The Effect of Diabetic Nephropathy on Cell Adhesion and Atherosclerosis

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Declaration

This thesis details the results of original investigations undertaken by myself as a candidate for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Sydney. The experimental work was completed between the years 1999-2004 in the Department of Medicine (Endocrinology), the University of Sydney and the Heart Research Institute, the Royal Prince Alfred Hospital, Sydney, Australia.

The work presented was conducted under the supervision of my principle supervisors, Professor Dennis Yue and Dr Alison Death, and my associate supervisor, Professor David Celermajer.

Although the experimental work, data analysis and this manuscript were my own work, I would like to thank the many people and centres, outlined in the acknowledgements, whose contributions and assistance enhanced the quality of the research presented.

The results presented herein have not been submitted previously for a degree or diploma at any other university; however, many results have been presented at international scientific meetings and/or have been accepted or submitted for publication, as detailed below.

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Abstract

Atherosclerosis is the leading cause of mortality in the Western world. Diabetic patients, especially those with kidney disease, have rates of cardiovascular (CV) disease far in excess of the general population (up to a 50-times increase, in the case of end-stage diabetic nephropathy). Yet, the mechanisms underlying these risks are unclear.

Recently, attention has been focused on novel risk factors for atherosclerosis and one such set of risk factors are the cell adhesion molecules (CAMs); two examples of these CAMs are ICAM-1 and VCAM-1. The CAMs are important because they participate in the earliest stage of atherosclerosis: the adhesion of circulating leukocytes to the vascular endothelium. The overall hypothesis of this thesis is that one underlying cause for the increased risk of CV disease is the potential for the diabetic and nephropathic milieu to up-regulate the endothelial expression of ICAM-1 and VCAM-1, thus initiating a chain of events that leads to atherosclerosis. The aim of this thesis is to explore the effects of the diabetic environment (as represented by hyperglycaemia) and the nephropathic environment (as represented by the serum of patients with diabetic nephropathy) on the expression and regulation of ICAM-1 and VCAM-1 in endothelial cells.

Chapter 3 investigates the effect of glucose, serum of diabetic subjects with increasing degrees of diabetic nephropathy, and the combination of these conditions on the endothelial cell culture expression of ICAM-1 and VCAM-1.

Using several different methods, the expression of both CAMs was found to be elevated by both high glucose and uraemic serum at the mRNA and protein levels, leading to a functional increase in monocyte-to-endothelial adhesion.

Chapter 4 explores the regulation mechanisms that are responsible for the effects seen in chapter 3. Again, by using a variety of different experimental techniques, it was demonstrated that both hyperglycaemic and uraemic conditions increase NF-κB activation, which in turn stimulates the promoter region of both ICAM-1 and VCAM-1. There was found to be no change in mRNA stability (except for a slight decrease in VCAM-1 mRNA stability under high glucose).

Chapter 5 finds significant correlation between the novel *in vitro* results presented in the previous chapters with the more commonly used soluble adhesion molecule assays, as well as many other well-established clinical markers of CV disease.

Finally, chapter 6 investigates the potential of HDL supplementation to profoundly suppress the endothelial expression of the CAMs and block the effects of hyperglycaemia and uraemic serum, thus pointing to a potential novel approach in preventing CV disease in diabetic subjects with nephropathy.

In conclusion, this thesis presents a variety of observations and mechanisms by which diabetic kidney disease may influence atherogenesis and highlights a possible new approach in the prevention of vascular disease in this very high risk population.

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Publications and Presentations

Publications

Macrovascular disease: the Sword of Damocles in Diabetes.

Ted Wu, Belinda Brooks, Dennis K. Yue

Diabetes in the New Millennium. Editors: JR Turtle, T Kaneko, S Osato. Sydney: The Endocrinology and Diabetes Research Foundation of the University of Sydney; 1999. p. 403-414.

Cardiovascular disease in diabetic nephropathy patients: cell adhesion molecules as potential markers?

Ted Wu, Kristine C.Y. McGrath, Alison K. Death

Vascular Health and Risk Management, 2005: 1(4) p. 309-316

HDL suppresses the expression of ICAM-1 and VCAM-1 in endothelial cells stimulated by hyperglycaemia and uraemia. (submitted)

Ted Wu, Kristine C.Y. McGrath, Raj Puranik, Alison K. Death, Dennis K. Yue

The effect of different stages of diabetic nephropathy and high glucose on endothelial expression of ICAM-1. (in submission)

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The expression and regulation of endothelial VCAM-1 by hyperglycaemia and diabetic nephropathy. (in submission)

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Presentations

American Diabetes Association, Annual Scientific Sessions, 2005, San Diego, USA. Late breaking abstract.

Suppression of ICAM-1 and VCAM-1 expression by HDL in endothelial cells stimulated by hyperglycaemia and uraemia.

Ted Wu, Kristine C.Y. McGrath, Raj Puranik, Alison K. Death and Dennis K. Yue

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Oral presentation

The effect of different stages of diabetic nephropathy and hyperglycaemia on the binding of monocytes to the endothelium: potential acceleration of early steps of atherogenesis.

Alison K. Death, Ted Wu and Dennis K. Yue

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The effect of different stages of diabetic nephropathy and hyperglycaemia on endothelial expression of cellular adhesion molecules.

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Ted Wu, Alison K. Death, Kristine C.Y. Chan, David S. Celermajer, Dennis K. Yue and John R. Turtle

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The effect of high glucose and interleukin-1ß on the endothelial expression of cellular adhesion molecules.

Ted Wu, Alison K. Death, Shirley Nakhla, Kristine C.Y. Chan, David S. Celermajer, Dennis K. Yue and John R. Turtle

Abbreviations

18S	18S ribosomal RNA
ANOVA	Analysis of variance
AP-1	Activator protein-1
ApoA-I	Apolipoprotein A-I
BAEC	Bovine aortic endothelial cell
bp	Base pairs
CAM	Cell adhesion molecule
CRP	C-reactive protein
Ctrl	Control
CV	Cardiovascular
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electophoretic mobility shift assay
HAEC	Human aortic endothelial cell
HBSS	Hanks balanced salt solution
HDL	High density lipoprotein
HEPES	N-2-Hydroxyehtylpiperazine-N'-2-ethanesulfonic acid
HG	High glucose
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule-1
IL-1β	Interleukin-1 beta
M199	Media 199
Mann	Mannitol (high osmotic control)
Micro	Microalbuminuria
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor-kappa B
NG	Normal glucose
nHDL	Native high-density lipoprotein
PCR	Polymerase chain reaction
Prot	Proteinuria
rHDL	Reconstituted high-density lipoprotein
RPMI	Roswell Park Memorial Institute media
RT-PCR	Reverse transcription polymerase chain reaction
sCAM	Soluble cell adhesion molecule
sICAM-1	Soluble intercellular adhesion molecule-1
sVCAM-1	Soluble vascular cell adhesion molecule-1
Ur	Uraemia
VCAM-1	Vascular cell adhesion molecule-1

Chapter 1

Introduction and Literature Review

1.1 Impact of diabetes and diabetic nephropathy on cardiovascular risk

Atherosclerosis, and the resultant increase in vascular events, is the main cause of mortality in Western societies. Macrovascular disease is also a major chronic complication of both type 1 and type 2 diabetes mellitus. Diabetes mellitus is associated with an approximately two to four times increased risk of vascular events as compared with control populations[1, 2].

As well as macrovascular complications, diabetes is associated with microvascular complications, one of which is diabetic nephropathy. While microvascular complications in general do not impact as greatly on the overall mortality of diabetic subjects as macrovascular complications do, this is not the case with kidney disease: the combination of diabetes and nephropathy increases the risk of vascular events by twenty- to fifty-fold[3-5].

Despite this dramatic potentiation of vascular disease, the molecular mechanisms underlying the atherogenic propensity of diabetes and diabetic nephropathy remains largely unclear. Although the disease process of diabetes mellitus is most strongly characterised by high plasma glucose, it does not automatically follow

that the hyperglycaemia itself is the pathogenesis of the atherosclerosis. The Diabetes Control and Complications Trial (DCCT) showed no significant benefit in macrovascular outcomes from the intensive treatment of glucose levels in type 1 diabetic subjects[6]. Similarly, on an intention to treat basis, the United Kingdom Prospective Diabetes Study also failed to show statistically significant reduction in myocardial infarction, stroke or cardiovascular (CV) mortality with intensive control of glucose levels in type 2 diabetes[7]. However, in a prospective observational analysis, the UKPDS found a strong correlation between exposure to hyperglycaemia and increased incidence of macrovascular complications[8]; and very recently, the DCCT/EDIC (Epidemiology of Diabetes Intervention and Complications) has shown an approximately 50% reduction in macrovascular events in type 1 diabetes patients previously randomised to intensive glucose control[246]. Thus, glucose itself must have some, but not a total role, in the development of atherosclerosis in diabetes.

As noted above, the presence of renal disease greatly increases the risk of atherosclerotic complications in diabetic patients. Though the increase in risk quoted above relates to the end stage of diabetic nephropathy, this rise in macrovascular disease seems to occur even in the earliest stages of kidney disease, where the only abnormality is that of small amounts of the protein albumin in the urine. Albuminuria in diabetes is a known marker for the development of further renal disease; however, there is increasing evidence that it can also be a marker for CV disease as well.

1.1.1 ALBUMINURIA AS A MARKER FOR RENAL DISEASE

Diabetic nephropathy can be classified on its severity. Initially, people with diabetes have no kidney disease at all (which will be abbreviated in this study as 'DM'). The earliest detectable level of kidney disease is microalbuminuria, where there is a minute amount of protein that is excreted in the urine ('Micro'). This level of protein leak is so small that it is not easily detectable by simple bedside testing – classically via a urine dipstick test – and requires more sensitive laboratory tests for confirmation. The next stage is proteinuria ('Prot'), which is defined as easily measurable levels of protein in the urine, but without disturbance of measures that generally mark renal failure (high serum creatinine and urea). Lastly, the disease process may develop into renal failure, otherwise known as uraemia ('Ur').

Albuminuria is usually measured either by albumin excretion rate (AER) or albumin-to-creatinine ratio (ACR). Microalbuminuria is defined as:

AER 30-300 μg/24 hours

20-300 μg/minute

ACR 3-40 mg/mmol in males

3.5-35 mg/mmol in females

Proteinuria is defined as albumin excretion greater than these figures, but without measurable disturbance in other measures of renal function such as serum creatinine, serum urea or creatinine clearance. In renal failure/uraemia, the latter two measures are, of course, abnormal.

Patients who eventually develop end stage diabetic renal failure usually progress through the stages of normal renal function, microalbuminuria and proteinuria before reaching uraemia. While not every patient who has microalbuminuria will end up with renal failure, it is nonetheless true that microalbuminuria indicates incipient nephropathy in both type 1 and type 2 diabetes. It is very well established that even slightly increased levels of albuminuria is a very large risk factor for the development of nephropathy both in primary studies [9, 10] and in meta-analysis[11]. In addition, the aggressive treatment of microalbuminuria with antihypertensives[12] and tight glucose control[6, 7] has been shown to decrease the progression of renal disease, and in some cases reverse microalbuminuria. Thus, it has long been standard practise to use microalbuminuria as a target for treatment in the prevention of diabetic nephropathy. Less clear, until recently, is the role of microalbuminuria as a marker and therapeutic target in vascular disease.

1.1.2 ALBUMINURIA AS A MARKER FOR VASCULAR DISEASE

A post-hoc analysis of three recent large clinical trials shows that in diabetes, proteinuria not only determines renal outcomes, but also CV outcomes [13-15]. In one of these three studies, the reduction of albuminuria with therapeutic interventions resulted in protection against CV disease as well as the development of progressive renal impairment[13].

This relationship is not only true for the diabetic population, but it also seems to be true for the non-diabetic population as well. Several studies indicate that CV risk increases in a continuous fashion along with the higher levels of albuminuria. Two studies that used self reported incidences of CV disease compared with investigator initiated measurement of urinary albumin found that the urine microalbumin was an independent risk factor for CV disease[16] and CV mortality[17]. Another prospective study looking at mortality in the four years following urine protein determination also concluded that urine albumin was an independent risk factor for CV death and that this risk increased stepwise with increasing stages of albuminuria[18]. Lastly, in a prospective 5 year survey of more than 20,000 subjects in the UK, microalbuminuria, and especially proteinuria, was independently associated with risk of CV disease and death[19].

1.1.3 POSSIBLE MECHANISMS LINKING ALBUMINURIA TO CARDIOVASCULAR DISEASE

The link between end-stage renal disease and CV disease is well established. Because of the manifold physiological abnormalities that are attendant in the uraemic state, possible mechanisms for the link between macrovascular disease and renal failure are relatively easy to identify[5]. High on the list of possible mechanisms are factors such as hypertension, anaemia[20], dyslipidaemia, activation of the renin-angiotensin system[21], calcification of the vascular tree, malnutrition[22], a pro-thrombotic state and inflammation.

However, as noted above, the increase in risk of macrovascular complications rises before end-stage renal failure and uraemia. Indeed, all current evidence points to a rise in risk even in the earliest stages of microalbuminuria, both in diabetic and non-diabetic populations. In these early stages of albuminuria, none of the adverse factors present in end-stage nephropathy are universally, or even usually, present. It is thus unclear what mechanisms underlie microalbuminuria being an independent risk factor for CV disease.

One possibility is that albumin itself is pro-inflammatory and damaging to the vasculature. There is recent evidence to suggest that albumin is associated with endothelial dysfunction. The leakage of even minute quantities of albumin from the renal vessels must therefore damage these local vessels over time. While this would explain the association of microalbuminuria and the eventual development of more severe renal disease[23], it does not explain why albumin appearing in the urine damages the vascular tree in the rest of the body to give rise to CV disease.

At present, the assumption is that the albumin in the urine indicates endothelial dysfunction within the vasculature of the kidney, and that this reflects generalised vascular dysfunction. This endothelial dysfunction can be measured by such methods as vascular reactivity, c-reactive protein and other novel risk factors.

1.1.4 NOVEL RISK FACTORS

Due to the massively raised incidence of atherosclerotic complications in diabetic subjects with kidney disease, much research has been carried out recently to identify the risk factors that can act as early markers for atherosclerosis. There are several well known, conventional risk factors such as blood pressure, dyslipidaemia, age, male sex and diabetes. However, these conventional risk factors alone cannot explain the increased risk of CV disease, as evidenced by the underestimation of total CV risk by the Framingham coronary point score[24], which is a conglomerate of conventional risk factors.

This has lead to the realisation that factors such as von Willebrand factor (vWF), intima-medial thickness, low level microalbuminuria, vascular reactivity, vascular compliance, anaemia and, perhaps most notably, c-reactive protein (CRP) are also markers for CV disease – the so-called novel risk factors for CV disease. One group of novel risk factors that has gained prominence recently are the cell adhesion molecules (CAMs). Two such adhesion molecules are intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).

1.2 Role of cell adhesion molecules in the development of atherosclerosis

Cell adhesion is not only a crucial step in the development of atherosclerosis, it is also the earliest[25]. Normally, both leukocytes and red blood cells circulate in the blood stream unhindered; however, under certain circumstances, the white cells may adhere to the endothelial cells lining the vessel walls. Circulating leukocytes first become tethered to the endothelium via low affinity binding. This transient binding results in a series of engagements and disengagements between the leukocytes and endothelium that causes the leukocytes to roll along the endothelium. After the initiation of rolling, it is possible for the leukocyte to initiate a higher-affinity interaction with the endothelium, which results in adhesion, then flattening of the leukocytes. Finally, the adherent leukocytes transmigrate into the subendothelial space. This last step sets the stage for the development of the next stage of atherosclerosis, the fatty streak (see figure 1).

Cell adhesion molecules (CAMs) expressed on the surface of endothelial cells mediate all the steps outlined above: tethering, rolling, adhering and transmigration. In physiological terms, these CAMs are vital for the efficient functioning of the immune system in protecting the body from infectious organisms. The cells of the immune system, primarily the leukocytes, circulate in the blood and lymph, and migrate as adherent cells into target tissues when necessary. Rapid transition between adherent and nonadherent states is the key to the surveillance and responsiveness of the immune system, and it is the CAMs that modulate this transition. While CAMs may be up-regulated in a protective

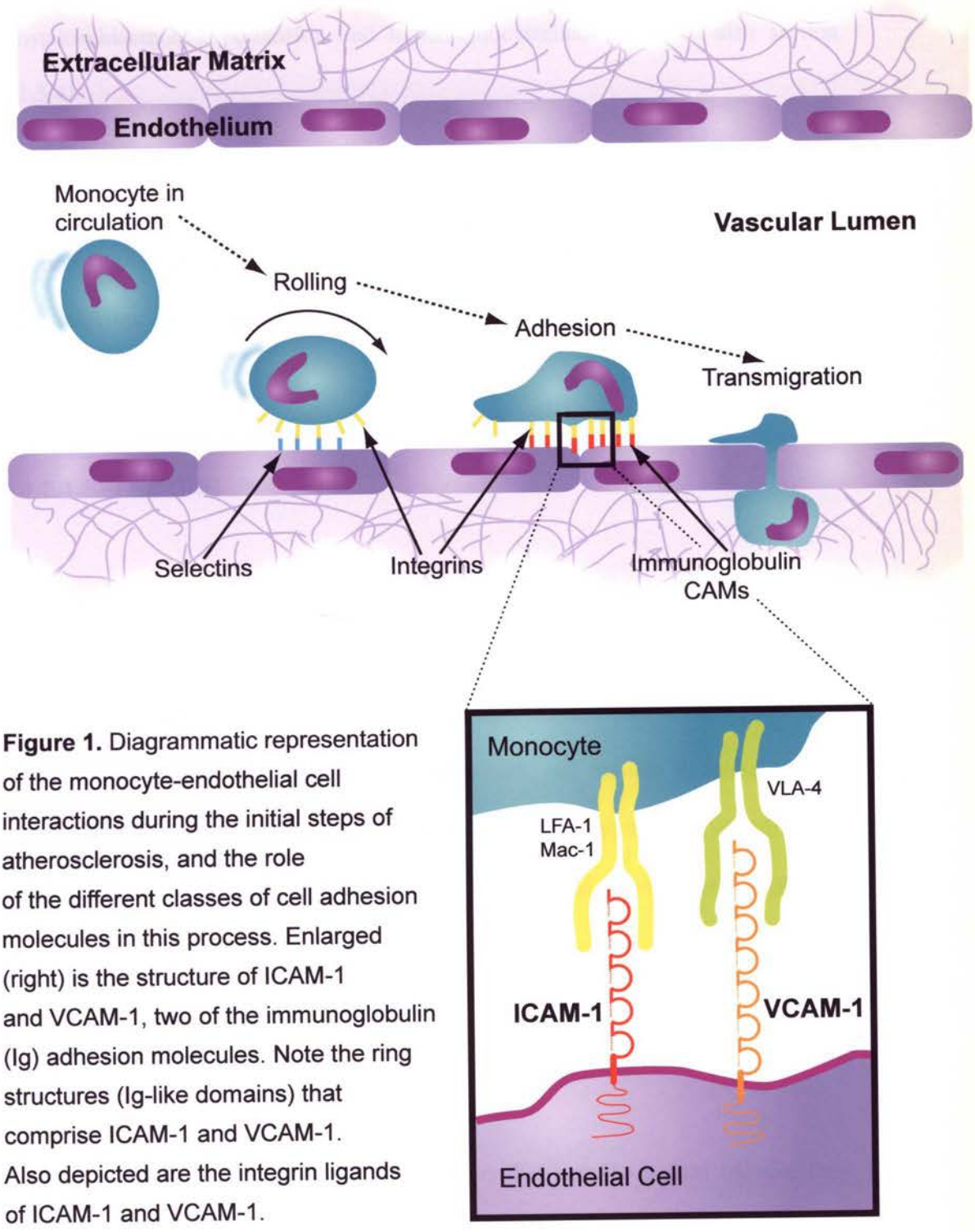


Figure 1. Diagrammatic representation of the monocyte-endothelial cell interactions during the initial steps of atherosclerosis, and the role of the different classes of cell adhesion molecules in this process. Enlarged (right) is the structure of ICAM-1 and VCAM-1, two of the immunoglobulin (Ig) adhesion molecules. Note the ring structures (Ig-like domains) that comprise ICAM-1 and VCAM-1. Also depicted are the integrin ligands of ICAM-1 and VCAM-1.

manner in such instances as localised infection or trauma, they may also be raised pathologically by known risk factors for atherosclerosis, such as smoking, hyperlipidaemia, hypertension and hyperhomocysteinaemia[26] (see also section 1.5.4).

There are many different types of CAMs and virtually every cell expresses one form of CAM or another. The CAMs can be classified into three major families: the selectins, the integrins and the immunoglobulin superfamily.

1.2.1 SELECTINS

Selectins are involved in the tethering of leukocytes to the endothelium. They are also involved in the subsequent rolling of leukocytes on the endothelial cell surface[27]. E-selectin is expressed on endothelial cells, and can be up-regulated by IL-1β and TNF-α[28]. P-selectin is synthesised and stored in platelets and endothelial cells, and can be rapidly expressed in response to such stimuli as IL-1β and TNF-α[26, 29]. L-selectin is constitutively expressed on endothelial cells[30].

1.2.2 INTEGRINS

Integrins are a family of membrane receptors that were so named because they were thought to "integrate" the cytoskeleton of cells with other cells or with the extracellular matrix. Importantly, some integrins are also receptors for the

immunoglobulin superfamily of adhesion molecules (see section 1.2.3). The integrins are transmembrane proteins that are composed of non-covalently linked heterodimeric molecules, with alpha (α) and beta (β) subunits[31]. There are at least 14 α subunits and 8 β subunits and many combinations are possible.

One subclass of integrins is the so-called 'very late activation' integrins (VLA) because some of them (VLA-1 and VLA-2) are expressed on lymphocytes only after 2 weeks of exposure to antigens *in vitro*. Structurally, the VLA subclass members all express the α_4 subunit. Perhaps the most important VLA in regards to cell adhesion is VLA-4[32] (structurally $\alpha_4\beta_1$), which is expressed on resting monocytes and lymphocytes, but not neutrophils. VLA-4 is the ligand for vascular cell adhesion molecule-1 (VCAM-1).

Another subclass of integrins has the β_2 subunit and, not surprisingly, are called β_2 integrins. Two β_2 integrins are particularly important in cell adhesion. Lymphocyte function-related antigen (LFA-1, $\alpha_L\beta_2$) is expressed on all leukocytes, while Mac-1 ($\alpha_M\beta_2$) is found only on monocytes, macrophages and granulocytes[33]. Both LFA-1 and Mac-1 are ligands for intercellular adhesion molecule-1 (ICAM-1).

1.2.3 IMMUNOGLOBULIN SUPERFAMILY

Physically, the immunoglobulin (Ig) superfamily of CAMs are made up of varying numbers of Ig-like domains of 70 to 100 amino acids, giving rise not only

to their name, but also to the molecular structure that binds their respective ligands. Amongst other things, the Ig superfamily of CAMs is involved in the high-affinity adhesion of leukocytes to endothelial cells that takes place after tethering and rolling. The Ig superfamily of CAMs also mediate subsequent cell migration and signal transduction[34, 35, 26]. They include the intercellular adhesion molecules (ICAM-1 through to ICAM-5), vascular cell adhesion molecule-1 (VCAM-1) and platelet-endothelial cell adhesion molecule (PECAM).

ICAM-1 and VCAM-1 are expressed on endothelial cells. Both are critical in the firm adhesion of lymphocytes and monocytes to endothelial cells[36] and the subsequent white cell extravasation. PECAM is constitutively expressed at the intercellular junctions of endothelial cells, and is involved in the transmigration of adherent leukocytes into the subendothelial space[37].

1.2.3.1 ICAM-1

The ICAM-1 molecule, a 76 to 114 kDa glycoprotein, consists of a long extracellular region that consists of five Ig-like domains which is anchored to the cell wall via a transcellular and intracellular region, as shown in figure 1. The extracellular region can be cleaved off via proteolytic processes[38] to form soluble ICAM-1 (sICAM-1) that can circulate in the bloodstream[39]. Whereas ICAM-1 on endothelial cells can only be measured by direct biopsy of tissues or in *in vitro* models, the soluble form of ICAM-1 can be simply measured in the plasma of living subjects.

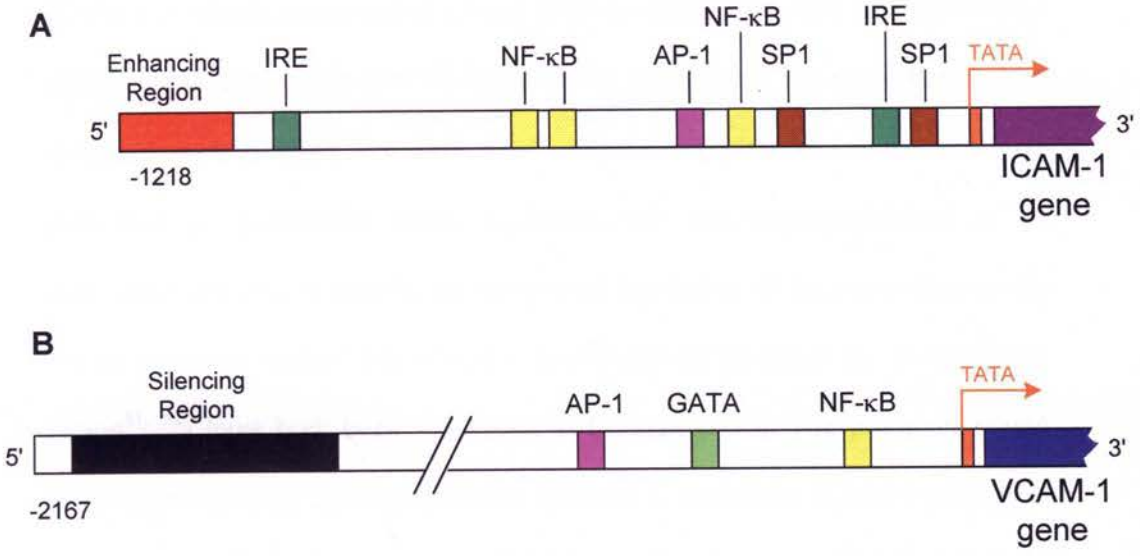


Figure 2. The structures of the 5' upstream regulatory ("promoter") regions for the **(A)** ICAM-1 and **(B)** VCAM-1 genes. Recognised sites of nuclear binding factors are shown in coloured boxes for each promoter region.

As outlined in section 1.2.2, the main ligands for ICAM-1 are the integrins LFA-1 and Mac-1. Because each ICAM-1 molecule contains multiple Ig-like domains, one molecule can bind more than one ligand at a time. Dimerisation, or the formation of even larger multimers, is commonly observed for ICAM-1 and may increase its binding affinity with its ligands[40].

It has been well demonstrated that atherosclerotic lesions in humans exhibit local increased expression of ICAM-1[41-45], making this CAM an obvious target for research into the development of atherosclerosis (see sections 1.3-1.6). An indication of the importance of ICAM-1 is demonstrated in mouse models with deficiencies in ICAM-1 genes in which there is significantly less macroscopic atherosclerosis in the aorta than wild-type mice [46, 47].

ICAM-1 is widely expressed at a basal level on endothelial cells and some white cells, but its expression can be dramatically upregulated by pro-inflammatory cytokines such as IL-1β and TNF-α[48, 49]. Even though ICAM-1 is constitutively expressed by endothelial cells, significant up- and down-regulation of its expression does occur and the transcriptional regulation of its expression occurs via its promoter region. The ICAM-1 gene[50] and its promoter (or 5'-regulatory region[51, 52]) have both been sequenced and characterised. The promoter region spans approximately 2000 bp upstream from the 5' end of the ICAM-1 gene, with many nuclear factor binding sites within this promoter region[53] (see figure 2). There are three NF-κB (nuclear factor-kappa B) binding sites starting at positions -208, -495 and -572. In addition, there are: two SP1 binding sites at -29 and -175; two IRE (insulin response elements) at -39 and -1090; and one AP-1 binding site at -253. The ICAM-1 promoter region also contains three enhancer regions (one starting at -1352, one somewhere between -2400 and -1352, and one downstream of -277) and an active silencing region (between positions -339 and-290)[54]. There appears to be a complex interplay between the silencing and enhancing regions that mediates cell type specific expression and regulation of ICAM-1[54].

1.2.3.2 VCAM-1

The VCAM-1 molecule is a 90-110 kDa glycoprotein that consists of seven Ig-like domains, although a six Ig-like domain isoform was the first to be cloned[55]. The two isoforms of VCAM-1 result from alternate mRNA splicing[56]. The 7 domain isoform has two functional VLA-4 binding sites (see figure 1), while the 6 domain form only has one[57]. As well as being involved in the firm adhesion of white cells to the endothelium, VCAM-1 is the only CAM that may play a role in

all stages of leukocyte adhesion, from rolling (early, weak leukocyte-endothelial interaction)[58] through to transendothelial migration[35].

Unlike ICAM-1, there is little basal expression of VCAM-1 on endothelial cells; however, levels of VCAM-1 are strongly inducible by pro-inflammatory cytokines[59, 49], not just on endothelial cells, but also macrophages, smooth muscle cells[60] and dendritic cells[61].

Like ICAM-1, focal increased expression of VCAM-1 has been found in human atherosclerotic lesions [42, 60, 44]. In addition, in a process parallelling ICAM-1, the extracellular region of VCAM-1 can be cleaved off via proteolytic processes[62, 63] to form soluble VCAM-1 (sVCAM-1). VCAM-1 expression is known to be increased by atherogenic factors such as oxidized LDL[64-66]. Therefore VCAM-1, like ICAM-1, has properties which allow it to be important in the study of the pathogenesis of early atherosclerosis (see sections 1.3-1.6). As an example, VCAM-1 deficiency in the form of a mutant VCAM-1 gene results in significantly less atherosclerotic lesions in the aorta than wild-type mice[67].

The VCAM-1 gene[68] and its promoter[69, 70] have been sequenced and characterised, with the promoter region driving the pattern of VCAM-1 expression. The VCAM-1 promoter includes the region approximately 2300 bp upstream from the transcriptional start site and features regulatory motifs for the transcription factors AP-1, GATA and NF-κB, as well as silencing regions[69] (figure 2). Two NF-κB binding sites are found at positions -63 and -77 bp, two GATA boxes are present at positions -259 and -245 bp in opposite transcriptional

orientations and a binding site for AP-1 at position -495 bp. Powerful silencing regions are located between -755 and -2167 bp, and are responsible, in part, for the low basal expression of VCAM-1 in unstimulated cells.

1.2.3.3 NF-κB

The promoter regions of ICAM-1 and VCAM-1 both contain multiple binding sites for the transcription factor NF-κB. Transcription factors are regulatory proteins that bind short, specific *cis*-acting DNA sequences within and near gene promoters, regulating RNA polymerase activity, either by direct interaction with the basal transcription apparatus or via co-factors[71]. Transcription factors may be ubiquitous or cell type-specific, with maximal transcriptional activity being mediated by the co-ordinated activity of several intracellular pathways, transcription factors and co-factors.

NF-κB itself is a family of dimeric transcription factors. While several transcriptionally active dimers have been identified, the most commonly described is the p50-p65 heterodimer. The dimeric consensus sequence for NF-κB is 5'-GGG(G/A)NNT(C/T)CC-3' (where N is any nucleotide).

In the inactive state, the NF-κB dimers are complexed to members of a family of inhibitory proteins referred to as inhibitory-kappa B (IκB) and these complexes are stored in the cell cytoplasm. Upon cell stimulation, IκB is phosphorylated and then degraded. The subsequent dissociation of IκB from the NF-κB dimer allows the latter to translocate from the cytoplasm to the nucleus, where it binds its specific sequence in promoters of target genes, stimulating gene transcription. NF-

κB itself activates the IκB gene, thus replenishing its own inhibitor and resulting in autoregulation[72-74].

NF-κB mediates the transcriptional activation of many pro-inflammatory and immune molecules, including ICAM-1, VCAM-1 and cytokines such as IL-1β and TNF-α[75]. Hyperglycaemia has been shown to directly increase activation of NF-κB in endothelial cells[76], as well as in vascular smooth muscle cells[77]. There are currently no studies that have looked at the effect of diabetic nephropathy, or any other type of nephropathy, on the endothelial activation of NF-κB (other than the identification of increased NF-κB expression as measured by immunohistochemistry in the glomerular endothelium of patients with IgA nephropathy[78]).

Because NF-κB appears to be the most important nuclear binding site in the regulation of both genes of interest[69, 79] and indeed is common to both promoter regions, the experiments in this thesis regarding the regulation of ICAM-1 and VCAM-1 have focused on NF-κB.

1.2.4 LATER STEPS IN THE DEVELOPMENT OF ATHEROSCLEROSIS

As a pathological process, atherosclerosis is gradually progressive. The molecular mechanisms described so far represent only the very earliest stage of atherosclerosis: the leukocyte (mainly monocyte) adhesion to and transmigration through the endothelium. Some time thereafter, the monocytes differentiate into

macrophages. At this point, there are still no macroscopic physical lesions detectable.

