their main function is management of innate immunity in humans by regulating pro-

inflammatory responses. Macrophages or monocytes are the main source of IL-1 β and IL-1 α (203). These cytokines are also secreted by other cell types such as epithelial cells, endothelial cells and fibroblasts(204, 205).

A brief description of the 11 members is shown in the following table.

Table: The IL-1 Family

Family name	Name	Receptor	Property
IL-1F1	IL-1 α	IL-1RI	Proinflammatory
IL-1F2	IL-1 β	IL-1RI	Proinflammatory
IL-1F3	IL-1Ra	IL-1RI	Antagonist for IL-1 α , IL-1 β
IL-1F4	IL-18	IL-18R α	Proinflammatory
IL-1F5	IL-36Ra	IL-1Rrp2	Antagonist for IL-36 α , IL-36 β , IL-36 γ
IL-1F6	IL-36 α	IL-1Rrp2	Proinflammatory
IL-1F7	IL-37	?IL-18R α *	Anti-inflammatory
IL-1F8	IL-36 β	IL-1Rrp2	Proinflammatory
IL-1F9	IL-36 γ	IL-1Rrp2	Proinflammatory
IL-1F10	IL-38	Unknown	Unknown
IL-1F11	IL-33	ST2	Th2 responses, proinflammatory

Adapted from: Dinarello CA (2011). "Interleukin-1 in the pathogenesis and treatment of inflammatory diseases". Blood 117 (14): 3720–32. (? : Pending confirmation)

Interleukin-1 β and Interleukin-1 α were the first members of the IL-1 family to be described and the best studied of the 11 members. They induce many different genes in multiple types of cells (204). They also stimulate further IL-1 production causing a positive feedback loop by up-regulating their own genes (203, 206). There are three main limiting steps controlling the multiple ways in which IL-1 promotes inflammation. These are 1) control of synthesis and release, 2) control of membrane receptors and 3) regulation of signal transduction downstream of the activated receptors. In response to ligand binding to the receptor, a complex sequence of phosphorylation and ubiquitination events results in activation of nuclear factor κ B signalling and the JNK

and p38 mitogen-activated protein kinase pathways, which, co-operatively, induce the expression of IL-1 target genes (such as IL-6, IL-8, MCP-1, COX-2, IL-1 α , IL-1 β , MKP-1) by transcriptional and post-transcriptional mechanisms. Of note, most intracellular components that participate in the cellular response to IL-1 also mediate responses to other cytokines (207). Several studies have demonstrated low levels of circulating IL-1 β in human disease. (208) IL-1 β binds to the type I IL-1 receptor (IL-1R1) the main receptor for IL-1 β activity. It also binds to an accessory receptor called the IL-1 receptor accessory protein (IL-1RAcP), which serves as a co-receptor required for signal transduction of IL-1/IL-1RI complexes. This co-receptor is also necessary for activation of IL-1R1 by other IL-1 family members.(207) The type II IL-1 receptor (IL-1R2) binds IL-1 α and IL-1 β but lacks a signalling-competent cytosolic part and thus serves as a decoy receptor (208). IL-1Ra (IL-1 Receptor antagonist) blocks IL-1 surface receptors, which are present on all nucleated cells, primarily by occupancy of the ligand-binding IL-1RI; in fact, IL-1Ra binds to this receptor with a greater affinity than IL-1 β .

IL-1 β plays an important role in regulation of HAS 2. Transcriptional regulation of the human *HAS* genes has been studied. Sp1 and Sp3 were found to be principal mediators of HAS2 constitutive transcription.(174) Work in our lab has also shown that IL-1 mediated HAS 2 up-regulation is transcriptionally controlled by Sp 1 and Sp 3. Knocking down Sp1 and Sp3 expression led to suppression in IL-1 β mediated HAS 2 mRNA induction.

ICAM-1 induction by inflammatory cytokines such as IL-1 β , TNF- α , and IFN- γ has also been revealed in various cell types such as human umbilical vein endothelial cells, pulmonary artery endothelial cells (209), intestinal epithelial cells (210), keratocytes (211), and renal tubular epithelial cells. (212)

5.1.2 TUMOR NECROSIS FACTOR α

Tumour necrosis factor alpha (TNF α) is also a mediator of inflammatory and immune functions. It regulates growth and differentiation of a wide variety of cell types. TNF α is secreted by activated monocytes and macrophages, and many other cells, including B cells, T cells and fibroblasts. The gene for TNF α is mapped to 6p21.3. TNF α is synthesised as a 26 kDa (233 amino acids) membrane-bound propeptide (pro-TNF α) and is secreted after cleavage by TNF α -converting enzyme (TACE). The 26 kDa form is also functional and binds to TNFRII via direct cell-to-cell contact.(213)

There are two types of TNF α receptors: Type I and type II. Type I receptors are found on most cells but there are few which don't such as RBCs and resting T cells. Type II is restricted to haematopoietic cells. The two types of receptors are very different to each other, suggesting different signalling mechanisms.

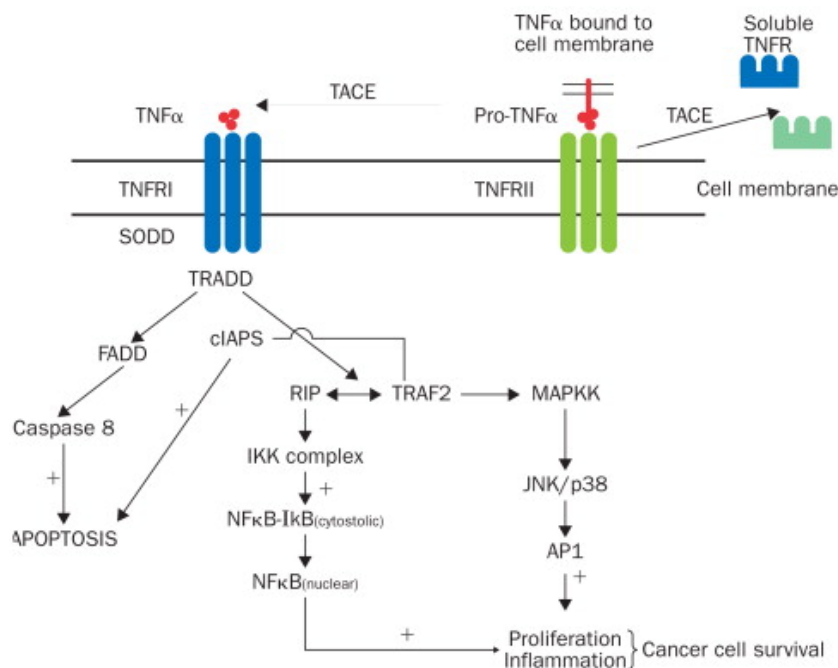


Figure 5-2 TNF α binds to TNFRI and TNFRII activating several pathways associated with inflammation, apoptosis, and cell survival. From THE LANCET Oncology Vol 4 September 2003

Given their central role in inflammation, $\text{TNF } \alpha$ antagonists have been developed as effective therapies for rheumatoid arthritis and inflammatory bowel disease.

Both $\text{TNF } \alpha$ and Interleukin-1 β are secreted by similar cells and in response to similar stimuli. Both cause activation of integrins on leucocytes as well as induction of adhesion molecules on endothelial cells (206). In chronic fibrotic diseases, such as idiopathic fibrosis (IPF) or liver cirrhosis, the persistent production of $\text{TNF } \alpha$ and recruitment of mononuclear cells is maintained (214). $\text{TNF } \alpha$ appears to be a major factor for inducing and maintaining fibrotic responses, even in the absence of an apparent inciting agent (215). It has been shown U937 cells differentiated by phorbol ester were able to release these two cytokines and, in the case of the co-culture, mRNAs for both cytokines were highly expressed in the U937 cells (121).

5.2 *AIM*

The aim of this chapter was to investigate in detail, the response of fibroblasts to conditioned medium. Experiments with membrane inserts and conditioned medium have shown in the previous chapter, the possibility of a soluble factor involved in the interaction between fibroblasts and U 937 cells. In this chapter, I investigated $\text{TNF } \alpha$ and IL-1 β as the potential soluble factors, given the strong evidence for these cytokines in up-regulating ICAM 1 and HAS 2 expression from previous work in the lab.

5.3 RESULTS

5.3.1 TIME DEPENDENT INDUCTION OF ICAM 1 AND HAS 2 IN FIBROBLASTS by IL 1 β AND TNF α

Initial experiments were designed to confirm that stimulation of fibroblasts with TNF α and IL1 β led to similar up-regulation of ICAM-1 and HAS2, as seen in previous chapters. Fibroblasts were plated in 6 well plates and growth arrested for 48 hours when near confluence. The growth-arrested monolayer was then treated with

1×10^{-12} M TNF α , 1×10^{-9} IL-1 β or both in serum free medium. Medium was aspirated at pre set time points; cells were washed with PBS and mRNA extracted. ICAM 1 and HAS 2 expression was assessed with Reverse Transcription and Q-PCR.

HAS 2 and ICAM mRNA expression showed up-regulation in a time-dependent manner, with maximum up-regulation with 2-hour incubation in the presence of IL 1 β , TNF α or both. The up-regulation in expression of both HAS 2 and ICAM 1 mRNA was more when fibroblasts were incubated with IL 1 β and TNF α together. Up-regulation of HAS 2 and ICAM 1 seen with the combination was significantly more than when the cytokines were used individually. ICAM 1 induction at 2 hours was also significantly increased in the presence of TNF α and IL 1 β individually in comparison to resting fibroblasts (p value for both being < 0.001). HAS 2 induction was significantly increased at 2 hours in the presence of IL 1 β (p value < 0.001), marginally increased in the presence of TNF α (p value=0.560) and significantly increased when both IL 1 β and TNF α were used (p value < 0.001) (*Figure 5-1*)

This is important as this again proves the synergistic effect of various cytokines in any interaction especially in this co-culture model where more than one soluble factor may be involved. It also supports the idea that these two cytokines may be involved in the fibroblast/monocyte interaction.

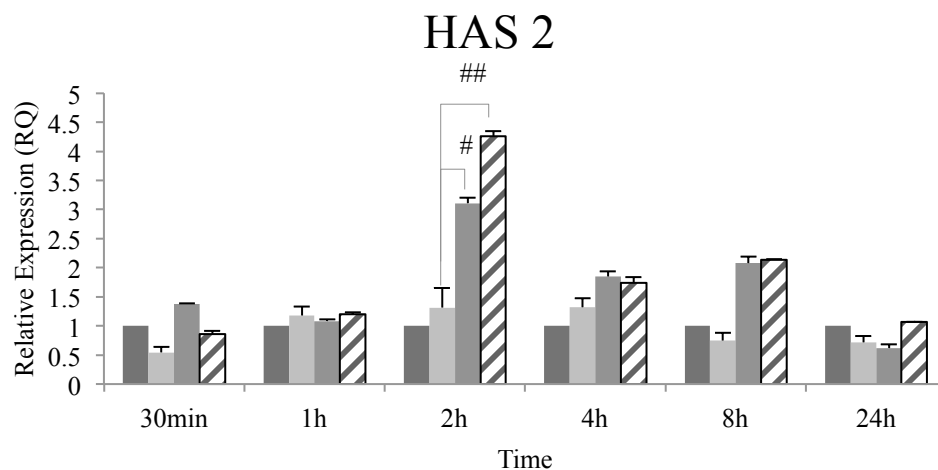
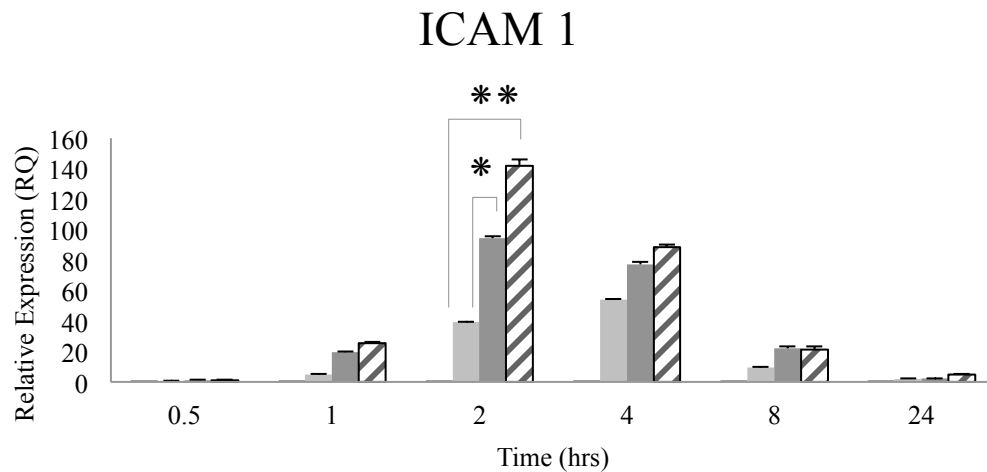


Figure 5-3: TNF and/ or IL 1 β stimulation of Fibroblasts

Fibroblast
 Fibroblasts + 1×10^{-12} M TNF α
 Fibroblasts + 1×10^{-9} M IL 1 β
 Fibroblasts + 1×10^{-12} M TNF α and 1×10^{-9} M IL 1 β

Growth arrested fibroblast monolayer were incubated with 1nmol of IL 1 β and / or 1picomolar TNF α . Cells in predetermined plates were washed with PBS \times 3 times at 30 minutes to 24 hours. mRNA was extracted as described before. QPCR results for ICAM 1 and HAS 2 expression are represented as mean + SE of mean normalised to rRNA (n = 3). P value: * <0.001 , ** <0.001 , # <0.001 , ## <0.001

5.3.2 EXPRESSION OF TNF α AND IL1 β IN FIBROBLAST AND U937 CELLS CO-CULTURE

The main aim for experiments in this chapter was to ascertain whether TNF α and IL 1 β might be soluble factors responsible for the up-regulation of ICAM-1 and HAS2 in fibroblast and U 937 cell co-culture. Therefore it was important to first see if there was any up-regulation of these cytokines in the co-culture model.