The next stage in the development of atherosclerosis, and the first physical defect, is the development of fatty streaks which primarily consist of lipid-laden macrophages (foam cells[80]) and are demonstrable in adolescence [81]. Progression of the lesion leads to more diffuse intimal thickening, with leukocyte and smooth muscle cell recruitment, deposition of collagen and other extracellular matrix proteins. This results in the characteristic formation of a plaque, with a fibrous cap overlying a lipid rich core. Advanced lesions may impinge on the arterial lumen, altering blood flow and may be complicated by plaque ulceration, rupture, thrombosis and haemorrhage[82].

The late clinical manifestations of atherosclerosis typically involve the coronary arteries (angina, myocardial infarction and sudden death), the cerebrovascular circulation (stroke and transient ischemic attacks) and/or the peripheral circulation (claudication, limb ischaemia, aneurysms and gangrene).

1.3 *In vitro* effects of glucose on human endothelial cell adhesion molecule expression

No matter how well controlled, all diabetic patients experience, at one time or another, some level of hyperglycaemia; that is, concentrations of plasma glucose that are higher than normal (greater than 7mmol/l). Because it lines the interior surface of blood vessels, it follows that the endothelium is very exposed to the effects of high plasma glucose. One common approach to studying the effect of hyperglycaemia on the endothelial expression of ICAM-1 and VCAM-1 is to expose cell cultures of endothelial cells to media containing high glucose concentrations and compare them to cells exposed to normal glucose concentrations; this method is used by the majority of articles reviewed in this section. End-points of study include the adhesion of monocytes to endothelial cells, the measurement of CAM protein using enzyme linked immunosorbent assay (ELISA), and RNA using polymerase chain reaction (PCR).

1.3.1 ADHESION STUDIES

There have been many studies that have demonstrated that high glucose increases the adhesion of primary monocytes[83, 84], monocyte cell lines[85-87] and neutrophils[88-90] to human endothelial cells. The concentration of glucose used as the hyperglycaemic condition varied from 25 mmol to 33 mmol in all of these studies, though one group demonstrated that the effect was present in concentrations of glucose up to 55 mmol[89, 90]. The magnitude of the rise in

adhesion varied from a 1.2-fold increase as compared to control[88] up to a 4-fold increase[87], with the other studies falling in between these two extremes. Some of these authors were able to demonstrate that the increase in adhesion could be blocked by either anti-ICAM-1 antibody[83, 87] or anti-VCAM-1 antibody[87, 84] thus indirectly indicating that both ICAM-1 and VCAM-1 are mediating this increased adhesion in high glucose conditions.

1.3.2 ICAM-1 EXPRESSION STUDIES

More direct evidence that glucose affects ICAM-1 and VCAM-1 expression can be gathered by direct measurement of their mRNA or protein levels in endothelial cell culture using a variety of methods.

Using either ELISA or fluorescence activated flow-cytometry (FACS), ICAM-1 protein expression in endothelial cells has been shown to be increased by incubation with high glucose by many authors[85, 86, 89-95]. The range of this increase in the various studies is between 16%[91] and 110%[88] above baseline.

Similarly, endothelial ICAM-1 has been found to be upregulated by glucose at the mRNA and gene level by groups using northern blot [85], reverse transcription-PCR[92] and gene microarrays[96]. The magnitude of the elevation was up to a 3.5-fold increase[92] over the gene expression in normal glucose.

1.3.3 VCAM-1 EXPRESSION STUDIES

There are relatively few groups who have studied the effect of glucose on the expression of VCAM-1 in endothelial cell culture. Those that have used FACS to study the protein expression of VCAM-1, and these studies show an increase of VCAM-1 in high glucose conditions[84, 94, 95]. Indeed, the most recent of these studies demonstrated that *in vitro* high glucose conditions resulted in a 7-fold increase in VCAM-1 protein expression in HUVECs[95].

To date, no authors have used real-time RT-PCR to quantitate the expression of ICAM-1 or VCAM-1 in endothelial cell culture exposed to glucose.

1.3.4 DIVERGENT RESULTS IN GLUCOSE STUDIES

There are three studies that have results which diverge from some of the data discussed in sections 1.3.2 and 1.3.3. The two earliest studies to look at adhesion, Kim *et al* (1994) and Taki *et al* (1996), did not show increased ICAM-1[83] or VCAM-1[83, 85] protein as measured by ELISA, even though both groups demonstrated increased monocyte adhesion to endothelial cells exposed to high glucose. One possible explanation is that the enhanced adhesiveness is mediated by cell adhesion molecules other than ICAM-1 or VCAM-1. However, arguing against this is the fact that in the case of Kim *et al*, the increased adhesion was blocked by anti-ICAM-1 antibodies, indicating that ICAM-1 must be involved. Being the two earliest studies, it is also possible that the methods used were not

yet sensitive enough to detect the rise in protein – all subsequent studies do show a rise in either protein or gene expression.

The one exception to this is a 2003 article in which human retinal vascular endothelial cells were incubated with 22 mmol of glucose for 24 hours. In this instance, neither ICAM-1 nor VCAM-1 protein was raised as compared to normal glucose conditions[97]. It is perhaps significant that this was the only study to use retinal vascular endothelial cells; all other studies outlined above used either HUVECs or HAECs. The latter two cell types are well established models for the study of atherosclerosis, whereas retinal endothelial cells are not. It is possible that the difference in cell types used explains the lack of CAM upregulation in this study.

Overall, however, it would seem from the weight of recent evidence that in most *in vitro* models, glucose does stimulate monocyte to endothelial adhesion, as well as endothelial ICAM-1 and VCAM-1 expression.

1.3.5 OSMOTIC MECHANISMS

There are many mechanisms that may cause the up-regulation of ICAM-1 and VCAM-1 in cells exposed to glucose, but one of the most obvious is the osmotic effect of glucose. As well as being a carbohydrate that can enter the cell and either act directly on cellular mechanisms or be metabolised into other active products, glucose can act as an osmotic molecule. The accepted method of distinguishing

between the metabolic and osmotic effects of glucose is to run an osmotic control within the experiment, that is, use an equimolar concentration of a sugar that is non-metabolisable. Examples of such sugars are mannitol and raffinose.

Five of the studies discussed above included osmotic controls. However, there is little agreement in the results. Two of the studies show that high osmotic conditions either had no effect on monocyte adhesion[83] or else actually decreased ICAM-1 expression[86] as compared to normal glucose controls. This implies that glucose was not acting through an osmotic pathway at all.

On the other hand, Manduteanu *et al* found that while 33 mmol of glucose will increase monocyte adhesion to endothelial cells 4-fold, incubation in osmotic control (27.5 mmol mannitol plus 5.5 mmol glucose) results in a 2.7-fold rise in adhesion[87]. This would indicate that the glucose is acting partially (but not completely) through an osmotic pathway. Similarly, Kado *et al* demonstrated that the increased ICAM-1 mRNA and protein in HAECs is partially osmotically dependent[92].

Lastly, one article indicates that both raffinose and mannitol osmotic conditions increased ICAM-1 protein expression and monocyte cell line adhesion as much as high glucose[85], implying that glucose is acting solely via an osmotic mechanism. There are no equivalent osmotic studies for VCAM-1.

Thus, taken in totality, the current literature is unable to determine whether glucose's effect on endothelial cell adhesion molecules is acting via an osmotic or metabolic pathway.

1.3.6 ADVANCED GLYCATION END-PRODUCTS

High plasma levels of glucose can have effects other than direct short-term metabolic effects or osmotic effects. High glucose levels over prolonged periods of time can also result in the irreversible modification of proteins into advanced glycation end-products (AGEs). AGEs are thought to be at least partially responsible for the full spectrum of microvascular as well as macrovascular complications of diabetes, though the precise mechanism of their pathological potential have not been fully elucidated.

Certainly, in the *in vitro* environment, Schmidt *et al* have been able to show that AGE-albumin can increase VCAM-1 protein expression 7-fold in endothelial cells, with a concomitant rise in the adhesiveness of these cells to leukocytes[98]. In this study, AGE-albumin did not cause a rise in ICAM-1 protein. The mechanism of the up-regulation of VCAM-1 was determined to be NF-κB dependent and the effect was able to be blocked by co-incubation with anti-RAGE-Ab (anti-receptor for AGE-Ab).

In a similar result, a more recent study has incubated endothelial cells in high glucose for up to 8 weeks, a period of time that is sufficient for the development

of AGEs. 24 hour incubation with glucose was sufficient to increase VCAM-1 expression and monocyte adhesion to the endothelial cells, but these effects were magnified at the 8 week time-point[84]. The increase in adhesion was blocked by anti-RAGE-Ab, but only at the 8 week time-point, indicating that the long term increase in adhesion was as a result of AGE formation.

Therefore, while glucose has direct effects on the endothelium in the short term to increase ICAM-1 and VCAM-1 expression, the longer term effects of hyperglycaemia on these CAMs may also occur via the development of AGEs.

1.4 *In vitro* effects of nephropathy on human endothelial cell adhesion molecule expression

In contrast to the strong evidence that glucose stimulates *in vitro* ICAM-1 and VCAM-1 expression in human endothelial cells, there is almost no human data regarding the corresponding effect of renal disease in such models. The only extensive study in this area is by Serrandell *et al* and was published in 2002[99]. There were 10 subjects chosen with end-stage renal disease on haemodialysis. The causes of the renal failure were polycystic kidneys, chronic glomerulonephritis, chronic pyelonephrosis, nephrosclerosis, haemolytic uraemic syndrome and bilateral nephrectomy; none of the patients had diabetic nephropathy. HUVECs were grown in cell culture and exposed to the serum of nephropathic patients at a concentration of 50% in the media. Immunogold labelling demonstrated a rise in ICAM-1 and VCAM-1 expression on the surface of the cells exposed to uraemic serum versus control serum. There was also shown to be a rise in mRNA levels by (non-quantitative) reverse transcription-PCR. It was noted that the peak rise in mRNA for both adhesion molecules seemed to be at a 4 hour time point (though this was a qualitative comparison). Soluble cell adhesion molecules were measured in the media supernatants after 24 hours. This showed that levels rose 59% in the case of sICAM-1, and 34% for sVCAM-1.

Though this is the most complete *in vitro* study of the effects of uraemic serum on endothelial cells, there are some flaws. Firstly, no effort was made to quantitate the rise in ICAM-1 or VCAM-1 expression, except for the soluble assays. Secondly, the measurement of the soluble adhesion molecules was not ideal:

because the cells were exposed to a 50% concentration of uraemic serum from patients, it is unclear what proportion of soluble CAMs that was measured came from the patient and what proportion was produced *de novo* by the cells in culture. To determine this, one simple method would be to measure the sCAMs directly from the serum of the test subjects. Another advantage of this approach is that it would then be possible to correlate the *in vivo* sCAM measures with the *in vitro* measures. However, the lack of quantitation in this study again makes this comparison impossible.

Nonetheless, Serradell *et al* did manage to demonstrate that uraemic serum stimulates ICAM-1 and VCAM-1 expression *in vitro*. There are many chemical disturbances in nephropathy. One component of uraemic serum, p-cresol, was found to decrease ICAM-1 and VCAM-1 expression in HUVEC's[100], thus this was not the agent responsible.

There are no *in vitro* studies of CAMs in diabetic nephropathy. There are also no studies looking at the combined effects of high glucose and any kind of nephropathy.

1.5 Observations *in vivo* of cell adhesion molecule levels in diabetic and nephropathic patients

As discussed above (section 1.2), although the CAMs are firmly anchored on the cell membranes of endothelial cells, CAMs may be broken off into the circulation forming the so-called soluble ICAM-1 (sICAM-1) and soluble VCAM-1 (sVCAM-1). Such soluble CAMs (sCAMs) are easily measurable in plasma and can therefore serve as simple *in vivo* indicators of CAM expression. As a result, much work has been done in the last 10 years focusing on the role of sICAM-1 and sVCAM-1 in the pathogenesis of CV disease in diabetes and diabetic nephropathy.

1.5.1 sICAM-1 AND sVCAM-1 IN DIABETES MELLITUS

It is now well established that both sICAM-1 and sVCAM-1 are increased in diabetes. There are a large number of studies that show higher levels of both sICAM-1 and sVCAM-1 simultaneously in type 2 diabetes [101-112] and type 1 diabetes [113-116]. Although there are more studies that support this observation for type 2 diabetes, the evidence is still strong for type 1. Additional evidence is found in the many other articles that looked at only one of the adhesion molecules, and still found them to be raised in diabetes: raised sICAM-1 in type 2 diabetes [117-122], sVCAM-1 in type 2 diabetes[98, 123-126], sICAM-1 in type 1 diabetes [127, 128] and sVCAM-1 in type 1 diabetes [129]. While CAM expression tends to increase slightly with age, it is interesting to note that people with type 1 diabetes

who are undergoing puberty have higher sICAM-1 levels than either pre-pubertal or post-pubertal type 1 diabetic subjects[130].

There are a small number of papers that do not find a rise in either sICAM-1 [129] or sVCAM-1[117, 131, 120, 132] in diabetes. However, these studies were either relatively small[117, 131] or used young, healthy type 1 diabetics as subjects[132]. Despite these dissenting findings, the weight of evidence at present is that both sICAM-1 and sVCAM-1 are increased in diabetes mellitus (types 1 and 2).

There also appears to be a relationship between the severity of diabetes and the level of the circulating CAMs, but only for type 2 diabetes. In type 2 diabetic subjects, the level of either or both sICAM-1 and sVCAM-1 has been found to correlate with many different measures of diabetes severity, which include HbA_{1c}[118, 119, 109], fasting glucose level[101, 133, 126], fasting insulin[133], fasting c-peptide[126], glucose level 2 hours post-oral glucose tolerance test (OGTT) [109, 111], and insulin resistance as measured by the homeostasis model (HOMA)[101, 134, 126, 135]. In the two studies that looked at this relationship in type 1 diabetes, there was found to be no correlation between HbA_{1c} and sICAM-1[113] or sVCAM-1[113, 136].

1.5.2 sICAM-1 AND sVCAM-1 AS A PREDICTOR FOR FUTURE DIABETES

As well as being raised in established diabetes, sICAM-1 and sVCAM-1 have been found to be sometimes increased in pre-diabetic states, thus making them

potentially useful in predicting future onset of diabetes. For example, sICAM-1 levels are elevated in subjects at risk of developing type 1 diabetes[127]. Both sICAM-1[137, 104] and sVCAM-1[137, 104] have been found to be raised in people with impaired glucose tolerance (which can be considered to be pre-type 2 diabetes). In people with no previous history of diabetes, the sICAM-1[138, 139] and sVCAM-1[139] levels correlate with the 2 hour post OGTT glucose and insulin levels, which are markers for the level of disturbed glucose metabolism. sVCAM-1 is also raised in non-diabetics who have a first-degree relative with diabetes, and are thus at increased risk of developing diabetes.[104]. Women with gestational diabetes are also at risk of developing type 2 diabetes later in life; there are persistent elevations in sVCAM-1 in gestational diabetic women as compared to healthy controls, even 12 weeks after delivery of the baby [140]. Despite the evidence outlined above, the association between sCAMs and pre-diabetic states is only indirect evidence that measuring these factors can predict the development of diabetes.

Two very recent articles confirm that sCAMs can predict the future onset of diabetes. Meigs *et al.* found that in a longitudinal survey of more than 30,000 women over the course of 10 years, subjects who developed type 2 diabetes had higher levels of both sICAM-1 and sVCAM-1 at baseline[141]. In another study, antibody positive (but not yet diabetic) relatives of type 1 diabetic subjects were observed over 10 years with regular assessment of sICAM-1. sICAM-1 was elevated in those who progressed to type 1 diabetes, especially in the 18-24 months before diagnosis[142]. These results indicate that sICAM-1 and sVCAM-1 can indeed predict the development of type 2 diabetes, while sICAM-1 predicts

the future onset of type 1 diabetes. Given the time course of the rise, it may be postulated that the increased sCAMs are indicative of endothelial dysfunction associated with the metabolic syndrome in the case of type 2 diabetes and autoimmunity in the case of type 1 diabetes. This is confirmed in another study in children by Toivonen *et al.* that shows the level of sICAM-1 correlates with the IA-2 antibody level (an autoantibody that is often raised in type 1 diabetes) [143].

However, it should be noted that sICAM-1 and sVCAM-1 was not predictive for the development of type 2 diabetes in Pima Indians[144]. The Pima Indians have one of the highest prevalences of type 2 diabetes in any population in the world and it is possible to speculate that this massive underlying risk far outweighs the relatively minor contribution that a rise in sCAM level confers.

1.5.3 sICAM-1 AND sVCAM-1 IN DIABETIC NEPHROPATHY

While diabetes is associated with increased sCAMs, there appears to be an even greater relationship between sICAM-1/sVCAM-1 and diabetic nephropathy. Clausen *et al.*[145] from Denmark found that in type 1 diabetic subjects, there was a general increasing trend in both sICAM-1 and sVCAM-1 with increasing levels of diabetic kidney disease (diabetes with no kidney disease, microalbuminuria, macroalbuminuria with normal creatinine, and macroalbuminuria with elevated serum creatinine, see figure 3). Similar studies, from Turkey and Japan respectively, also show the same relationship between increasing levels of diabetic kidney disease and increasing sICAM-1[146] and sVCAM-1 levels[147].

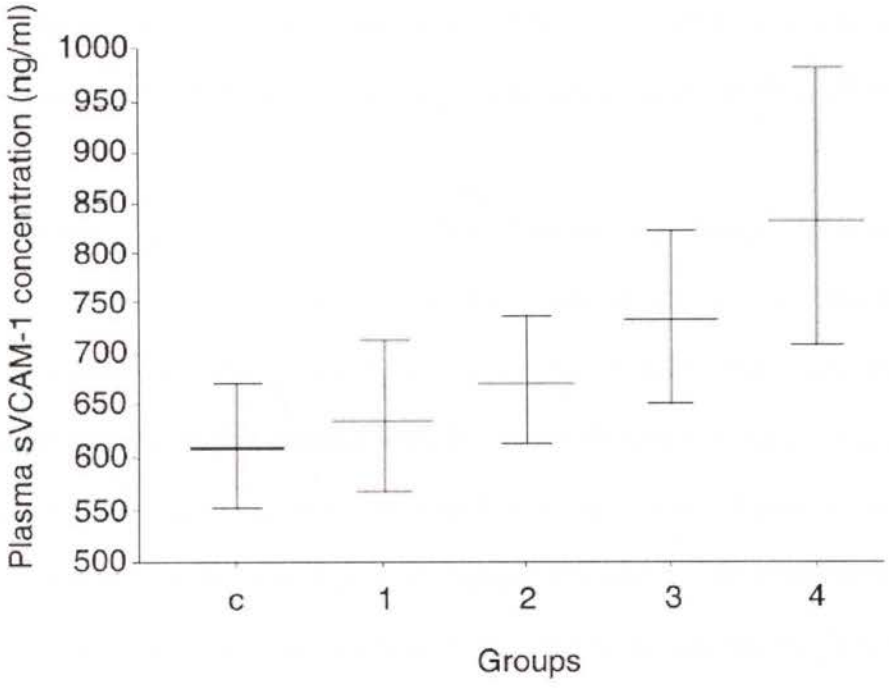


Figure 3. Means and 95% confidence intervals of sVCAM-1 levels from (c) control patients without diabetes, (1) type 1 diabetic subjects with no kidney disease, (2) microalbuminuria (3) macroalbuminuria with normal creatinine and (4) macroalbuminuria with raised creatinine. From Clausen *et al*[145].

In the Hoorn Study, sVCAM-1 was significantly associated with microalbuminuria and decreasing GFR in type 2 diabetic subjects[133]. Other studies have shown that as compared to controls, microalbuminuric type 2 diabetic subjects demonstrated enhanced expression of sVCAM-1[148, 113, 119, 149, 101] and sICAM-1[149, 101]. Koga *et al.*[150] found that while sVCAM-1 is raised in type 2 diabetic subjects with microvascular complications as a whole, only nephropathy (and not retinopathy, nor neuropathy) is independently associated with raised sVCAM-1 in a multivariate analysis. This study also showed a significant

correlation between sVCAM-1 and $\log_{10}$ urinary albumin excretion in patients with normal serum creatinine levels ($r = 0.51$, $p < 0.0001$) and a significant correlation between sVCAM-1 and $\log_{10}$ serum creatinine ($r = 0.83$, $p < 0.0001$).

A minority of authors disagree with these findings. For example, Yoshizawa *et al.*[151] showed that while there was a direct relationship between proliferative diabetic retinopathy and raised sVCAM-1 levels in type 2 diabetic subjects, there was no such rise in the subjects with diabetic nephropathy in their study group. Another group found that while sICAM-1 is raised in type 1 diabetes, there was no further rise in the subgroup with diabetic retinopathy, microalbuminuria or proteinuria[113]. However, the last two studies seem to be isolated findings and on the whole there is good evidence that diabetic nephropathy is associated with even higher levels of sICAM-1 and sVCAM-1 than diabetes without the nephropathy. In support of this, similar increases in sCAMs are seen in other microvascular complications of diabetes, such as retinopathy[152, 129, 153].

The CAMs are also raised in renal disease other than diabetic nephropathy. sICAM-1 and sVCAM-1 are simultaneously increased in haemodialysis patients in general [154] and specifically in IgA nephropathy[155]. sVCAM-1 is associated with lupus nephritis[156, 157]. Raised renal ICAM-1 expression in the kidneys is found in reflux nephropathy[158], Henoch-Schonlein purpura[155], [159] and mebrano-proliferative glomerulonephritis[159]. Similarly, there is increased kidney expression of VCAM-1 in cresentic nephritis, minimal change nephritis, lupus nephropathy and amyloid[160].

1.5.4 CORRELATIONS WITH CONVENTIONAL RISK FACTORS FOR ATHEROSCLEROSIS

sICAM-1 and sVCAM-1 show good correlation with most of the well established CV risk factors. Hypertension alone has been shown to up-regulate both sICAM-1 and sVCAM-1 levels[107, 110], though the combination of hypertension and diabetes has an additive effect on both soluble adhesion molecules[107]. The Hoorn Study also found an association between hypertension and sVCAM-1[133].

In terms of lipoproteins, Ridker showed a slight, but significant correlation between sICAM-1 and HDL and triglyceride levels[161]. sICAM also correlated with oxidised LDL levels in non-diabetic subjects[138] and sVCAM-1 correlated with LDL levels in type 1 diabetic subjects with microvascular disease[113].

There is also a link between obesity and circulating CAMs. Body-mass index (BMI) is positively associated with both sICAM-1 and sVCAM-1 in type 2 diabetic subjects[135]. In Pima Indians, sVCAM-1 was positively correlated with BMI; and both sICAM-1 and sVCAM-1 were correlated with waist circumference[144]. Another author showed that sICAM-1 (but not sVCAM-1) was independently correlated with the BMI[162]. However, in one study, sICAM-1 and sVCAM-1 were no different between obese and non-obese type 2 diabetics[163].

Smoking not only has a large impact on CV disease, but it affects the circulating CAMs as well. In diabetic subjects, smoking has been shown to elevate sICAM-1[103, 128, 108] and sVCAM-1[133, 108] levels to even higher than those measured in

diabetic non-smokers. This appears to be a dose-dependent effect[103, 128]. sICAM-1 remains higher in ex-smokers than in diabetic subjects who have never smoked[103, 108], thus indicating that cessation of smoking does not fully reverse the detrimental effects of tobacco on the vasculature.

While one study found an association between increased sVCAM-1 and male sex[133], another found that women had higher levels of sICAM-1 than men[103]. The significance of this is not clear. What does seem clear is that sICAM-1 and sVCAM-1 show a close association with conventional risk factors for CV disease such as hypertension, dyslipidaemia, obesity, smoking, and, of course, diabetes mellitus.

1.5.5 CORRELATIONS WITH NOVEL RISK FACTORS FOR ATHEROSCLEROSIS

The CAMs are just one of a number of emerging makers for CV risk. The most well established of these novel risk factors is CRP. Both sICAM-1[161] and sVCAM-1[164, 133] have been shown to be significantly correlated with CRP in the diabetic setting.

Another prominent non-conventional marker of early atherosclerosis is carotid intima-media thickness (IMT) as measured by ultrasound. Again, sICAM-1[108, 165] and sVCAM-1[123, 102, 166] are positively correlated in multiple studies. Further,

Otsuki *et al.* noted that this relationship between sVCAM-1 and carotid IMT existed only for the diabetic subjects studied, not the non-diabetic subjects[123].

There are many other novel risk factors that are associated with the sCAMs: advanced glycation end-products (AGEs) are positively related with sVCAM-1[167, 136, 168]; sVCAM-1 is associated with decreased flow-mediated vasodilation of the brachial artery[132]; sVCAM-1 increases with plasma vWF and homocysteine [133]; there are significant correlations between sICAM-1 and homocysteine[161, 169], fibrinogen and tissue-type plasminogen-activator antigen[161]. Microalbuminuria is also considered a novel risk factor, and its positive correlation with sICAM-1 and sVCAM-1 has already been detailed above.

1.5.6 IMPLICATIONS FOR THE DEVELOPMENT OF ATHEROSCLEROSIS AND MORTALITY

While the association between sICAM-1 and sVCAM-1 with other known CV risk factors (both conventional and non-conventional) is impressive, this is not in itself evidence that they are actually predictive of atherosclerosis or CV mortality. Even the observation that the aortas and internal mammary arteries from diabetic subjects showed more *in-situ* expression of ICAM-1 and VCAM-1 than non-diabetics is not direct evidence[170]. However, several recent large, long-term studies have demonstrated that both sICAM-1 and sVCAM-1 are good predictors of clinical CV disease.

Hwang *et al* were able to show that sICAM-1 predicted carotid artery atherosclerosis (as assessed by ultrasound) and clinically significant coronary heart disease in a group of 792 subjects followed over 5 years, independent of other known CV risk factors[103]. This was closely followed by a very large study of almost 15,000 subjects, which found that sICAM-1 was a very good predictor of future myocardial infarction in the general population[161]. Another recent study has shown that in type 2 diabetic subjects high sICAM levels, independent of known CV risks, predicted all cause as well as CV mortality over 10 years[122].

Increased levels of sVCAM-1 are also associated with increased risk of mortality in type 2 diabetes, as seen in the Hoorn Study[133]. Over 8 years of follow-up, 631 type 2 diabetic and control subjects with higher sVCAM-1 levels at the beginning of the study period had increased risk of CV death even after adjustment for age, sex, glucose tolerance, hypertension, CV disease, HDL, LDL, homocysteine, microalbuminuria, vWF, CRP and glomerular filtration rate. This effect was magnified in subjects with type 2 diabetes, thus indicating that sVCAM-1 is independently associated with the risk of CV mortality, especially in type 2 diabetes[133]. Stehouwer *et al.* also showed that high sVCAM-1 levels are independently associated with increased mortality in type 2 diabetic subjects followed for a mean of 9 years[171]. Interestingly, the earlier Hwang study did not find this relationship between sVCAM-1 and atherosclerosis[103], but this is possibly because this study did not specifically examine a diabetic population (unlike the Hoorn study).

There are also several cross-sectional studies that confirm the findings of the longitudinal studies. Both sICAM-1 and sVCAM-1 levels are higher in elderly type 2 diabetic subjects with known cerebrovascular disease[102]; and sVCAM-1 levels are higher in type 2 diabetic patients with symptomatic CV disease as compared to those with no known CV disease[123]. However, one very recent study did not demonstrate any correlation between sICAM-1 and sVCAM-1 and known CV disease nor with the degree of intima-media thickening[112]. It is important to keep in mind that this last survey was smaller than the longitudinal studies discussed above. Also, its cross-sectional nature does not allow it to analyse the predictive nature or otherwise of sCAM levels, a point that the authors acknowledge[112].

Taken as whole, the current literature indicates that sICAM-1 and sVCAM-1 levels are predictive for the future development of CV disease and mortality, especially in diabetic subjects.

1.5.7 CORRELATION BETWEEN *IN VIVO* AND *IN VITRO* STUDIES

In reviewing the various studies on the CAM expression in diabetes and nephropathy, it is clear that for the most part, they focus on either the *in vitro* protein/RNA expression or *in vivo* soluble CAM levels, but almost never both. The only exception to this is the mouse study by Bro *et al*[172] which did measure both the tissue levels of ICAM-1 and VCAM-1 mRNA and was able to correlate their expression with the sICAM-1 and sVCAM-1 levels, respectively, in the same mice (see section 1.6).

In all other instances, it has been assumed that an increase in the expression of the CAMs measured in cell culture experiments are equivalent in magnitude and functional significance as the higher levels of circulating sCAMs found in clinical studies. However, this is not necessarily so, especially in the case of nephropathy. The sCAM level in circulation is determined not only by the production of CAMs on the surface of endothelial cells, but also the rate of disposal from the body. While *in vitro* experiments are able to measure the rate of synthesis by endothelial cells, it is more difficult to determine the rate of disposal. This factor is especially significant in patients with the end stages of kidney disease because the removal of many circulating substances is markedly impaired. To date, no group has correlated the *in vivo* levels of sCAMs in individual human subjects with the ability of their serum to increase *de novo* production of surface CAMs on endothelial cells.

There is also the question of the functional significance of increased levels of circulating sCAMs. While cell surface CAMs undoubtedly affect leukocyte adhesion and atherosclerosis, it is not hard to imagine that high levels of circulating sCAMs may compete for ligand binding with the surface CAMs and may have a paradoxically protective effect. This is implied by one study that showed that high levels of sICAM-1 may actually down-regulate the inflammatory response of mononuclear cells and T-cells[173]. Significantly, there are not *in vitro* studies of white cell adhesion to endothelial cells exposed to uraemic serum, which has higher levels of sCAMs than either control populations or diabetic subjects without nephropathy.

1.6 Animal studies of diabetes and nephropathy cell adhesion molecules

For the most part, mouse and rat models parallel the *in vitro* human cell and *in vivo* human clinical studies detailed in the preceding sections in that glucose increases endothelial ICAM-1 and VCAM-1. Rat mesentery exposed to 25 mmol of glucose for 12 hours exhibited increased leukocyte rolling and adherence to the postcapillary venules, an effect that was blocked by inhibition of protein kinase C (PKC) activity[174]. Diabetic rats have also been found to express increased ICAM-1 expression in their retinal vessel endothelium[175]. In rat mesangial cells, high glucose and high osmotic control increases leukocyte to mesangial cell adhesion and ICAM-1 (but not VCAM-1) protein and mRNA[176]. The effect was found to occur via a NF-κB and PKC dependent pathway.

Finally in mice that were surgically nephrectomised, sICAM-1 and sVCAM-1 rose after 12 weeks as compared to mice which were sham operated[172]. The aortic expression of ICAM-1 and VCAM-1 mRNA was also found to be raised in the nephropathic mice, and the increase correlated with the sICAM-1 and sVCAM-1 levels, respectively[172].

1.7 Treatment and cell adhesion molecules

Many different therapeutic interventions have been shown to modify both tissue and soluble CAMs to varying degrees. Some of these treatments are specific to diabetes or diabetic nephropathy, some are not.

1.7.1 GLUCOSE CONTROL AND WEIGHT LOSS

The effect of glucose control on sICAM-1 and sVCAM-1 is not consistent between studies, with only a minority showing decreasing levels with normalisation of glucose. One study demonstrated that the correction of hyperglycaemia in diabetic subjects by intensive treatment with insulin decreased sVCAM-1 levels to control levels after 14 days[177]. Overnight normalisation of glucose in type 2 diabetic subjects with the use of an artificial pancreas suppressed the sICAM-1 (but not sVCAM-1) to normal levels[120]. The PPAR-γ receptor agonist troglitazone decreases sICAM-1 in obese type 2 diabetic subjects and has other profound anti-inflammatory effects (such as decreases in NF-κB in mononuclear cells)[178].

On the other hand, 90 days of intensive glucose control resulted in a small, non-significant drop in sVCAM-1 and no effect with sICAM-1[179]. Two other studies found no significant decrease in sVCAM-1 levels with correction of hyperglycaemia in type 2 diabetic subjects with either insulin, glibenclamide, metformin or a combination of these agents[180, 181]. Lastly, 6 weeks treatment with

rosiglitazone (a PPAR-γ receptor agonist) did not result in a fall in sICAM-1 levels in obese diabetic patients, despite reductions in CRP and TNF-α[182]. Even though hyperinsulinaemia is associated with increased sVCAM-1 (see above), there was no rise in sVCAM-1 demonstrated during insulin infusions in type 2 diabetic subjects[125].