Fibroblasts and U937 cell co-culture was carried out as detailed previously. Expression of IL 1 β and TNF α was evaluated in addition to HAS 2 and ICAM 1 mRNA expression. These experiments demonstrated definite up-regulation in expression of these two cytokines in the co-culture model. The induction was statistically significant at 4 hours, with p value < 0.001 for both IL1 β and TNF α mRNA. ICAM 1 and HAS induction was again found to be significant (Figure 5-2a)

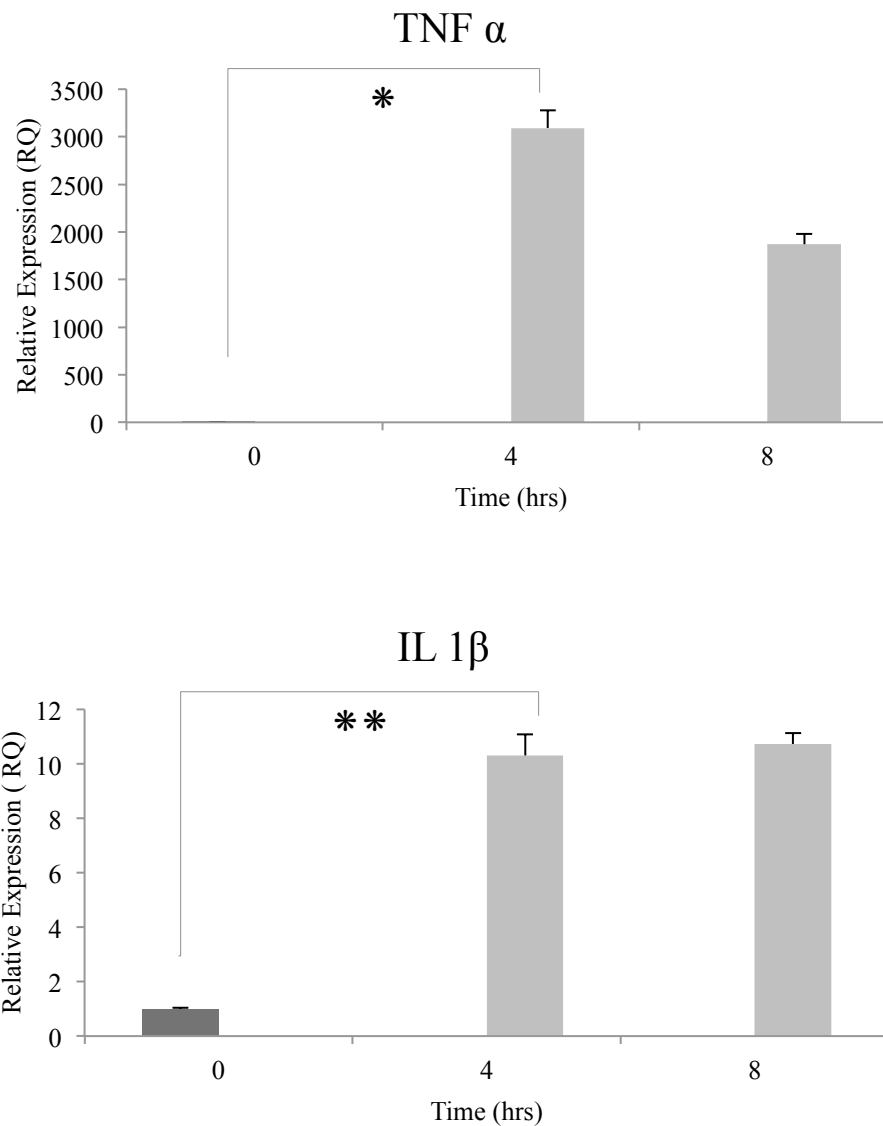


Figure 5-4:-a *TNF α and IL 1 β mRNA expression following Lung Fibroblasts and U937 cell co-culture*

Growth arrested fibroblasts were incubated with 5×10^5 U937 cells for times up to 24 hours. Cells were washed with PBS ($\times 3$ times) to wash the U937 cells off and mRNA extracted as described. RT and q-PCR for TNF α and IL 1 β were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=6), p value:* <0.01,** <0.001

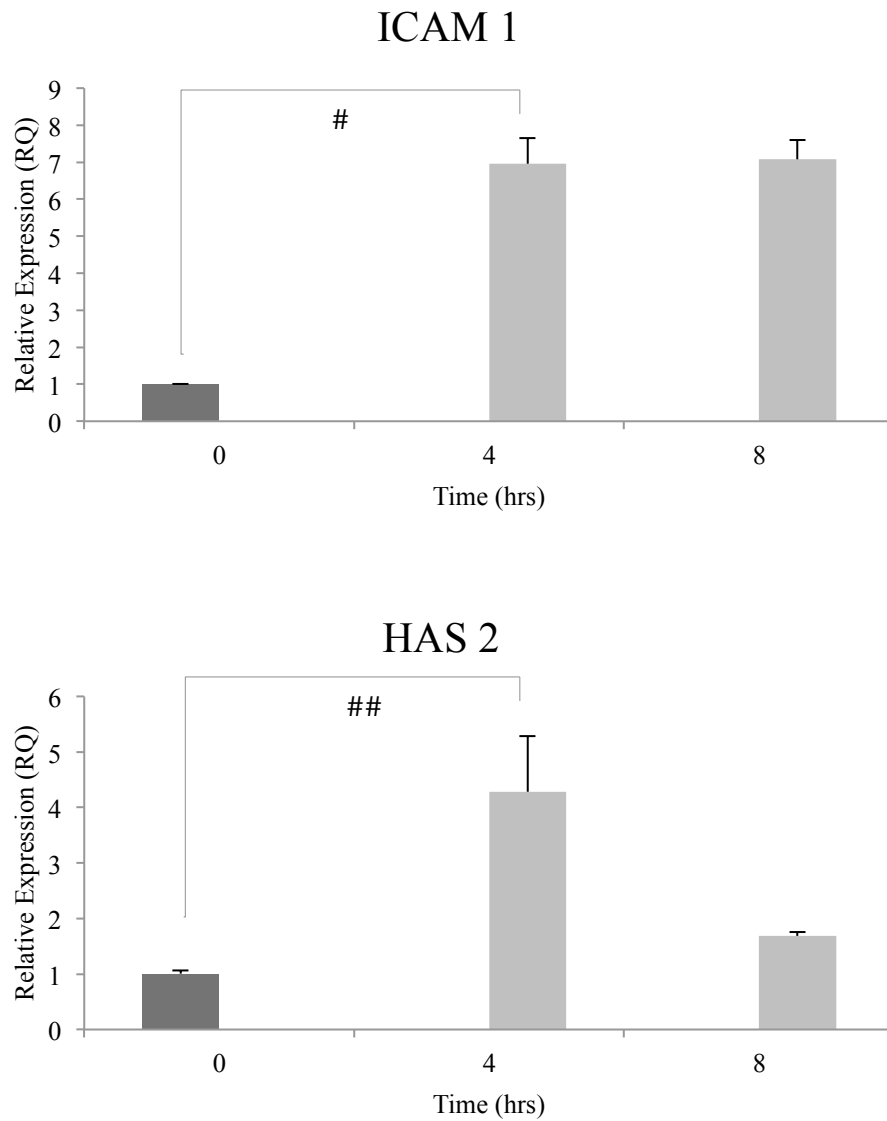


Figure 5-2b: ICAM 1 and HAS 2 mRNA expression following Lung Fibroblasts and U937 cell co-culture

Growth arrested fibroblasts were incubated with 5×10^5 U937 cells for times up to 24 hours. Cells were washed with PBS ($\times 3$ times) to wash the U937 cells off and mRNA extracted as described. RT and q-PCR for ICAM-1 and HAS 2, TNF α and IL 1 β were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=6), p value # < 0.001, ##= 0.030

5.3.3 EXPRESSION OF TNF α AND IL 1 β IN FIBROBLASTS AND U937 CELLS CO-CULTURE IN THE PRESENCE OF sICAM 1

The role of binding between U937 cells and fibroblasts was previously shown to be ICAM and CD18 dependent using sICAM 1 (Experiment 4.3.2). U 937 cells were suspended in DMEM F12 HAM medium for 1 hour in the presence of varying concentrations of soluble ICAM. After 1 hour 5×10^5 U937 cells/ml were added to growth arrested fibroblasts monolayer. RNA was then extracted as described. Expression of ICAM 1, HAS 2, TNF α and IL 1 β mRNA were then assessed using Reverse Transcription and Q PCR. TNF and IL1 first

The results again showed visible reduction in the upregulation of ICAM1 (55% reduction in the presence of 800ng/mL of sICAM1, p value of 0.048), HAS 2 (~ 60% reduction in the presence of 800ng/mL of sICAM1, p value 0.030).

IL 1 β and TNF α induction was also suppressed. IL 1 β induction was reduced by 67% in the presence of sICAM-1, but this was statistically not significant (p value 0.059). TNF α induction was suppressed by 70% approximately in the presence of 800ng/mL sICAM 1 (p value 0.030) (*Figures 5-3 and 5-4*).

This confirms that TNF α and IL 1 β induction in U937 cell and fibroblast co-culture is also dependent on ICAM 1 binding. The presence of some mRNA induction could suggest incomplete nullification of ICAM1 binding or the presence of other mechanisms contributing to the process.

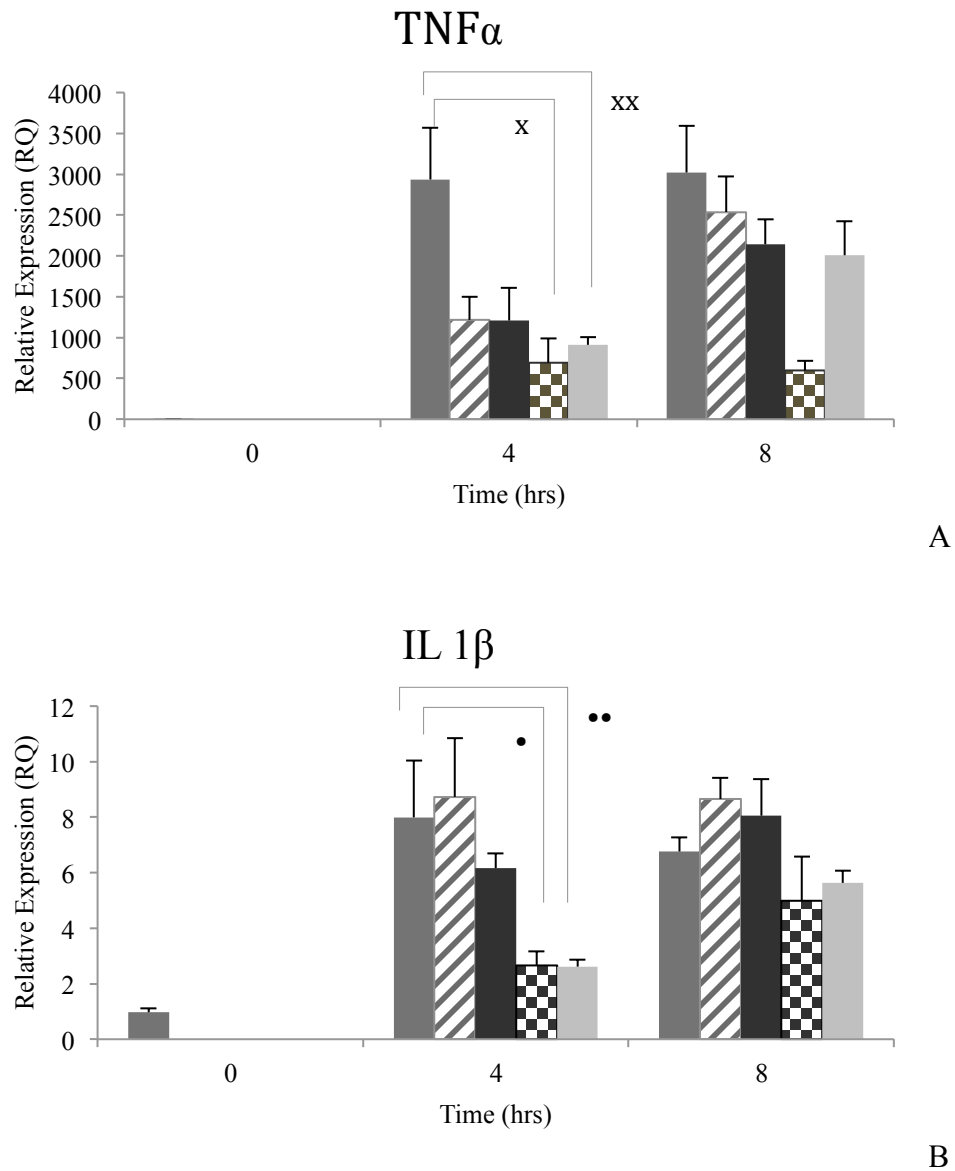
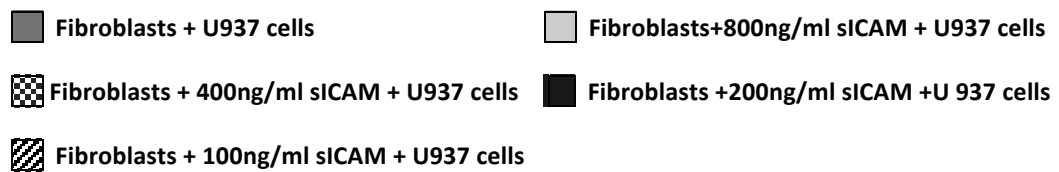
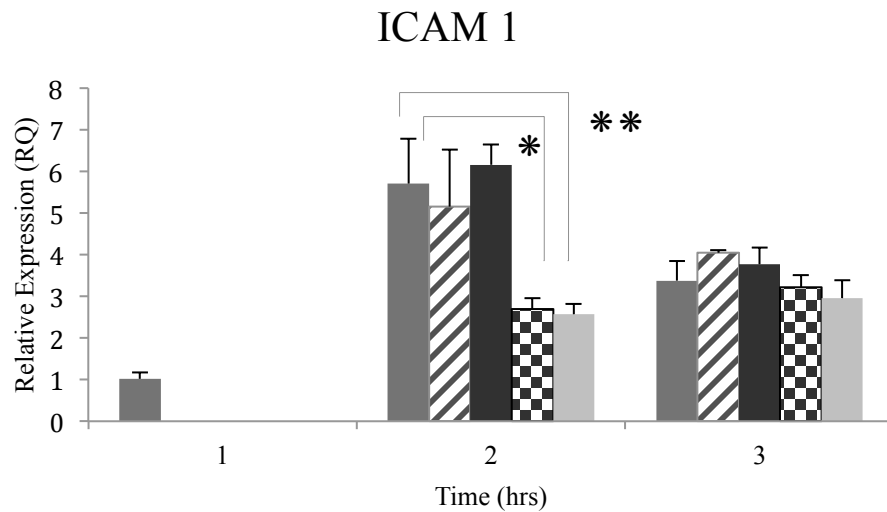


Figure 5-5: Expression *TNF* and *IL 1* in fibroblast-U937 cells co-culture in the presence of *sICAM*

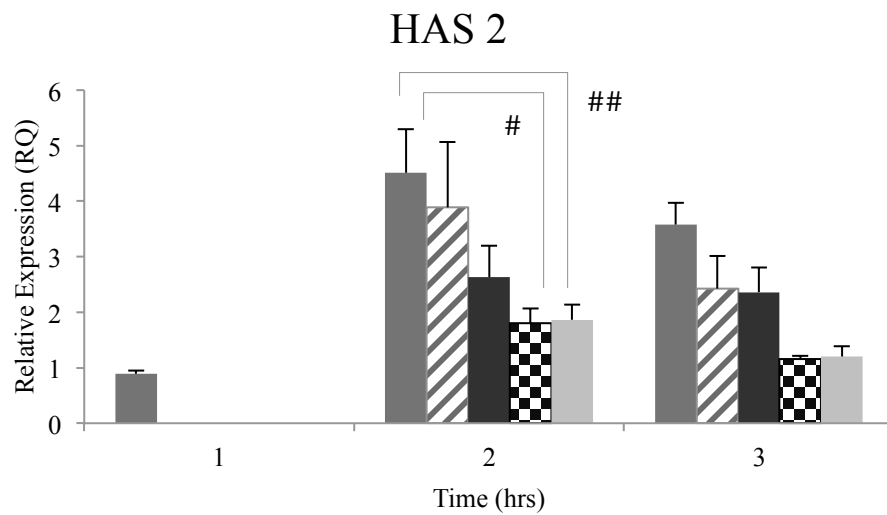


U 937 cells were suspended in varying concentrations of sICAM in DMEM F12 HAM medium for 1 hour. These media were then used to incubate growth arrested fibroblasts monolayers for 4 and 8 hours with 5×10^5 U937 cells in 12 well plates. RNA was extracted as detailed. mRNA expression for TNF α (A), IL 1 β (B), ICAM 1(C) and HAS 2(D), was assessed using reverse transcription and Q PCR techniques.