At the tissue level, there seems to be a greater effect of glucose control on ICAM-1. Gliclazide (a commonly used sulphonylurea), but not glibenclamide, nateglinide or glimepiride, has been found to reduce ICAM-1 expression in cultured endothelial cells (HUVECs) and to reduce neutrophil adhesion[89, 90, 183]. Animal studies confirm these findings: gliclazide (but not glibenclamide) has been shown to down-regulate ICAM-1 expression in renal tissue to control levels, as well as decreasing macrophage migration in the glomeruli of diabetic rats[184]. There is even animal evidence that troglitazone prevents type 1 diabetes in NOD mice by decreasing ICAM-1 expression on pancreatic cells[185].

Weight loss may also help to suppress sICAM-1. In a study of obese type 2 diabetic patients, those that lost weight also showed significant falls in sICAM-1 levels down to the level of lean controls[186]. Those who decreased HbA_{1c} only without weight loss experienced no decrease in sICAM-1 levels, indicating that weight loss may be more important than HbA_{1c} in regulating sICAM-1 levels.

1.7.2 OTHER TREATMENTS COMMON IN DIABETES

Glucose control alone may not be enough to significantly decrease the sCAM levels in diabetic patients, but there are other treatments used in the context to diabetes that can modulate sCAM expression.

Hypertension is a common problem in people with diabetes, and blockade of the renin-angiotensin system is a common method used to achieve control of blood pressure. An ACE inhibitor (fosinopril) administered for 12 weeks to microalbuminuric type 2 diabetic subjects resulted in a decrease in sVCAM-1 to control levels, but no change in sICAM-1 levels[149]. In a crossover trial, an ACE inhibitor (enalapril) and an angiotensin receptor II blocker (losartan) both decreased sVCAM-1, but not sICAM-1, in type 2 diabetic subjects[187]. Even though both decreased blood pressure and urine albumin excretion by the same amount, the enalapril was found to be more effective than losartan in decreasing the level of sVCAM-1[187]. However, ACE inhibition with quinapril did not decrease sVCAM-1 in a group of type 1 diabetic subjects[188], probably because the subjects were all normotensive to begin with and otherwise healthy.

Management of dyslipidaemia with HMG-coA reductase inhibitors is also common in diabetes. Three months of simvastatin therapy was able to decrease both sICAM-1 and sVCAM-1 levels significantly[111]. In addition, simvastatin was able to significantly suppress the acute rise in sICAM-1 and sVCAM-1 seen after a glucose load and/or high-fat oral load[111]. Atorvastatin significantly decreases

sVCAM-1 in type 2 diabetic subjects as compared to placebo[189]. This is confirmed by an *in vitro* experiment which shows that both pravastatin and fluvastatin inhibit the glucose-stimulated increase in ICAM-1 expression and adhesion in HUVECs[88]. In streptozocin diabetic rats, cerivastatin is able to downregulate ICAM-1 expression via an NF-κB dependent pathway[190].

The influence of diet on the sCAMs can be demonstrated by a study in which a high-fat load significantly enhanced the acute increase in sICAM-1 and sVCAM-1 post-OGTT (high glucose load)[111]. This effect was present in both the type 2 diabetic and the non-diabetic subjects in the trial. Many attempts have been made to modify diet to best decrease the sCAM expression in people with diabetes. For example, the ingestion of vitamins E and C immediately after a meal can significantly suppress the rapid rise in sICAM-1 and sVCAM-1 seen after a high fat meal[191]. Another group of diabetic subjects given RRR-alpha-tocopheol therapy for 3 months showed a significant fall in sICAM-1 and sVCAM-1[106]. However, this effect is inconsistent. When given alone, neither vitamin E[132], nor vitamin C[192], has any effect on sVCAM-1 in patients with type 2 diabetes. In well-controlled type 2 diabetic subjects, a combination tomato juice, vitamin E, and vitamin C for 4 weeks resulted in no change in sICAM-1 and sVCAM-1 levels as compared to placebo[193]. n-3 polyunsaturated fatty acid supplements in the diet of type 2 diabetic subjects did not decrease sICAM-1[121].

Apart from oral supplements, modification of food composition may also be important. A high-fat meal will result in significantly higher sICAM-1 and sVCAM-1 levels than a high-carbohydrate meal of the same energy value [191].

High oral intake of dietary AGEs in diabetic patients results in increased sVCAM-1. A low-AGE diet will decrease the sVCAM-1 within 6 weeks[168]. This is confirmed by the finding of a rise in sVCAM-1 in dialysis patients fed a high-AGE diet, which can be reversed with a low-AGE diet[194].

Antioxidants such as N-acetyl-L-cysteine can decrease sVCAM-1[124] and glutathione can decrease sICAM-1[195] in type 2 diabetic subjects. If given during an OGTT, glutathione can also abolish the acute rise in sICAM-1 associated with this test[195].

Aspirin, used as an anti-platelet agent, decreases sICAM-1 levels in patients with unstable angina[196]. Another anti-platelet agent, sarpogrelate, also decreases sICAM-1 in diabetic patients[197].

Hormone replacement therapy, whether oestrogen only[198] or continuous combined hormone replacement[199], has not been shown to have any effect on sICAM nor sVCAM in post-menopausal type 2 diabetic women.

Finally, experimental agents that target diabetic complications directly such as epalrestat (an aldose reductase inhibitor)[89] and aminoguanidine (an inhibitor of AGE formation)[92] have both been demonstrated to decrease ICAM-1 expression in endothelial cell cultures.

1.7.3 DIALYSIS

Two of the mainstays of treatment in end stage diabetic nephropathy are haemodialysis and peritoneal dialysis. Dialysis has an unquestionably positive impact on the quality of life and the morbidity of people with renal failure. However, haemodialysis has been shown to have mixed effects on CAM expression.

One study has shown that a single three hour session of haemodialysis with a cuprophane membrane significantly decreases circulating sICAM-1 and sVCAM-1 levels by about 50% [200]. But this stands in contrast with many other studies which show an increase in sICAM levels-1[201] and sVCAM-1[201-203] levels during haemodialysis. Others still show no change in sICAM-1[204, 203] or sVCAM-1[204] between the pre-haemodialysis and the post-dialysis end-points. Attempts have been made by various authors to differentiate between the effects of membranes that the dialysis was performed on, but the results are either inconsistent or show no difference between types of membrane used[201, 204].

A rise in soluble CAMs seems almost paradoxical, given that dialysis is a process where circulating particles, solutes and fluids are removed from the blood stream. The most likely explanation lies in activation of leukocytes during the haemodialysis process which then results in the secretion of pro-inflammatory cytokines such as IL-1β and TNFα[205], both of which up-regulate the production of CAMs.

Peritoneal dialysis may be considered as having a less pro-inflammatory effect on circulating leukocytes, but it has been far less studied in regards to its effects on CAM expression. One study found no significant difference between the pre-dialysis levels of sICAM-1 and sVCAM-1 in patients who were either receiving peritoneal or haemodialysis[204], though both groups showed significant elevation as compared to a healthy control population. Although the effect of a single session of haemodialysis was investigated (see above), the effect of a session of peritoneal dialysis was not.

1.7.4 HIGH DENSITY LIPOPROTEIN

High density lipoprotein (HDL) is known to be protective against CV disease. At the same time, HDL levels are often low in diabetes, especially in type 2 diabetes. It is known that patients with low plasma HDL concentrations tend to have higher levels of sICAM-1[206, 207] and sVCAM-1[201, 207] than those with high HDL levels, implying that HDL may suppress VCAM-1 expression *in vivo*.

The accepted mechanism of the atheroprotective effect of HDL is the reverse transport of cholesterol by the HDL particles. At least three different underlying mechanisms are involved: passive diffusion, facilitated diffusion and apolipoprotein mediated efflux. The latter effect is the most important and involves the apolipoprotein A-1 (ApoA-I), the major protein component of HDL.

While weight loss, exercise and low levels of alcohol consumption are able to increase HDL, there are relatively few drug interventions that actively raise HDL. The fibrates are one of the few pharmacological agents that are used in diabetes which significantly raise HDL levels, but concerns have been raised regarding their use in patients with severe renal impairment[208-210]. Statin drugs increase HDL levels less effectively than fibrates[211, 212].

A novel approach is to administer HDL directly to patient. It is well established that HDL given orally reduces atherosclerotic lesions in rabbits[213]. Further, ApoA-I administration is also protective against atherosclerosis in both mice[214] and rabbit[215] models. There is also emerging data in humans that synthetic HDL (ApoA-I Milano/ETC-216 complex) is a potent agent in the regression of atherosclerotic lesions in humans when given weekly by intravenous injections[216]. The last study is startling in the large magnitude of atheroma regression – 4.2% for total atheroma volume – after just 5 weeks of treatment. A comparable study using simvastatin and niacin reported only a 0.4% decrease in regression of coronary artery stenosis after 3 years of therapy[217]. It should, however, be noted that the ApoA-I Milano study is relatively small (57 subjects) and the findings are yet to be confirmed by other investigators.

At a molecular level, HDL has been found to inhibit the cytokine induced expression of ICAM-1[218-221] and VCAM-1[218, 220-226] mRNA and protein in endothelial cells. Others have found that neutrophil-to-endothelial adhesion[219, 227] and even neutrophil transmigration[227] is decreased by incubation with HDL. These cellular studies are supported by animal studies that show HDL treatments

attenuates ICAM-1 expression in injured rat kidneys[228, 229] and VCAM-1 expression in injured mice carotids[230]. There is evidence that the downregulation of the CAMs occurs by decreased NF-κB[231, 226] and AP-1[226] signalling.

There are, however, two groups of researchers that show a failure of HDL to suppress ICAM-1[225] and VCAM-1[232, 225] in endothelial cell cultures. The main difference is that these two studies both used "native HDL" (nHDL) which is derived from different subjects with minimal processing (centrifugation), and thus exhibits experiment to experiment variability depending on the donor[222]; the majority of the positive studies summarised above used "reconstituted HDL" (rHDL), a more highly purified form that shows little subject-to-subject variability. Some studies used both rHDL and nHDL and showed suppression of CAM expression for both types of HDL[218]. It should be noted that when one of the negative studies used rHDL, VCAM-1 (but not ICAM-1) protein expression was downregulated. The other major difference is that the two negative studies used human coronary artery endothelial cells[232] and human aortic endothelial cells[225], unlike the human umbilical vein endothelial cells used in the positive studies.

In general, however, it seems well established that HDL is able to decrease endothelial expression of both CAMs in cell culture and when given intravenously *in vivo*. Despite this strong evidence, the role of HDL in modulating ICAM-1 or VCAM-1 expression in diabetes, hyperglycaemia and diabetic nephropathy has not been studied.

1.8 Summary, Hypothesis and Aims

Diabetic patients, especially those with nephropathy, have a much higher incidence of CV disease and mortality than the general population. The overall hypothesis of this thesis is that one underlying cause for this increased risk is the potential for the diabetic and nephropathic milieu to up-regulate the endothelial expression of ICAM-1 and VCAM-1, thus initiating a chain of events that leads to atherosclerosis.

Review of the current literature shows that there is increasing recognition that the CAMs are good predictors of atherosclerotic disease and CV mortality, and that both sICAM-1 and sVCAM-1 are raised in diabetes. On a cellular level, this increased expression occurs at the RNA, protein and adhesion levels in response to glucose. Even though there is some data showing that the sCAMs are raised in diabetic nephropathy, there is almost no data regarding the direct effect of uraemic serum on endothelial CAM production. And while many of the treatments commonly used in the management of diabetes have the ability to decrease CAM expression, dialysis (the mainstay of treatment in end-stage diabetic nephropathy) seems to have little effect.

There are some other aspects of the relationship between the CAMs and diabetic nephropathy that are still unresolved. For example, the magnitude of the effect of uraemic serum on endothelial cell CAM expression is unknown. Further, it is not clear whether uraemic serum or hyperglycaemia has the greater effect, nor is it known whether their presence in combination has an effect on CAM expression in

endothelial cell culture. The question of whether glucose acts via an osmotic pathway in its up-regulation of the CAMs remains uncertain. There have been no studies into the effect of either glucose or nephropathy on regulating gene transcription of the CAMs. It has yet to be ascertained whether a correlation exists between cellular up-regulation of CAMs and their soluble levels in either diabetes or nephropathy in humans. Finally, it is unknown whether HDL is able to modulate the influence of glucose and uraemia on endothelial cells.

Thus, the experiments described in this thesis will measure the effects of hyperglycaemia and uraemic serum on endothelial ICAM-1 and VCAM-1 expression, designed in part to answer the questions outlined above. The specific aims of each chapter within this thesis are:

Chapter 3 Aims to establish the effect of glucose, serum of diabetic subjects with increasing degrees of diabetic nephropathy, and the combination of hyperglycaemia and end-stage nephropathic serum on the endothelial cell culture expression of ICAM-1 and VCAM-1. mRNA expression will be measured using real-time RT-PCR, protein expression by ELISA and monocyte-to-endothelial cell binding by adhesion studies. The question of whether glucose acts via an osmotic pathway will also be studied.

Chapter 4 Aims to investigate the molecular regulatory mechanisms responsible for the effect seen in chapter 3. The regulation of ICAM-1 and

VCAM-1 gene expression will be studied at the level of mRNA stability, promoter activity and NF-κB activation.

Chapter 5 Aims to correlate the *in vitro* findings of chapter 3 with the clinically used measure of soluble adhesion molecules in order to investigate the validity of comparing existing laboratory based studies with clinical studies. Soluble adhesion molecules will be measured and compared with the *in vitro* findings for each individual subject in the study. Baseline clinical markers such as HbA_{1c} and existence of known CV disease will also be correlated with the CAM levels to confirm the relevance of CAM estimation in clinical practice.

Chapter 6 Aims to investigate whether HDL is able to suppress ICAM-1 and VCAM-1 mRNA/protein expression in endothelial cell cultures, thus highlighting a possible method of prevention and treatment of CV disease in diabetic subjects with and without kidney disease.

In designing these experiments, it is hoped that new insights will be found to help explain the vastly increased CV risk in diabetic nephropathy.

Chapter 2

General Methods

2.1 Collection of diabetic and nephropathic serum

Serum was obtained from patients in the Diabetes Centre of Royal Prince Alfred Hospital. Patients were chosen randomly, depending on their nephropathic status.

Control subjects were chosen to be young, healthy, non-diabetic subjects to minimise the possibility of subclinical macrovascular disease. Baseline clinical characteristics (age, duration of diabetes, HbA_{1c}, spot urine albumin, serum creatinine and clinical presence of CV disease) were collected for all subjects. The baseline characteristics of the subjects are summarised in table 2 in chapter 3.
All subjects gave informed consent, and the protocols for patient selection and collecting blood were approved by the Ethics Committee of the Royal Prince Alfred Hospital.

Serum was obtained by drawing 60 ml of blood in clotted tubes. The blood was transported on ice, and then allowed to clot. The blood was spun at 2000 G for 5 minutes to separate the serum and clotted cells. The serum was separated into two fractions. One fraction was frozen immediately at -20°C for further processing for sCAM levels, the other fraction was heat treated at 55°C for 30 minutes before freezing. The heat treated serum was used to treat cell cultures for the *in vitro* experiments and was filtered with 0.22 μm filters before storage at -20°C.

2.2 Cell culture

2.2.1 HUVEC

Human umbilical vein endothelial cells (HUVECs) were harvested from healthy male and female infant umbilical cords under sterile conditions by the collagenase digestion method[233]. HUVECs were established as primary cell cultures in M199 (Invitrogen, Melbourne, Victoria, Australia) supplemented with 1% glutamine, 0.5% endothelial cell growth factor (Starrate Products, Bethungra, NSW, Australia), and 20% filtered foetal calf serum (FCS, Gibco BRL, Gaithersburg, MD, USA). Powdered medium was reconstituted with endotoxin-free water and filtered with a 0.2 μm filter.

Gelatin-coated plastic cell culture containers were prepared by using 1ml of 1:250 Gelofusine(B Braun Australia, Bella Vista, NSW):PBS solution per $5cm^2$ of cell culture surface and incubated for 1 hour at 37°C, with excess solution removed before use. HUVECs were propagated on gelatin-coated flasks in M199 media, supplemented as above, and used between passages 2-4. Subsequently, cells were trypsinised and seeded at a cell count of 0.1-0.2 x 10^6 cells/ml onto gelatin-coated 6 well plates for RT-PCR experiments, 12 well plates for adhesion studies and 96 well plates for ELISAs.

2.2.2 BAEC

Bovine Aortic Endothelial Cells (BAECs) were purchased from Cell Applications Inc. (San Diego, CA, USA) and maintained in RPMI (Invitrogen, Melbourne, Victoria, Australia), supplemented with 1% glutamine, 1% penicillin-streptomycin and 10% filtered FCS. Powdered medium was reconstituted with endotoxin-free water and filtered at 0.2 μm.

BAECs were propagated on non-gelatin coated flasks in RPMI (5 mmol/l glucose) media, supplemented as above, and used between passages 10-24. Subsequently, cells were trypsinised and seeded at a cell count of 0.1-0.2 x 10^6 cells/ml onto 12 well plates for transfection.

2.2.3 HAEC

Human Aortic Endothelial Cells (HAECs) were purchased from Cell Applications (San Diego, CA, USA) and maintained in Endothelial Cell Growth Medium (Cell Applications). HAECs were propagated on non-gelatin coated flasks in RPMI (5 mmol/l glucose) media, supplemented as above, and used between passages 8 and 20. Subsequently, cells were trypsinised and seeded at a cell count of 0.1-0.2 x 10^6 cells/ml onto 6 well plates for RT-PCR experiments and 96 well plates for ELISAs.

2.3 Real-time RT-PCR for mRNA expression

2.3.1 mRNA EXTRACTION

1 ml of TRI reagent (Sigma, St Louis, MO, USA) stored at 4°C was added to each well of 6 well plates containing endothelial cells. After an incubation at room temperature for 1 minute, each well was scraped with a sterile cell scraper. The cell and TRI reagent mix was transferred to sterile 1.5 ml eppendorf tubes and kept on ice. 100 µl of 1-brom-3-chloropropan was added to each tube, then the tubes sealed and vortexed. After centrifugation at 14000G for 15 min at 4°C, the mixture separates into three layers. The top (clear) layer is carefully transferred to a new 1.5 ml eppendorf tube, to which 500 µl isopropan-2-ol is added. After another vortex and a 30 minute incubation at 4°C, the samples are centrifuged at 14000G 4°C for 15-20 min. At this point, a small RNA pellet forms at the bottom of the plastic tube. The isopropan-2-ol is removed, 900 µl of 70% alcohol (-20°C) added, centrifuged again and alcohol removed. Each pellet is dissolved in 60 µl of nuclease free water and stored at -80°C until needed.

2.3.2 RNA ASSAY

RNA was quantitated and normalised for each set of reverse transcription reactions with the SYBR Green II assay, using serial dilutions of RNA ladder (1 µg/µl) as the standard. 5 µl of SYBR Green II (Molecular Probes, Eugene, OR, USA) was added to every 50 ml of TE buffer (pH 8), with the RNA quantitation

utilising the Ascent software (version 2.6, Thermo Labsystems). For all RT experiments, the aim RNA concentration was 100 ng/μl, though concentrations of at least 60 ng/μl were usable. Within each RT experiments, there was less than ±5% variation in the RNA concentrations between different conditions.

2.3.3 REVERSE TRANSCRIPTION

Reverse transcription of mRNA into cDNA was achieved using iScript reverse transcriptase (Bio-Rad, Hercules, CA, USA). After dilution with the RT enzyme, RT buffer and nuclease free water, the concentration to total RNA at the start of the RT reaction was 50 ng/μl. The thermal cycler protocol used was 5 min at 25°C, 30 min at 42°C, 5 min at 85°C and then hold at 4°C. Samples were stored at -20°C until PCR amplification was performed.

2.3.4 REAL-TIME PCR

Real-time PCR was used to quantify the mRNA expression levels of ICAM-1 and VCAM-1. To control for RNA concentration and degradation, mRNA levels of the housekeeping genes, 18S RNA or β2-microglobulin, were simultaneously measured. Real-time PCR amplification was performed using the iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA, USA) in an iCycler iQ RealTime thermocycler detection system (Bio-Rad Laboratories) which incorporates a SYBR Green I dsDNA fluorescent dye system. The cycling parameters were 95°C

for 30 seconds (denaturing), 62°C for 30 seconds (annealing) and 72°C for 30 seconds (extension) for 40 cycles, and real-time data collected at each cycle.

The primer sequences were designed to cross introns so that genomic DNA could not be amplified in the PCR reactions. The primers used were as follows:

ICAM-1 Sense: 5'-CCA TCT ACA GCT TTC CGG CGC

ICAM-1 Anti-Sense: 5'-CTC TGG GGT GGC CTT CAG CA

VCAM-1 Sense: 5'-ATG TAG TGT CAT GGG CTG TG

VCAM-1 Anti-Sense: 5'-GGA ATG AGT AGA GCT CCA CC

β2-microglobulin Sense: 5'-CAT CCA GCG TAC TCC AAA GA

β2-microglobulin Anti-Sense: 5'-GAC AAG TCT GAA TGC TCC AC

18S Sense: 5'-CGG CTA CCA CAT CCA AGG AA

18S Anti-Sense: 5'-GCT GGA ATT ACC GCG GCT

Serial dilutions using a sample RT product showed efficient real-time PCR amplification with these primers. When amplification data is plotted against expected results from the serial dilutions, there was a good correlation with all genes (r^2>0.99 in all cases). Melt temperature analysis confirmed tight clustering in the PCR products of the experimental samples, confirming specificity. Mean melt temperatures for the genes were: ICAM-1 89.94 (CV 0.0018), VCAM-1 86.51 (CV 0.0016), 18S 86.67 (CV 0.0027). The PCR products were also run on an agarose gel to confirm the presence of only one product for each set of primers. The product sizes for the primer sets were: ICAM-1 153 bp, VCAM-1 153 bp, β2-microglobulin 180 bp and 18S 110 bp.

Relative quantitation of mRNA expression was determined by the comparative ($\Delta\Delta C_T$) method[234], where levels of expression are given relative to housekeeping gene (18S) values. Briefly, the C_T value is the threshold cycle at which a significant increase in gene expression is first detected. The difference between C_T values of the genes of interest and the housekeeping gene is given by ΔC_T. The baseline ΔC_T is set as the ΔC_T for control conditions (in all experiments, control serum with normal glucose). The comparative $\Delta\Delta C_T$ calculation was determined by subtracting each sample's ΔC_T and the baseline ΔC_T. The absolute value for comparative expression level is then given by the formula $2^{-\Delta\Delta C_T}$.

2.4 ELISA for protein expression

Confluent endothelial cell monolayers grown in 96 well plates were exposed to treatment conditions for 24 hours, after which, the wells were washed twice with HBSS. The cells were incubated for 30 minutes with monoclonal antibodies to VCAM-1 and ICAM-1; negative control wells were incubated with isotype mouse IgG (ICN Immunobiologicals, Irvine, CA, USA) at a concentration of 0.1 μg/100 μl of HBSS with heat-inactivated human serum. The wells were then washed 3 times with HBSS and 0.05% Tween 20 before incubation with sheep-anti-mouse antibody-horseradish peroxidase conjugate (Amersham International, Buckinghamshire, UK) at a concentration of 1:500 in 100 ml HBSS with 10% heat-inactivated human serum and 0.05% Tween 20 for 30 minutes. Wells were then washed with HBSS four times, before 150 ml of ABTS substrate (Kirkegaard

& Perry Laboratories, Gaithersburg, MD, USA) was added to each well and incubated for 15 minutes. Results are expressed as units of optical density measured at 405 nm with a Sunrise plate reader (Tecan Instruments, Maennedorf, Switzerland) using XRead Plus software (version 4.04, Tecan Instruments).

2.5 Adhesion studies for monocyte-to-endothelial adhesion

2.5.1 ISOLATION OF HUMAN MONOCYTES

Concentrates of white blood cells ("buffy coats") were obtained from the peripheral blood of healthy human volunteers and obtained from the Red Cross Blood Bank. Within 24 hours of collection, monocytes were removed from the buffy coats by density gradient separation using Lymphoprep (Nycomed Pharma, Roskilde, Denmark). This was followed by counterflow centrifugation elutriation at 20°C using a Beckman J2-21 M/E centrifuge equipped with a JE-6B elutriation rotor and a 4.2 ml elutriation chamber (Beckman Instruments, Fullerton, CA, USA). The elutriation buffer was HBSS without calcium or magnesium supplemented with EDTA (0.1 g/l) and 1% heat-inactivated human serum. The tubing and elutriation rotor were washed sequentially with 250 ml each of 70% ethanol, 6% hydrogen peroxide, endotoxin-free water and elutriation media. The lymphoprep derived mononuclear cell fraction was loaded into the elutriation rotor chamber, with the rotor revolving at 2020 rpm at 20°C and the tubing flow rate set at 9 ml/min. Flow rate was increased by 1 ml/min every 10 minutes and the effluent from 15 to 18 ml/min collected in separate fractions. Collected

fractions were examined by a Cytospin system (Shandon, Pittsburgh, PA, USA) and Wright's stain (Diff-Quik, Laboratory Aids, Narrabeen, NSW, Australia) for monocyte purity. Fractions with monocyte purity >90% were assessed for viability with Trypan blue exclusion, and accepted for use if viability was >95%. The monocytes were resuspended in phenol red-free RPMI and used immediately for adhesion studies.

2.5.2 MONOCYTE-TO-ENDOTHELIAL ADHESION STUDIES

Confluent HUVECs in 12 well plates were exposed to various conditions of interest for 24 hours (see chapter 4). After this time, the media containing the conditions was aspirated, $1x10^6$ monocytes in 1ml of RPMI was added to the HUVEC monolayer and incubated for 1 hour at 37°C under 5% carbon dioxide in air. The non-adherent monocytes were removed by gentle washing with a 1ml pipette, and the 1 ml suspension stored on ice in Teflon coated tubes[84]. The cells in suspension were counted by a Neubauer haemocytometer (Weber Scientific, Cambridge, UK). The percentage of adherent monocytes was calculated by comparison with the initial concentration. This method has a low intra-observer error and co-efficient of variation (<5%)[235].

2.6 Stability for mRNA degradation

ICAM-1 and VCAM-1 mRNA stability was determined by stimulating HUVECs with IL-1β for 24 hours before blocking transcription by treatment with actinomycin D (5 μg/ml) at the designated time of zero hours. The cells were then incubated in the conditions of interest. At time 0 hours and at each of following time points, RNA was extracted and analysed by real-time RT-PCR as described in section 2.3. The ICAM-1 or VCAM-1 mRNA levels at the time of addition of actinomycin D (ie. 0 hours) was set at 100% and the mRNA levels of the subsequent time-points compared to this.

2.7 Promoter studies

2.7.1 ICAM-1 AND VCAM-1 PROMOTER-REPORTER VECTOR CONSTRUCTS

The methods section of chapter 4 will detail the construction of the ICAM-1 and VCAM-1 promoter region-luciferase reporter vector constructs.

2.7.2 TRANSFECTIONS

BAECs were used for the transfection assays because of their higher transfection efficiency[236]. BAEC were grown in RPMI media (Invitrogen, Melbourne,

Victoria, Australia) containing 5 mmol/l glucose, 20% foetal calf serum and glutamine (0.1%). The cells were grown to confluence in $75cm^2$ tissue culture flasks and passaged every 2-3 days and used between passages 10 and 24. For transfection, the cells were subcultured onto 12 well plates and grown for a further 24 hours in fresh media. The cells were then co-transfected with the promoter-luciferase constructs and a *renilla* luciferase plasmid (pRL-TK) using Effectene (Qiagen, Hilden, Germany) to control for transfection efficiency. *Renilla* is a species of sea-pansy, and like fireflies they are able to produce bioluminescence. *Renilla* was used as an internal control for two reasons: firstly, the *renilla* luciferase can be assayed independently from the firefly luciferase and secondly, the *renilla* gene activity does not alter in the different conditions as it is constitutively expressed via a CMV-promoter.

After 4-6 hours, the cells were washed with PBS and again put into fresh media. The cells were then stimulated with IL-1β (4 units/ml) and exposed to the experimental conditions for 4 hours. Cells were then harvested by passive lysis buffer (Promega Corporation, Sydney, NSW, Australia) and one freeze-thaw cycle.

2.7.3 LUCIFERASE ASSAY

Luciferase activity of the cell extracts harvested by passive lysis were analysed using the Dual Luciferase Reporter System (Promega Corporation, Sydney, NSW, Australia). Bioluminescence was measured by a Fluoroskan Ascent FL

luminometer (Thermo Labsystems, Waltham, MA, USA) using 20 µl of each cell extract in a 96 well format. Results are expressed as luciferase activity corrected for transfection efficiency (firefly/*renilla* luciferase).

2.8 EMSA for NF-κB activation

The gel shift assay provides a simple and rapid method for detecting DNA-binding proteins. The assay is based on the observation that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel more slowly than free DNA fragments or double stranded oligonucleotides.

2.8.1 NUCLEAR EXTRACTION

HUVECs were cultured to confluence in 75cm^2 gelatin-coated flasks and exposed to control serum (± 25 mmol/l glucose) and uraemic serum (± 25 mmol/l glucose) for 4 hours. Cells were harvested with trypsin and washed with cold PBS after centrifugation. The cells were then lysed by a 10 minute incubation in 400 µl ice-cold hypotonic buffer A (see materials, section 2.11) to which a 1:100 concentration of protease inhibitor had been freshly added. After centrifugation, buffer A was removed and the cells resuspended in 50 µl Buffer B mix (again, to which a 1:100 concentration of protease inhibitor had been freshly added). This was followed by a 20 minute incubation on ice, and then another centrifuge 2 minutes at maximum speed at 4°C.

The resulting supernatant, which contains the nuclear extract, is quickly aliquoted into pre-chilled eppendorf tubes, each tube holding enough for one experiment. The aliquots were then immersed in liquid nitrogen for 2 minutes and stored at -70°C until needed.

2.8.2 PROTEIN ASSAY FOR EMSA

Protein concentration of the nuclear extracts was performed using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). One part of dye reagent concentrate was diluted in five parts deionised water. A standard curve was obtained by means of serial dilution of 10 mg/ml bovine serum albumin. The samples were loaded onto a clear 96 well plate and the diluted dye reagent added. After a 5-10 minute incubation at room temperature, the absorbance was measured at a wavelength of 595 nm on a Sunrise microplate reader (Tecan Instruments, Maennedorf, Switzerland) using XRead Plus software (version 4.04, Tecan Instruments).

2.8.3 OLIGONUCLEOTIDE LABELLING

2 µl of 1.75 pmol/µl NF-κB oligonucleotide (Promega Corporation, Sydney, NSW, Australia) was assembled in a 1.5 ml eppendorf tube along with T4 Kinase Buffer, ^{32}P-ATP, T4 Kinase Enzyme (Promega) and enough nuclease free water to make up a total 10 µl volume. This mixture was incubated for 10 minutes,

during which time the T4 Kinase phosphorylated the NF-κB oligonucleotide, and in doing so, ^{32}P-labelled the oligonucleotide. The reaction was stopped by the addition of 1μL 0.5M EDTA, and then diluted in 89 μL of TE buffer. The labelled oligonucleotide was stored at 4°C until needed.

Labelled SP-1 oligonucleotide (non-specific competitive control for the EMSA reaction) was made by substituting SP-1 oligonucleotide for NF-κB oligonucleotide. "Cold" oligonucleotide for NF-κB (specific competitive control for the EMSA reaction) was also made in a similar fashion to the reaction detailed above, but without the addition of ^{32}P.

2.8.4 EMSA

The volumes required for 2 μg of nuclear extract was determined for each sample using the results of the protein assay (section 2.8.2). This was mixed in a 1.5 ml eppendorf tube with 5x binding buffer (see materials), labelled NF-κB oligonucleotide and enough nuclease free water to make a total reaction volume of 10 μl. After a 30 minutes incubation at room temperature, 1 μl of gel loading buffer (see materials) was added to each reaction and the final mixture loaded onto a polyacrylamide gel and run at 100 V for 1 hour. Complexes of labelled oligonucleotide and DNA migrate through the polyacrylamide gel more slowly than free DNA fragments or double stranded oligonucleotides. Larger bands labelled oligonucleotide-DNA complexes (near the top of the gel) indicate increased NF-κB DNA binding activity.