P values: $x = 0.032$, $xx = 0.034$, $\bullet = 0.064$, $\bullet\bullet = 0.059$

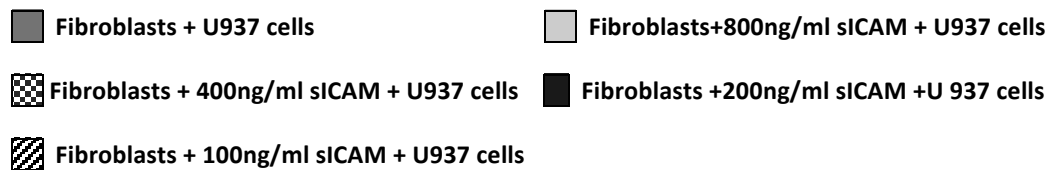


C



D

Figure 5-6: Expression of *ICAM 1* and *HAS 2* in fibroblast-U937 cells co-culture in the presence of *sICAM*



U 937 cells were suspended in varying concentrations of sICAM in DMEM F12 HAM medium for 1 hour. These media were then used to incubate growth arrested fibroblasts monolayers for 4 and 8 hours with 5×10^5 U937 cells in 12 well plates. RNA was extracted as detailed. mRNA expression for ICAM 1(A), HAS 2(B), TNF α (C) and IL 1(D) was assessed using reverse transcription and Q PCR techniques.

P values: * = 0.054, ** = 0.048, # = 0.033, ## = 0.030

5.3.4 EXPRESSION OF TNF α AND IL 1 β IN FIBROBLAST AND U937CELLS CO-CULTURE IN THE PRESENCE OF MEMBRANE INSERTS

The role of TNF α and IL 1 β in the fibroblast and U937 cell co-culture model was further evaluated, using membrane inserts as before. This was to see if preventing cell-to-cell contact between fibroblasts and U937 cells affected the expression of TNF α and IL 1 β , which in turn may be responsible for the differential up-regulation of ICAM 1 and HAS

There was a significant reduction in the expression of TNF α (p value<0.001), but no significant change in the up-regulation of IL 1 β mRNA (p value = 0.345) or HAS 2 as before (p value=0.747) (*Figure 5-5*). This suggested that TNF α may have a greater role in regulation of ICAM 1 expression, which appears to be more cell-to-cell contact dependent than HAS 2 expression.

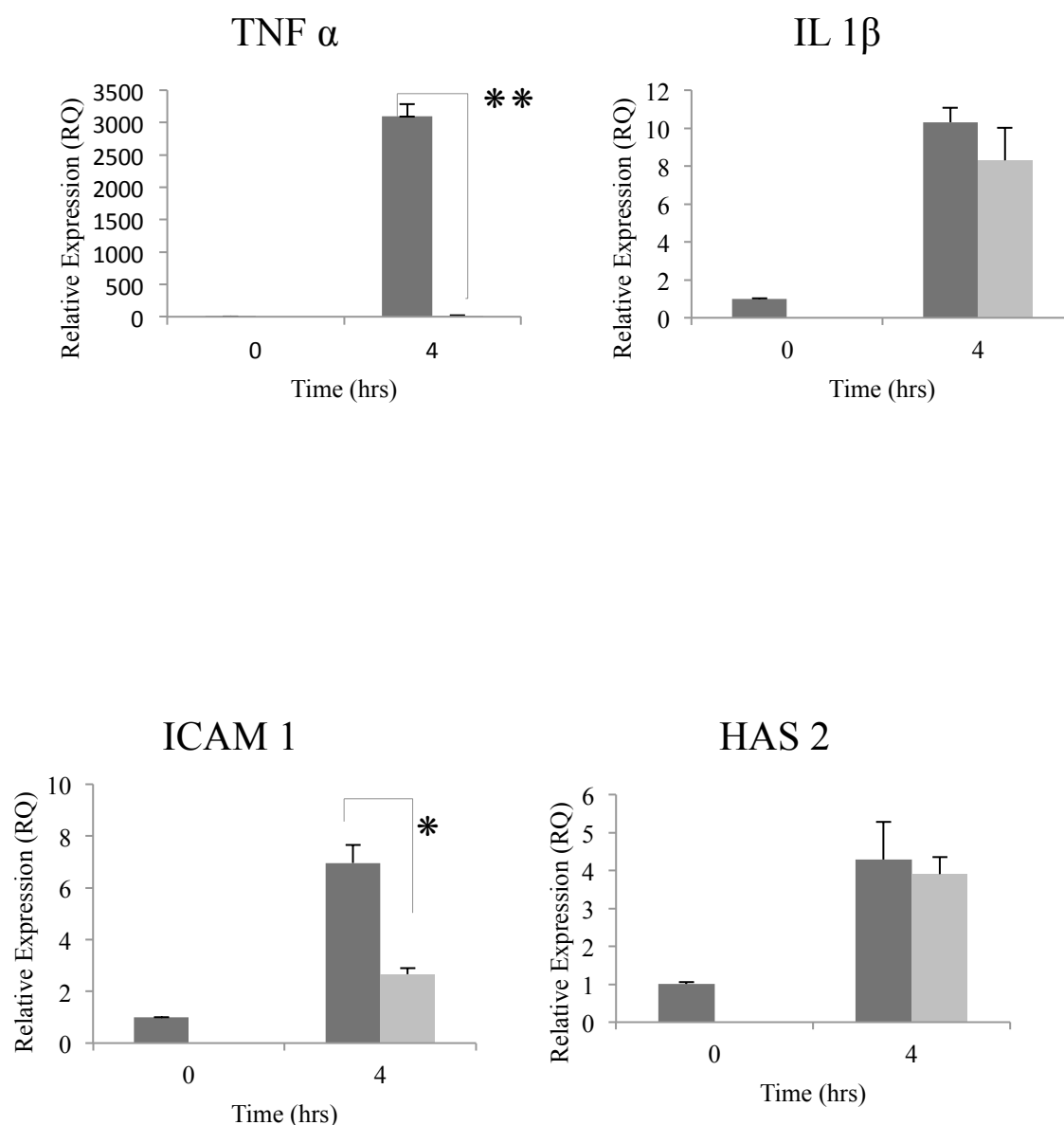


Figure 5-7: Fibroblasts and U937 cells incubation in the presence/ absence of 0.4μ membrane inserts.

■ Fibroblasts+U937 cells □ Fibroblasts+ U937 cells in presence of 0.4μ membrane inserts

Growth arrested fibroblasts and 5×10^5 U937 cells/ml were incubated for 4 and 8 hours as control. 0.4μ membrane inserts were suspended over Fibroblasts monolayer and 5×10^5 U937 cells/ml were added into the insert in serum free DMEM F12 HAM medium. Medium was added outside the insert to allow contact between the 2 phases to allow movement of solutes.

Cells were washed with PBS (×3 times) to wash the U937 cells off and mRNA extracted as described before. RT and qPCR for ICAM-1, HAS-2 IL-1β and TNF α were performed and the results are expressed as mean + SEM of the measurements corrected for rRNA (n=6)

P value: * < 0.001, ** < 0.001

5.3.5 DETERMINATION OF IL 1 β AND TNF α PROTEIN CONCENTRATION IN FIBROBLASTS AND U937 CELLS CO-CULTURE

ELISA experiments were set up to evaluate the protein concentration in the fibroblast-U937 co-culture and to investigate the changes in the concentration when the environment was changed, such as in the presence of membrane inserts. Although I have shown the upregulation of TNF α and IL 1 β at mRNA level, it was important to see if the protein levels were also raised to cause or promote the upregulation of ICAM 1 and/ or HAS 2. Fibroblasts were plated in 6 well plates. 5×10^5 U937 cells/ml in 2 ml medium were added to growth arrested fibroblasts. Medium was aspirated at set time points (Time 0, 2hrs, 4hrs, 8hrs and 24 hours). Similarly experiment was designed to assess TNF α levels in a setting where U 937 cells were not in contact with the fibroblasts using membrane inserts as before.

The aspirate was centrifuged at 7000rpm for 7 minutes and supernatant used for ELISA to look for cytokine concentration. Enzyme linked immunosorbent assay (ELISA) was used to analyse TNF α and IL 1 β concentrations in cell culture. This was assessed by using a commercial available kit (TNF α , R&D). ELISA was done as described in the methods chapter.

The results showed a time dependent increase in TNF α concentration and this induction was reduced in the presence of membrane inserts suggesting dependence of TNF α upregulation on cell-to-cell contact. IL 1 β concentration was not reliably measurable because the concentration was most likely below the threshold for the kit used.

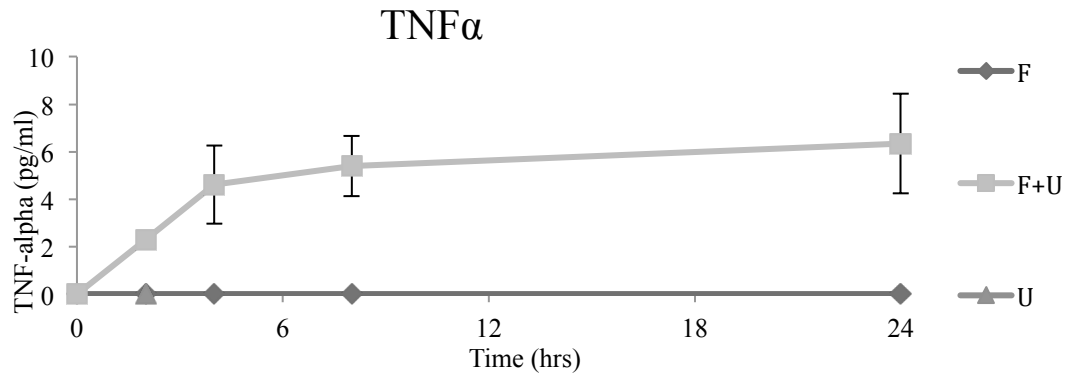


Figure 5-8: *TNF estimation in fibroblast and U937 cell co-culture*

Fibroblasts were incubated with 5×10^5 U937 cells/ml in 6 well plates. As control, fibroblasts and U937 cells were incubated in 6 well plates. Medium was aspirated at 0, 2, 4, 8 and 24 hours, centrifuged and supernatant analysed for TNF by ELISA as described in the methods chapter. (n=3)

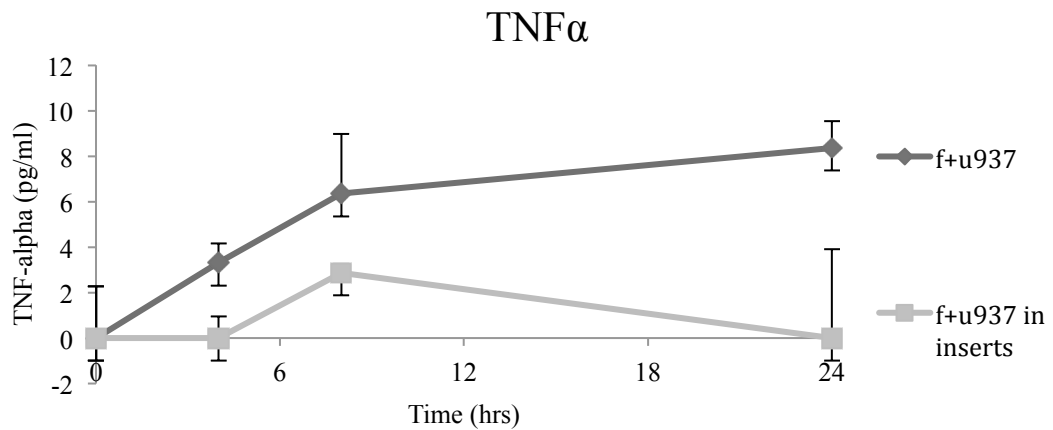


Figure 5-9: *TNF estimation in Fibroblast and U937 cell co-culture and comparison with co-culture in the presence of membrane inserts*

Fibroblasts were incubated with 5×10^5 U937 cells in 6 well plates allowing cell-to cell contact. At the same time fibroblasts were incubated U937 cells suspended in a membrane insert. Medium was aspirated at 0, 4, 8 and 24 hours. This was centrifuged and supernatant used for protein estimation using ELISA as per manufacturer's protocol. (n=3)

IL-1

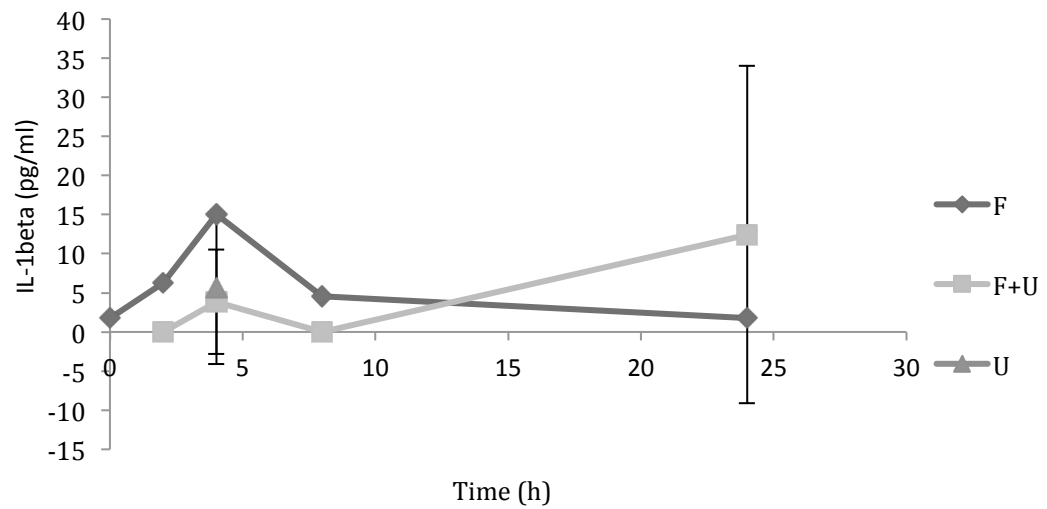


Figure 5-10: *IL 1 β estimation in Fibroblast and U937 cell co-culture , fibroblasts and U937 cells plated in isolation*

Fibroblasts were incubated with 5×10^5 U937 cells in 6 well plates allowing cell-to cell contact. At the same time fibroblasts and U937 cells were incubated as control Medium was aspirated at 0, 4, 8 and 24 hours. This was centrifuged and supernatant used for protein estimation using ELISA as per manufacturer's protocol.