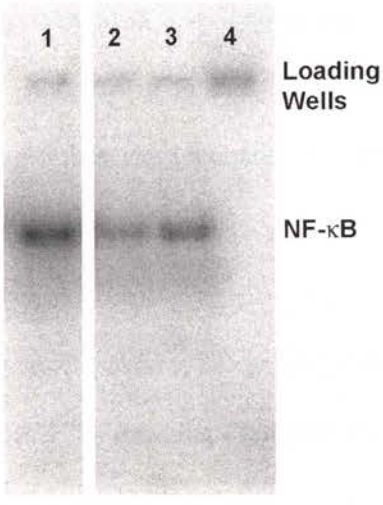


Figure 4. Sample EMSA demonstrating specificity of ^{32}P- labelled NF-κB oligonucleotide. **Lane 1** is nuclear extract run with ^{32}P-labelled NF-κB oligonucleotide. **Lane 2** is nuclear extract run with ^{32}P-labelled NF-κB oligonucleotide and the addition of "cold" (unlabelled) NF-κB oligonucleotide, showing slightly decreased band density, as expected. **Lane 3** is nuclear extract run with ^{32}P-labelled NF-κB oligonucleotide and the addition of a non-specific labelled oligonucleotide (SP-1), showing greater band density than lane 2 as expected. **Lane 4** is ^{32}P-labelled NF-κB oligonucleotide only, showing no band at all, again as expected. Loading wells show up due to residual radioactive label. Unbound labelled oligonucleotides are detected at the bottom of the gel (not shown here, but present in figure 24).

Specificity for the assay was determined by including a 2-fold excess of unlabeled "cold" oligonucleotide or non-specific oligonucleotide (SP-1) competing in the binding mixture. These were mixed with the appropriate labelled and non-labelled control oligonucleotide, incubated and loaded onto a polyacrylamide gel in a similar fashion to the samples of interest. Retarded bands disappear in the presence of a competing unlabeled sequence and remain in the presence of the irrelevant sequence (see figure 4).

Phosphoimager plates were exposed to the polyacrylamide gels for two hours, and scanned using a Fujix Bas 1500 Imaging Plate Reader (Fuji Photo Film Company, Tokyo Japan) using ImageReader software (version 1.8E, Fuji Photo Film Company). Band densities were calculated from the resulting captured images using QuantityOne software (version 4.2.3, Bio-Rad, Hercules, CA, USA).

2.9 Soluble assays for sICAM-1 and sVCAM-1

Commercially available kits for the assaying of sICAM-1 and sVCAM-1 were purchased from R&D Systems (Minneapolis, MN, USA). The assays are based on a quantitative sandwich ELISA technique, where any sCAMs present in a sample is sandwiched by immobilised antibody and the enzyme-linked monoclonal antibody specific for the CAM of interest.

Briefly, samples of serum (100 µl) are added to the wells of the prepared 96-well plate provided with the kit, along with 100 µl of conjugate solution specific to the

sCAM of interest. This is incubated at room temperature for 1.5 hours and then each well washed 6 times with a wash buffer to remove unbound antibodies. Following this, a substrate solution is added to the reactions and colour develops in proportion to the amount of sCAM bound. After 20-30 minutes of development, a stop solution is added to cease the colour development. The intensity of the colour in each well is determined using a Sunrise (Tecan Instruments, Maennedorf, Switzerland) microplate reader set to 450 nm, with wavelength correction set to 620 nm. A set of standards is supplied with each kit, and each set of reactions were run with the standards to obtain a standard curve for concentration. Each serum sample was measured in duplicate.

2.10 Statistical analysis

Descriptive data are expressed as mean ± SE. Groups were compared by ANOVA, with Fisher's procedure used to determine significance; p-values of <0.05 were assumed to represent statistical significance. Multiple data points for each condition (eg triplicates for real-time RT-PCR) were condensed to one mean value and used as one data point for further analysis. Statview 5.0.1 for Windows (SAS Institute Inc, Cary, NC, USA) was used for statistical analysis.

2.11 Materials

PBS

137mM NaCl
2.7mM KCl
4.3mM Na_2HPO_4
1.47mM KH_2PO_4

HBSS

5mM KCl
0.3mM KH_2PO_4
138mM NaCl
4mM $NaHCO_3$
0.3mM Na_2HPO_4
5.6mM D-glucose

Media 199 (M199)

M199 powder (Invitrogen, Melbourne, Victoria, Australia) to make up 5 litres of media
11g $NaHCO_3$
24g HEPES
Deionised water to make a volume of 5 litres
NaOH to balance pH to 7.5
Filter with 0.2 μm filter

RPMI Media 1640

RPMI powder (Invitrogen, Melbourne, Victoria, Australia) to make up 5 litres of media
10g $NaHCO_3$
24g HEPES
Deionised water to make a volume of 5 litres
NaOH to balance pH to 7.5
Filter with 0.2 μm filter

Penicillin/streptomycin concentrate

3g Penicillin
5g Streptomycin
Deionised water to make a volume of 500 ml
To be added 1:100 to media

High glucose concentrate

4.5 g D-glucose
100 ml media (M199 for HUVECs, RPMI for BAECs)
To be added 1:10 to media for high glucose conditions

Mannitol concentrate

3.64 g mannitol
100 ml media (M199 for HUVECs, RPMI for BAECs)
To be added 1:10 to media for high osmotic control conditions

TE buffer (pH 8)

10mM Tris-HCl
1mM EDTA

LB agar (per litre)

10g NaCl
10g Tryptone
5g Yeast extract
15g Agar
Deionised water to make a volume of 1 litre

LB broth media (per litre)

10g NaCl
10g Tryptone
5g Yeast extract
Deionised water to make a volume of 1 litre

LB-ampicillin agar

1 litre of LB Agar
Autoclaved
Cooled to 55°C
Add filter-sterilised ampicillin to a final concentration of 100 μg/ml

LB-ampicillin agar with IPTG/X-Gal

Prepare LB-ampicillin agar as above, plated onto Petri dishes
Spread the following over cooled plates:
100 µl of 100 mM IPTG
20 µl of 50 mg/ml X-Gal
Allow 30 minutes at 37°C to absorb, prior to use

Buffer A for EMSA

0.2383 g HEPES
0.07455 g KCl
0.030995 g $MgCl_2$
0.007715 g DTT
100 ml Water
Prior to use, add 1:100 dilution of fresh protease inhibitor cocktail (Sigma)

Buffer B for EMSA

0.28596 g HEPES
0.009149 g $MgCl_2$
0.004629 g DTT
1.4727 g NaCl
450 ml Water
15 ml Glycerol
26.67 µl 0.45M EDTA
Prior to use, add 1:100 dilution of fresh protease inhibitor cocktail (Sigma)

5x EMSA binding buffer

500 µl 1M HEPES
2.5 ml 1M KCl
20 µl 0.5M EDTA
12.5 µl 1M DTT
5 ml Glycerol
25 µl Nonindet P-40
1.83 ml Water

EMSA Gel loading buffer

2.5 ml 1M Tris, pH 7.5
4 ml 40% Glycerol
3.5 ml Water

NB. Add 0.02 g 0.2% Bromophenol blue for negative control ("cold" oligonucleotide) wells

10x Gel Running buffer for EMSA

50 ml 1M Tris, pH 8.3
28.52 g Glycine

Polyacrylamide gel for EMSA

1 ml 10x gel running buffer for EMSA
3 ml 37.5:1 40% acryl/bis acrylamide solution
15.8 ml water
200 µl 10% ammonium persulfate (freshly made)
20 µl TEMED

Chapter 3

CAM Expression under the influence of Glucose and Nephropathy

3.1 Introduction

This chapter examines the effect of glucose and various degrees of diabetic nephropathy on the expression of ICAM-1 and VCAM-1 in endothelial cells. As discussed in Chapter 1, it is known that endothelial CAM expression is increased in high glucose conditions. However, very little is known about their expression profile in nephropathic conditions. The only other study in this area examined nephropathy, but did not investigate diabetic nephropathy and did not quantify their results[99]. No research has been done into the effect of a combination of high glucose and uraemic serum, and the mechanism by which glucose acts has not been clearly elucidated (see section 1.3.5).

The experiments described in this chapter have been designed to investigate potential differential effects of the various levels of diabetic kidney disease on the CAMs. The effect of glucose (alone and in combination with uraemic serum) was also studied, as was the mechanism by which it acts.

3.2 Methods

3.2.1 DIABETIC AND NEPHROPATHIC SERUM

Serum was collected from subjects with various degrees of diabetic nephropathy. The subjects were selected and serum obtained as per the methods described in section 2.1. The subjects in the study were classified into five groups: controls (Ctrl) with neither diabetes nor kidney disease; those with diabetes mellitus (DM) and no kidney disease; diabetic subjects with microalbuminuria (Micro); diabetic subjects with proteinuria (Prot) but normal serum creatinine; and diabetic subjects with end-stage nephropathy and uraemia (Ur) as indicated by raised serum creatinine.

3.2.2 CELL CULTURE CONDITIONS

HUVECs were exposed to various conditions. The conditions are summarised in table 1. The concentration of serum used was 10%. Thus, for a total well volume of 1 ml, 100 µl of serum was used.

For the glucose conditions, 200 mmol/l solutions of both glucose and mannitol were prepared (see Materials, section 2.11). M199 media was used for cell culture, which has a glucose concentration of 5 mmol/l. Thus, for a total well volume of 1 ml, 100 µl of the appropriate concentrate was added to the media, resulting in a final concentration of 25 mmol/l glucose (HG, high glucose) or 5

mmol/l glucose + 20 mmol/l mannitol (Mann, high osmotic control). Normal glucose (NG) had a glucose concentration of 5 mmol/l.

The different conditions (summarised in table 1) were used in various experiments as described in the experimental setup below (section 3.2.3).

Abbreviation	**Serum used**	**Final Glucose concentration**	**Final Mannitol concentration**
Ctrl NG	Control	5 mmol/l	-
Ctrl HG	Control	25 mmol/l	-
Ctrl Mann	Control	5 mmol/l	20 mmol/l
DM	Diabetic, no kidney disease	5 mmol/l	
Micro	Microalbuminuric	5 mmol/l	-
Prot	Proteinuric	5 mmol/l	-
Ur NG	Uraemic	5 mmol/l	-
Ur HG	Uraemic	25 mmol/l	-
Ur Mann	Uraemic	5 mmol/l	20 mmol/l

Table 1. Summary of conditions used for cell culture.

3.2.3 EXPERIMENTAL SETUP

ICAM-1 and VCAM-1 expression was measured at the mRNA and protein levels, by real-time RT-PCR and ELISA, respectively. Additionally, the functional significance of ICAM-1 and VCAM-1 expression was determined by a monocyte-to-endothelial cell adhesion assay.

3.2.3.1 Real-time PCR

HUVECs were grown to confluence in 6 well plates and used between passages 2 and 4. The conditions used have been described in table 1. The final volume of media and conditions used was 1 ml. Incubation time was 4 hours and the mRNA was extracted as described in the general methods in section 2.3. Real-time PCR for each condition was performed in triplicate to assay mRNA expression.

In the initial set of experiments, only the Ctrl NG, Ctrl HG and Ctrl Mann conditions were used to examine the effect of glucose on adhesion molecule expression. In further experiments the serum representing the increasing stages of nephropathy were used (Ctrl, DM, Micro, Prot, Ur) in normal glucose conditions. Lastly, the combination of Ur and HG/Mann conditions were tested against Ctrl serum.

The serum within each group was *not* pooled (with the exception of control serum), and thus each subject's serum was tested separately. This allowed comparison of expression between subjects and between different assay methods (eg. compared to soluble measures or clinical indicators within an individual). Each subject's serum was tested in two independent experiments. Because there were roughly five subjects in each group, there were a total of 10 experiments in each group of real-time experiments.

3.2.3.2 ELISA

HUVECs (between passages 2 and 4) were seeded onto 96 well plates. The conditions used have been described in table 1. The final volume of media and conditions used was 100 µl/well. Incubation time was 24 hours, after which the cells were washed and ELISA for ICAM-1 and VCAM-1 protein was performed as described in section 2.4.

The experimental setup was to use all 9 conditions described in table 1 on one plate. Each condition was repeated in quadruplicate for both ICAM-1 and VCAM-1. Like the mRNA experiments, each group was *not* pooled (with the exception of control serum). Four independent ELISA experiments were performed.

3.2.3.3 Adhesion

HUVECs were seeded onto 12 well plates with a media volume of 500 µl. The cells were exposed to conditions for 24 hours before being washed with PBS, then incubated with human monocytes derived from elutriation of a buffy coat of one blood donor (see elutriation, chapter 2). After one hour, the media containing the monocytes was carefully aspirated and the monocytes counted via the two methods described in chapter 2 (see adhesion assay). Lower monocyte numbers are indicative of increased adhesion. The adhesion experiments were repeated four times.

The adhesion studies focused on the conditions that were most effective in increasing CAM expression, which were the uraemic serum conditions in normal

and high glucose. Control serum with high glucose was also included. Thus, the conditions used were Ctrl NG, Ctrl HG, Ur NG and Ur HG.

3.2.4 STATISTICAL ANALYSIS

Descriptive data are expressed as mean ± SE. Groups were compared by ANOVA with Fisher's procedure used to determine significance; p-values of <0.05 were assumed to represent statistical significance. Multiple data points for each condition (eg triplicates for real-time PCR and quadruplicates for ELISA) were condensed to one mean value and used as one data point for further analysis. Statview 5.0.1 for Windows (SAS Institute Inc, Cary, NC, USA) was used for statistical analysis.

3.3 Results

3.3.1 BASELINE CLINICAL CHARACTERISTICS

A total of 25 subjects were recruited into the study. The control subjects were chosen to be young, healthy subjects without any clinical evidence of CV disease. The baseline characteristics are summarised in table 2.

	Ctrl	**DM**	**Micro**	**Prot**	**Ur**
n	6	5	5	4	5
Age	34.2 ± 9.7	62.2 ± 9.7 *	63.9 ± 7.5 *	61.3 ± 13.2 *	60.1 ± 5.9 *
Duration of diabetes	N/A	18.6 ± 14.4	11.4 ± 1.14	8.5 ± 6.6	17.2 ± 7.5
HbA_{1c}	4.0 ± 0.6	7.8 ± 1.3 *	6.9 ± 1.8 *	8.7 ± 1.4 *	7.1 ± 1.9 *
Spot Albumin	5.2 ± 2.5	7.2 ± 5.45	38.0 ± 14.0	1017.8 ± 947.0 *	1483.0 ± 1257.3 **
Serum Creatinine	78.0 ± 16.0	76.0 ± 18.9	63.6 ± 8.3	68.5 ± 20.3	280.2 ± 72.7 ***
Known CV Disease	0	2	4	1	3

Table 2. Baseline characteristics of subjects recruited for the experiments in this study (mean ± SD). Units of measurement: HbA_{1c} – %; spot albumin – mg/l; serum creatinine – μmol/l. Significance tested by ANOVA: *p<0.05 vs Ctrl; **p<0.05 vs Ctrl, DM and Micro; ***p<0.05 vs Ctrl, DM, Micro and Ur.

3.3.2 REAL-TIME PCR RESULTS

There were a total of 10 real-time RT-PCR experiments. The results are expressed as fold difference over baseline (control serum with normal glucose concentrations of 5 mmol/l, Ctrl NG) and adjusted for the housekeeping gene β2-microglobulin (by dividing raw expression by β2-microglobulin expression).

3.3.2.1 The effect of glucose on CAM mRNA expression

In serum from subjects with neither diabetes nor nephropathy (control serum), glucose increases the expression of both ICAM-1 (figure 5A) and VCAM-1 (figure 5B) mRNA. There is a 1.50-fold rise over baseline for ICAM-1 and VCAM-1 is elevated 1.55-fold, both increases are statistically significant.

Incubation in 20 mmol/l of mannitol and 5 mmol/l of glucose (high osmotic control) resulted in a small rise in both ICAM-1 (1.19-fold) and VCAM-1 (1.38-fold) mRNA. However these increases were both smaller than the upregulation seen in the high glucose condition and were not significantly different to the normal glucose condition.

3.3.2.2 The effect of nephropathic serum on CAM mRNA expression

As HUVECs were exposed to sera from subjects of increasing severity of diabetic nephropathy, there was a steady increase in both ICAM-1 (figure 6A) and VCAM-1 (figure 6B) mRNA expression. The significance of this trend was strong, with a p value of 0.0094 for ICAM-1 and 0.0227 for VCAM-1.

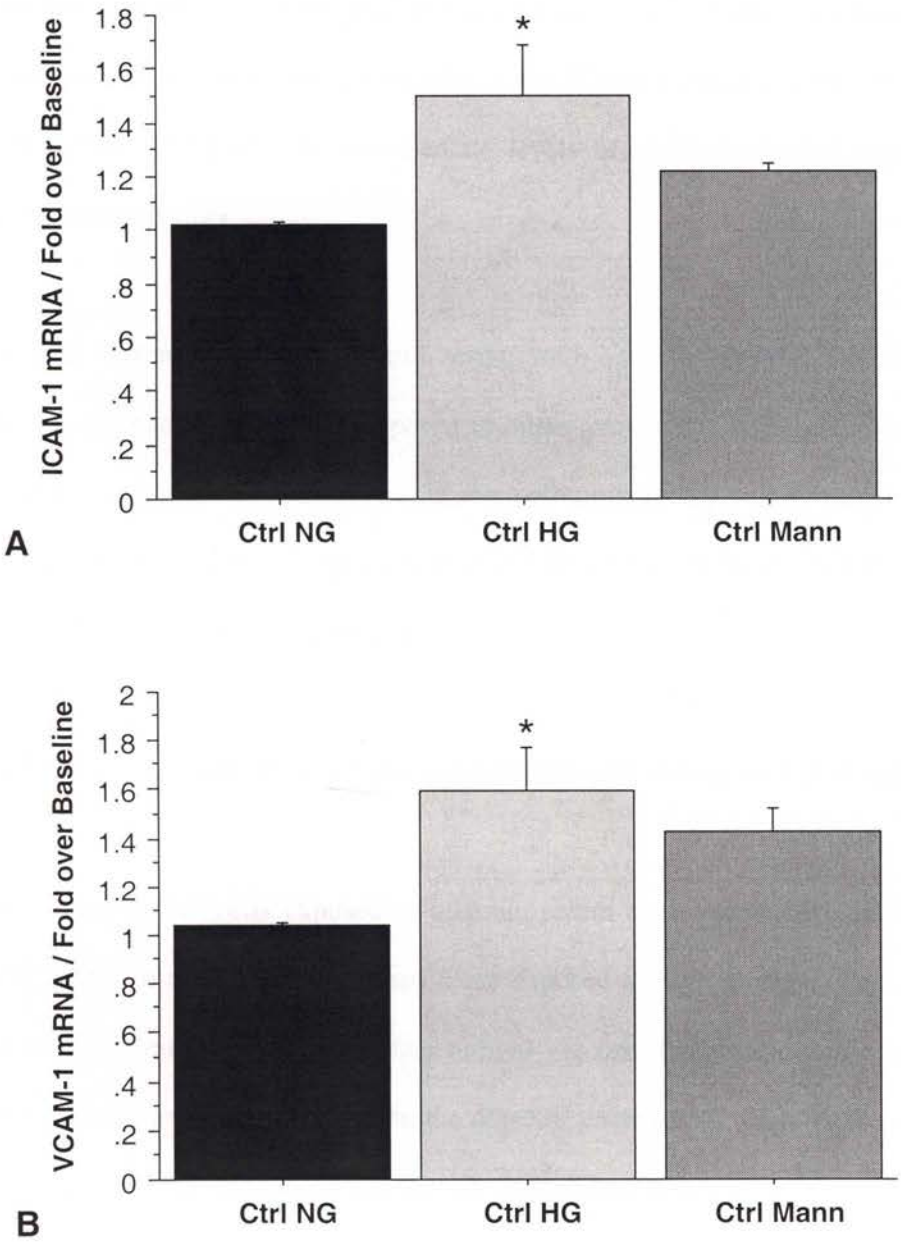


Figure 5. (A) ICAM-1 mRNA and **(B)** VCAM-1 mRNA as measured by real time RT-PCR. Expression is expressed as fold difference over baseline (Ctrl NG). Bars represent means ±SE. *$p<0.05$ vs Ctrl NG.

Diabetes, with no kidney disease, increases ICAM-1 by 1.97-fold over control serum. For VCAM-1, this upregulation was by a factor of 2.13-fold. The maximal rise was seen for uraemic serum for both CAMs: ICAM-1 was elevated 3.65-fold and VCAM-1 3.32-fold. The intermediate levels of nephropathy fell between these respective values.

Significant differences versus control serum were seen in ICAM-1 expression when the endothelial cells were exposed to either proteinuric or uraemic serum. VCAM-1 was significantly increased in microalbuminuric as well as proteinuric and uraemic serum. Uraemic serum was also statistically divergent from diabetic serum for ICAM-1 mRNA expression.

3.3.2.3 The combined effects of glucose and uraemic serum on CAM mRNA expression

Figure 7 shows that cells exposed to uraemic serum overexpress both ICAM-1 and VCAM-1 to a greater extent than those exposed to high glucose alone, and expression is significantly greater than normal glucose. Because uraemic serum increased CAM expression more than the other types of serum, high glucose was added to uraemic serum in order to study their combined effect.

Combining uraemic serum with high glucose resulted in an even greater rise in CAM expression than uraemic serum alone: ICAM-1 mRNA increased 4.84-fold as compared to control and VCAM-1 mRNA by 3.97-fold. The trend for this rise in expression from control normal glucose to control high glucose to uraemia

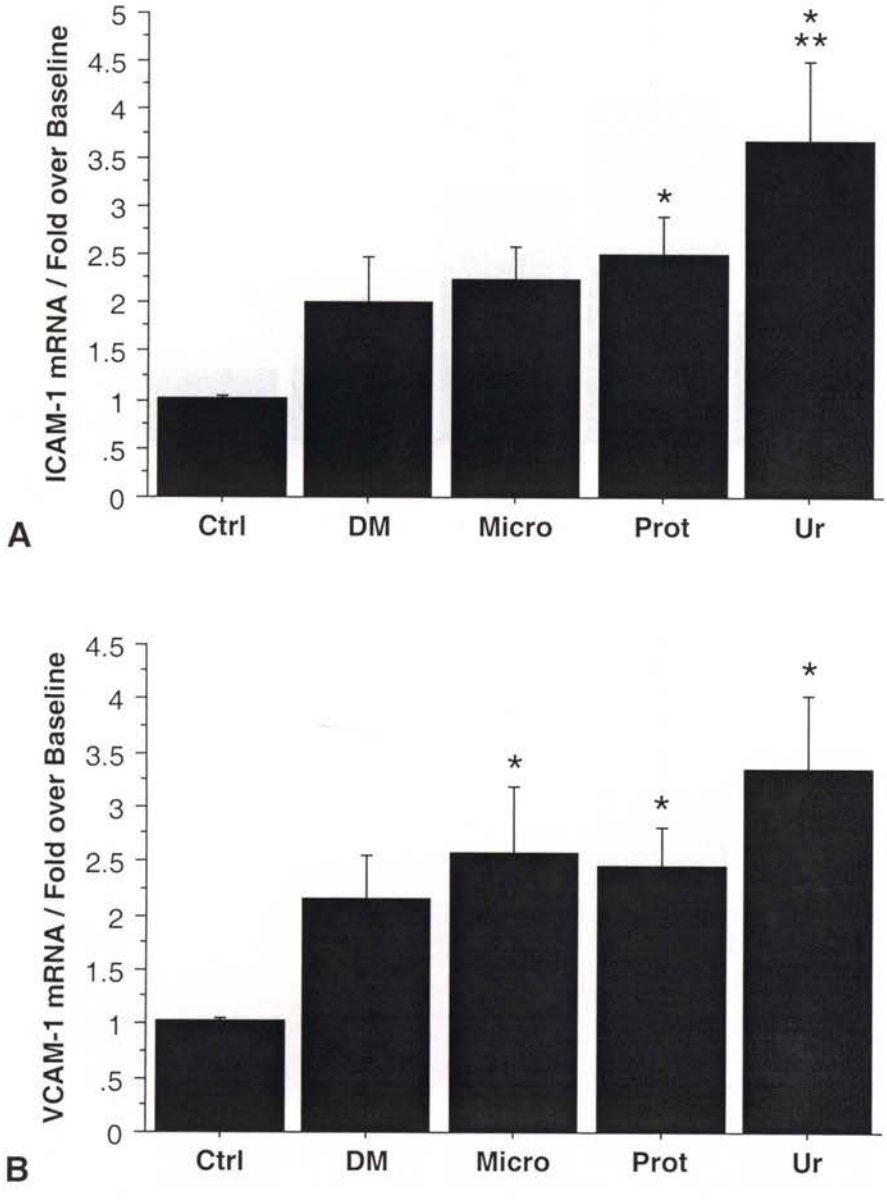


Figure 6. (A) ICAM-1 mRNA and **(B)** VCAM-1 mRNA as measured by real time RT-PCR. p for increasing trend **(A)** 0.0094 **(B)** 0.0227. Bars represent means ±SE. *p<0.05 vs Ctrl, **p<0.05 vs DM.

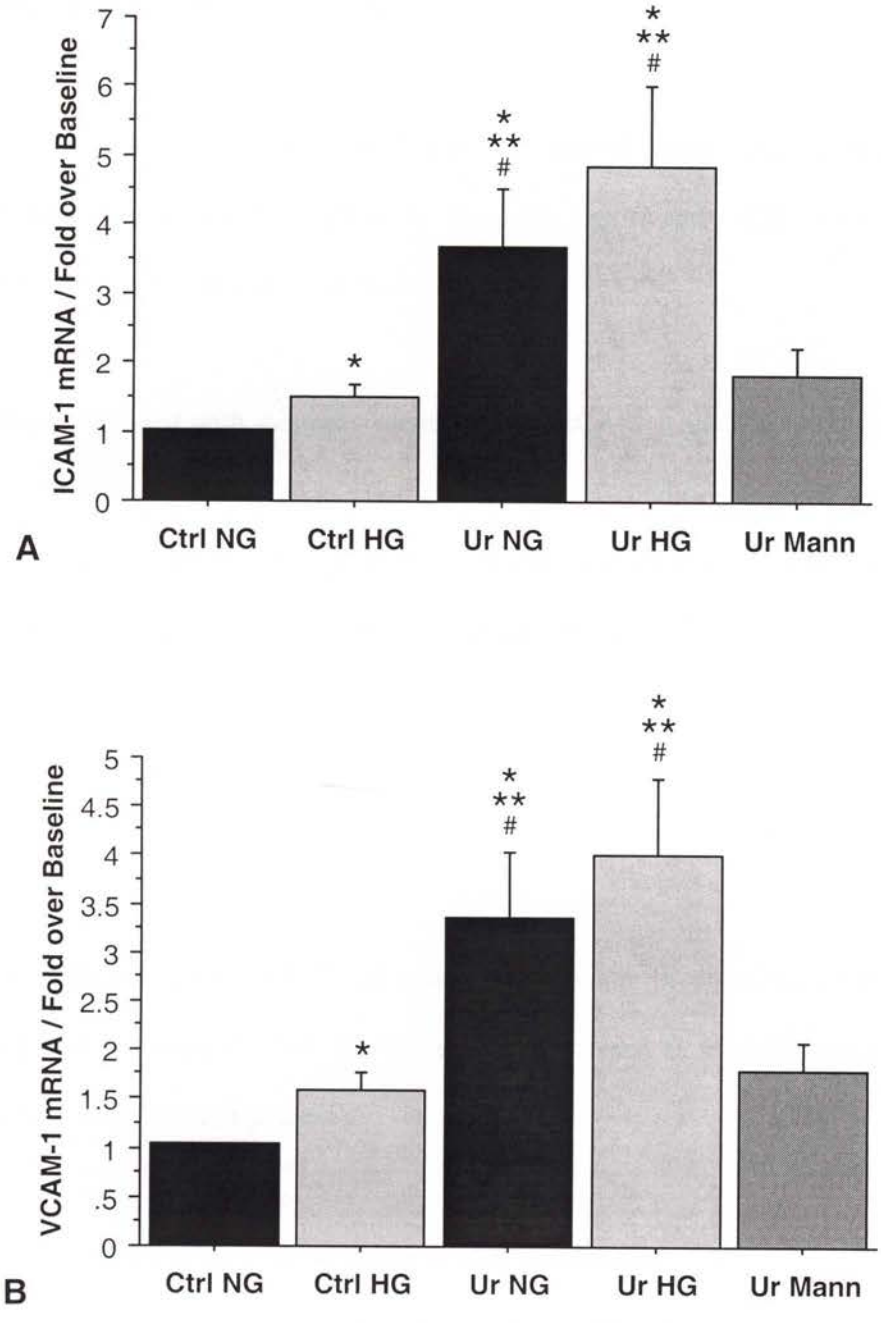


Figure 7. (A) ICAM-1 mRNA and **(B)** VCAM-1 mRNA as measured by real time RT-PCR. p for increasing trend **(A)** 0.0250 **(B)** 0.0233. Bars represent means ±SE. *p<0.05 vs Ctrl NG, **p<0.05 vs Ctrl HG, #p<0.05 vs Ur Mann.

normal glucose to uraemia high glucose was significant for both ICAM-1 ($p=0.0250$) and VCAM-1 ($p=0.0233$).

In addition to being statistically different from control normal glucose, uraemia (with both normal and high glucose) was also significantly different to both control high glucose and uraemia mannitol.

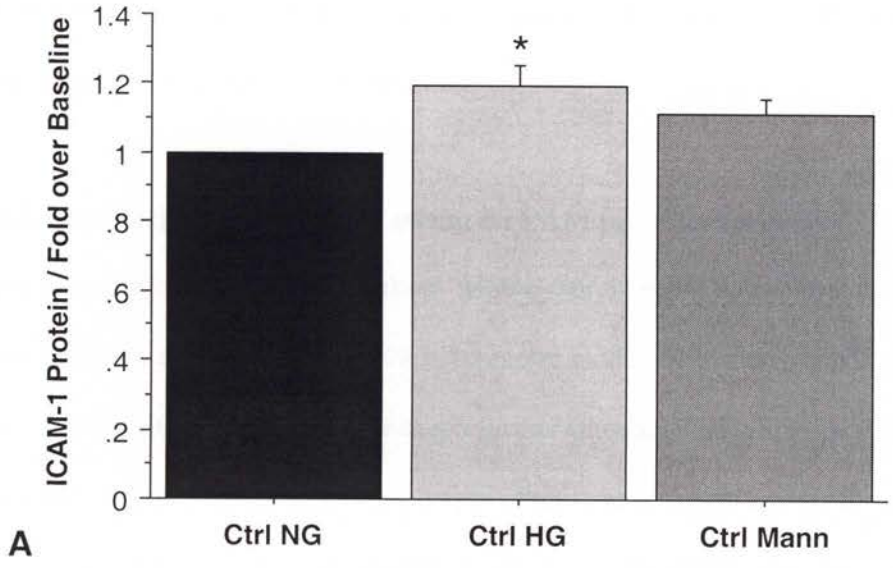
Uraemia combined with mannitol increased both ICAM-1 and VCAM-1 mRNA expression by only 1.57-fold as compared to control conditions, which was less than the expression change measured for uraemia normal glucose. This increase was not significantly different to control normal glucose.

3.3.3 ELISA

The experiments measuring CAM protein expression in endothelial cells by ELISA were repeated 4 times. The results are expressed as fold difference over baseline (control normal glucose).

3.3.3.1 The effect of glucose on CAM protein expression

High glucose significantly increased ICAM-1 protein expression by 1.20-fold over baseline (figure 8A). Although VCAM-1 protein expression was more than 1.25-fold raised above baseline, this result was not statistically significant, due to greater variability in results (figure 8B).



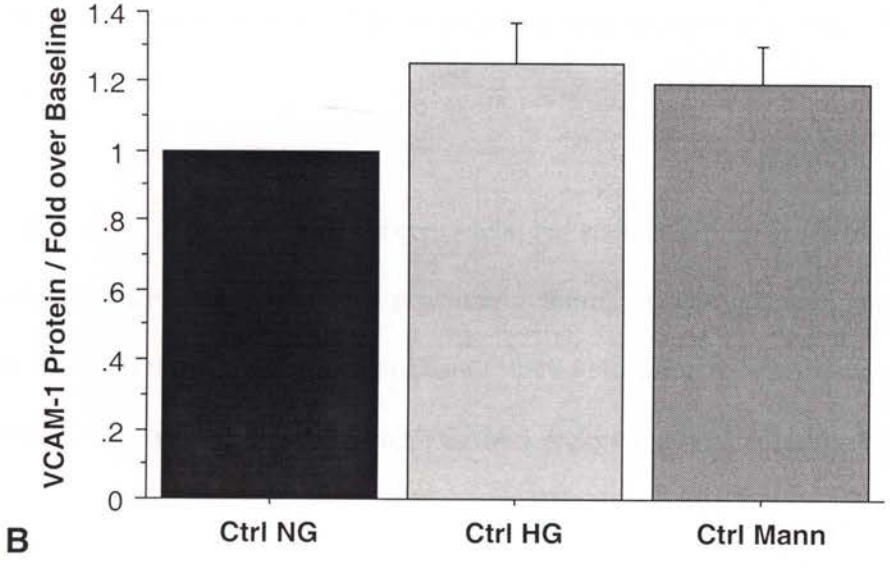


Figure 8. (A) ICAM-1 protein and **(B)** VCAM-1 protein as measured by ELISA. Bars represent means ±SE. *p<0.05 vs Ctrl NG

Mannitol elevated ICAM-1 protein by 1.11-fold and VCAM-1 protein by 1.19-fold, but these values were not statistically significant from either the control normal glucose or high glucose conditions.