5.3.6 EXPRESSION OF ICAM 1 AND HAS 2 IN FIBROBLAST AND U937 CELL CO-CULTURE IN THE PRESENCE OF IL 1 RECEPTOR ANTAGONIST

Fibroblast and U937 cell co-culture experiments in the presence of the IL1 receptor antagonist were designed to investigate the role of IL 1 β in ICAM 1 and HAS 2 upregulation. Fibroblast monolayers were co-cultured with 5×10^5 U937 cells/ml which had been pre-treated with varying concentrations of IL1 receptor antagonist (IL 1RA, Recombinant Human IL-1ra/IL-1F3, 280-RA-010/CF, R&D Systems) for an hour. After 4h and 8h, wells were washed with PBS 3 times, RNA extracted with with tri-reagent as described before, and analysed for expression of ICAM 1 and HAS 2.

One would expect that presence of IL 1RA would neutralise the IL 1 β present in the co-culture environment and therefore suppress any up-regulation of ICAM 1 and HAS 2, if IL 1 β was responsible for the same. I was able to show a dose dependent reduction in HAS 2 expression (p value 0.047) but with no significant affect on ICAM 1 expression (*Figure 5-9*).

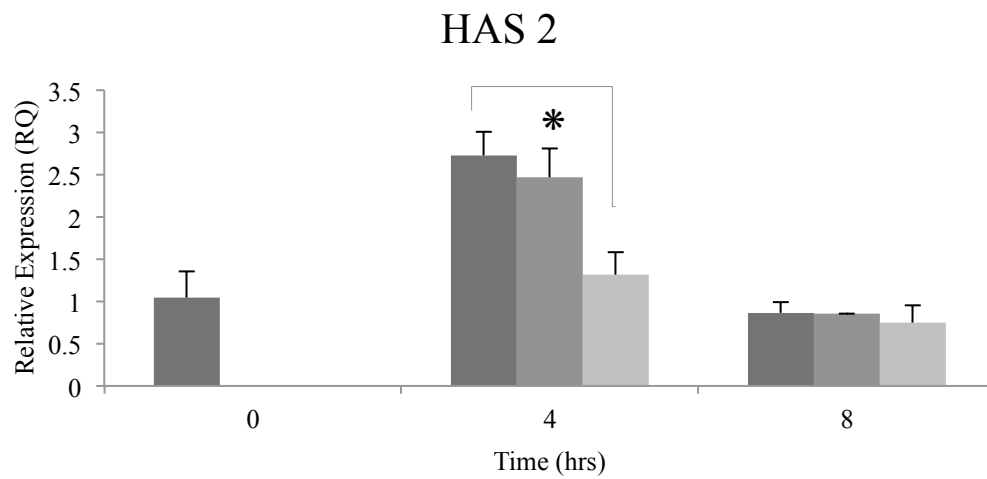
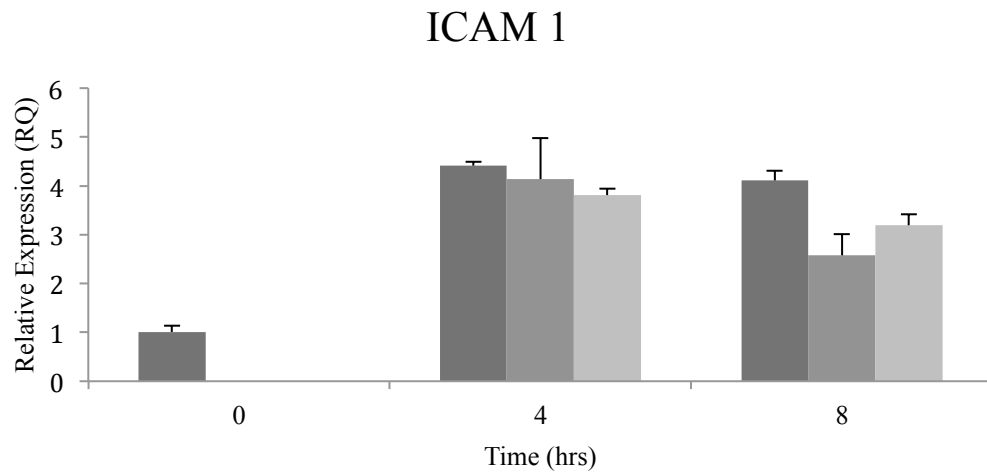


Figure 5-11: Co-culture in the presence of IL 1 receptor antagonist IL-1ra/il-1F3 (R&D Systems)

- Fibroblasts + 5 x 10⁵ U937 cells + 1mcg/ml IL-1RA
- Fibroblasts + 5 x 10⁵ U937 cells + 500ng/ml IL-1RA
- Fibroblasts + 5 x 10⁵ U937 cells

Fibroblasts monolayer were co cultured with 5 × 10⁵ U937 cells/ml which had been pre treated with varying concentrations of IL 1 receptor antagonist for an hour. After 4h and 8h respective wells were washed with PBS × 3 times and subjected to treatment with tri reagent and mRNA extracted as described before. RT and qPCR data for ICAM 1 and HAS 2 is presented as mean + SEM (n=3). P value *=0.047

5.3.7 EXPRESSION OF ICAM-1 AND HAS2 IN FIBROBLAST AND U 937 CELL CO-CULTURE IN THE PRESENCE OF ANTI TNF α

As above, fibroblast and U 937 co-culture experiments were designed to analyse the role of TNF α by using anti TNF α antibodies. Growth arrested fibroblast monolayer was co-cultured with 5×10^5 U937 cells/ml which had been pre-treated with varying concentrations of anti TNF α antibodies (AB-210-NA, R&D Systems) for 1 hour. After 4h and 8h, respective wells were washed with PBS x 3 times and RNA extracted as discussed and analysed for ICAM 1 and HAS 2 expression using reverse transcription and Q PCR.

A reduction in the expression of ICAM-1 was expected, based on the results from previous experiments. The results, however, showed more reduction in HAS2 expression, though not statistically significant (p value 0.069, 0.094 and 0.125 with 10 μ g/ml, 5 μ g/ml and 1 μ g/ml of TNF α respectively) but no change in ICAM-1 mRNA expression.

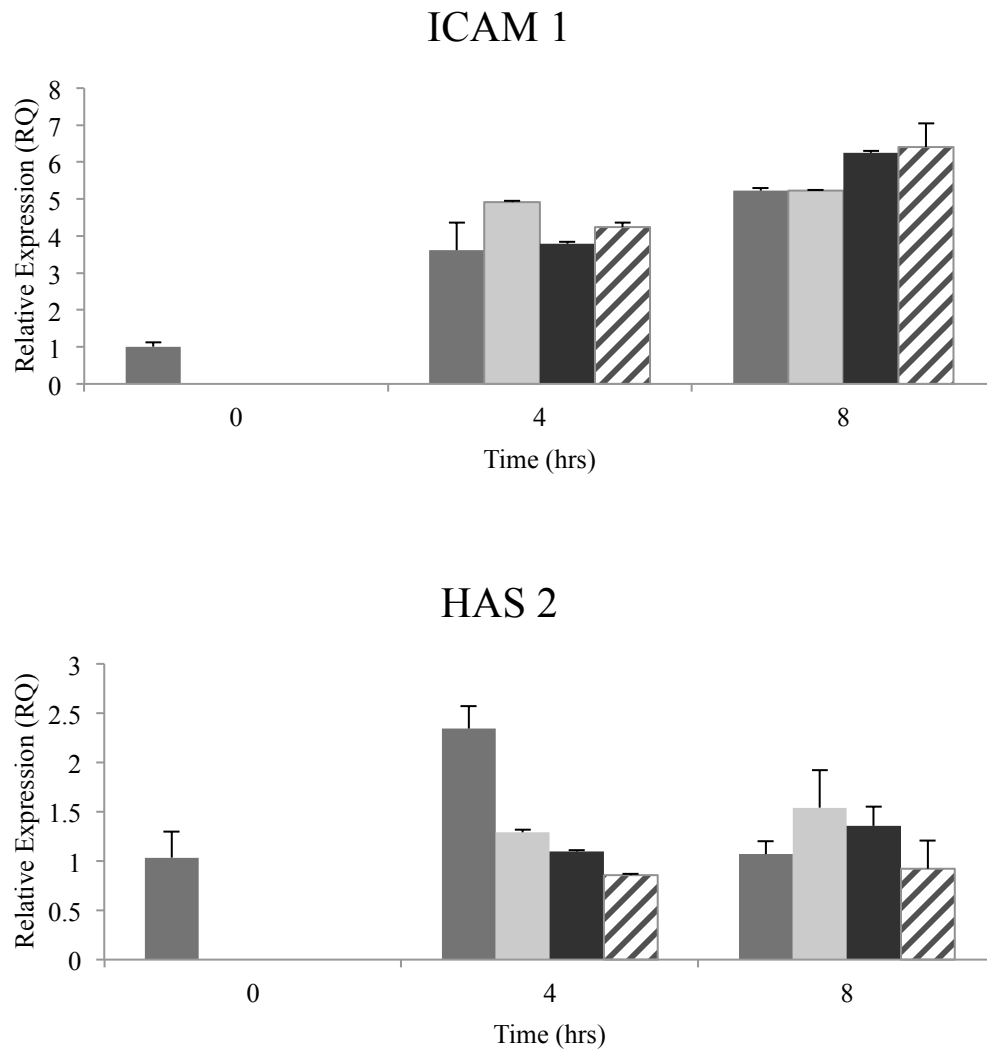


Figure 5-12: *Fibroblast and U937 cells Co-culture in the presence of anti TNF α antibodies*

Growth arrested Fibroblasts monolayer was co cultured with 5×10^5 U937 cells/ml which had been pre treated with varying concentrations of anti TNF α antibodies (AB-210-NA, R&D Systems) for 1 hour. (1mcg/ml, 5 mcg/ml, 10mcg/ml anti TNF α)

After 4h and 8h respective wells were washed with PBS x 3 times and subjected to treatment with tri reagent and mRNA extracted as described. RT and qPCR data for ICAM 1 and TNF α is presented as mean + SE of mean (n=3).

5.3.8 EXPRESSION OF ICAM 1 AND HAS 2 IN FIBROBLAST AND U937 CELL CO-CULTURE IN THE PRESENCE OF BOTH ANTI TNF α AND IL 1 RA

The next step was to design a co-culture experiment in the presence of both anti TNF α antibodies and IL1RA, as there is evidence that a combination of cytokines leads to more significant upregulation of ICAM 1 than with individual cytokines.

Fibroblasts monolayers were co-cultured with 5×10^5 U937 cells/ml and U937 cells which had been pre-treated with 5 mcg/ml anti TNF alpha antibodies (AB-210-NA, R&D Systems) and 500ng/ml IL 1 RA(Recombinant Human IL-1ra/IL-1F3, 280-RA-010/CF, R&D Systems). After 4h and 8h, respective wells were washed with PBS 3 times and subjected to treatment with tri-reagent, mRNA extracted and analysed for ICAM 1 and HAS 2 expression as described.

The results showed a trend to a reduction in the upregulation of ICAM 1 and HAS 2 mRNA expression. This was more pronounced for HAS 2 in comparison to ICAM 1 expression, though not statistically significant (ICAM-1 P-value = 0.89, HAS 2 P-value = 0.052) (*Figure 5-11*).

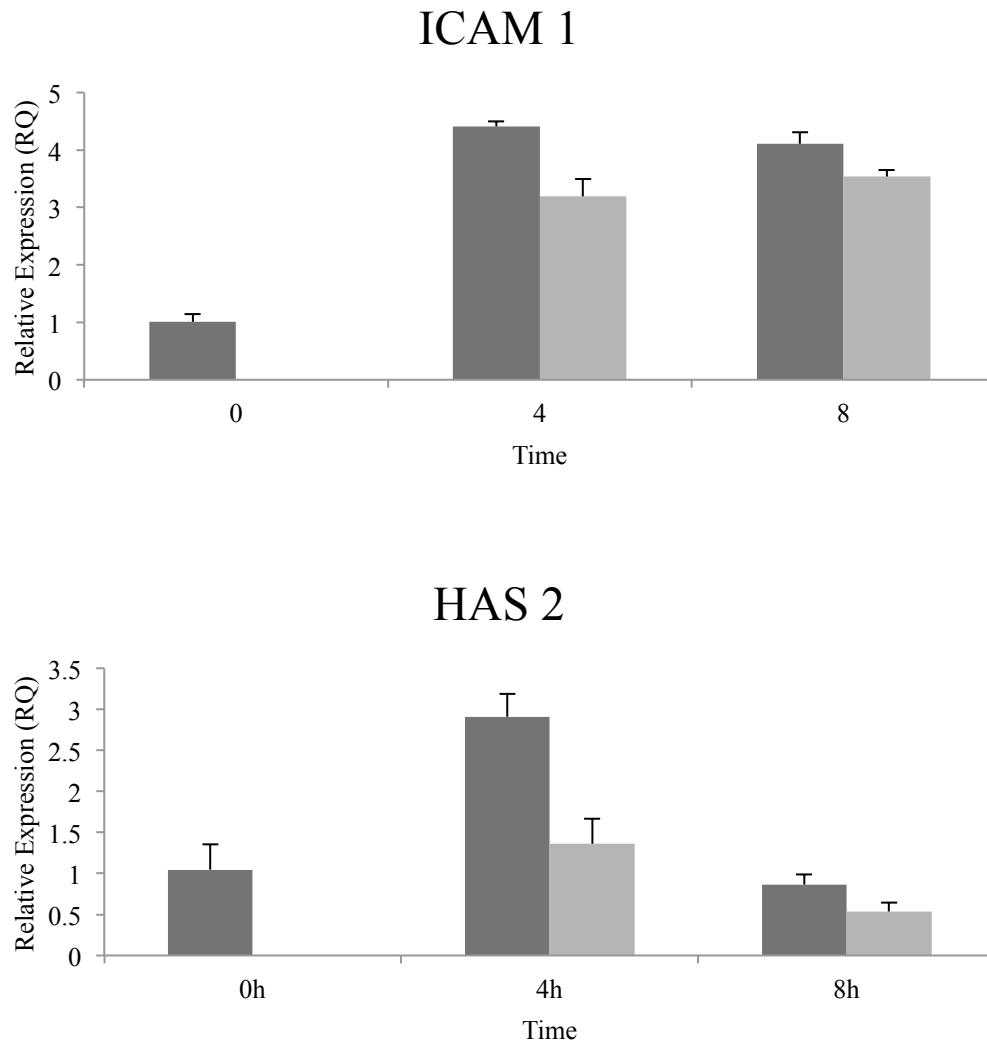


Figure 5-13: *Co-culture in the presence of IL 1 receptor antagonist and anti TNF α*

Fibroblasts monolayer were co cultured with 5×10^5 U937 cells/ml (■) and U937 cells which had been pre treated with 5 mcg/ml anti TNF α antibodies and 500ng/ml IL 1 RA (□). After 4h and 8h respective wells were washed with PBS \times 3 times and subjected to treatment with tri reagent and mRNA extracted as described. RT and qPCR data for ICAM 1 and HAS 2 is presented as mean + SE of mean (n=3).

5.3.9 EXPRESSION OF TNF α AND IL 1 β IN FIBROBLASTS AND ACTIVATED U 937 CELLS CO-CULTURE IN PRESENCE OF sICAM 1

Since, in the presence of activated U937 cells, there was exponential activation of fibroblasts, the effect of activated U937 cells on the induction of TNF α and IL 1 β mRNA was next assessed in the presence of sICAM-1 as before.