3.3.3.2 The effect of nephropathic serum on CAM protein expression

Figure 9A shows that diabetic (without nephropathy) serum alone will increase ICAM-1 protein expression by 34.8%. The lesser grades of diabetic nephropathy do not increase ICAM-1 expression much further (though it should be noted that proteinuric serum does result in statistical significance versus control serum, whereas diabetes and microalbuminuria do not). However, uraemic serum significantly increases ICAM-1 protein expression by 74.7%. The p-value for the overall trend of increasing ICAM-1 expression with increasing nephropathy is 0.0254.

Similarly, VCAM-1 protein showed consistent, but statistically insignificant, rises in diabetic, microalbuminuric and proteinuric serum. It was not until uraemic serum was used that there was a significant (66.4%) elevation in VCAM-1 protein expression. The overall trend in rising VCAM-1 expression with nephropathy was 0.0208.

3.3.3.3 The combined effects of glucose and uraemic serum on CAM protein expression

Like the mRNA results, uraemia (normal glucose) enhanced ICAM-1 and VCAM-1 protein expression more than high glucose alone. The addition of high glucose to uraemic serum increased both ICAM-1 and VCAM-1 protein further

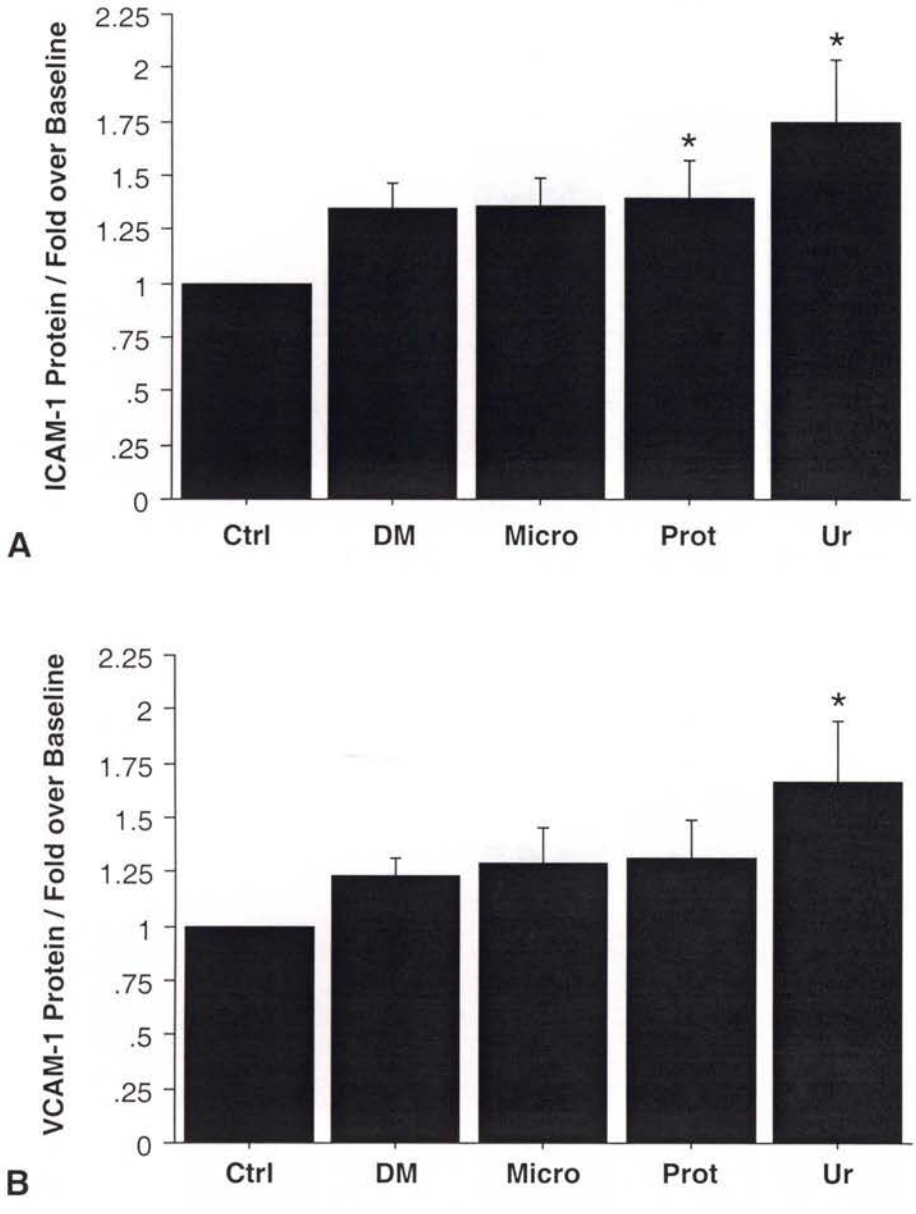


Figure 9. (A) ICAM-1 protein and **(B)** VCAM-1 protein as measured by ELISA. p for increasing trend **(A)** 0.0254 **(B)** 0.0208. Bars represent means ±SE. *p<0.05 vs Ctrl NG.

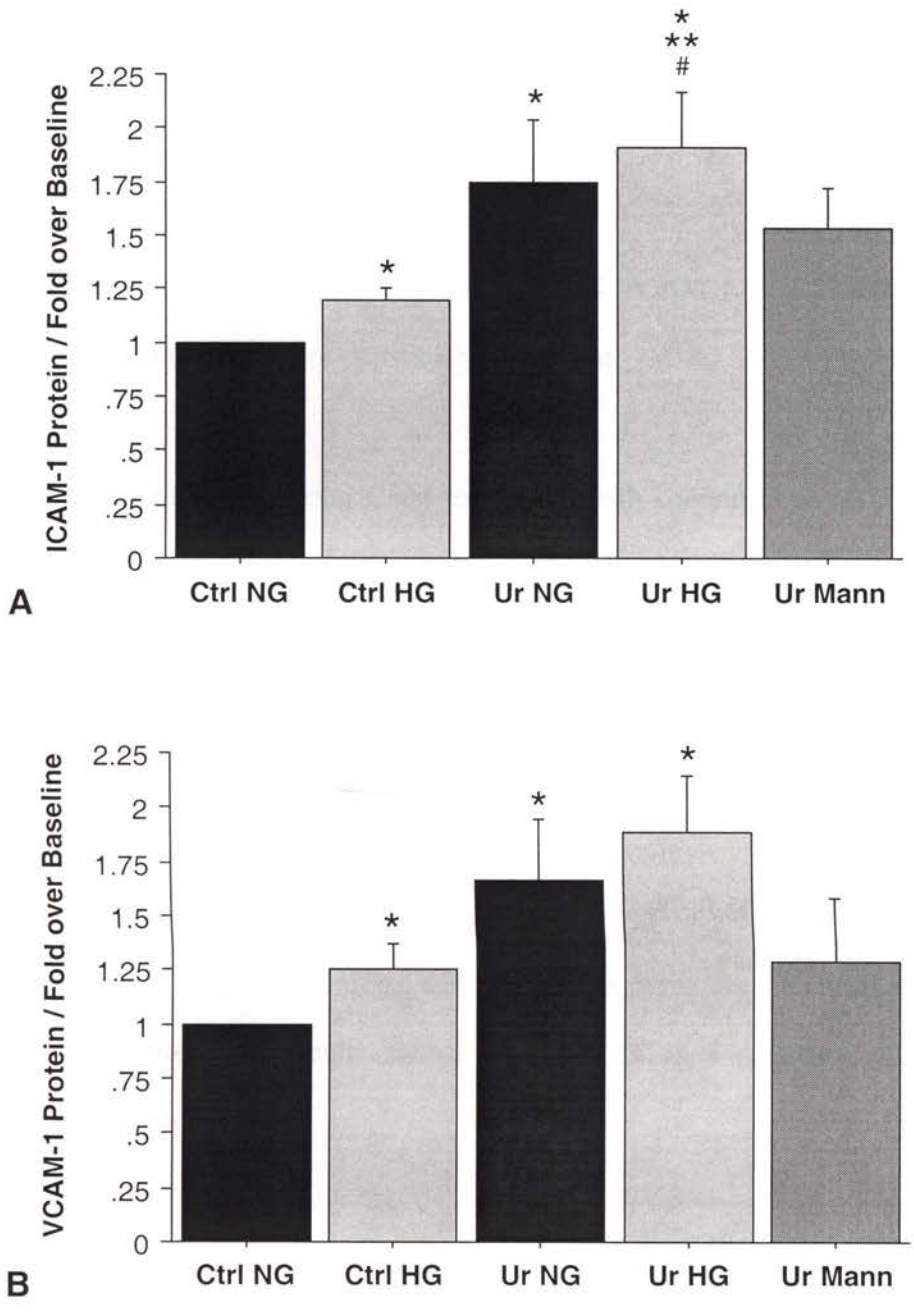


Figure 10. (A) ICAM-1 protein and **(B)** VCAM-1 protein as measured by ELISA. p for increasing trend **(A)** 0.0247 **(B)** 0.0065. Bars represent means ±SE. *p<0.05 vs Ctrl NG, **p<0.05 vs Ctrl HG, #p<0.05 vs Ur Mann.

than uraemic serum alone (upregulation by 1.91-fold and 1.89-fold, respectively). In the case of ICAM-1, uraemic serum plus high glucose was significantly different to control normal glucose, control high glucose and uraemia mannitol (see figure 10).

Uraemia mannitol raised ICAM-1 by 1.20-fold and VCAM-1 by 1.29-fold, and neither of these increases were statistically significant.

The overall trend of increasing CAM expression with combinations of glucose and uraemic serum was $p=0.0247$ for ICAM-1 and $p=0.0065$ for VCAM-1.

3.3.4 ADHESION STUDIES

Monocyte adhesion to HUVECs exposed to combinations of glucose and uraemic serum showed a very strong trend that reflected patterns seen in mRNA and protein expression. The results shown in figure 11 is a summary of four independent experiments.

High glucose alone increases monocyte adhesion by 1.18-fold ($p<0.008$ vs control normal glucose). Uraemic serum alone raises adhesion by 1.39-fold ($p<0.0001$ vs control normal glucose, $p<0.003$ vs control high glucose). Finally, the combination of uraemic serum with high glucose enhances monocyte adhesion 1.54-fold, and this is statistically significantly different to the other three conditions (including uraemia normal glucose).

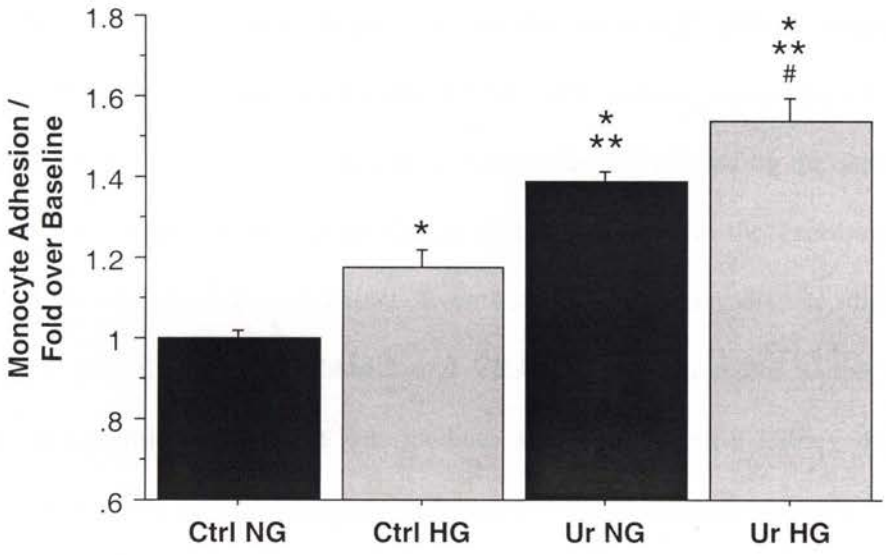


Figure 11. Monocyte adhesion to HUVECs exposed to combinations of control serum, uraemic serum, normal glucose and high glucose conditions. Bars represent means ±SE. *p<0.01 vs Ctrl NG, **p<0.01 vs Ctrl HG, #p<0.02 vs Ur NG.

3.4 Discussion

The results presented in this chapter indicate that while high glucose conditions alone will upregulate the endothelial mRNA and protein expression of both ICAM-1 and VCAM-1, the uraemic environment (as represented by the serum of subjects with chronic renal failure due to diabetes) will raise the expression of both CAMs to a much greater extent. Even without any kidney disease, diabetes alone will slightly increase ICAM-1 and VCAM-1 levels, but not to the same extent as uraemic serum. The intermediate stages of diabetic kidney disease (microalbuminuria and proteinuria) raise both CAMs to degrees between that of diabetes alone and uraemia, with a significant trend towards a stepwise increase in levels of CAMs with each increasing stage of diabetic nephropathy. Finally, the combination of high glucose and uraemic serum elevates ICAM-1 and VCAM-1 mRNA/protein expression even further than uraemia alone.

Osmotic mechanisms explain part, but not all of the induction of CAM expression by high glucose alone. However, when combined with uraemic conditions, a high osmotic environment seems to downregulate ICAM-1 and VCAM-1 expression slightly when compared to the uraemia normal glucose condition, indicating that in this instance, glucose is acting through a non-osmotic pathway. Indeed, the alternate (possibly metabolic) pathways that glucose is acting through must be able to overcome the mild inhibitory effect of its osmotic properties. It is possible that this is why the combination of glucose and uraemia seems to have additive, as opposed to synergistic, effects on CAM expression.

In order to test the functional significance of the increased mRNA and protein expression, adhesion was also studied. As has been noted in the literature review, both ICAM-1 and VCAM-1 are central in the adhesion of monocytes to the endothelium. As the results demonstrated, adhesion is significantly raised in high glucose alone and uraemia normal glucose, but is even more markedly elevated with the combination of high glucose and uraemia. This indicates that the CAM increase that was demonstrated at the mRNA/protein level was having a functional impact on the initial pathological mechanism of atherosclerosis, namely, monocyte adhesion to the endothelium.

3.4.1 CORRELATION WITH CURRENT LITERATURE

While this is the first study to use real-time RT-PCR to quantitate endothelial CAM expression under the influence of glucose, the results do accord with the one study to use semi-quantitative RT-PCR to determine that ICAM-1 mRNA increases in high glucose conditions[92].

The ELISA results match the existing literature on stimulatory effect of glucose on ICAM-1[85, 86, 88-95] and VCAM-1[84, 94, 95] protein expression. In the case of ICAM-1 protein, our estimation of a 20% increase in expression lies within the 16-110% increase that has been found in previous studies. Taken as a whole, the expression data argues against the small minority of prior research that indicated that endothelial ICAM-1 and VCAM-1 are not influenced by high glucose[83, 85, 97].

The adhesion results also confirm the many studies that indicate that high glucose alone increases monocyte adhesion to endothelial cells[83-87]. The magnitude of the increase in adhesion were in keeping with the lower end of estimates of leukocyte to endothelial adhesion[88].

Because there are no other *in vitro* studies into the effect of diabetic nephropathy on endothelial cells, there is no prior research to compare the nephropathic data (alone, and in combination with high glucose) presented in this chapter. However, the findings of markedly increased ICAM-1 and VCAM-1 expression in endothelial cells exposed to the serum of uraemic subjects does accord with Serradell *et al,* which showed the qualitative rise in CAM expression in endothelial cells treated with the serum of non-diabetic renal failure patients[99]. Because the Serradell *et al* study did not quantitate the rise in CAM levels, it is not possible to compare the magnitude of the increase found between these two studies.

Even though it may not be possible to directly compare *in vitro* cellular CAM expression with serum levels of sCAMs, it is nonetheless interesting to note that the near stepwise increase in both ICAM-1 and VCAM-1 mRNA (figure 6) and protein (figure 9) with increasing degrees of diabetic nephropathy seems to mirror the stepwise increase in sICAM-1 and sVCAM-1 concentrations seen in previous clinical studies[145-147], as demonstrated in figure 3. Chapter 5 will further detail the correlation between *in vitro* and *in vivo* measures of the CAMs.

Finally, as noted in section 1.3.5, there is no consensus in the current literature as to whether glucose acts to increase the CAMs through an osmotic pathway or not. Our data would support the previous studies that indicate that high glucose acts only partially through an osmotic pathway[87, 92]. There has been no previous research into the combination of uraemic conditions and a hyperosmolar environment to allow a comparison with the results presented in this chapter.

3.4.2 CLINICAL IMPLICATIONS

Knockout animal models have demonstrated that both ICAM-1[46, 47] and VCAM-1[67] play a central role in the development of atherosclerotic lesions. Thus, upregulation of either CAM would have a direct impact on the future probability of CV disease.

The results presented in this chapter indicate that hyperglycaemia itself could contribute to the increased incidence of atherosclerotic disease in diabetes mellitus by stimulating the expression of ICAM-1 and VCAM-1 on endothelial cells. This finding coincides with the recent observation that HbA_{1c} is strongly associated with the development of such macrovascular complications such as acute myocardial infarction, stroke, heart failure and peripheral vascular disease[8], and not just microvascular disease as had previously been thought[6, 7]. It is worthwhile to point out that the mRNA levels were increased within four hours on introduction of glucose, implying that even short term hyperglycaemia that a

diabetic patient may typically experience can be sufficient to induce CAM production.

Our finding that uraemic serum enhances ICAM-1 and VCAM-1 expression more than hyperglycaemia alone is also in accordance with the observation that while diabetes itself increases the risk of atherosclerosis two- to four-fold, the corresponding risk for diabetic nephropathy is at least ten times higher again.

These experiments highlight the sensitivity of the endothelium to the additive effects of the hyperglycaemic and uraemic milieu. The finding that hyperglycaemia in combination with uraemic serum act together to increase CAM expression further speaks to the importance of excellent glucose control in patients with diabetic nephropathy (though this is already standard practice aimed at preventing the development of microvascular complications).

It is reasonable to speculate that given their importance, ICAM-1 and VCAM-1 would be logical targets for therapy in the prevention of atherosclerosis in diabetes and diabetic nephropathy. One possible intervention (HDL) that may inhibit CAM expression will be investigated in chapter 6.

3.4.3 STUDY LIMITATIONS

This was only a small study with 25 subjects, however, it was still able to provide useful data regarding the effect of glucose and diabetic nephropathy on

endothelial CAM expression. However, as with any small study, patient selection has a large effect on the end results. The subjects were selected randomly from patients attending the Diabetes Centre at Royal Prince Alfred Hospital and it is possible that a different set of patients may have resulted in a different outcome.

One aspect of the subject group that may have influenced the results was that of the age of the subjects. The control group was significantly younger than any of the diabetic groups. This was a deliberate choice as it helped to ensure that the control group consisted of young, healthy individuals with no history of CV disease. sICAM-1 levels are known to be unrelated to age in the adult population[237, 103, 238]. Indeed, one of these studies found a slight negative correlation between age and sICAM-1 levels[238], though the subject group ranged in age from 4-55 years, a slightly younger group than the subjects presented herein. While VCAM-1 is correlated with advancing age[237, 103], this increase is minor in comparison to the increase found in VCAM-1 expression found with diabetic nephropathy. It should be noted that in our experiments, the subjects from the diabetes group through to the uraemia group had no variation in age: yet, within this group, there was still a clear trend to increasing CAM expression with increasing kidney disease. Chapter 5 (especially section 5.4) will analyse the relationship between age and the CAMs further.

Another potential limitation was the definition of microalbuminuria and proteinuria. At the time of patient recruitment, albumin excretion rate (AER), rather than albumin-creatinine ratio (ACR), was the accepted clinical measure of early diabetic kidney disease. Soon after patient recruitment, ACR became

accepted as the better measure of urine protein leak. In using AER, there was a possibility that some of the subjects were misclassified. The importance of this factor is mitigated by several facts. Firstly, it does not affect the definition of the controls or uraemia subjects. Secondly, there are only small differences between the expression of the CAMs in the diabetes, microalbuminuria and proteinuria groups. Thirdly, it would not be expected that many of the subjects were misclassified.

In order to rule out the differing methods of measurement an issue, the data was reanalysed using ACR instead of AER. Using ACR, there was one microalbuminuric subject who would have been proteinuric, one proteinuric subject who would have been microalbuminuric. Changing these two subjects and reanalysing the data did not materially affect the results.

Lastly, while the results clearly show that there are factors within diabetic nephropathic serum that stimulates CAM expression, the experiments were not designed to identify the component responsible. This is an obvious area for future research.

3.5 Conclusion

High glucose significantly increases ICAM-1 and VCAM-1 expression in endothelial cells at the mRNA and protein level via a partially osmotic mechanism. Uraemic serum stimulates the expression of both CAMs much more than high glucose. The intermediate degrees of diabetic nephropathy also upregulate the CAMs, and there is a strong trend to a stepwise increase in CAM expression with increasing kidney disease. Combining high glucose with uraemic serum results in the highest levels of both ICAM-1 and VCAM-1; in this instance, the stimulatory effect of glucose did not occur via an osmotic pathway. The increased expression of the CAMs resulted in a functional increase in monocyte adherence to endothelial cells.

The upregulation of these two CAMs in high glucose and nephropathic serum and the resultant increase in monocyte adhesion helps to explain the increased risk of atherosclerosis in diabetic patients, especially those with diabetic nephropathy.

Chapter 4

The Effect of Glucose and Uraemic Serum on the Regulation of ICAM-1 and VCAM-1 Gene Expression

4.1 Introduction

Chapter 3 established that glucose and uraemia increase ICAM-1 and VCAM-1 expression in endothelial cells at the mRNA and protein levels. This led to an increase in the physiologically important end-point, monocyte adhesion to endothelial cells. The aim of this chapter is to determine the regulatory mechanisms that underlie these findings.

In order to do this, the effect of glucose and uraemia on the transcriptional activity of the 5' regulatory regions of the ICAM-1 and VCAM-1 genes was investigated. These regions, called promoter regions, are sections of DNA that are a few thousand bp in length that lie upstream from the 5' end of the genes they control. They exert their influence on their associated genes by either stimulating or inhibiting the transcription of the encoding DNA into mRNA.

Activity of the promoter regions is controlled by several nuclear factors that are able to enter the nucleus from the cytoplasm and bind to specific sites within the DNA of the promoter region. Such nuclear factors can increase or decrease promoter activity, and thus influence the production of many genes.

Post transcription, mRNA can last for variable amounts of time in the cytoplasm before it is degraded. Different stimuli can increase or decrease half-life of mRNA; conditions that increase mRNA stability will result in greater expression of the protein encoded by the mRNA sequence. Therefore, this represents another way in which extracellular conditions, such as hyperglycaemia and nephropathy, can influence protein expression levels.

The experiments described in this chapter were designed to examine the effect of glucose and nephropathy on ICAM-1 and VCAM-1 mRNA stability, ICAM-1 and VCAM-1 promoter activity and the activation of the nuclear factor NF-κB.

4.2 Methods

4.2.1 CONSTRUCTION OF PROMOTER FRAGMENTS

The activity of the respective promoter regions was assessed by use of the firefly luciferase reporter gene plasmid vector pGL3-Enhancer (Promega). Firefly (*Photinus pyralis*) luciferase allows rapid and sensitive quantitative analysis of factors that regulate mammalian gene expression, assayed by the measurement of light production upon addition of luciferin and ATP[239].

Sea pansy (*Renilla reniformis*), like fireflies, are able to produce bioluminescence which can be assayed independently from firefly luciferase activity. pRL-TK, a constitutively expressed *Renilla* luciferase plasmid, was co-transfected with the

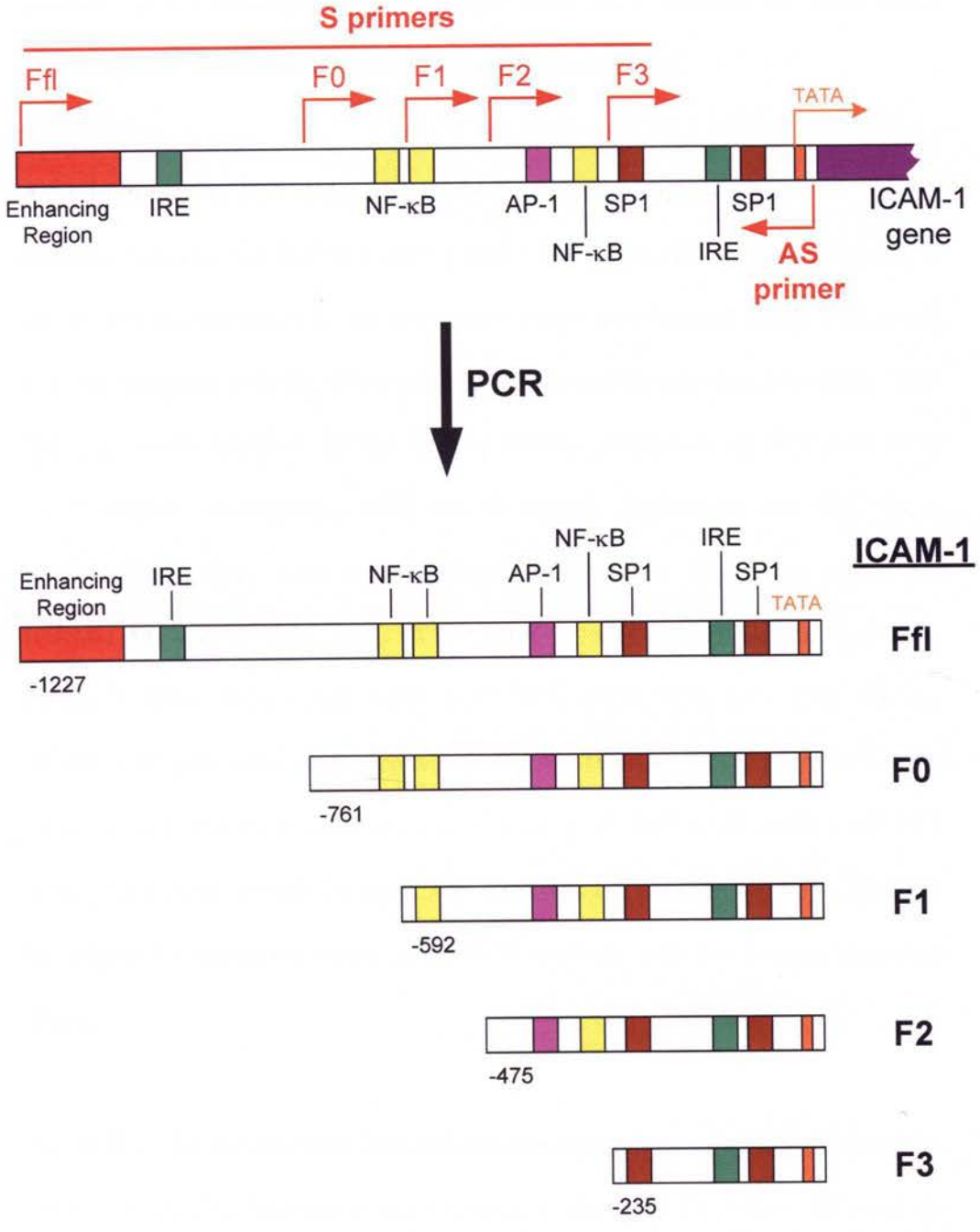


Figure 12. Design of a set of ICAM-1 promoter region fragments (Ffl through to F3) using a series of nested deletions. Five different sense (S) primers were designed and used with a common anti-sense (AS) primer. The S primers all included a *Mlu* I restriction site, and the AS primer included a *Xho* I site to assist in directional cloning (seen in figure 16). Numbers indicate bp length.

promoter-pGL3-Enhancer constructs and used as a control for transfection efficiency. All results are corrected for this internal control.

4.2.1.1 Cloning of ICAM-1 and VCAM-1 promoter regions

The functional human ICAM-1 and VCAM-1 full-length (Ffl) promoter regions in the 5' regions upstream of the respective genes were cloned using PCR using primers designed with the Oligo program (National Biosciences, Plymouth, MN, USA) as shown in figure 12 and 13. The cycling parameters for PCR were 94°C for 5 seconds (denaturing), 65°C for 30 seconds (annealing) and 72°C for 3 minutes (extension), with amplification for 35 cycles. The sense primer for ICAM-1 Ffl was 5'-CGC AGC CCC CTT CCC CGA TAG and the anti-sense primer 5'-GTA GCA GAG AGG AGC TCA GCG TCG ACT GG. For the VCAM-1 Ffl, the sense sequence was 5'-GAA TTC AAA ATG ATA TTT CAG TGG and the anti-sense sequence was 5'-GAC CAT CTT CCC AGG CAT TTT AAG. The product length for the ICAM-1 Ffl was 1227 bp and VCAM-1 Ffl 2167 bp. Figure 14 shows the result of the PCR reactions with the primers described above.

The PCR products were then inserted into the reporter vectors as detailed below. All 5' primers also included a *Mlu* I restriction site (5'-ATA ACG CGT) and the 3' primers had a *Xho* I restriction site (5'-TAA CTC GAG).

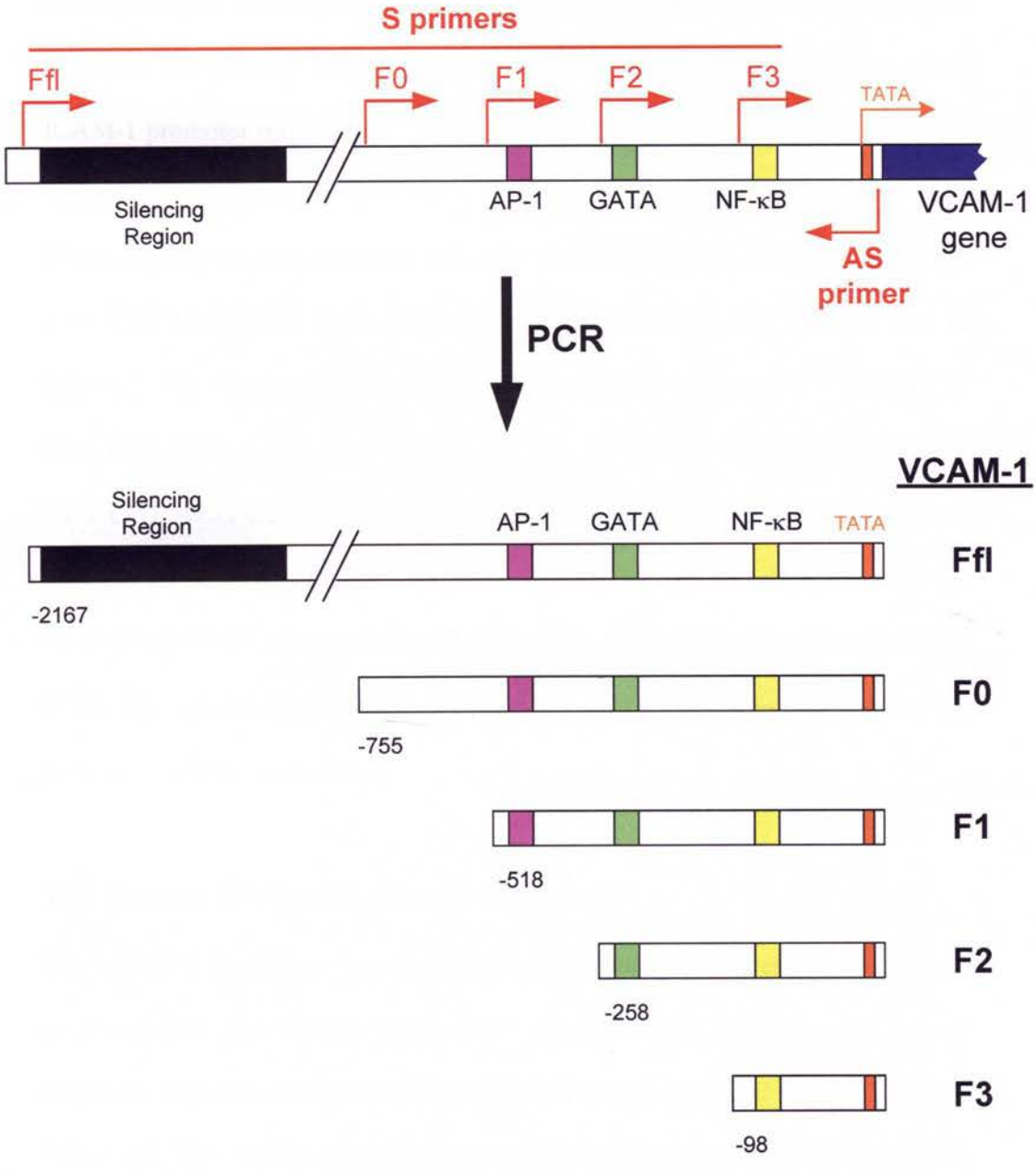


Figure 13. Design of a set of VCAM-1 promoter region fragments (Ffl through to F3) using a series of nested deletions. Five different sense (S) primers were designed and used with a common anti-sense (AS) primer. The S primers all included a *Mlu* I restriction site, and the AS primer included a *Xho* I site to assist in directional cloning (seen in figure 16). Numbers indicate bp length.

4.2.1.2 Design of ICAM-1 promoter fragments

Promoter region analysis from Stade *et al*[53] was analysed with the Oligo program (National Biosciences) to find primers near the areas of interest, yielding novel ICAM-1 promoter fragments.

The anti-sense primer remained the same as for the ICAM-1 Ffl fragment (5'-TAA CTC GTA GCA GAG AGG AGC TCA GCG TCG ACT GG), as seen in figure 12. The sense primers for each fragment were as follows: F0, 5'-CTC CGC CAA GGA CGA AAG; F1, 5'-CCC GTG TCA GCT AGG TGT GG; F2, 5'-GGC CTG CGT AAG CTG GAG AG; and F3, 5'-GCG CCG ATT GCT TAA GCT TG. The product sizes for each fragment was: F0 761 bp, F1 592 bp, F2 475 bp, F3 235 bp. All 5' primers also included a *Mlu* I restriction site (5'-AAT ACG CGT). The anti-sense primer was the same as used for the full length ICAM-1 Ffl promoter fragment (see above).

4.2.1.3 Design of VCAM-1 promoter fragments

The VCAM-1 fragments were selected from previously described constructs used to characterise the VCAM-1 promoter region[69]. While the anti-sense primer remained the same as for the VCAM-1 Ffl fragment (5'-GAC CAT CTT CCC AGG CAT TTT AAG), the sense primers for each fragment were as follows: F0, 5'-AGA GAT TTG CCA CTT CAG ATG GAT T; F1, 5'-GAA GTT ATG GTG TCC CTT TTT TAA A; F2, 5'-CCT TTA TCT TTC CAG TAA AGA TAG C; and F3, 5'-TAA ACT TTT TTC CCT GGC TCT GCC C. The product sizes for each fragment was: F0 755 bp, F1 518 bp, F2 258 bp, F3 98 bp, as shown in figure 13. Again, all 5' primers also included a *Mlu* I restriction site (5'-AAT

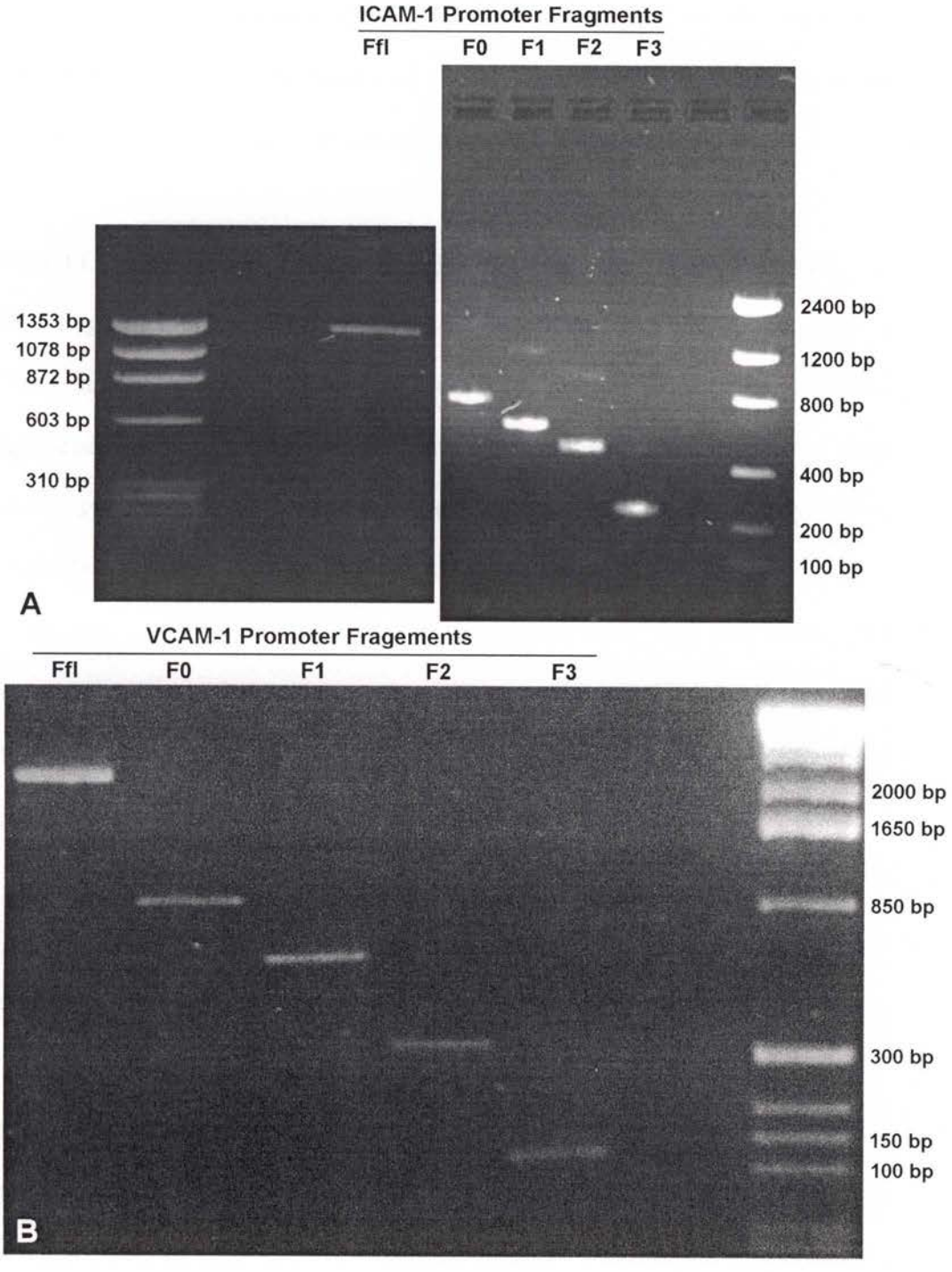


Figure 14. PCR products of (A) ICAM-1 and (B) VCAM-1 promoter fragments.

Promoter fragment design is outlined in figures 12 and 13.

ACG CGT). The anti-sense primer was the same as used for the full length VCAM-1 Ffl promoter fragment (see above). Figure 14 shows the result of the PCR reactions with the primers described in this, and the previous, section.

4.2.1.4 Construction of Promoter Fragment-Luciferase Reporter Vectors

The fragments described above were all amplified with *Taq* polymerase, a thermostable DNA polymerase which adds deoxyadenosine in a template-independent fashion to the 3'-ends of amplified fragments[240]. The pGEM-T Easy vector system (a 3018 bp plasmid from Promega Corporation, Sydney, NSW, Australia) contains 3'-thymidine overhangs at the insertion site, providing a compatible overhang for PCR products generated using *Taq* polymerase (see figure 15A). Thus, after PCR, each fragment was gel purified using a Wizard PCR Prep kit (Promega) and ligated into the multi-cloning site (MCS) of linearised pGEM-T Easy plasmids using T4 DNA ligase (Promega).

The host bacterial strain, JM109 *E.coli* High Efficiency Competent Cells (Promega) was transformed with the promoter fragment constructs. Transformation, the process by which foreign DNA (in this case the promoter-plasmid construct) is taken up by bacteria was performed using the following protocol: 50 µl of competent JM109 bacteria was transferred into 10 ml tubes (Falcon 2059) containing 2 µl of ligation reaction from the previous step. The tubes were gently flicked, then placed on ice for 20 minutes, prior to heat-shock for 45 seconds in a 42°C water bath, then immediately returned to ice. After 2 minutes, bacteria were recovered by the addition of 950 µl of room temperature LB broth, and incubation at 37°C for 90 minutes, with gentle shaking at 150 rpm.

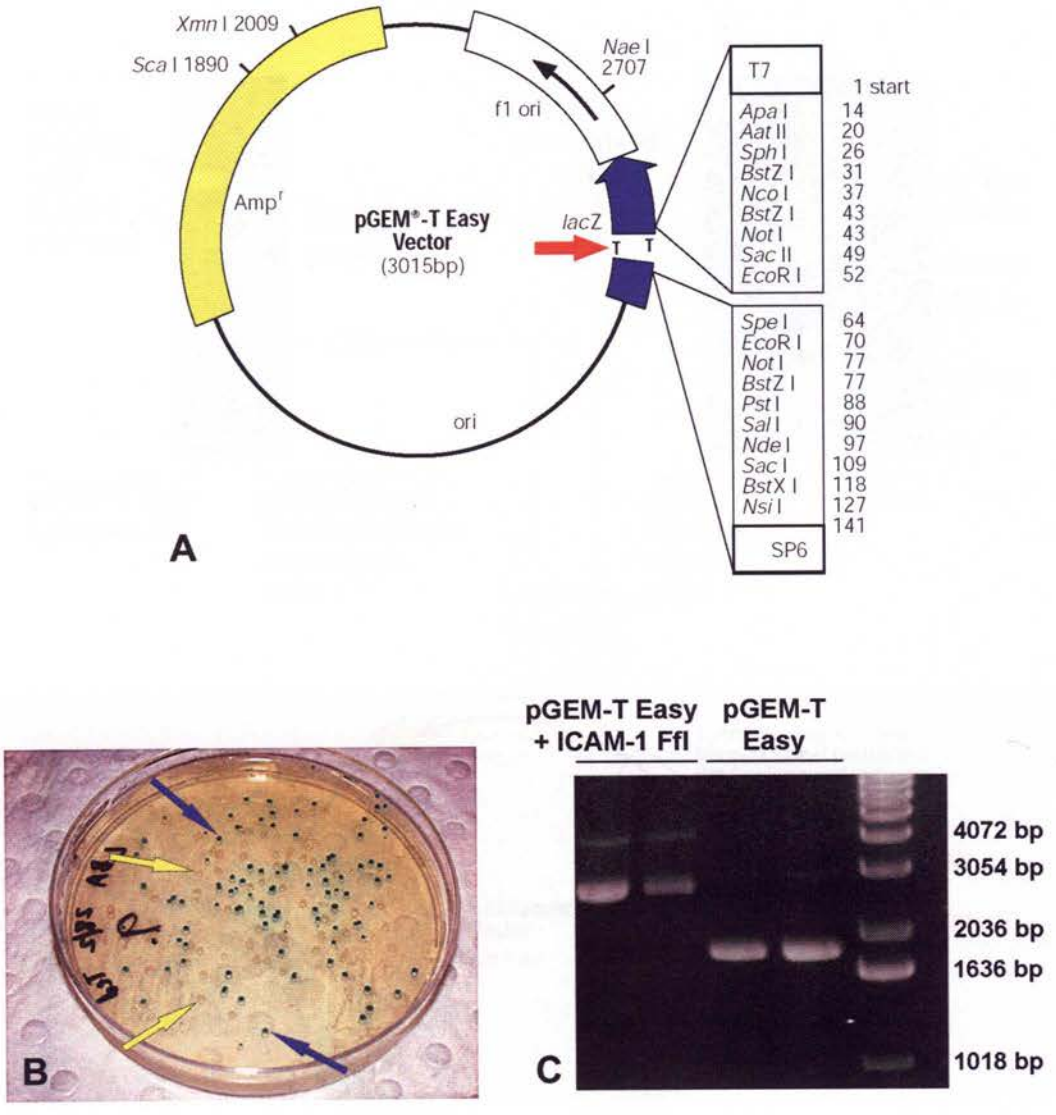


Figure 15. (A) pGEM-T Easy vector. The promoter fragments were inserted into the location indicated by the red arrow (flanked by *lacZ* gene). **(B)** Example of blue/white screening on LB-ampicillin agar supplemented with IPTG/X-Gal. Blue colonies (blue arrow) contain no inserts; intact *lacZ* gene enables colonies to produce blue colouration. White colonies (yellow arrow) contain promoter inserts, disrupting the *lacZ* gene. **(C)** Plasmids from white colonies (pGEM-T Easy+promoter fragment, in this case ICAM-1 Ffl) are retarded as compared to those from blue colonies (pGEM-T Easy only) when run on an agarose gel due to greater size. Similar gels exist for the other fragments.

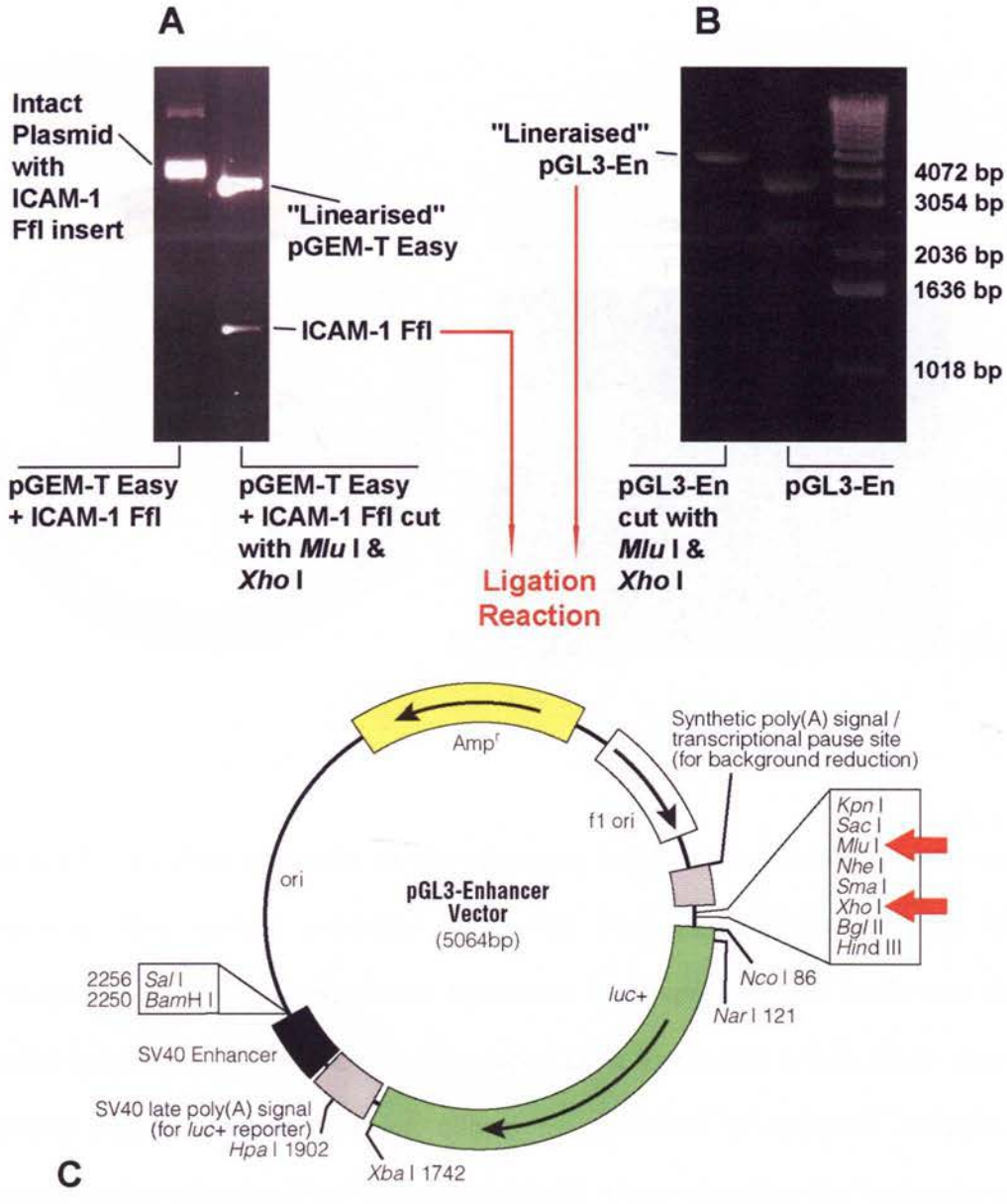


Figure 16. (A) ICAM-1 promoter fragment digested from pGEM-T Easy construct with restriction enzymes *Mlu* I and *Xho* I (restriction sites were encoded in the sense and anti-sense primers, respectively). Similar results exist for the other fragments. **(B)** pGL3-Enhancer (pGL3-En) luciferase reporter vector digested with *Mlu* I and *Xho* I. The linearised plasmid was ligated with the digested promoter fragments. **(C)** Gene map of pGL3-En. Note the positions of restriction sites in the MCS, which were digested by *Mlu* I and *Xho* I, allow directional cloning of the promoter fragments between the red arrows.

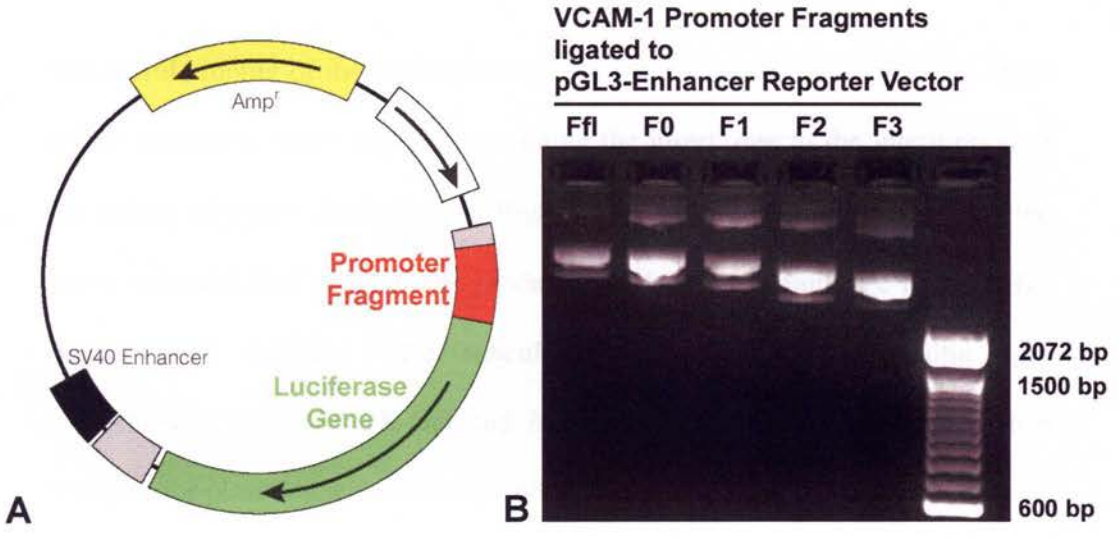


Figure 17. (A) Final structure of the promoter fragment-luciferase reporter vector constructs. The various promoter fragments (red) lies upstream of the firefly luciferase gene (green), and these modulate the expression of luciferase under different conditions, which can be measured by a luciferase assay. Note also the ampicillin resistance gene (which allows for selection of bacterial colonies on selective media) and the SV40 Enhancer (black) which amplifies the luciferase gene and increases the sensitivity of this model. **(B)** The different VCAM-1 promoter fragment-pGL3-En constructs extracted from host bacterial (JM109) colonies. These purified plasmid constructs are ready for transfection into endothelial cells. Note the retardation of the larger constructs (eg. Ffl) when run on an agarose gel. Similar gel exists for the ICAM-1 promoter fragments.

100 µl of the transformed bacteria were plated on LB-ampicillin agar plates supplemented with IPTG/X-Gal (see section 2.11) and incubated overnight.

Successful cloning of the inserts into pGEM-T Easy was assessed by blue-white colour screening: white colonies containing the insert, due to the interruption of the coding sequence (*lacZ* gene) of β-galactosidase; blue colonies containing the non-interrupted *lacZ* gene able to produce the blue colouration (see figure 15B). Single white colonies were subcultured onto fresh LB-ampicillin agar supplemented with IPTG/X-Gal and incubated overnight. Colonies were then screened for plasmids with inserts by observing their mobility through an agarose gel (see figure 15C) as well as using a PCR reaction with the appropriate ICAM-1 or VCAM-1 fragment primers.

Plasmid constructs were then extracted from the bacteria using GeneElute Plasmid Miniprep Kits (Sigma, St Louis, MO, USA) and run on an agarose gel to confirm product size. The inserts encoding the promoter fragments were excised from the pGEM-T Easy vector using the restriction enzymes *Mlu* I and *Xho* I (see figure 16A) and further gel-purified using the Wizard PCR Prep kit (Promega).

pGL3-Enhancer (5010 bp) vector (Promega) was also linearised with *Mlu* I and *Xho* I (see figure 16B), which is possible because the MCS of pGL3-Enhancer contains DNA sequences cut by these restriction enzymes (as depicted in figure 16C). The 5'-recessed ends of the linearised plasmid were dephosphorylated with 0.2 units Thermosensitive Alkaline Phosphatase (Gibco BRL, Gaithersburg, MD, USA) to prevent vector recircularisation. The purified promoter fragments

(previously cut out with the restriction enzymes) were then directionally sub-cloned into the pGL3-Enhancer (5010 bp) vector (Promega) using T4 DNA liagase (Promega), yielding promoter-luciferase reporter vector constructs, as depicted in figure 17A.

The constructs were again transformed into JM109 using the protocol as outlined above and cultured on LB-ampicillin agar plates. Single bacterial colonies were screened by PCR for the presence of promoter fragment inserts with the appropriate original PCR primers and suitable colonies recultured on fresh LB-ampicillin agar plates, as well as being stored in glycerine stocks (50% glycerine, 50% LB broth) at -80°C for future use.

The plasmids were isolated from the bacteria using the Wizard Miniprep kit (Promega) and diluted to 100-400 ng/μl in nuclease free water for nucleotide sequencing (Newcastle DNA, Newcastle, NSW, Australia). Once the sequences were confirmed, midipreps (Qiagen, Hilden, Germany) of the promoter fragment-luciferase reporter vector constructs were made, and used for transfection into endothelial cells (see section 2.7.2).

4.2.2 CELL CULTURE CONDITIONS

Conditions for the incubation of endothelial cell cultures used in these experiments have been described in section 3.2.1 and outlined in table 1. The

different conditions were used in various experiments as described in the experimental setup below.

4.2.3 EXPERIMENTAL SETUP

4.2.3.1 mRNA stability assay

ICAM-1 and VCAM-1 mRNA stability in the presence of the treatment conditions was determined by the addition of the RNA polymerase inhibitor actinomycin D (5 μg/ml) to culture wells at the same time as the test conditions were added.

HUVECs (passage 2 to 4) were seeded onto 12 well plates. The final volume of media and conditions used was 0.5 ml. The conditions used were Ctrl NG, Ctrl HG, Ur NG, Ur HG. These conditions were chosen to investigate the effects of hyperglycaemia and uraemic serum on the stability of mRNA. In these experiments, both control and uraemic serum was pooled.

Incubation was continued for 2, 4, 8, 12 and 24 hours. At time 0 hours and at each of following time points, RNA was extracted and analysed by real-time RT-PCR (see chapter 2 for further details). The ICAM-1 or VCAM-1 mRNA levels at the time of addition of actinomycin D (ie. 0 hours) was set at 100%. Each condition was done in independent triplicates and two separate experiments were performed.

4.2.3.2 Promoter assays

Cell cultures of BAECs were grown to confluence in 12 well plates and used between passages 10 and 24. The cells were transfected with the promoter pGL3-Enhancer construct of interest using Effectene reagent (Qiagen, Hilden, Germany). After a 4.5 hour incubation, the cells were washed twice with PBS and 0.5 ml of RPMI media added. After overnight incubation, treatment conditions were added. The treatment conditions were: 1) Ctrl, DM, Micro, Prot and Ur, all in normal glucose and 2) Ctrl LG, Ctrl HG, Ctrl Mann, Ur LG, Ur HG and Ur Mann. 4 hours after the addition of these conditions, the cells were lysed in passive lysis buffer and analysed for both types of luciferase activity with the Dual Luciferase Reporter System (Promega), and adjusted for internal transfection control (*Renilla* luciferase). See section 2.7 for more details. Like the mRNA experiments, the serum was not pooled (with the exception of control serum). All subjects were tested twice in independent experiments. There were ten separate experiments performed for each promoter construct.

4.2.3.3 Promoter Fragment assays

Promoter fragments were constructed using serial deletions of the Ffl sequence and inserted into the pGL3-Enhancer luciferase reporter plasmid as described in sections 4.2.1.2 and 4.2.1.3. Analysis of the response of the different fragments allow the identification of regions within the promoter region that are particularly responsive to a given condition.

4.2.3.4 NF-κB EMSA

To test the effect of the conditions on the activation of the NF-κB transcription factor, EMSA was performed. HUVECs were grown to confluence in 25 cm^2 cell culture flasks and used in passages 2-4. Final media volume for each flask was 5 ml. Incubation time was 4 hours, after which the cells were harvested by trypsinisation. Further cell processing and EMSA methodology are described in chapter 2. The conditions used were Ctrl NG, Ctrl HG, Ur NG, Ur HG. These conditions were chosen to investigate the effects of hyperglycaemia and uraemic serum on the DNA binding activity of NF-κB. The results are the analysis of four independent experiments.

4.2.4 STATISTICAL ANALYSIS

Descriptive data are expressed as mean ± SE. Groups were compared by ANOVA, with Fisher's procedure used to determine significance; p-values of <0.05 were assumed to represent statistical significance. Multiple data points for each condition (eg triplicates for stability) were condensed to one mean value and used as one data point for further analysis. Statview 5.0.1 for Windows (SAS Institute Inc, Cary, NC, USA) was used for statistical analysis.

4.3 Results

4.3.1 mRNA STABILITY

There was no significant difference in the rate of degradation of ICAM-1 mRNA between any of the treatment conditions at any of the time points. The polynomial curve-fits did not differ significantly between the treatment groups (see figure 18A). At the 4 hour point, about 78% of the original ICAM-1 mRNA was still present. By 24 hours, only 27% was left.

In a normal glucose environment, control serum and uraemic serum had similar effects on the rate of VCAM-1 mRNA degradation at all times points examined. However, at the 12 hour and 24 hour points, the cells exposed to high glucose conditions exhibited significantly decreased mRNA levels as compared to either of the normal glucose conditions (see figure 18B). For the 24 hour time point, the normal glucose conditions had 28% of VCAM-1 mRNA still present, whereas in the high glucose conditions, there was only 15% left. Within the high glucose conditions, there was again no difference between the control serum and uraemia groups.

Thus, while ICAM-1 mRNA stability is not affected by either glucose or uraemic serum, VCAM-1 mRNA exhibits decreased stability in a high glucose environment.

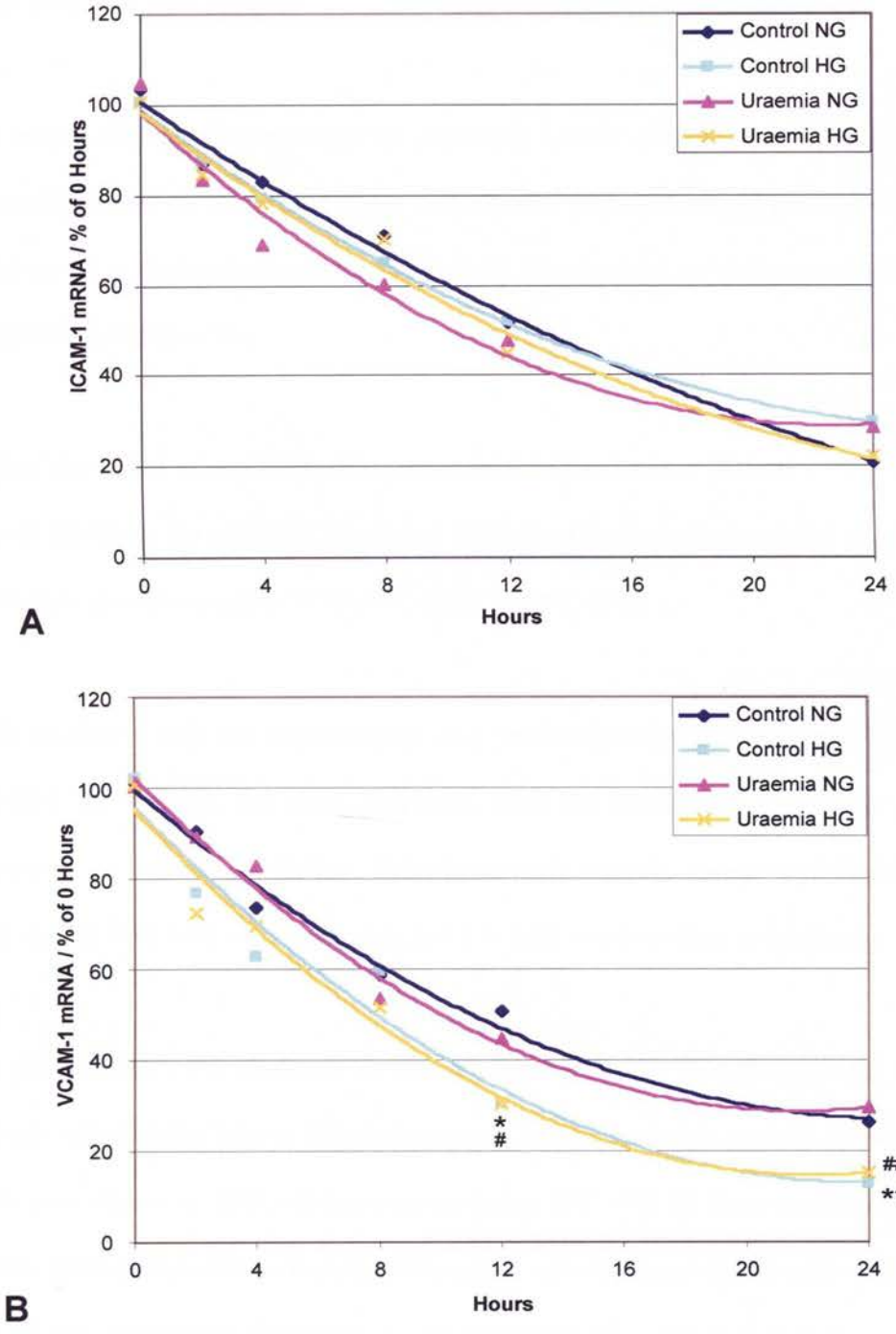


Figure 18. Determination of **(A)** ICAM-1 and **(B)** VCAM-1 mRNA stability after the concomitant addition of the RNA polymerase II inhibitor, Actinomycin D and the glucose/uraemia condition. *p<0.05 vs Ctrl NG 12 hours, #p<0.05 vs Ur NG 12 hours, **p<0.05 vs Ctrl NG 24 hours, ##p<0.05 vs Ur NG 24 hours.

The results for these experiments are expressed as fold difference over baseline for firefly luciferase reporter activity, and adjusted for *renilla* luciferase activity, which acted as the internal transfection control. These results are a summary of 10 independent experiments.

4.3.2.1 The effect of nephropathic serum on CAM promoter activity

Figure 19 shows the effect of increasing degrees of diabetic nephropathy on the full length promoter regions (Ffl) of ICAM-1 and VCAM-1.

Both diabetic (with no nephropathy) and microalbuminuric serum increased ICAM-1 Ffl activity, but these increases were not statistically significant as compared to control conditions. Proteinuria and uraemic serum significantly increased ICAM-1 Ffl activity by 2.0- and 2.1- fold over baseline, respectively.

For the VCAM-1 Ffl promoter construct, there was only a slight increase in activity with diabetic serum. Microalbuminuric serum increased activity by 30%, proteinuric serum by 51% and uraemic serum by 70% with all three results being significantly different from baseline. The effect of uraemic serum also showed a statistically significant difference as compared to the effect of diabetic and microalbuminuric serum.

While there was a general trend towards increasing activity with progression of renal disease for both full-length fragments, the trend was stronger for VCAM-1

Ffl and ICAM-1 Ffl. However, it should also be noted that the magnitude of the increase was greater for ICAM-1 Ffl than VCAM-1 Ffl (as can be seen in figure 19).

4.3.2.2 The combined effects of glucose and uraemia on CAM promoter activity

Glucose increased ICAM-1 Ffl activity by 48% over baseline, as seen in figure 20A. Like the expression data, uraemia has more of an effect than glucose alone: uraemia induced promoter activity by 110% increase (as described in section 4.3.2.1). The combination of uraemia and high glucose raised ICAM-1 Ffl further, to 121% over control normal glucose. All of these three results were significantly different to control normal glucose. Both the uraemic conditions were also statistically higher than control high glucose.

Figure 20B shows that the pattern of glucose response for VCAM-1 Ffl matches that of ICAM-1 Ffl. High glucose is significantly more than normal glucose (12%), uraemia higher again (70%) and uraemia high glucose showing the greatest increase (86%). Like the nephropathic serum data, the magnitude of this difference was larger for ICAM-1 Ffl than VCAM-1 Ffl. Not shown in the graphs is the fact that the mannitol in control serum increases VCAM-1 Ffl activity by 10% (slightly less than high glucose, not significantly different to normal glucose); and uraemia with mannitol increased activity by 23% (less than uraemia normal glucose).