There was definite exponential up-regulation of TNF α and IL 1 β mRNA and of ICAM-1 and HAS2 mRNA. This was significantly more than when the fibroblasts were incubated with inactive U 937 cells. The magnitude of up-regulation in the expression of TNF α and IL 1 β was reduced in the presence of sICAM. This was more pronounced for TNF α and ICAM-1, potentially suggesting a greater role of cell-to-cell binding in the regulation of these genes (*Figure 5-12 and 5-13*). The reduction in expression in the presence of soluble ICAM-1 in the medium would again suggest a role for the CD18/ICAM-1 binding interaction and the lack of 100% effect could also suggest the inability of soluble ICAM to saturate all the integrin receptors on the surface of the activated U 937 cells.

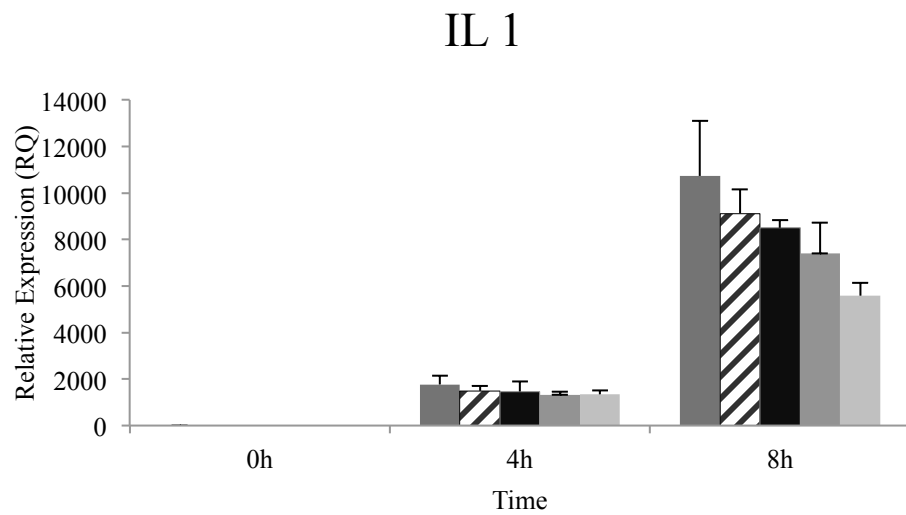
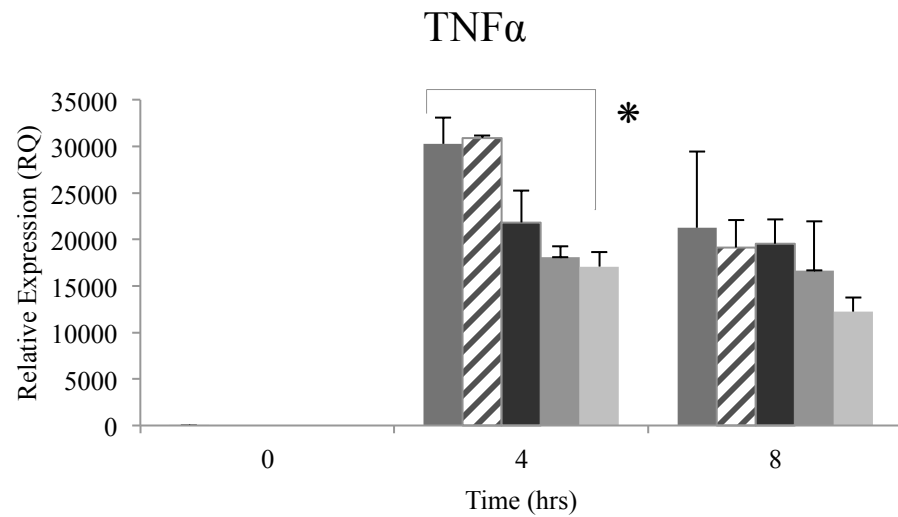


Figure 5-14: : Expression of TNF α and IL 1 β in fibroblast and PMA activated U937 cells co-culture in the presence of sICAM 1

- Fibroblasts + activated U937 cells
- Fibroblasts+800ng/ml sICAM + act U937 cells
- Fibroblasts + 400ng/ml sICAM + act U937 cells
- Fibroblasts + 200ng/ml sICAM + act U 937 cells
- Fibroblasts + 100 ng/ml sICAM + act U937 cells

PMA activated U 937 cells were suspended in varying concentrations of sICAM containing DMEM F12 HAM medium for 1 hour. These media were then used to incubate growth arrested fibroblasts monolayers for 4 and 8 hours in 12 well plates. RNA was extracted as detailed. mRNA expression for ICAM 1, HAS 2, TNF a and IL 1 was assessed using reverse transcription and Q PCR techniques. P values * = 0.015

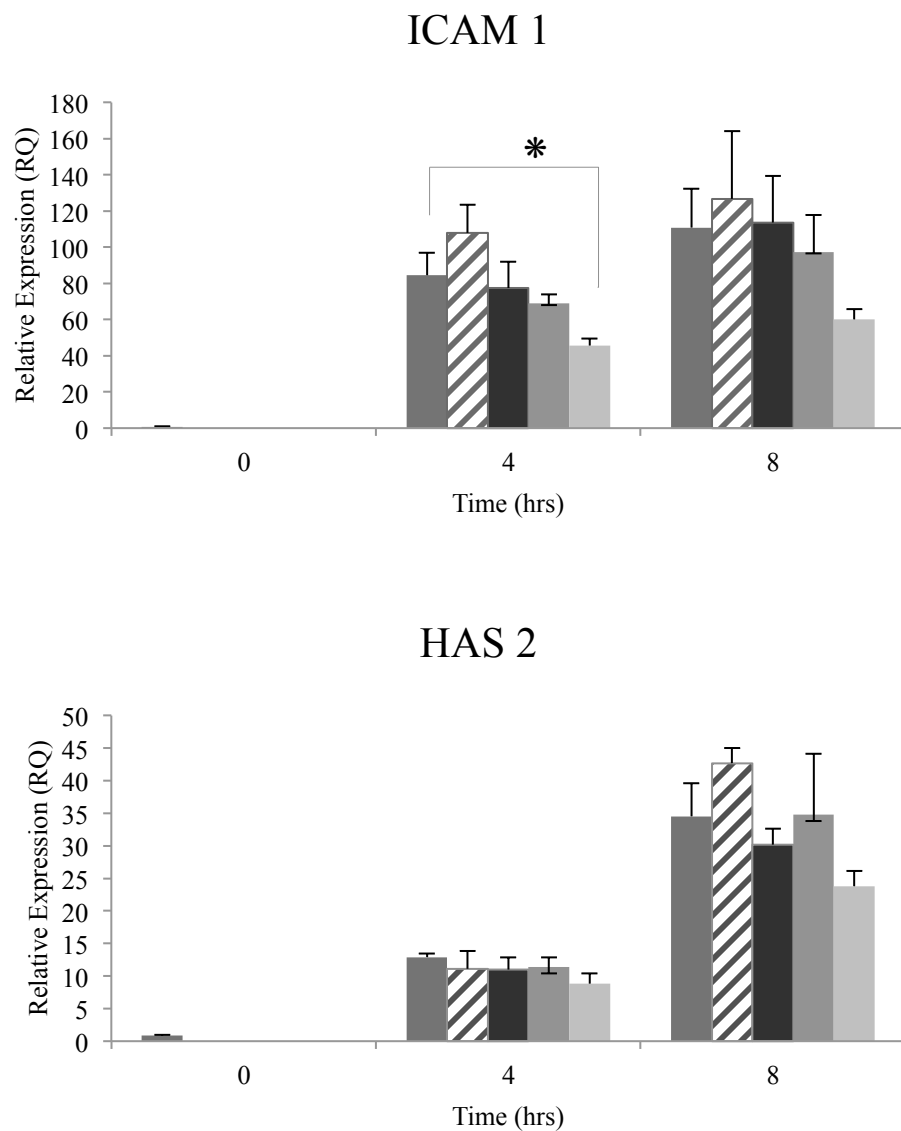


Figure 5-15: Expression of ICAM 1 and HAS 2 in fibroblast and PMA activated U937 cells co-culture in the presence of sICAM 1

- Fibroblasts + activated U937 cells
- Fibroblasts+800ng/ml sICAM + act U937 cells
- Fibroblasts + 400ng/ml sICAM + act U937 cells
- Fibroblasts + 200ng/ml sICAM + act U937 cells
- Fibroblasts + 100 ng/ml sICAM + act U937 cells

PMA activated U 937 cells were suspended in varying concentrations of sICAM containing DMEM F12 HAM medium for 1 hour. These media were then used to incubate growth arrested fibroblasts monolayers for 4 and 8 hours in 12 well plates. RNA was extracted as detailed. mRNA expression for ICAM 1, HAS 2 , TNF and IL 1 was assessed using reverse transcription and Q PCR techniques. P value * = 0.043,

5.3.10 EXPRESSION OF IL 1 β IN FIBROBLASTS AND PMA ACTIVATED U 937 CELLS CO-CULTURE IN PRESENCE OF MEMBRANE INSERTS

Inactive and activated U 937 cells behave differently. I have shown that there is an exponential increase in IL 1 β , TNF α , ICAM 1 and HAS 2 up-regulation when lung fibroblasts are cultured with activated U 937 cells. PMA activated U 937 cells induced more IL 1 β (216) and also caused increased ICAM-1-dependent binding (217). It was therefore important to confirm the importance of cell-contact in the co-culture experiments.

Insert experiments were set up as before using activated U937 cells. RNA extracted was then evaluated for not only IL 1 β but also ICAM 1 and HAS 2. There was significant reduction in the expression of ICAM 1, HAS 2 and IL 1 β (p value < 0.001, < 0.001 and 0.037 respectively) (*Figure 5-14 and 5-15*). This again highlights the significance of cell-to-cell binding in the presence of activated U937 cells.

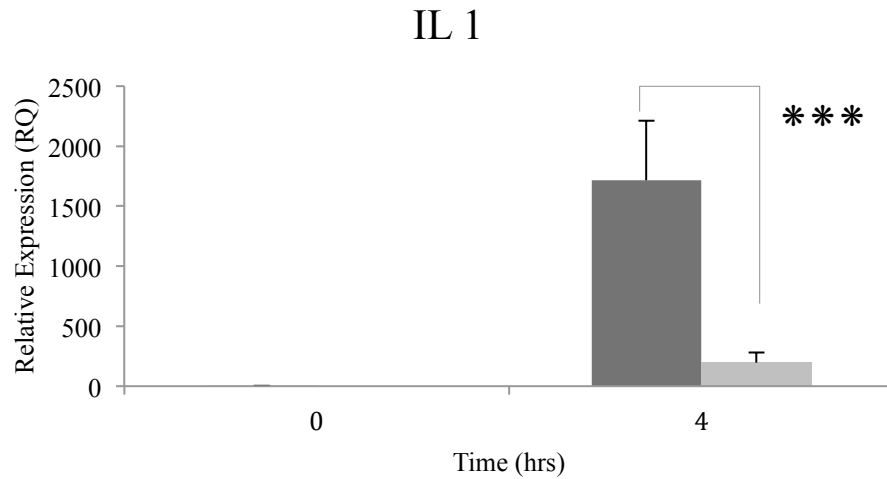


Figure 5-14: *IL 1 β expression in Fibroblasts and PMA activated U937 cells co-culture in the presence/absence of 0.4 μ membrane inserts.*

Fibroblasts+ activated U937 cells
 Fibroblasts+ activated U937 cells in presence of 0.4 μ membrane inserts

Growth arrested fibroblasts and 5×10^5 activated U937 cells/ml were incubated for 4 hours as control. 0.4 μ membrane inserts were suspended over Fibroblasts monolayer and 5×10^5 U937 cells/ml were added into the insert in serum free DMEM F12 HAM medium. Medium was added outside the insert to allow contact between the 2 phases to allow movement of solutes.

Cells were washed with PBS ($\times 3$ times) to wash the U937 cells off and mRNA extracted as described before. RT and qPCR for ICAM-1, HAS-2 IL-1 β and TNF α were performed and the results are expressed as mean + SEM of the measurements corrected for rRNA (n=3)

P value: * < 0.001, ** < 0.001, *** = 0.037

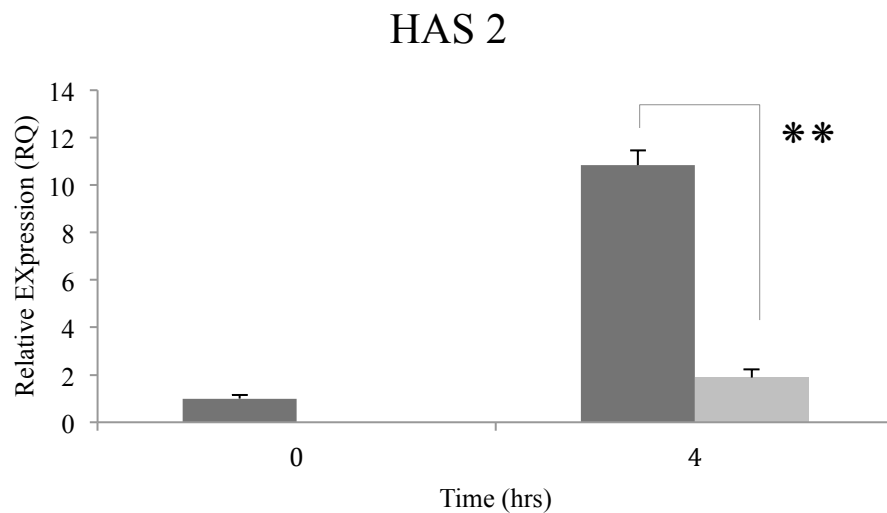
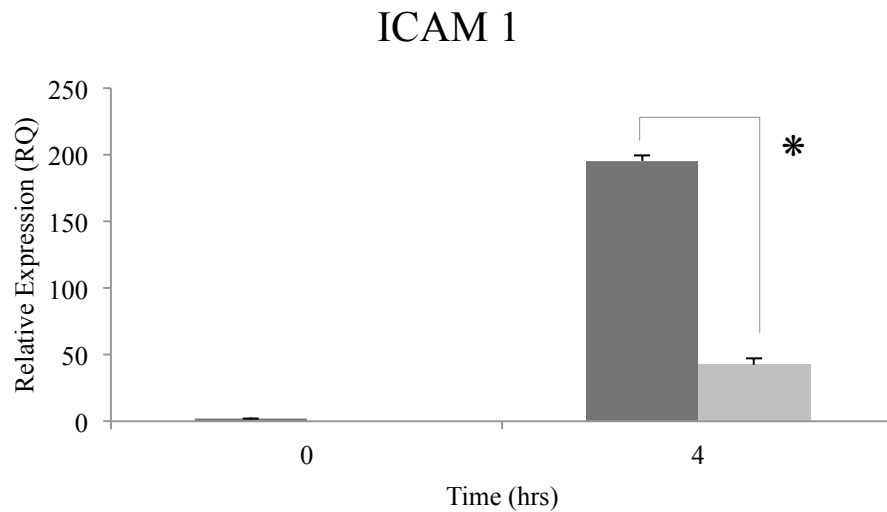


Figure 5-15: Fibroblasts and PMA activated U937 cells incubation in the presence/ absence of 0.4μ membrane inserts.