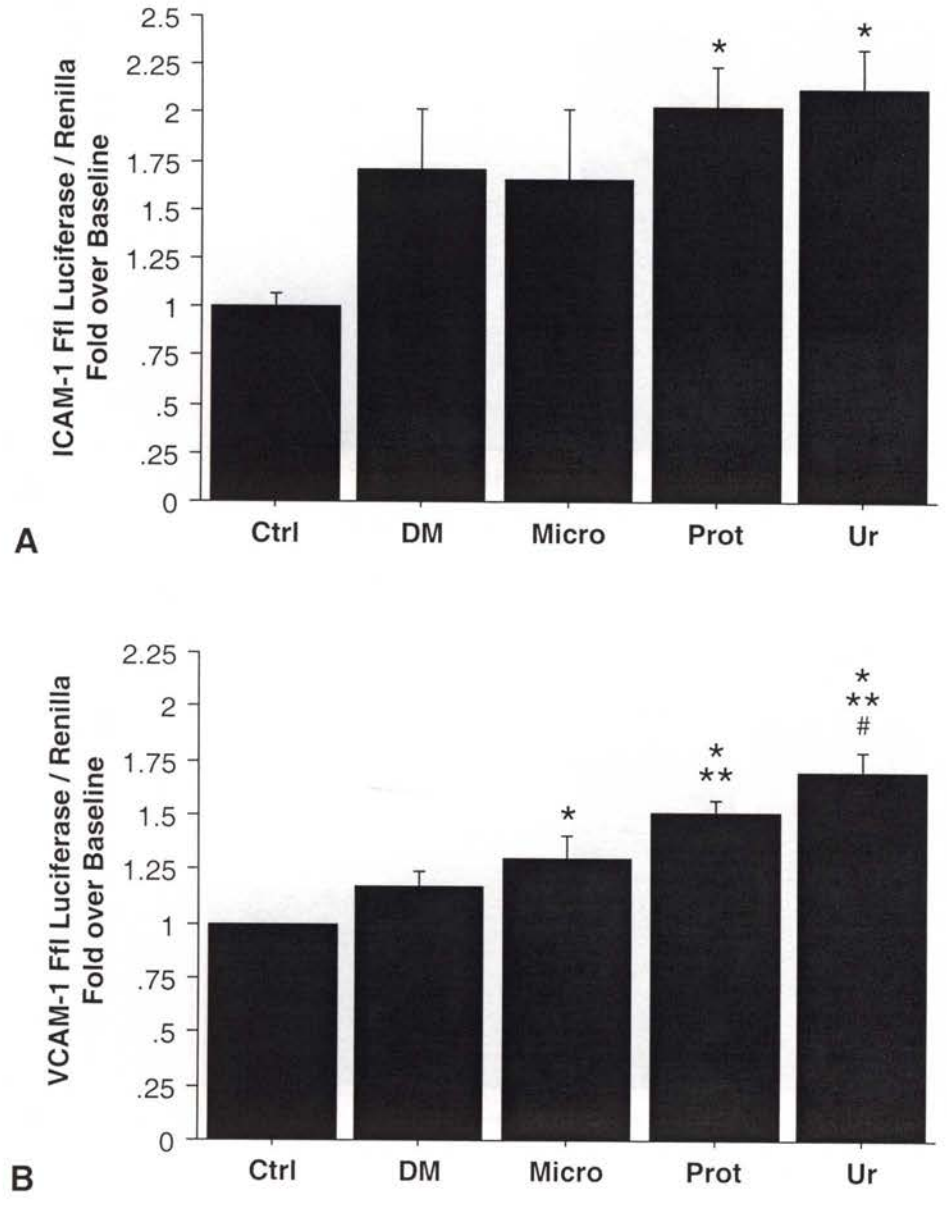


Figure 19. The effects of various degrees of nephropathic serum on luciferase activity for the "full-length" promoter/luciferase reporter vector constructs for the **(A)** ICAM-1 promoter and **(B)** VCAM-1 promoter. Bars represent means ±SE, adjusted for renilla (transfection control) expression. *p<0.05 vs Ctrl, **p<0.05 vs DM, #p<0.05 vs Micro.

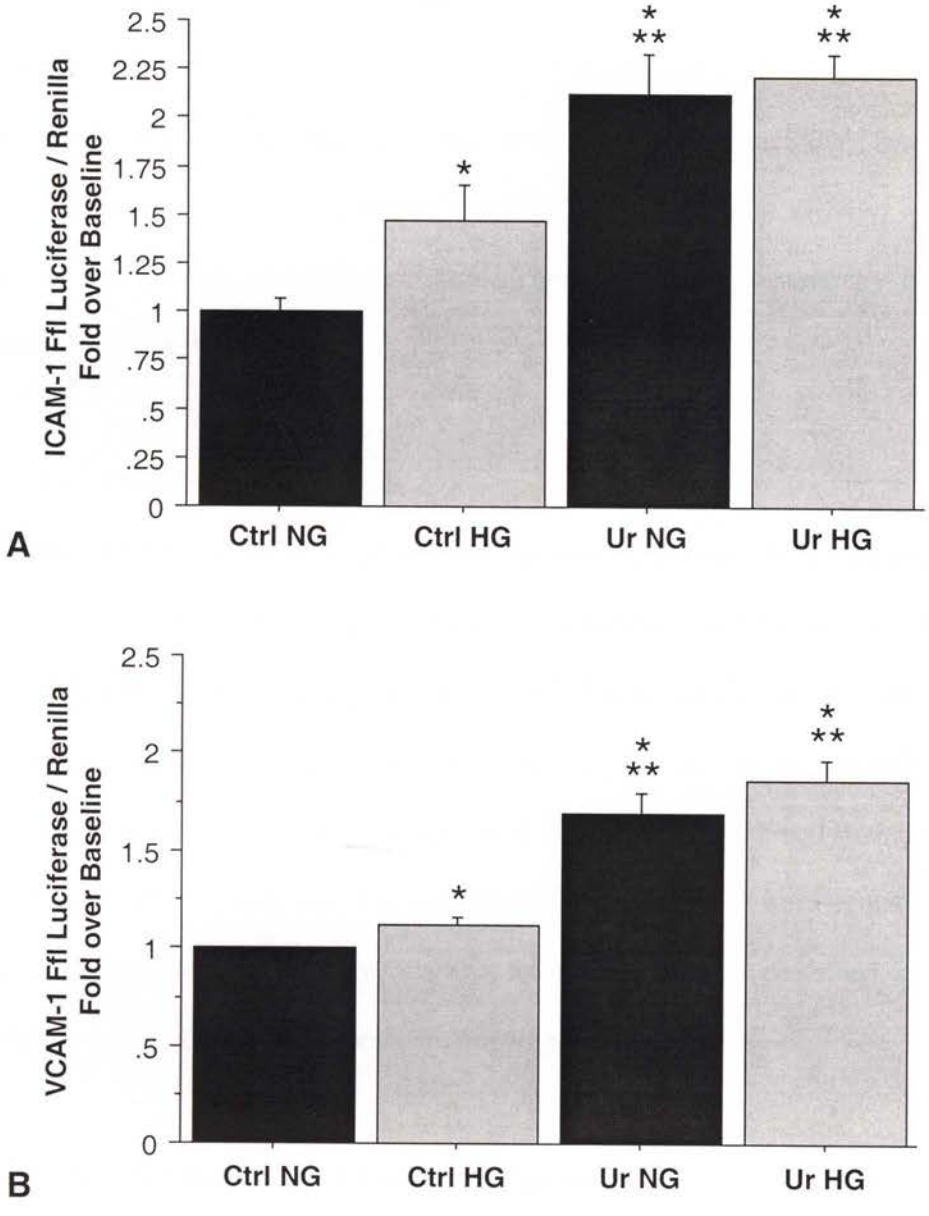


Figure 20. The combined effects of high glucose and uraemic serum on luciferase activity for the "full-length" promoter/luciferase reporter vector constructs (Ffl) for the **(A)** ICAM-1 promoter and **(B)** VCAM-1 promoter. Bars represent means ±SE, adjusted for renilla (transfection control) expression. *p<0.05 vs Ctrl NG, **p<0.05 vs Ctrl HG.

The results for these experiments are expressed as fold difference over baseline for luciferase reporter activity, and adjusted for *renilla* luciferase activity, which acted as the internal transfection control. These results are a summary of 10 independent experiments.

4.3.3.1 ICAM-1 fragment analysis

After the ICAM-1 promoter fragment-reporter vector plasmids were constructed as described in the methods, transfection into endothelial cells failed to produce any luciferase response for any of the ICAM-1 fragments. *Renilla* activity was present as expected, showing that this was not a failure of transfection. Transfection was repeated by an independent investigator not directly related to this project and nil response was confirmed. The constructs were remade by another independent investigator, starting from the first step described in the methods section. These experiments are ongoing.

4.3.3.2 VCAM-1 fragments and nephropathic serum

In contrast to the ICAM-1 fragments, the VCAM-1 promoter fragment constructs showed good luciferase activity. Figure 21 depicts the response of the various VCAM-1 promoter fragment-luciferase reporter vector constructs to the serum of increasing degrees of diabetic nephropathy. Each graph in this figure has an accompanying diagram which shows the size and binding sites present for each fragment. Although there is a lot of data presented in this figure, the most important aspects can be summarised by the following points:

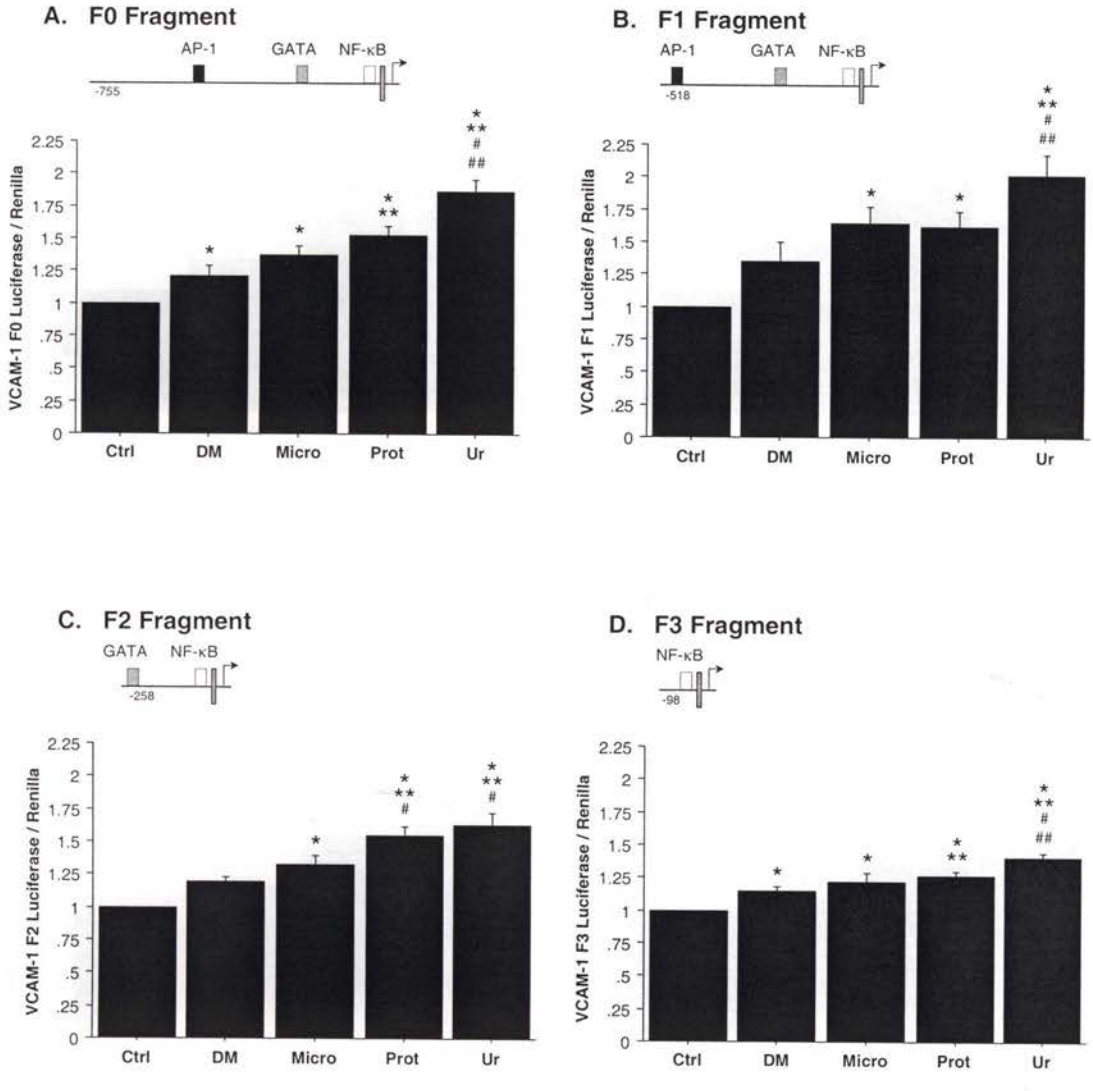


Figure 21. The effects of various degrees of nephropathic serum on luciferase activity for the VCAM-1 promoter fragment/luciferase constructs **(A)** F0 Fragment **(B)** F1 Fragment **(C)** F2 Fragment **(D)** F3 Fragment. Diagrammatic representations of each fragment show nuclear binding sites and size of fragment. Bars represent means ±SE, adjusted for renilla (transfection control) expression. *p<0.05 vs Ctrl, **p<0.05 vs DM, #p<0.05 vs Micro, ## p<0.05 vs Prot.

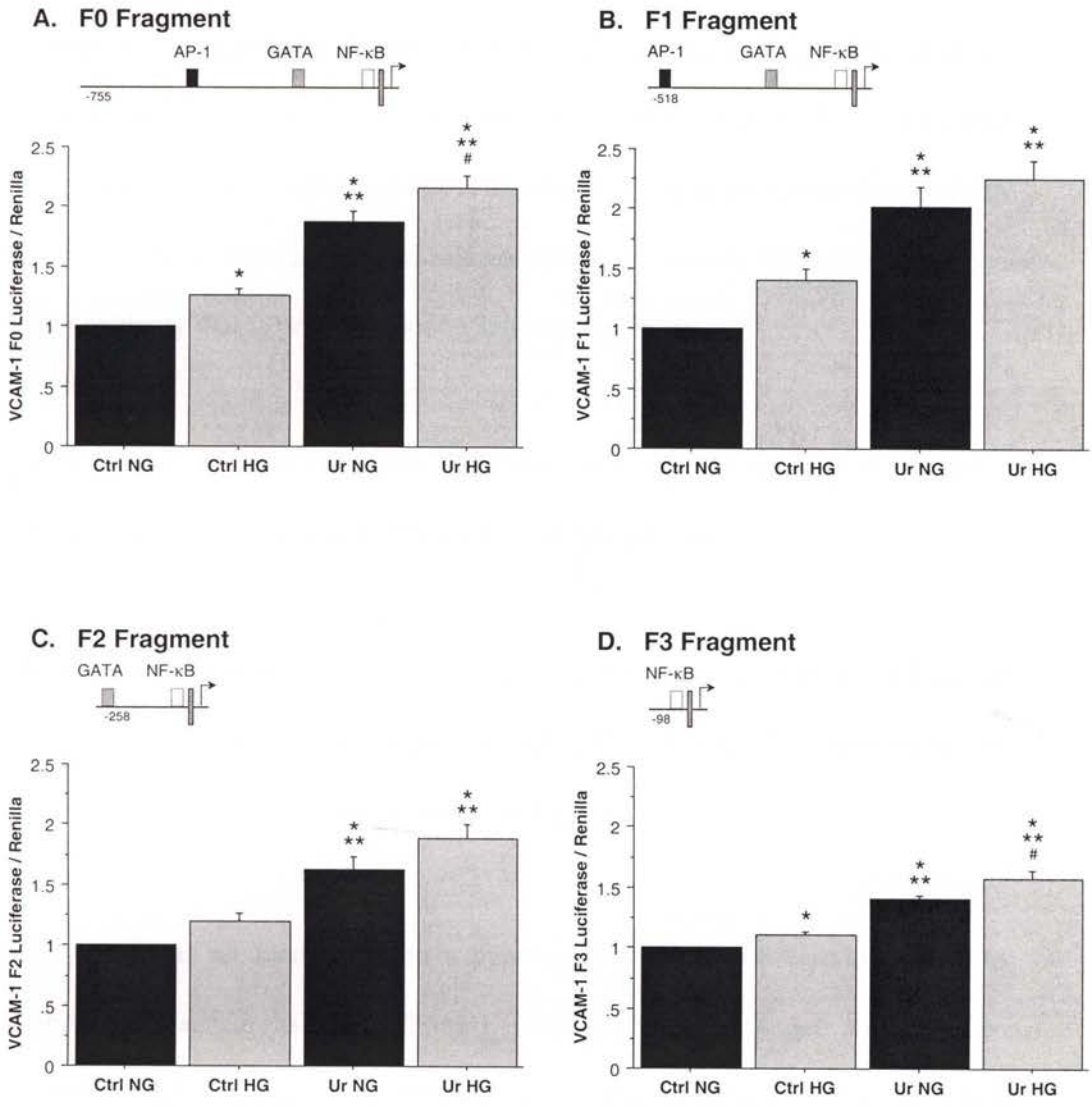


Figure 22. The combined effects of high glucose and uraemic serum on luciferase activity for the VCAM-1 promoter fragment/luciferase constructs (A) F0 Fragment (B) F1 Fragment (C) F2 Fragment (D) F3 Fragment. Diagrammatic representations of each fragment show nuclear binding sites and size of fragment. Bars represent means ±SE, adjusted for renilla (transfection control) expression. *p<0.05 vs Ctrl NG, **p<0.05 vs Ctrl HG, #p<0.05 vs Ur NG.

Firstly, each of the fragments behaved like the VCAM-1 Ffl fragment in that there is generally increasing luciferase activity with increasing degree of diabetic nephropathy. This can be easily seen when the graphs in figure 21 are compared to Figure 19B. The exception to this is that for the F1 fragment, proteinuric serum has a slightly smaller effect than microalbuminuric serum; however, this was a minor anomaly that is probably due to experimental error.

Secondly, the increased promoter activity seen in any nephropathic serum, especially uraemic serum, is highly statistically significant.

Thirdly, the pattern of the stepwise increases in promoter activity, as well as the statistical significance, extends right through to the VCAM-1 F3 fragment which only contains the NF-κB nuclear factor binding site.

Lastly, there is an increase, then a general decrease in the responsiveness to nephropathic serum as the VCAM-1 promoter fragments get smaller. The fragment which showed the greatest increase in responsiveness to uraemic serum was the F1 fragment (102% increase in activity with uraemic serum), closely followed by the F0 fragment (87% rise). Both of these were greater than the Ffl fragment, which, as described in section 4.3.2.1, increased activity by 70% in uraemic serum. The fragment with the least response was the F3 fragment with which uraemic serum enhanced promoter activity by 41% over baseline. This data is represented graphically in figure 23 (dark bars). As can be ascertained from this diagram, the F1 fragment was statistically significantly more responsive to uraemic serum than all fragments other than the F0 fragment.

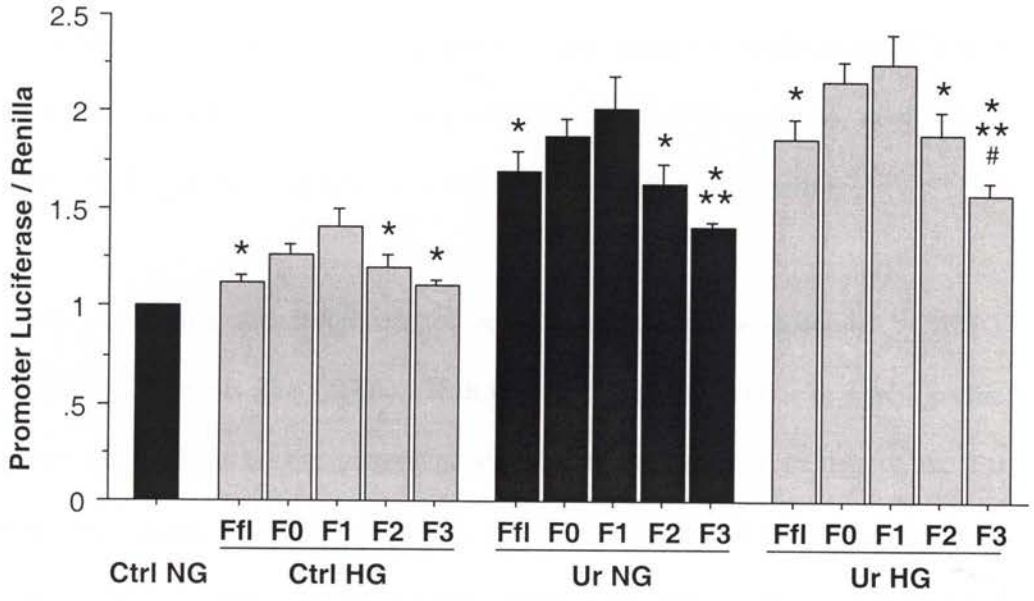


Figure 23. Graph showing the relative magnitude of promoter stimulation for each VCAM-1 fragment construct in any given glucose condition. *p<0.05 vs F1 fragment (within same glucose condition), **p<0.05 vs F0 fragment (within same glucose condition), #p<0.05 vs F2 (within same glucose condition).

4.3.3.3 VCAM-1 fragments and glucose combined with uraemia

High glucose on its own significantly increases activity of all VCAM-1 promoter fragments. The combination of uraemia and high glucose stimulated the VCAM-1 promoter fragments more than either uraemia or high glucose alone, as shown in figure 22. For the F1 fragment, this increase was 126% over baseline.

When comparing the magnitude of response between the different VCAM-1 promoter fragments, the situation with high glucose was similar to nephropathic serum alone. That is: the pattern of stimulation was similar to that of the Ffl fragment (figure 20B); the increased promoter activity was highly statistically significant; the pattern of responsiveness extends through to the F3 fragment; and the F1 fragment exhibited the greatest increase to glucose (± uraemia), as seen in figure 23.

4.3.4 NF-κB EMSA

In cells exposed to high glucose, NF-κB activity was increased by 15% above that in normal glucose conditions ($p<0.01$). Uraemia alone raised NF-κB by 18%. Uraemia in combination with high glucose enhances NF-κB the most (32% over baseline), a result that is statistically significantly different to all other conditions. These results are plotted in figure 25. A representative EMSA gel is shown in figure 24. This gel clearly demonstrates increased NF-κB DNA binding in glucose and uraemic conditions.

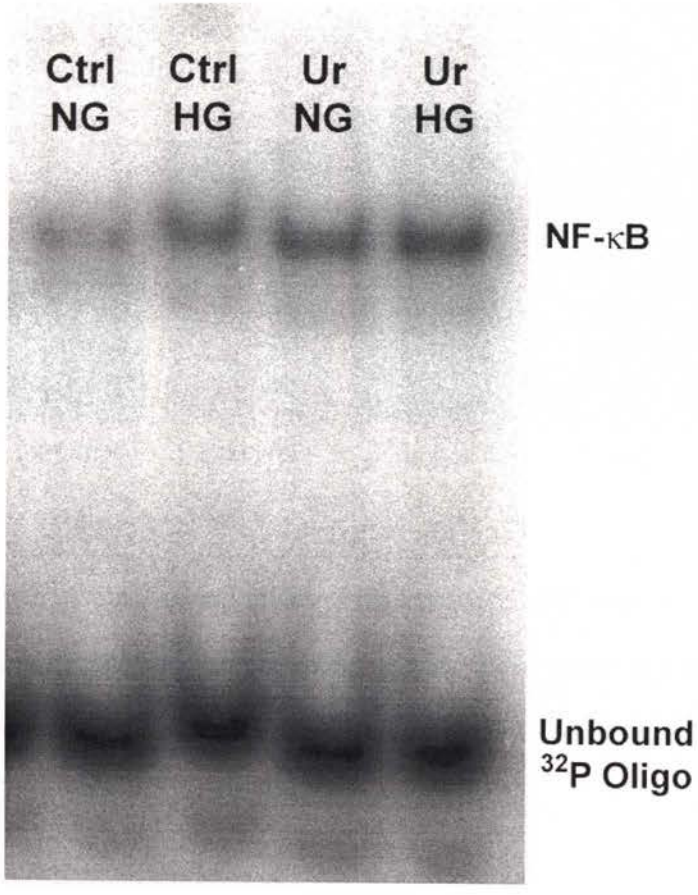


Figure 24. Gel of a typical NF-κB EMSA experiment. Increasing density of NF-κB bands show evidence of increasing NF-κB DNA binding activity in endothelial cells exposed to hyperglycaemia and uraemic serum. The combination of the two conditions stimulates NF-κB DNA binding activity than either one alone. Four experiments are summarised in figure 25.

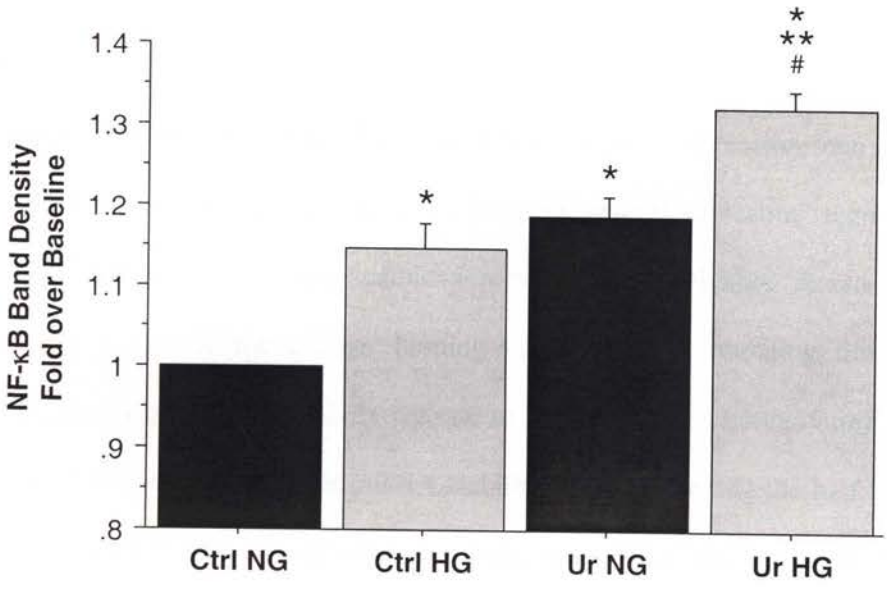


Figure 25. Summary of the combined effect of high glucose and uraemic serum on NF-κB band densitometry as measured by EMSA. Bars represent means ±SE, adjusted for background. *p<0.05 vs Ctrl NG, **p<0.05 vs Ctrl HG, #p<0.05 vs Ur NG.

4.4 Discussion

The increased expression of ICAM-1 and VCAM-1 gene expression seen in the experiments in chapter 3 may have its basis in several molecular regulatory mechanisms. Glucose or nephropathic serum could conceivably increase the expression of one of the nuclear binding factors (thus stimulating promoter activity), increase promoter activity for one of the genes (thus upregulating gene expression) or else may increase mRNA stability (thus prolonging the half-life of a gene's mRNA). Any, or all, of these could account for the increased CAM expression that was seen in the previous chapter.

The stability experiments described in this chapter show that the regulation of the CAMs by glucose or uraemia does not occur at the mRNA stability level. Uraemia has no effect on the half-life of either ICAM-1 or VCAM-1 mRNA. While hyperglycaemia does not affect ICAM-1 mRNA stability, VCAM-1 mRNA half-life is actually slightly shortened in a high glucose environment. The implication is that there is no post-transcriptional regulation of ICAM-1, and the only modification of VCAM-1 mRNA is a downward regulation in hyperglycaemia. Therefore, the increased CAM mRNA seen in high glucose/uraemic conditions must be due to regulatory mechanisms at the transcriptional level; indeed, for VCAM-1, these mechanisms must overcome the decreased stability of VCAM-1 mRNA in high glucose. The mechanism of transcriptional regulation is most likely to be found in the promoter regions of the genes of interest.

When the promoter regions were examined, the "full-length" promoters of both ICAM-1 and VCAM-1 were stimulated by increasing degrees of nephropathic serum, glucose and the combination of uraemic serum and hyperglycaemia. The trends of response (stepwise increase with degree of nephropathy, smaller increases with glucose and large increase with uraemia, largest effect with uraemia/high glucose) mirror that of the mRNA and protein results in chapter 3. In addition, glucose again seemed to be acting partly via an osmotic mechanism when used alone, as discussed in chapter 3. Taken together, the Ffl promoter assays indicate that the regulation of mRNA (and subsequent protein) levels is occurring at the promoter level for both ICAM-1 and VCAM-1.

Deletion of segments of the promoter fragments were then able to show the nuclear binding site within the VCAM-1 promoter region that was involved in the regulation. Because the pattern of both glucose and nephropathic responsiveness stayed constant out to the VCAM-1 F3 promoter fragment, this indicated that the NF-κB nuclear binding site must be the area within the promoter region that is central to the VCAM-1 gene's upregulation by glucose and uraemic serum.

This finding was confirmed by the EMSA studies which demonstrated a highly significant increase in NF-κB activity in high glucose, uraemia and especially uraemia with hyperglycaemia. Both the ICAM-1 and VCAM-1 Ffl promoter regions contain multiple NF-κB binding sites, and increased activity of NF-κB in hyperglycaemia and uraemic serum undoubtedly leads to stimulation of both promoter regions.

Further analysis of the promoter fragment and EMSA data does yield another interesting observation. Although the VCAM-1 F3 fragment is still responsive to glucose and uraemia, the maximum effect was seen with the F0 and F1 fragments. Not coincidentally, these are the VCAM-1 promoter fragments that have all three nuclear binding sites (AP-1, GATA and NF-κB) but without the powerful silencing region that has previously been described between -755 and -2167 bp[69]. This would imply that while NF-κB is the central nuclear factor involved in glucose/uraemic responsiveness, GATA and AP-1 must also play a role in general VCAM-1 transcription by acting in combination with NF-κB. This is supported by the fact that GATA is primarily known to act by interacting with NF-κB to help generate full VCAM-1 expression rather than acting by itself[69].

4.4.1 CORRELATION WITH CURRENT LITERATURE

While the majority of the data in this chapter represents novel findings, there are some results that can be compared with previously published studies. Primarily, the work presented here confirms the observation that hyperglycaemia increases the activation of NF-κB, a finding that has previously been demonstrated in human endothelial cells[76], vascular smooth muscle cells[77] and rat mesangial cells[176].

No other groups have studied the effect of diabetic nephropathy on NF-κB activation. There was one study showing increased NF-κB expression in the glomerular endothelium of patients with IgA nephropathy[78]. The investigation of

the combined effects of diabetic nephropathy and hyperglycaemia, the use of the promoter fragments to analyse these effects and the mRNA stability data are all novel and cannot be correlated with previous publications.

4.4.2 CLINICAL IMPLICATIONS

These results highlight the fact that changes present in the blood stream of diabetic nephropathy results in a cascade of intracellular signalling that lead to an increase in CAM expression. Moreover, the regulatory mechanisms studied in this chapter were induced by both high glucose and uraemic serum within four hours; this indicates that even the short term hyperglycaemia that is commonly experienced in diabetic patients (either with or without nephropathy) has a profound impact on the eventual development of atherosclerosis.

The elucidation of the underlying molecular regulatory mechanisms responsible for the upregulation of CAMs in diabetic nephropathy may also lead to new targets for therapy in the prevention of macrovascular disease.

Our VCAM-1 promoter fragment work also confirms the importance of the silencing region of the VCAM-1 promoter region in suppressing VCAM-1 gene expression *in vivo*[69].

4.4.3 STUDY LIMITATIONS

The most obvious limitation of these studies is the fact that the ICAM-1 promoter fragment constructs failed to show any response. It is, however, important to note that the ICAM-1 F0 fragment deletes an enhancing region that has previously been described at position -1352[54]; and because the ICAM-1 promoter fragments were designed using a series of nested deletions, all subsequent smaller fragments also lack this enhancing region. So while this result could represent a failure of experimental technique, it could also indicate that the ICAM-1 promoter becomes inactive and unresponsive once the enhancing region is deleted. Another technique that could be used to circumvent this problem would be to use site directed mutagenesis, with the aim of knocking out specific nuclear binding sites within the promoter region rather than just deleting sections.

Another possible concern is the use of BAECs for the promoter experiments, rather than the HUVECs used in the expression, stability and EMSA studies. There were many attempts to transfect HUVECs using a number of techniques including non-liposomal electroporation, a number of different transfection reagents and calcium phosphate transfection. However, no reliable results could be obtained from these transfections for HUVECs. The higher transfection efficiency of BAECs has also been noted by other authors[236]. The use of BAECs can be justified on the grounds that the promoter experiments are studying the effect of glucose and nephropathic serum on the promoter constructs themselves, and the transfected cells are merely acting as cellular vessels for the promoter-plasmid constructs. In addition, BAECs are still endothelial cells and thus would

contain similar intracellular machinery to HUVECs. The very similar patterns of response to glucose/uraemia in the promoter studies versus the HUVEC expression experiments support this notion.