Fibroblasts+ activated U937 cells
 Fibroblasts+ activated U937 cells in presence of 0.4μ membrane inserts

Growth arrested fibroblasts and 5×10^5 activated U937 cells/ml were incubated for 4 hours as control. 0.4μ membrane inserts were suspended over Fibroblasts monolayer and 5×10^5 U937 cells/ml were added into the insert in serum free DMEM F12 HAM medium. Medium was added outside the insert to allow contact between the 2 phases to allow movement of solutes.

Cells were washed with PBS (×3 times) to wash the U937 cells off and mRNA extracted as described before. RT and qPCR for ICAM-1, HAS-2 IL-1β and TNF α were performed and the results are expressed as mean + SEM of the measurements corrected for rRNA (n=3)

P value: * < 0.001, ** < 0.001, *** = 0.037

5.3.11 EXPRESSION OF TNF α AND IL 1 β FOLLOWING ICAM 1 CROSS LINKING

To confirm that it is the interaction with ICAM1 on the fibroblast surface that is inducing TNF α and IL 1 β samples from the cross-linking experiments (3.3.1.) were analysed for TNF α and IL 1 β mRNA. While there was an increase in the mRNA for both this was not statistically significant.

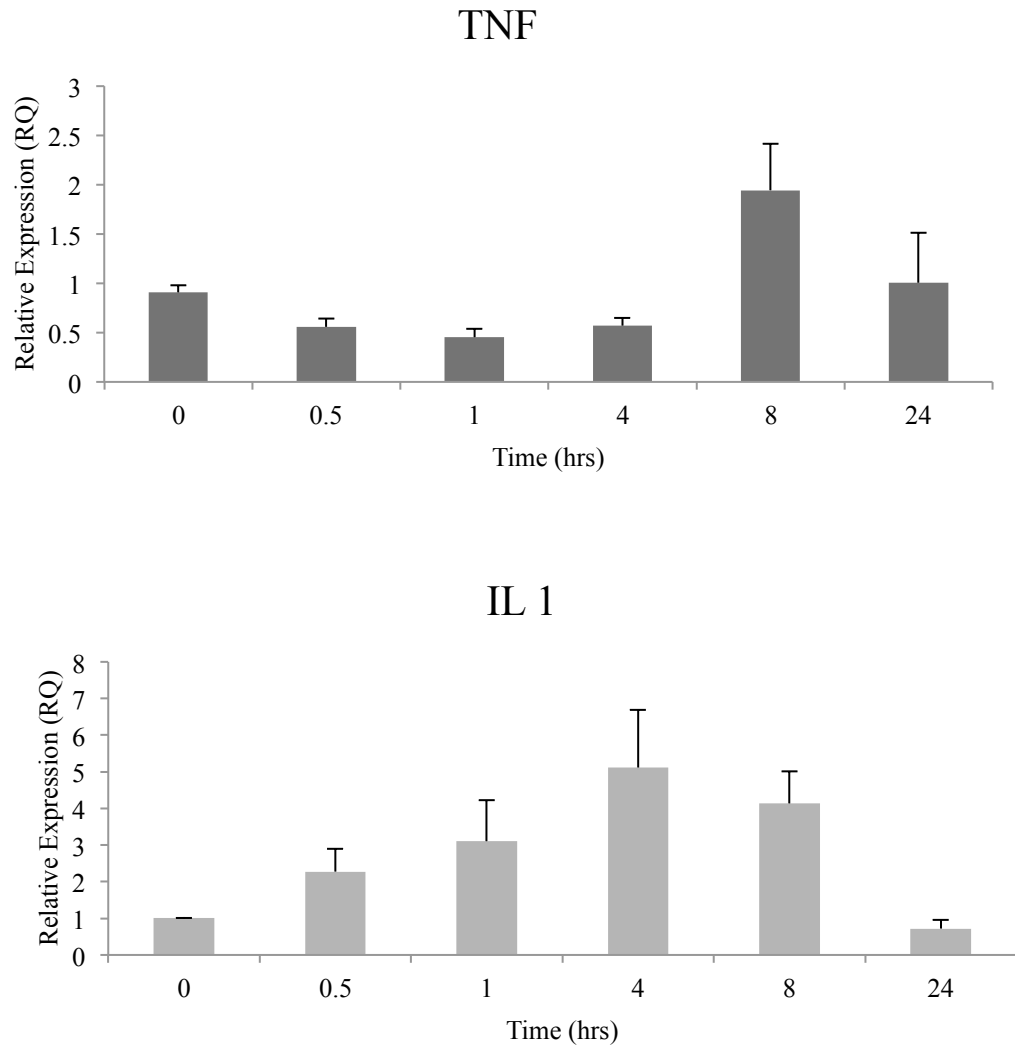


Figure 5-16: *TNF and IL 1 expression in response to ICAM 1 crosslinking*

Fibroblasts were incubated with 10^{-12} TNF α for 24 hours after growth arresting. Cells were washed with sterile PBS and incubated with anti – ICAM 1 monoclonal IgG (10 μ g/ml) in serum free DMEM F12 HAM for 1 hour. Cells were then washed with PBS (\times 3 times) and primary antibody was cross linked using goat anti mouse IgG antibody at a concentration of 10 μ g/ml. mRNA was extracted as discussed in methods. RT and qPCR for TNF α and IL 1 β were performed and the results are expressed as mean + SE of mean of the measurements corrected for rRNA (n=6).

5.3.12 EFFECT OF Sp1 AND Sp3 KNOCKDOWN ON ICAM 1 AND HAS 2 EXPRESSION

Having investigated the roles of cell contact and soluble factors in the up-regulation of ICAM 1 and HAS 2, the next stage of the study was to investigate the intracellular mechanisms that were involved. Various transcription factors play a role in cytokine mediated ICAM 1 and HAS 2 up-regulation. Sp1 and Sp3 have been proven to play an important role in IL 1 β mediated HAS 2 expression and TNF α mediated ICAM 1 up-regulation. The following experiment was designed to see if the up-regulation of HAS 2 and ICAM 1 mRNA was affected if the fibroblasts lacked the ability to activate these transcription factors.

The ability of siRNAs specific to Sp1 and Sp3 to knock down their respective mRNAs was investigated. Transient transfection of fibroblasts was performed with specific siRNA nucleotides (Ambion, US) targeting Sp1 and Sp3 using Lipofectamine 2000 transfection reagent (Invitrogen) as per the manufacturer's specified protocol.

The incubation time for ideal knockdown of the target genes is variable. Therefore it was important to first confirm the optimum transfection time. Fibroblasts were incubated with Sp1specific SiRNA, Sp3 specific RNA or scrambled control. Medium was changed to serum and antibiotic free medium at 24 hours. mRNA was then extracted at different time points. Sp1 and Sp 3 expression was then analysed relative to their expression in samples treated with scrambled control SiRNA for the particular time point. Based on this, I decided to perform transfection for 36 hours as the expression of both Sp1 and Sp3 was reduced by approximately 70% or more without significant changes in cell morphology and viability.

Co-culture experiments were then carried out and fibroblasts were incubated for 24 hours in serum- and antibiotic-free medium containing the transfection complexes for 24 hours. Medium was then aspirated and replaced with serum free and antibiotic free

medium for further 12 hours prior to co-culture with U 937 cells. Medium was then aspirated and mRNA analysed for HAS 2 and ICAM 1 as described before.

There was approximately 80% reduction in the up-regulation of HAS 2 with both SP1 and SP3 knockdown but this was not statistically significant. ICAM 1 upregulation, however, was significantly attenuated in fibroblasts with Sp1 knockdown but not Sp3 knockdown (p value 0.015). These results would strongly support a role for these transcription factors in the activation of ICAM 1 and HAS 2 expression following interactions with monocytes.

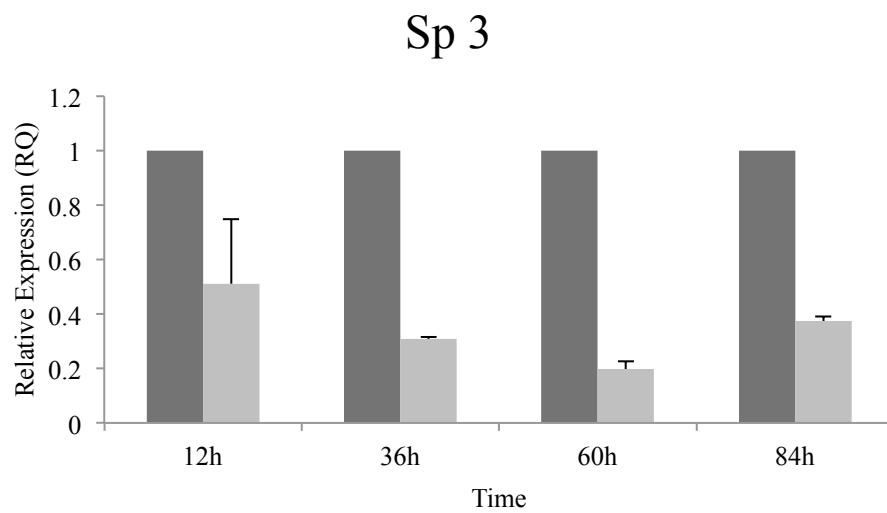
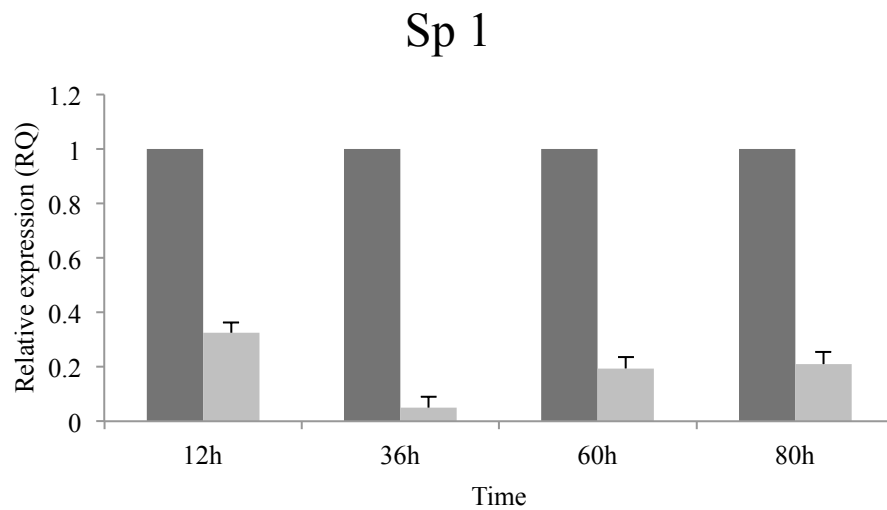


Figure 5-17: SiRNA Optimisation

Q-PCR Analysis of Sp1 and Sp3 mRNA expression following siRNA knockdown of Sp1 and Sp3. Relative expression in comparison with scrambled negative control siRNA of Sp1 following Sp1 siRNA treatment, Sp3 following Sp3 siRNA treatment. (n=3)

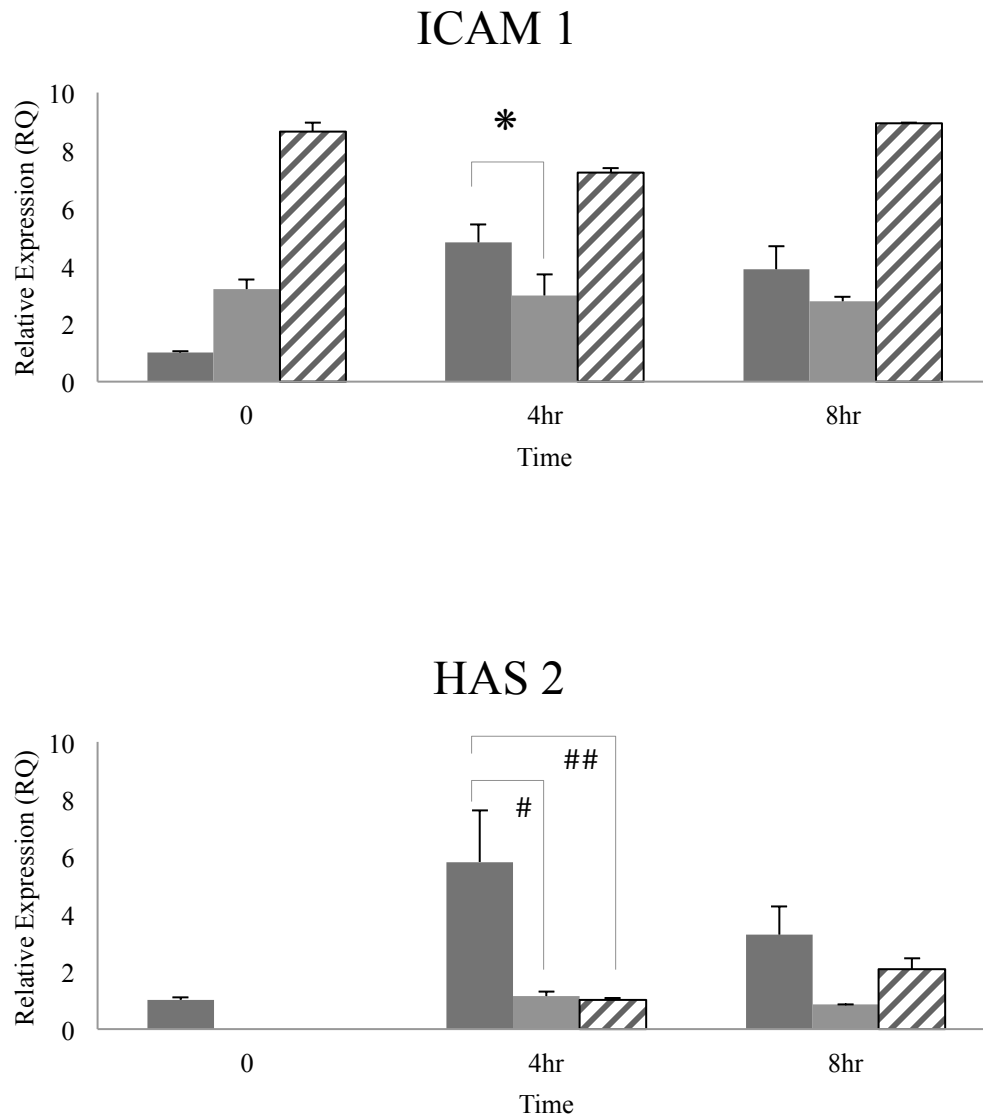


Figure 5-18: ICAM 1 and HAS 2 expression in Fibroblasts-U937 cell co-culture with Sp1 and Sp3 knockdown

qPCR analysis of ICAM 1 and HAS2 mRNAs expression following siRNA knockdown of Sp1 and Sp3 and treatment with U 937 cells. Relative expression in comparison with scrambled negative control siRNA of ICAM 1 and HAS 2 following Sp1 siRNA treatment and Sp3 following Sp3 siRNA treatment. Data is shown as mean + Standard error of mean (n=3). P value * = 0.015, # = 0.093, ## = 0.056.

5.4 DISCUSSION

I have shown in the previous chapter that there is a circulating soluble factor in Fibroblast and U 937 cell co-culture that influences ICAM 1 and HAS 2 up-regulation. I then went on to investigate whether this circulating factor could be $\text{TNF}\alpha$, $\text{IL } 1\beta$ or a combination of both based on the strong evidence I have presented in the discussion for chapter 4.

I first showed that $\text{IL } 1\beta$ and $\text{TNF } \alpha$ are capable of up-regulating mRNA expression of both ICAM 1 and HAS 2. I then demonstrated that $\text{IL } 1\beta$ and $\text{TNF } \alpha$ are induced in fibroblasts and U937 cells co-culture. Membrane insert experiments revealed that ICAM 1 and $\text{TNF } \alpha$ induction is suppressed, but not completely absent, when cell-to-cell contact was prevented using membrane inserts. In contrast, there was no change in the induction of HAS 2 and $\text{IL } 1\beta$. This would suggest a greater role of cell binding in the induction of ICAM 1 and $\text{TNF } \alpha$. This would also suggest a link between HAS 2 and $\text{IL } 1\beta$. The dose dependent reduction in the HAS 2 induction in the presence of $\text{IL } 1$ receptor antagonist (IL-1RA), with no effect on ICAM 1 induction, again strengthens the evidence and favours a relation between $\text{IL } 1$ and HAS 2 mRNA induction. HAS 2 induction was also suppressed in the presence of anti $\text{TNF } \alpha$ in a dose dependent manner. Surprisingly ICAM 1 induction was not suppressed in the presence of anti $\text{TNF } \alpha$.

ICAM 1 induction in the co-culture, which had shown no change in the presence of $\text{IL } 1\text{RA}$ or anti TNF , was suppressed in the presence of both anti TNF and $\text{IL } 1\text{RA}$ by 30% approximately.

I was also able to show that there was a dose dependent reduction in up-regulation of ICAM 1, HAS 2, $\text{TNF } \alpha$ and $\text{IL } 1\beta$ in the presence of soluble ICAM, again confirming the role of ICAM 1 binding in their regulation.

There is ample evidence to prove TNF dependent ICAM 1 up-regulation. This makes it difficult to explain the lack of TNF α up regulation in cross-linking experiments.

The up-regulation of IL 1 β and TNF α was exponential when PMA activated U937 cells were used for co-culture. TNF α induction was suppressed in the presence of sICAM 1.

There is strong evidence for the role of these two inflammatory cytokines in various inflammatory conditions. For instance, elevated levels of IL-1 β and TNF- α are predominant in the pathogenesis of rheumatoid arthritis.(218) It has been shown that IL-1 β led to an increase in ICAM- 1 dependent monocyte binding in proximal tubular cells.(165) It has also been shown that IL 1 β leads to up-regulation of Hyaluronan in proximal tubular cells.(219) Studies have demonstrated an up-regulation of TNF- α and ICAM-1 expression in the kidney in response to cisplatin. In animal models of cisplatin nephrotoxicity, blocking of ICAM-1 reduces the severity of cisplatin-induced renal injury.(220) The role of pro-inflammatory cytokines in macrophage and fibroblast interaction is well known in various inflammatory disorders involving not only kidneys, but also other organs and in multisystem disorders such as SLE(221). TNF- α is increased in various inflammatory renal diseases and in both mesangial and proximal tubular cells.(222, 223) TNF- α has been shown to induce a dose and time-dependent increase in ICAM-1 protein expression and U937 adhesion to A549 cells, which are alveolar epithelial cells.(126)

I first showed that IL 1 β and TNF α are capable of up-regulating mRNA expression of both ICAM 1 and HAS 2. It is well known that TNF α and IL 1 β up-regulate ICAM 1 expression in various cell types as previously mentioned.(209-211) For example, TNF α and IL-1 β mediate ICAM-1 induction via microglia–astrocyte interaction in CNS radiation injury. (167) It has been established that various cytokines together can increase ICAM-1 expression greater than either cytokine alone.(123) Similar synergistic

action could exist for TNF α and IL 1 β as revealed in the experiment when fibroblasts are stimulated with both these cytokines together.

It has been shown that ICAM-1 crosslinking induces activation of the transcription factor AP-1 and transcription of the IL-1 β gene using specific antibodies to cross-link ICAM-1 on a rheumatoid synovial cell line (E11 cells).(168) It has also been shown that ICAM-1 cross-linking leads to TNF α secretion. ICAM-1 binding induces cAMP accumulation and activation of the mitogen-activated protein kinase extracellular signal-regulated kinase.(224) My experiments indicate up-regulation in expression of TNF- α and IL 1 β mRNA in ICAM-1 cross-linking experiments that were done to mimic its interaction with its ligands. Similar results were seen in experiments involving lung fibroblast and U937 co-culture.

Research done elsewhere, with co-culture of differentiated U937 cells with fibroblast-like synoviocytes, failed to release detectable levels of IL-1 β and TNF α from the U937 cells. U937 cells differentiated by phorbol ester were able to release these two cytokines and, in the case of the co-culture, mRNAs for both cytokines were highly expressed.(121) The induction of ICAM-I and VCAM-I by the co-culture of human umbilical cord vein endothelial cells, HUVECs and monocytes was inhibited by the combination of anti-IL-1 α , anti-IL-1 β and anti-TNF Antibodies. This would suggest that the monocyte-endothelial cell interaction induces the expression of ICAM-1 and VCAM-I in endothelial cells partially through the production of IL-1 β and TNF α .(152) This was revealed in our co-culture model, where combination of IL 1RA and anti TNF α showed reduced up-regulation of ICAM 1, and more significantly for HAS 2.

Also, my experiments reveal reduced up-regulation of TNF α and ICAM-1 when fibroblast and U937 interaction is restricted using membrane inserts as before. IL 1 β and HAS 2 expression were unrestricted despite the membrane inserts. This suggests

that ICAM 1 and TNF α expression may be regulated in a different way in comparison to IL 1 β and HAS 2.

Finally, I have shown that presence of soluble ICAM in the fibroblasts and U 937 cell co-culture leads to reduced up-regulation of ICAM 1, HAS 2, TNF α and IL 1 β , again highlighting the role of cell-to-cell contact as well as the fact that there are other mechanisms involved in the above interaction responsible for some up-regulation.

The major intracellular signal transduction pathways involved in the regulation of ICAM-1 expression include protein kinase C (PKC), the mitogen-activated protein (MAP) kinases (ERK, JNK, and p38), and the NF- κ B signaling pathways.(225-227) Multiple transcription factors are involved in activation of ICAM-1 expression include AP-1, NF- κ B, STAT and Sp1.(124) The ICAM-1 promoter contains Sp1 binding sites. The Sp1 binding site in the proximal promoter has been shown to be required for basal transcription of the ICAM-1 gene.(228)

Work in our lab has shown Il-1 β mediated HAS 2 up-regulation is mediated by transcription factors Sp1 and Sp3. The rate-limiting step in the production of IL-1 β is transcription. I have demonstrated that sp1 and sp3 knockdown leads to reduced up-regulation of both HAS2 and ICAM 1 in fibroblast and U 937 cell co-culture. Also, there is reduced up-regulation of ICAM 1 in Sp1 knockout fibroblasts. These results strengthen the hypothesis that TNF α and IL 1 β are the likely circulating factors responsible for ICAM 1 and HAS 2 up-regulation.

The abundant signaling pathways and transcription factors involved in ICAM-1 transcription reflect the complex cell type-specific and stimulus-specific regulation of the ICAM-1 gene. This may also explain the inability to completely suppress the up regulation of ICAM 1 and HAS 2 in the fibroblast U 937 cell co-culture model in the presence of membrane inserts, soluble ICAM, anti TNF α antibodies and/or IL-1RA.

6 GENERAL DISCUSSION AND FURTHER WORK:

6.1 *Questions answered*

In previous studies we have shown that cross-linkage of ICAM 1 on the surface of fibroblasts leads to its up-regulation (119). The aim of the current study was to extend these findings to fibroblast/monocyte interactions and to investigate the mechanisms involved.

I have shown that the interaction of fibroblasts with U937 cells leads to up-regulation of ICAM 1 mRNA expression. This would suggest a role for this response in a pro-inflammatory feedback mechanism, allowing for more inflammatory cells to be pulled to the site of inflammation in order to enhance and prolong the inflammatory response. I have also demonstrated that this interaction leads to up-regulation of HAS2, the major synthase implicated in the pathological generation of hyaluronan. Further experiments showed that the up-regulation of ICAM 1 and HAS 2 takes place even when fibroblasts interacted with unstimulated U937 cells. Up-regulation of ICAM 1 and HAS 2 was significantly increased, however, when U937 cells were activated. This has important implications, as it would suggest that the presence of monocytes at an inflammatory site might be enough to maintain an ongoing inflammatory process by promoting interactions that would lead to adhesion molecule expression and continuous release of cytokines and chemokines even after the offending stimulus has been removed. These cytokines and chemokines can play an important role in prolonging inflammation, scarring and leading to fibrosis.

Further investigations of the potential mechanisms involved showed that cell-to-cell contact was important but not compulsory in the up-regulation of both ICAM 1 and HAS 2. Cell-to-cell contact dependent up-regulation was preferentially greater for ICAM 1 than for HAS 2. This was proved by using membrane inserts to prevent physical contact between the fibroblasts and U937 cells, and furthermore, by using soluble

ICAM and

further investigations of the potential mechanisms involved showed that cell-to-cell contact was important but not compulsory in the up-regulation of both ICAM 1 and HAS 2. Cell-to-cell contact dependent up-regulation was preferentially greater for ICAM 1 so responsible for the up-regulation of ICAM 1 and HAS 2 might differ, thus giving us the opportunity to manipulate the two responses independently.

The differential up-regulation of both ICAM 1 and HAS 2 in the presence of conditioned medium suggests a role for a soluble factor in the up-regulation of HAS 2 and ICAM 1. There is ample evidence for the role of TNF α and IL-1 β in the up-regulation of ICAM 1 and HAS 2 in various cell types as discussed in chapter 5. I therefore investigated the role of TNF α and IL 1 β as possible soluble factors responsible for the above results. My experiments demonstrated that TNF α and IL 1 β , both individually and together, caused up-regulation of both ICAM 1 and HAS 2.

Cross-linking ICAM 1 on the surface of fibroblasts led to up-regulation of ICAM 1 and HAS 2. It also induced up-regulation of TNF α and IL 1 β mRNA. While the level of up-regulation did not appear to be sufficiently high to suggest a causal relationship in these samples, the superimposed effect of cell-cell contact may play a role in ensuring that cytokines are presented to the target cell surface efficiently. My experiments demonstrated significant up-regulation of TNF α and IL 1 β mRNA in the fibroblast and U937 cell co-culture. The up-regulation was differentially affected in the presence of membrane inserts with the TNF α up-regulation being reduced, and IL-1 β up-regulation being unaffected. TNF α and IL-1 β mRNA up-regulation was also exponentially increased in the fibroblast activated U937 cell co-culture.

Anti TNF α antibodies and IL-1RA were used to reduce the exposure of fibroblasts and U937 cells to the cytokines. This was done to assess the role of these cytokines in the induction of ICAM 1 and HAS 2. These experiments showed greater reduction in HAS 2 up-regulation in comparison to ICAM 1 mRNA expression. They also showed

reduced $\text{TNF } \alpha$ and $\text{IL } 1 \beta$ mRNA expression. Reduced HAS 2 expression in the presence of IL 1RA proves the role of $\text{IL } 1 \beta$ in this interaction. The failure of these antibodies in reducing ICAM 1 mRNA expression is not clearly explained by these experiments. It could be explained by a possible positive feedback loop exerted by ICAM 1 induction on its own up-regulation as seen in cross linking experiments. It is also difficult to explain the reduction in HAS 2 mRNA expression in the presence of anti $\text{TNF } \alpha$ antibodies given the marginal increase in HAS 2 when fibroblasts were stimulated with $\text{TNF } \alpha$ (figure 5-1)

Sp1 and Sp3 are transcription factors known to be responsible for $\text{IL } 1 \beta$ mediated HAS 2 induction. I performed Sp1 and Sp3 knockdown experiments to prove that $\text{IL } 1 \beta$ is at least one of the soluble factors responsible for the up-regulation of HAS 2 in the fibroblast-U937 cell co-culture model. I was able to prove this by showing a significant reduction in the expression of HAS 2 mRNA in the co-culture model. There was also a noticeable reduction in ICAM 1 mRNA expression in fibroblasts with Sp1 knockdown, incubated with U937 cells. Sp1 is known to be one of the transcription factors responsible for $\text{TNF} \alpha$ mediated ICAM 1 up-regulation(124). This would confirm a causative role for $\text{TNF} \alpha$ and $\text{IL } 1 \beta$ in the up-regulation of ICAM 1 and HAS 2 mRNA in fibroblasts and U937 co-culture.

6.2 Implications of the study

Accumulation of LFA-1 (CD-11+ and CD18+) cells in areas of tubulointerstitial damage has been noted in experimental models, showing significant up-regulation of ICAM-1 in areas of renal damage. However, IL-1RA treatment resulted in a dramatic inhibition of interstitial ICAM-1 expression, interstitial leukocyte infiltration, and tubulointerstitial damage. This again highlights the importance of ICAM-1 in renal

disease and the role of IL-1 β (146). In rat models, it has been shown that up-regulation of periglomerular and/ or peritubular capillary ICAM-1 expression is important for mononuclear cell entry into the interstitium, while interaction with fibroblast-like cells may facilitate movement and subsequent focal accumulation of monocytes at sites within the interstitium (19). Therefore, by reducing up-regulation of ICAM-1 beyond a certain stage, it may be possible to reduce the prolonged exposure of fibroblasts to inflammatory cells.

Recent studies have shown reduced decline in renal functions among patients with rheumatoid arthritis (RA) and chronic kidney disease (CKD) treated with anti-TNF- α drugs. Anti-TNF- α drugs used in the study were adalimumab, etanercept or infliximab. This would suggest beneficial role of anti TNF α therapy for managing RA combined with CKD (229). Studies also suggest that targeted TNF- α blockades have the potential to improve renal function by attenuation of renal inflammation in CKD patients (230).

Diabetic nephropathy remains a leading cause of renal disease worldwide. In different experimental models of DN, renal macrophage accumulation correlates with the severity of glomerular and tubulointerstitial injury. ICAM-1 also appears to be a critical promoter of nephropathy in mouse type 2 diabetes by facilitating kidney macrophage recruitment (231, 232). Blocking IL-1 β has been shown to be effective in improving insulin secretion by pancreas in type 2 diabetes. The same study also showed improvement in the severity of joint erosions in rheumatoid arthritis (233, 234).

Pirfenidone is an anti-fibrotic drug for the treatment of idiopathic pulmonary fibrosis. It works by reducing lung fibrosis through down-regulation of the production of growth factors. It reduces fibroblast proliferation, TGF β production and TGF β induced collagen production. Pirfenidone as a prophylactic regimen reduces proteinuria in anti-GBM nephritis via preservation of podocytes (235, 236). It has also been shown to reduce production of inflammatory mediators such as TNF- α and IL-1 β .(237)

Combining therapy to reduce ICAM 1 up-regulation and reducing HA accumulation has been studied and found to be effective in inflammatory conditions such as Grave's ophthalmopathy(19). Ischemia-reperfusion injury (IRI) is a major cause of renal dysfunction in both native kidneys and renal allografts. ICAM 1 is known to play a significant role in inflammation in ischaemic reperfusion injury models (238). This has been postulated to be mediated in part by $\text{TNF } \alpha$ and $\text{IL-1 } \beta$ (239). The amount of accumulated HA also increases with increasing duration of ischemia and the gene expression of HAS2 is elevated (240). HA is said to contribute to oedema in the transplanted kidney and cause delayed graft function. Combining anti ICAM 1 therapy and reducing HA accumulation in this model of ischaemic reperfusion injury as seen in delayed graft function post renal transplant, offers a potential therapeutic option.

6.3 LIMITATIONS OF STUDY

6.3.1 Estimation of protein concentration of ICAM 1 and HAS 2

Although I have been able to show induction of HAS2 and ICAM1 at the messenger RNA level, it would be important to see if this message was transcribed into protein to cause functional changes. It would also be helpful to measure ICAM 1 to evaluate its functional implications.

6.3.2 Estimation of $\text{TNF } \alpha$ and $\text{IL } 1\beta$ concentration

An ELISA to look into $\text{TNF } \alpha$ concentrations did confirm induction above the detectable threshold and reduced concentration in the presence of inserts. However, the experiment analysing $\text{IL } 1\beta$ concentrations was unable to show detectable levels of $\text{IL } 1\beta$ induction above the threshold. This could not be repeated due to time and funding constraints towards the end of my research period.

6.3.3 HA type and concentration

As mentioned in the introduction, HA is synthesised by three synthases, HAS1, HAS2 and HAS3(73). Of the three, HAS 2 is the predominant synthase involved in HA production.

HAS 2 has been found to be the primary synthase whose expression is elevated in pathological diseases such as diabetes, ischaemic reperfusion injury, renal transplant rejection etc. In animal models it has been shown that HAS 2 expression is increased in diabetic animals with an increased medullary HA concentration(97).

It was therefore reasonable to look into the induction of HAS 2 in my model. Although I have shown the increased expression of HAS 2 in the fibroblast-monocyte co-culture model, I did not get an opportunity to characterise the hyaluronan produced. It is well known that HMW HA and LMW HA serve different purposes in inflammatory conditions. High-molecular-weight preparations of HA are without effect; however, when high-molecular-weight preparations of HA are digested with hyaluronidase, an adhesion-molecule-inducing activity can be elicited. Very small HA molecules such as HA hexamers, which represent the minimal binding motif for CD44, are also without effect on ICAM-1(153).

It would therefore be useful to confirm HA production. Once confirmed it would be useful to quantify and characterise the HA produced, on the basis of its molecular weight and distribution on cell surface.

6.3.4 Signalling pathways involved:

It is well known that ICAM 1, in addition to acting as an adhesion molecule, acts as a signal transducer. In various studies it has been demonstrated that ICAM 1 shows cell-type specific “outside-in” signalling. Multiple intracellular signal transduction pathways

are involved in the regulation of ICAM-1 expression include protein kinase C (PKC), the mitogen-activated protein (MAP) kinases (ERK, JNK, and p38), and the NF- κ B signalling. In a study using a rheumatoid synovial cell line, cross-linking of ICAM-1 activated the transcription factor AP-1 and subsequently induced IL-1 β transcription (168). ERK1/ERK2, as well as p38 MAPK, are activated upon ligation of ICAM-1 in astrocytes (241). ICAM-1 cross-linking demonstrated increase in TNF α -mediated RANTES (Regulated on Activation, Normal T-cell Expressed and Secreted) production involving activation of ERK in airway epithelial cells (242). However, work published from our lab has shown induction of p38, ERK1, and ERK2 MAP kinases were activated in a calcium dependent manner, with only p38 activation being essential for ICAM 1 induction (119). The abundant signalling pathways and transcription factors involved in ICAM-1 transcription reflect the complex cell type-specific and stimulus-specific regulation of the ICAM-1 gene. It is therefore important to try and ascertain the pathways involved in the fibroblast-monocyte interaction induced ICAM 1 activation. This could also suggest a link to the HAS 2 induction, thereby providing another point for possible intervention. I started looking into the role of MAP kinases, ERK (Extracellular-signal-regulated-kinases) and p38 Kinases. Initial experiments were suggestive of an ERK dependent activation. Results are attached in Appendix 1. I also investigated the role of NF- κ B activation in the up-regulation of ICAM 1 and HAS 2 but I decided to omit this result for now as this was only one experiment with n=1 and needs confirmation.

6.3.5 Role of CD44 receptors

The major HA receptor is CD44, a 90kDa cell surface molecule. In normal kidney these are found in the dendritic interstitial cells but in pathological conditions it is expressed significantly in the tubular epithelial cells and glomerular crescents (243). Cross-linking of CD44 on the cell surface of tubular epithelial cells has been shown to lead to up-

regulation of ICAM-1 and VCAM-1 (153). Subsequent to my work, our laboratory has shown that IL 1 β dependent stimulation of fibroblasts leads to spiculated protrusions of the HA coat on the cell surface. These spiculations co-localize CD44 and ICAM 1. Functionally, these HA-rich structures appear to enhance ICAM-1 and monocyte interactions and promote fibroblast-monocyte binding (244). It would therefore be interesting to evaluate CD44 dependent binding in my co-culture model, considering that I hypothesized IL 1 β to be one of the possible factors responsible for HAS 2 and ICAM 1 induction.

6.4 CONCLUSION

In conclusion the work presented shows a possible mechanism where unstimulated fibroblasts and mononuclear cells up-regulate ICAM 1 and HAS 2, thus providing a mechanism to perpetuate inflammation in the absence of an overt inflammatory stimulus. The mechanism responsible involves cell-to-cell contact using ICAM 1 dependent binding but also pathways independent of cell contact. There is a soluble factor that plays an important role in the interaction leading to ICAM 1 and HAS 2 induction in addition to cell-to-cell contact. Pro-inflammatory cytokines may play a role in this interaction in the form of these soluble factors.

I also demonstrated a significant increase in ICAM 1, HAS 2, IL 1 β and TNF α induction in the presence of activated/stimulated U937 cells, a surrogate of activated monocytes in inflammation. Cell-to-cell contact seems to play a more significant role in this model of interaction. It is possible that other receptors involved in cell-to-cell contact such as CD44 may have a role in this interaction but this needs to be evaluated. This study therefore gives us an opportunity to develop novel ways of intervening in the continuous process of inflammation and eventual fibrosis by therapeutically targeting different points in this interaction.

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APPENDIX 1

Role of MAP Kinases in induction of ICAM 1 and HAS 2

As discussed, extensive literature exists supporting role of MAP Kinases in the induction of HAS 2 and ICAM 1. There involvement of p38 and ERK has been shown to vary depending on the cells interacting with the monocytes and the environment they interact in. I looked into the role of these kinases in the induction of ICAM 1 and HAS 2 in fibroblast-U937 cells interaction. Work in our lab had previously shown p38 MAP Kinase activation , not ERK, is involved in ICAM 1 upregulation in ICAM 1 crosslinking experiments (119)

Fibroblasts were growth arrested when near confluence for 48 hours. U 937 cells co-incubated with varying concentrations of p38 inhibitor (SB203580, Cell Signalling technologies,Inc). Fibroblasts and fibroblasts with co-incubated with untreated U937 cells were used as control. Similarly Fibroblasts were incubated with U937 cells pretreated with ERK inhibitor (PD98059, Cell Signalling technologies,Inc.). Two concentrations were used, 10microM and 50 microM). RNA was extracted as discussed in methods chapter at 4 hours and mRNA assessed using RT and Q PCR.

The experiments showed almost 70% reduction in ICAM 1 and HAS 2 induction in the presence of ERK inhibition at both concentrations used but this was not statistically significant (p value= 0.34 with 10microM and 0.142 with 50microM ERK inhibitor) No significant reduction in the induction of ICAM 1 and Has 2 was seen with p38 inhibitors (SB 203580)

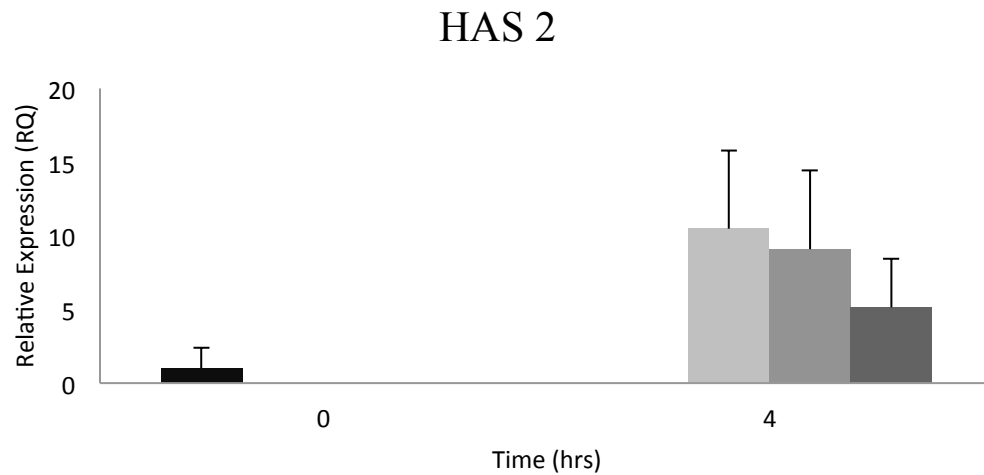
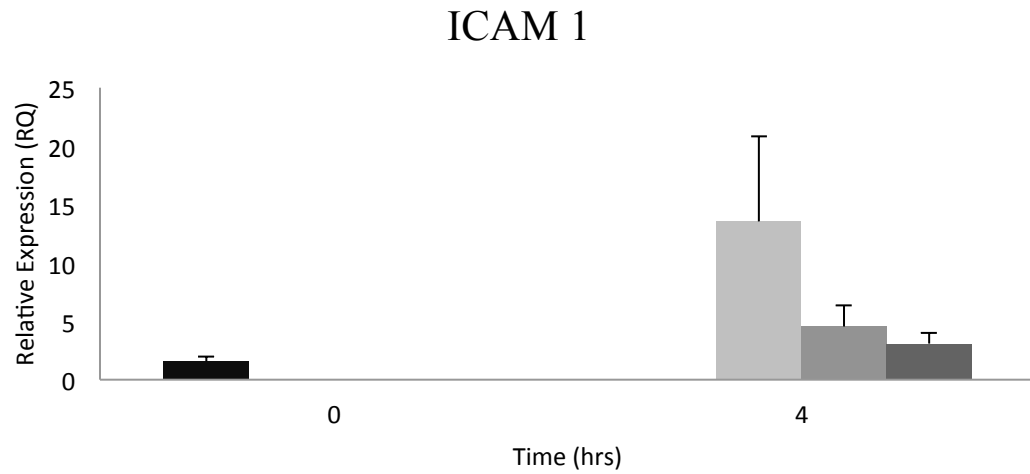


Figure A-0-1: Inhibition of ICAM 1 and HAS 2 induction in the presence of ERK inhibitor

Fibroblasts were grown to near confluence in 6 well plates. They were growth arrested for 48 hours before incubation with 5×10^5 U937 cells/ml pre treated with ERK inhibitor (PD98059). ■ Fibroblast and fibroblast incubated with U937 cells were used as control. Two concentrations of inhibitor for pretreating the U937 cells were used. □ 10μM ERK inhibitor and ■ 50μM were used. After 4 hours the cells were washed with PBS x 3 times and mRNA extracted as in Methods. Q PCR results for ICAM 1 are represented as mean + SE of mean (n=6).

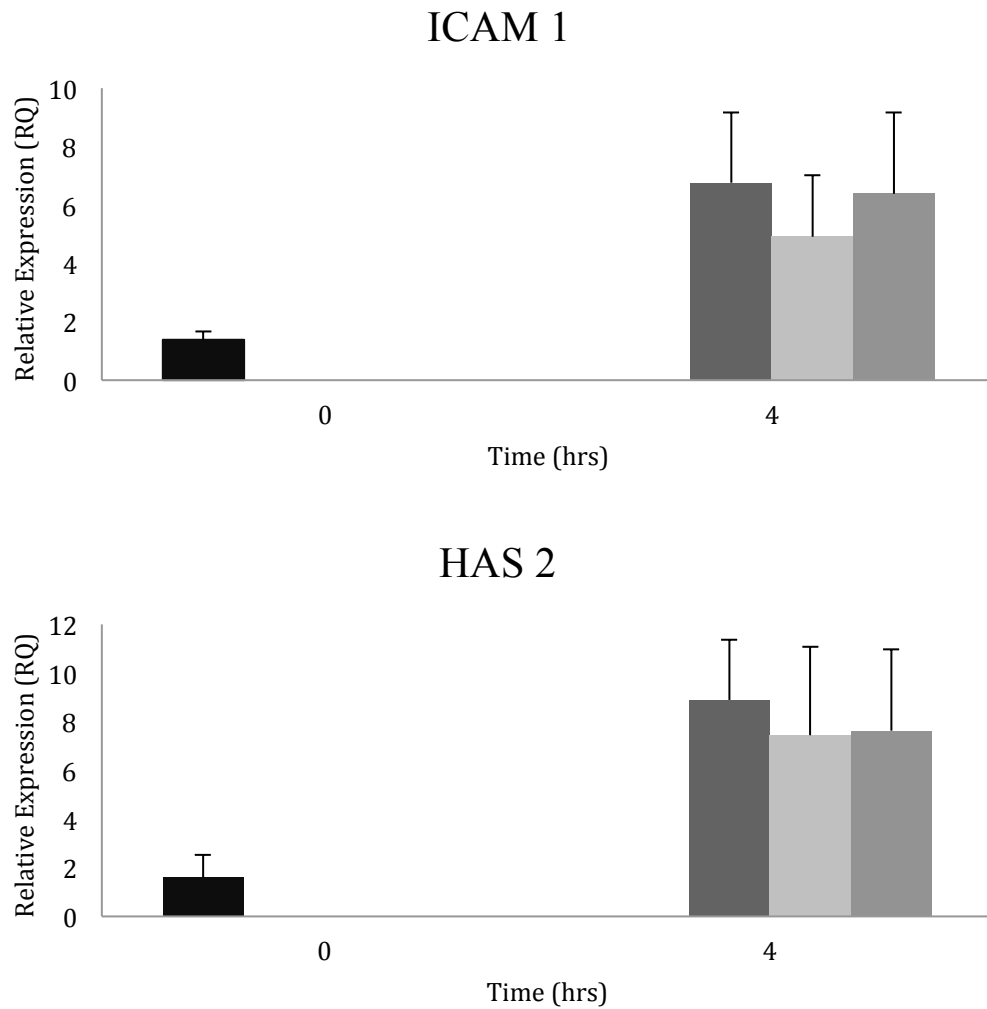


Figure A-0-2 Inhibition of ICAM 1 and HAS 2 in the presence of p38 Inhibitor

Fibroblasts were grown to near confluence in 6 well plates. They were growth arrested for 48 hours before incubation with 5×10^5 U937 cells/ml pre treated with p38 inhibitor (SB203580). ■ Fibroblast and ■ fibroblast incubated with U937 cells were used as control. Two concentrations of inhibitor for pretreating the U937 cells were used. ■ 500 nM inhibitor and ■ 1microM SB203580 were used. After 4 hours the cells were washed with PBS x 3 times and mRNA extracted as in Methods. Q PCR results for ICAM 1 are represented as mean + SE of mean (n=3).