The stability and EMSA studies focused on uraemia versus control serum only (± hyperglycaemia) because uraemic serum increased expression more than any other degree of nephropathy. While an exhaustive investigation of regulation could also have included stability and EMSAs for the intermediate severities of renal disease, this was thought to be unnecessary because the expression results clearly demonstrated that endothelial CAM response for diabetes alone, microalbuminuria and proteinuria always fell between control and uraemia. It was therefore postulated that NF-κB activity and mRNA stability for the other types of renal disease would fall somewhere between the response to control serum and uraemic serum. This hypothesis is supported by the promoter data in this chapter.

Lastly, although the experiments in section 4.3.3 and 4.3.4 clearly show that glucose and uraemic serum is acting via NF-κB, further work could be performed to examine the signalling pathways by which NF-κB is activated. This could include examination of NF-κB's cytoplasmic regulation by IκB, and the use of specific NF-κB inhibitors, dominant negative transfection or new siRNA technology.

4.5 Conclusion

The regulation of ICAM-1 and VCAM-1 in high glucose and nephrotic conditions occurs primarily at the level of the promoter regions of each gene. NF-κB is the principle nuclear binding factor involved, though there is evidence of support from other factors such as AP-1 and GATA. There is no post-transcriptional control of ICAM-1 mRNA, but there is some decrease in VCAM-1 mRNA stability in hyperglycaemia. This effect was relatively small, and was overcome by the increased activation of NF-κB and the VCAM-1 promoter region.

Chapter 5

Correlations between Clinical and Laboratory measures

5.1 Introduction

The methods described in chapters 3 and 4 used the serum from subjects with diabetic nephropathy to modify the expression of CAMs in cell culture. While this has been used by some other investigators to examine CAM expression in diabetes and nephropathy, these papers are in the minority and are far outnumbered by the studies that measure only soluble CAMs. Those studies that did use primarily *in vitro* methods to estimate CAM expression did not correlate their findings with sCAM concentrations. This brings into question the direct applicability of these laboratory based experiments to the clinical situation. In addition, increases in sCAMs in end-stage nephropathy may reflect not only synthesis of CAMs by endothelial cells, but also markedly decreased excretion in late renal failure; this raises questions about the relevance of sCAM studies in the uraemic environment.

This chapter attempts to correlate the physiological regulation of CAMs from chapters 3 and 4 with the more commonly used sICAM-1 and sVCAM-1 measurements. Comparisons will also be made to the baseline clinical markers of the study subjects to test the clinical relevance of CAM expression in the detection of established atherosclerotic risk factors.

5.2 Methods

5.2.1 SOLUBLE CELL ADHESION MOLECULE ASSAYS

The serum of each study subject was assayed for sICAM-1 and sVCAM-1 using commercially available kits from R&D Systems. These are ELISA based assays and the protocol used is described further in section 2.9. Each serum sample was tested in duplicate.

5.2.2 STATISTICAL ANALYSIS

The sCAM assay results were expressed as mean for each group ± SE. Groups were compared by ANOVA with Fisher's procedure used to determine significance; p-values of <0.05 were assumed to represent statistical significance.

The sCAM data was then taken and compared to the CAM expression data from chapter 3. sICAM-1 was correlated with the ICAM-1 mRNA and ICAM-1 ELISA results for individual subjects, and the same tests undertaken for VCAM-1. Comparisons were performed using simple regression analysis and statistical significance taken to be p-values of <0.05.

CAM expression data (sCAM, mRNA and ELISA) was further correlated with the clinical measures that were taken at baseline for each individual subject. For the continuous variables (age, HbA_{1c}, spot urine albumin, serum creatinine, duration

of diabetes), simple regression analysis was again used. For the known CV disease at entry into study (a nominal variable), the CAM expression was grouped according to the presence of CV disease and then compared by ANOVA; significance was tested by Fisher's procedure and significance set at $p<0.05$. Statview 5.0.1 for Windows (SAS Institute Inc, Cary, NC, USA) was used for statistical analysis.

5.3 Results

5.3.1 sICAM-1 AND sVCAM-1 ASSAYS

In general, the greater the degree of diabetic kidney disease, the greater the level of soluble adhesion molecule concentration in the serum of subjects (see figure 26). The results for sVCAM-1 show a strong stepwise increment with severity of nephropathy (figure 26B). While proteinuric subjects were significantly different from control subjects, uraemic subjects had sVCAM-1 levels which were much higher than – and also statistically different to – any other group, including proteinuria. On average, uraemic subjects had sVCAM-1 levels exactly 2-fold greater than those of control subjects.

sICAM-1 levels demonstrated a more gentle rise with progressive nephropathy (figure 26A). The microalbuminuric, proteinuric and uraemic groups were all significantly different from the control group; in addition, the uraemic and

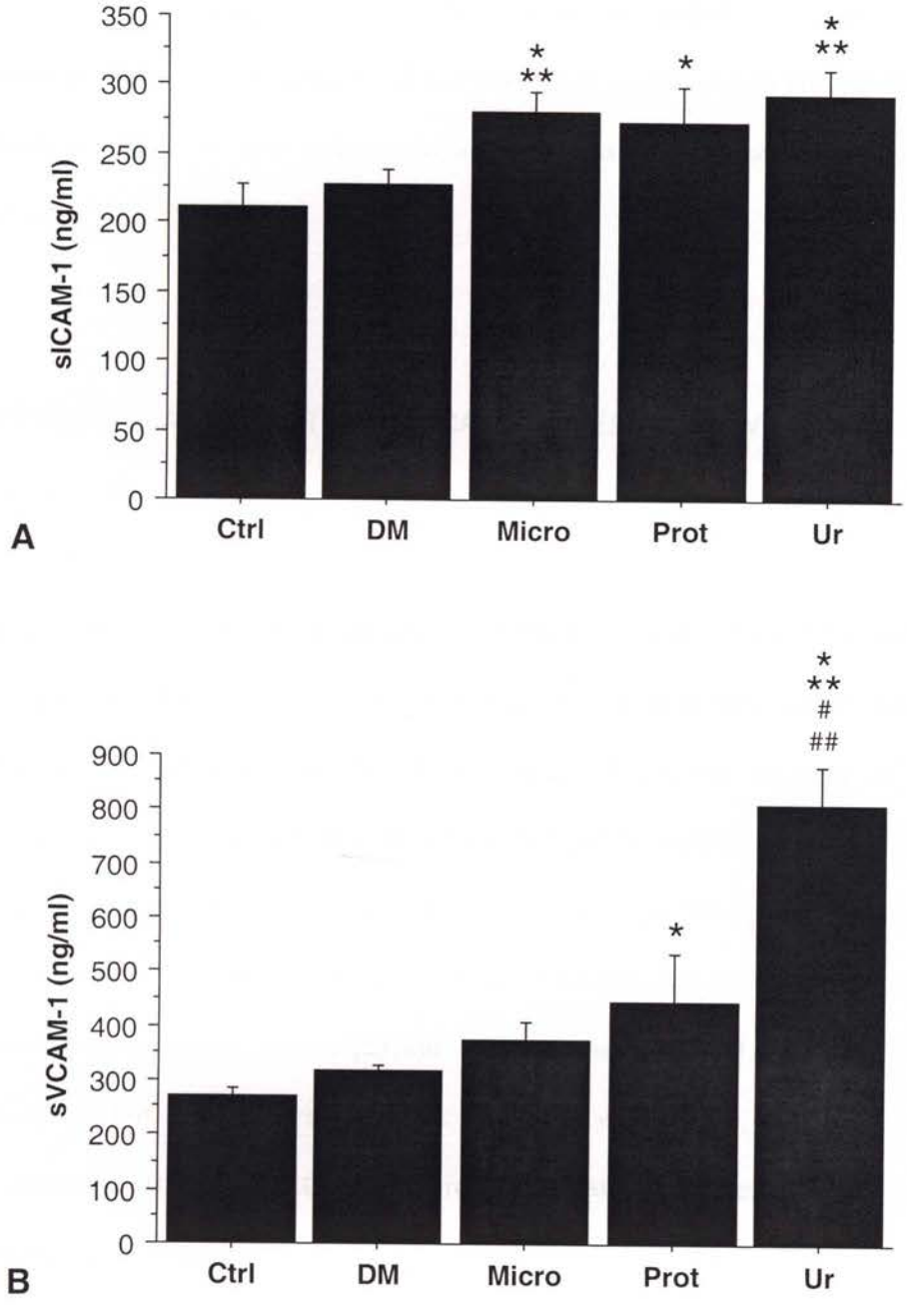


Figure 26. Soluble CAM assays for (A) sICAM-1 (B) sVCAM-1, stratified by nephropathic status. The serum measured was the same as that used to incubate the endothelial cells for the previous experiments. p for increasing trend (A) 0.0241 (B) 0.0457. Bars represent means ±SE. *p<0.05 vs Ctrl, **p<0.05 vs DM, #p<0.05 vs Micro, ## p<0.05 vs Prot

microalbuminuric groups were also different to the diabetic (without kidney disease) group. Uraemic subjects did not show such a great rise in sICAM-1 over baseline or indeed the other groups: overall, there was a 39% increase over control subjects.

5.3.2 CORRELATION BETWEEN sCAMS LEVELS AND *IN VITRO* MEASURES

Simple regression analysis of the data from chapter 3 and section 5.3.1 yielded significant correlations between the level of ICAM-1 protein measured by ELISA and sICAM-1 measured from the subjects' serum. There was an even stronger relationship between the real-time PCR VCAM-1 mRNA results and the sVCAM-1 estimations (table 3). Protein and mRNA results were positively correlated in all other instances with their respective soluble measures, but at lesser degrees of statistical significance. A comparison between the sICAM-1 and sVCAM-1 readings within individual subjects in the study yielded the most significant correlation ($r = 0.662$, $p = 0.0003$). Figure 27 illustrates the regression analyses of the significant results.

5.3.3 CORRELATION WITH OTHER CLINICAL MEASURES

Several of the clinical attributes that were measured at the start of the study were compared to the results that were obtained in chapter 3 and section 5.3.1. HbA_{1c}

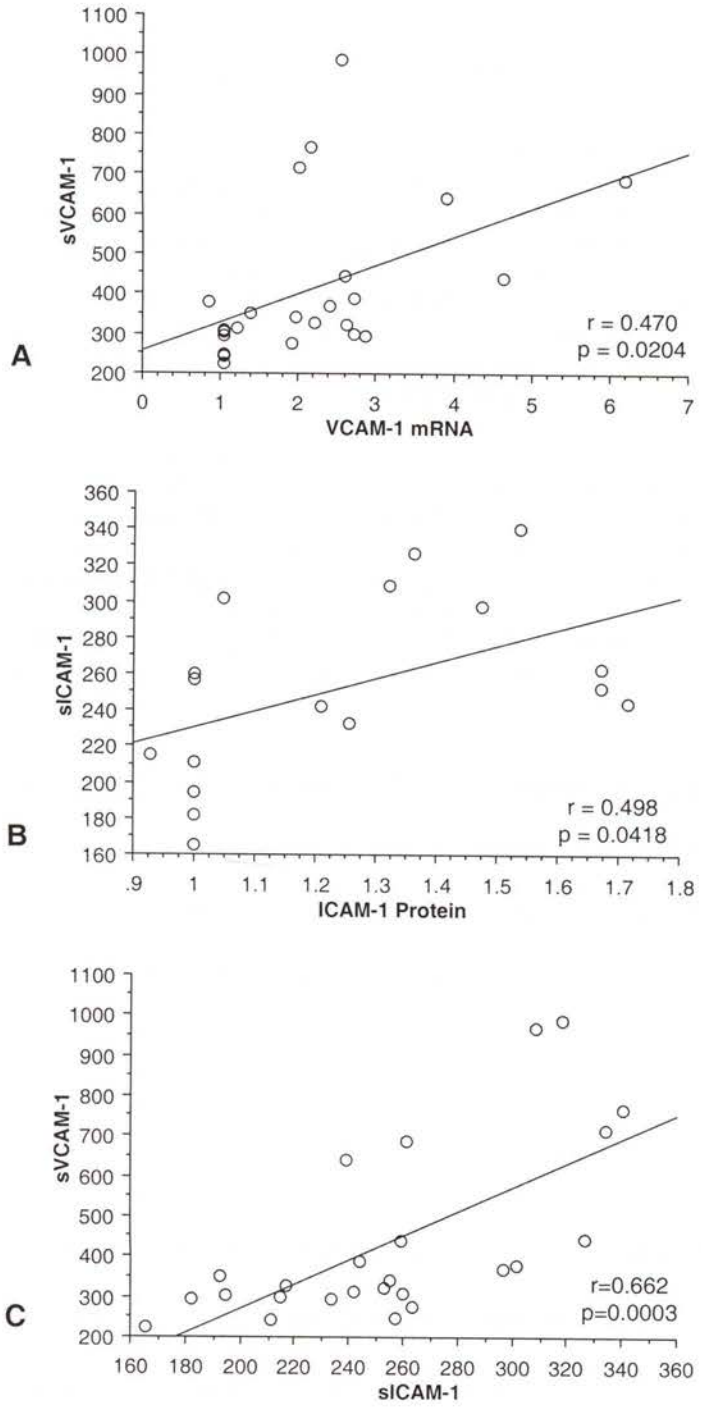


Figure 27. Regression plots of the significant findings summarised in Table 3. **(A)** sVCAM-1 vs VCAM-1 mRNA. **(B)** sICAM-1 vs ICAM-1 protein ELISA. **(C)** sVCAM-1 vs sICAM-1.

	sICAM-1	
	r	p
ICAM-1 mRNA	0.389	0.0603
ICAM-1 ELISA	0.498	0.0418 *
sVCAM-1	0.662	0.0003 *

	sVCAM-1	
	r	p
VCAM-1 mRNA	0.47	0.0204 *
VCAM-1 ELISA	0.325	0.2026
sICAM-1	0.662	0.0003 *

Table 3. Summary of simple regression analyses between sICAM-1 (top) and sVCAM-1 (bottom) and their respective in vitro measures for individual subjects in the study group. Note also the close relationship between sICAM-1 and sVCAM-1 within individuals. *p<0.05 = statistically significant.

correlated well with VCAM-1 mRNA, and to an even greater extent with ICAM-1 protein and VCAM-1 protein estimation (see table 4). Spot urine albumin demonstrated strong correlations with sICAM-1, ICAM-1 mRNA, sVCAM-1 and VCAM-1 mRNA. Similarly, serum creatinine at study entry was very significantly linked to sICAM-1 mRNA, sVCAM-1 and sVCAM-1 mRNA. The most significant of these relationships are plotted in figure 28.

Values for all three measures (soluble, mRNA and protein) for both ICAM-1 and VCAM-1 were higher in the group with known CV disease than in the group without known CV disease at entry into the study. While this reached statistical significance only for sICAM-1 (p=0.0097 via ANOVA, see figure 29), the overall trend was strong. When all three measurement methods were combined and averaged, overall ICAM-1 expression was 16% greater and VCAM-1 23% more in the group with known CV disease.

Of the clinical attributes taken at baseline, only duration of diabetes had no relationship with any of the laboratory estimations. Increasing age was barely positively correlated with CAM expression. Only ICAM-1 mRNA was significantly related, and even this was a weak relationship (r=0.411, p=0.0461, table 4, figure 30A).

However, given that the controls were significantly younger than any of the diabetic groups, this may have skewed the age correlations (controls were younger and had less CAM expression than any of the other groups). When the data was reanalysed without the control subjects, there was actually a negative correlation between age and CAM expression: the higher the age of the subjects, the lower the apparent CAM levels (figure 30 and table 5). This inverse relationship was true for both CAMs and all three types of measurement, with the comparison with protein measures being statistically significant (p=0.0053 for ICAM-1 and p=0.0047 for VCAM-1).

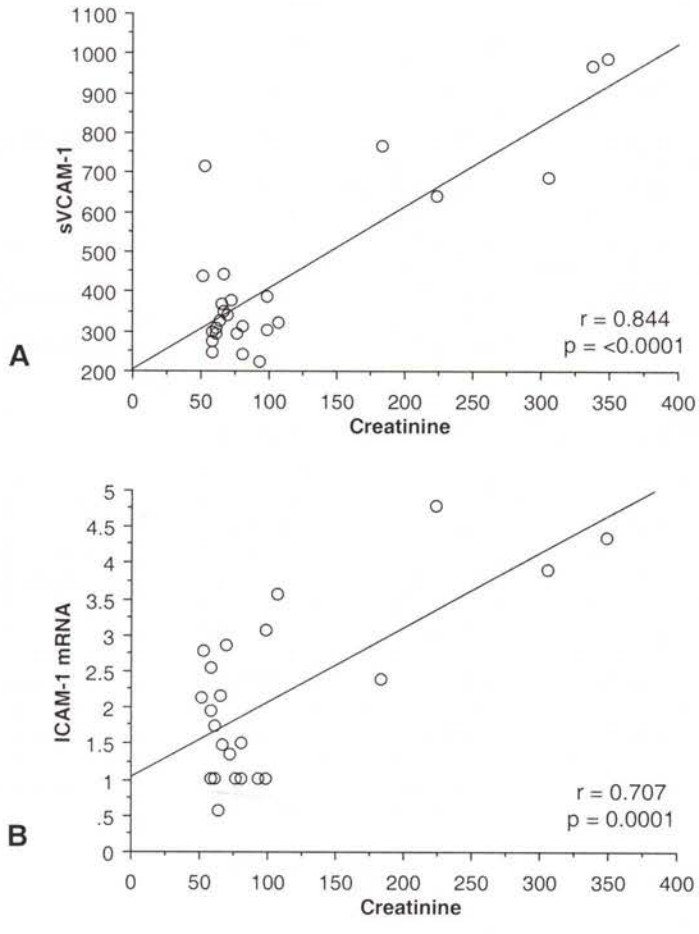


Figure 28. Regression plots of the significant findings summarised in Table 4. **A.** sVCAM-1 vs serum creatinine at study entry. **B.** ICAM-1 mRNA vs serum creatinine at study entry. Units: ICAM-1/VCAM-1 – fold difference over baseline; creatinine – μmol/l

	sICAM-1		sVCAM-1	
	r	p	r	p
Age	0.366	0.0721	0.345	0.0911
Duration of diabetes	0.186	0.446	0.053	0.8309
HbA1c	0.351	0.0853	0.235	0.2583
Spot Albumin	0.459	0.0209 *	0.631	0.0007 *
Serum Creatinine	0.329	0.1081	0.844	<0.0001 *
Known CV disease		0.0097 *		0.1652

	ICAM-1 mRNA		VCAM-1 mRNA	
	r	p	r	p
Age	0.411	0.0461 *	0.295	0.1618
Duration of diabetes	0.231	0.3557	0.177	0.4829
HbA1c	0.395	0.056	0.526	0.0084 *
Spot Albumin	0.519	0.0094 *	0.49	0.0150 *
Serum Creatinine	0.707	0.0001 *	0.507	0.0115 *
Known CV disease		0.396		0.8902

	ICAM-1 ELISA		VCAM-1 ELISA	
	r	p	r	p
Age	0.445	0.0737	0.326	0.2019
Duration of diabetes	0.092	0.787	0.145	0.6697
HbA1c	0.73	0.0009 *	0.581	0.0144 *
Spot Albumin	0.343	0.1778	0.241	0.3512
Serum Creatinine	0.216	0.4048	0.233	0.3685
Known CV disease		0.244		0.1601

Table 4. Summary of simple regression analyses between laboratory measures (columns) and clinical data for individual subjects at study entry (rows). *p<0.05 = statistically significant. See figure 29 for details regarding CV disease.

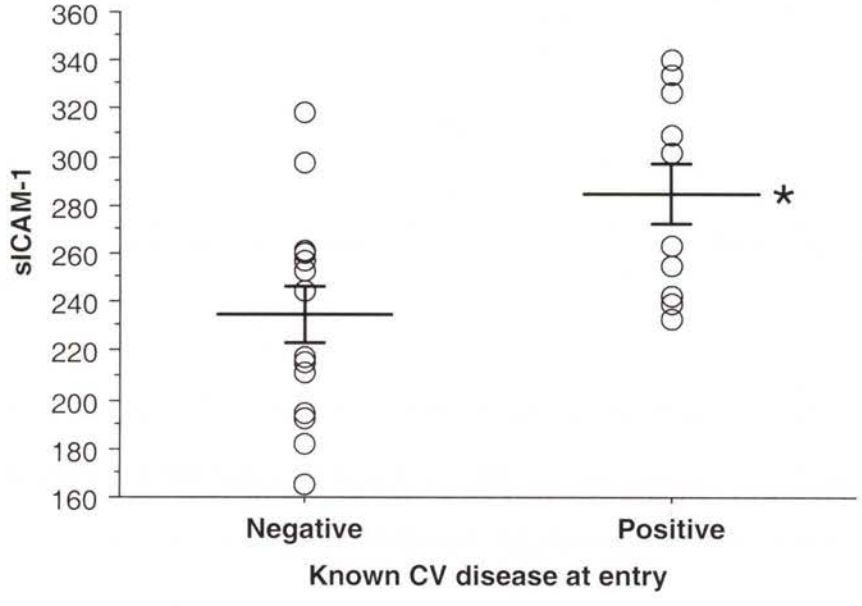


Figure 29. Scatter plot of sICAM-1 concentration split by known cardiovascular disease (ECG changes, history of cardiac disease or cardiac interventions) at study entry. Large lines denote group means ± SEM. *p<0.01 difference between groups by ANOVA. Similar data exists for sVCAM-1, ICAM-1/VCAM-1 mRNA and ICAM-1/VCAM-1 ELISA (not statistically significant)

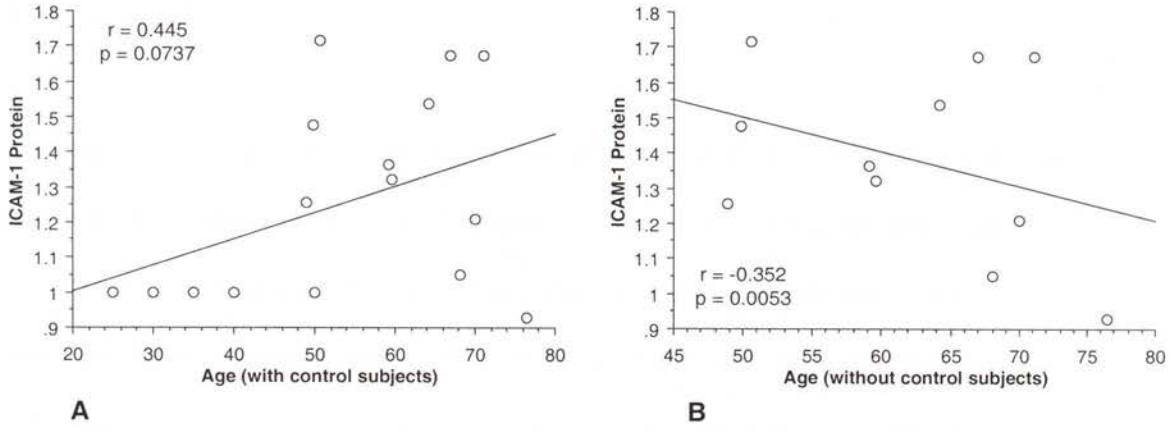


Figure 30. (A) Regression plot of ICAM-1 protein vs age of subjects with control subjects included. **(B)** Regression plot of ICAM-1 protein vs age of subjects with control subjects excluded. Note significant negative correlation when control subjects are excluded.

	Age (control subjects omitted)	
	r	p
sICAM-1	-0.033	0.8927
sVCAM-1	-0.043	0.1889
ICAM-1 mRNA	-0.099	0.1223
VCAM-1 mRNA	-0.346	0.1599
ICAM-1 ELISA	-0.352	0.0053 *
VCAM-1 ELISA	-0.411	0.0047 *

Table 5. Table summarises the relationship between age and various measures of CAMs with control subjects excluded. *p<0.05 = statistical significance.

5.4 Discussion

The finding of a stepwise increase in both sICAM-1 and sVCAM-1 shown in figure 26 with advancing degree of diabetic nephropathy is not surprising given the known literature (see section 5.4.1) and the results of chapters 3 and 4. However, more interesting observations result from the comparison between this data and the results of the expression results. The fact that the majority of experiments in this thesis were designed from the outset to use individual patient samples rather than pooled samples (as in some previously published work in this field) allowed comparison between the *in vitro* laboratory results and more commonly used clinical measures within individuals.

Perhaps the most clinically relevant of these correlations is that between the soluble CAM estimations and the laboratory measures. This is because in the current literature, other investigators either study the soluble CAM levels or else use laboratory measures, but only rarely both. The only example of the latter that was found in the current literature was one animal study by Bro *et al* in surgically nephrectomised mice[172] (see section 1.6). In all other cases, authors have assumed that an increase in protein or mRNA must equate automatically to an elevated level in soluble CAM expression. However, this may not be the case in end-stage renal disease where any raised circulating levels of the CAMs (as well as other proteins) would be significantly affected by decreased renal excretion as well as increased production.

The results presented in section 5.3.2 serve to demonstrate that the CAM mRNA results that were garnered correlate well with the measures of their respective soluble counterparts (VCAM-1 statistically significant, ICAM-1 very nearly so). This implies that the elevated sICAM-1 and sVCAM-1 concentrations found in the serum of diabetic, and especially diabetic nephropathic patients, results not just from decreased clearance of these proteins, but from increased *de novo* synthesis of ICAM-1 and VCAM-1 by stimulated endothelial cells. The correlation between the protein ELISA results and the soluble assays (significant for ICAM-1) underlines this result.

The correlations of the laboratory measures with the baseline subject clinical data were, if anything, even stronger. The relationship between the various measures of CAMs and spot albumin and serum creatinine are highly significant, but this result would have been expected given the stepwise increases demonstrated in the expression of ICAM-1 and VCAM-1 in chapter 3 and section 5.3.1. Somewhat more surprising for such a small study was the very strong trend for all types of measures of expression in both CAMs to be higher in the group that had known CV disease (albeit with only sICAM-1 being statistically significant for this relationship). HbA_{1c}, but not duration of diabetes, was also noted to be quite closely correlated with most of the measures of CAM expression.

Advancing age and its relationship with CAM levels depends on whether the control subjects were included or not. Because the control group were significantly younger overall than the other groups, and because their CAM expression was lower overall, their inclusion in the age vs CAM comparison led

to a general (but weak) positive trend towards increasing CAMs with advancing age (figure 30A and table 4); the only regression to reach significance was that between ICAM-1 mRNA. Without the control subjects, the trend was reversed. Within this study group of diabetic subjects (with any degree of renal disease) there was a tendency (significant for both ELISAs) for a negative relationship with advancing age (table 5). On the one hand, this shows that in the patients chosen, greater age does not necessarily automatically mean increasing CAM levels, thus lessening the impact of the difference in age between the control and the other groups (see section 3.4.3 for further discussion). However, on the other hand, it serves to highlight the effect of patient selection in a small study group.

5.4.1 CORRELATION WITH CURRENT LITERATURE

The comparison between the sCAM and *in vitro* measures of CAM expression confirm the animal study by Bro *et al* that CAM mRNA correlates with sCAM levels in end-stage kidney disease[172]. This is the first human study to show this, and the first one to demonstrate that it is also true for hyperglycaemia/diabetes.

As has already been noted in previous chapters, the results are also in keeping with the Serradell *et al* study that looked at the qualitative rise in ICAM-1 and VCAM-1 in endothelial cells exposed to non-diabetic uraemic serum[99]. The difference is that our study looked at purely *in vivo* adhesion molecules expression in comparison to mRNA expression, rather than their method of measuring

sCAMs in cell culture media (which was composed of 50% patient sCAM and 50% newly synthesised protein from the cell culture).

The raw sICAM-1 and sVCAM-1 results are in keeping with the findings by other authors regarding the stepwise increase of the soluble CAMs with progressing renal disease[145-147] and shown in figure 3. Many other studies also found an increase in either sICAM-1[149, 101] or sVCAM-1[148, 113, 119, 149, 101, 133] in diabetes with microalbuminuric subjects. The positive relationship between almost all types of CAM expression and serum creatinine or AER is confirmed by the sVCAM-1 work of Koga *et al*[150].

The positive correlation between the ELISA results and HbA_{1c} that was found in these experiments is also well established in the literature[118, 119, 109]. Certainly, other measures of diabetic control or degree of diabetes have been correlated with sICAM-1 and sVCAM-1 levels, and these include fasting glucose levels, fasting insulin, fasting c-peptide, glucose levels 2-hours post oral glucose tolerance test and insulin resistance measured by HOMA (detailed further in section 1.5.1).

The negative correlation between advancing age and ICAM-1 protein levels within the non-control group has been previously found for sICAM-1 in normal subjects by other investigators[238].

Finally, our observation that the CAM levels tended to be higher in our subjects with known CV disease at study entry coincides with two earlier studies that found the association for sICAM-1[102] and sVCAM-1[102, 123]. This is somewhat

surprising as our study consisted of only 25 subjects, while these other two papers described observations from 82[102] and 101[123] subjects. This is even more so when one considers that an even larger cross-sectional survey of 239 subjects failed to demonstrate a relationship between either sICAM-1 or sVCAM-1 with known CV disease.

5.4.2 CLINICAL IMPLICATIONS

The results summarised in this chapter are important clinically in that they demonstrate for the first time that *in vitro* studies into endothelial cell adhesion molecule expression correlate with the actual soluble CAM expression that is found in individual patients. The corollary of this is that studies that have used mRNA, ELISA, FACS and other laboratory methods can be compared directly to the more common studies that have just measured sCAM levels in populations.

The other clear indication from that data is the reinforcement of the fact that CAM levels are closely associated with known clinical markers for atherosclerosis such as HbA_{1c}, spot albumin, serum creatinine and the existence of established CV disease. This relationship exists whether the method of CAM measurement is an *in vitro*/cell culture based method or a simple blood assay for sCAMs. This confirms that measurement of sCAMs may become a useful screening tool in clinical practice for the detection and tracking of macrovascular risk factors.

5.4.3 STUDY LIMITATIONS

The most obvious limitation is once again the small size of the study population. As previously discussed in section 3.4.3, this situation may give rise not only to a lack of power, but also the possibility of skewed data depending on patient selection. Indeed, given the numbers, it was somewhat unexpected that there would be such strong correlations between the CAM estimations and the various clinical markers. However, this may merely be an indicator of the strength of the relationship between CAM expression and known cardiovascular risk factors. It is still the case that with a larger sample size, better correlations (r- and p-values for regression) would be expected for comparisons that just failed to reach statistical significance, such as sICAM-1 vs ICAM-1 mRNA, sVCAM-1 vs VCAM-1 ELISA and CAMs in general vs known CV disease.

It could be argued that our definition of known cardiovascular disease was flawed. There was no attempt made to actively screen for the presence of CV disease with measures such as ECG, stress tests, arterial ultrasound, coronary angiograms or spiral CT. However, if one of the test subjects did happen to have these tests, and they were positive, or there was otherwise a positive clinical history of CV disease, then they were classified as positive for CV disease. A more rigorous recruitment process would have actively used a screening test.

The previously described potential limitation of defining nephropathic status by AER vs ACR is not as important as in the previous chapters because of the fact that the majority of analyses in this chapter did not rely on splitting the subjects

into different stages of nephropathy; instead, a continuous comparison by regression analysis was used (the exception to this being the raw sCAM results in section 5.3.1).

Lastly, although we concluded from the data that the increased sCAM levels were due to increased production and not decreased excretion, no methods were devised to measure the actual excretion rate of sCAMs in nephropathic patients. In addition, another measure that could have been correlated to our data is the actual tissue expression of CAMs within individuals with varying degrees of diabetic kidney disease. However, given that this would require biopsy of vascular tissue from living subjects, the technical and ethical challenges would have been far greater than the simple drawing of blood used in this study.

5.5 Conclusion

We conclude from these results that the *in vitro* measures of ICAM-1 and VCAM-1 expression derived from the treatment of endothelial cell cultures correlate well with the clinical measurement of sICAM-1 and sVCAM-1 levels within individual subjects. This finding permits direct comparison of cell culture studies with clinical studies which measure only soluble adhesion molecule concentrations.

The levels of both the soluble and *in vitro* measures of CAMs were associated with several known risk factors for future cardiovascular disease including HbA_{1c}, spot urine albumin, serum creatinine and prior history of cardiovascular disease. These findings are confirmed by many previously published studies and underline the importance of ICAM-1 and VCAM-1 as markers of existent and future atherosclerosis.

Chapter 6

The Effect of HDL Treatment on ICAM-1 and VCAM-1 Expression

6.1 Introduction

As discussed in section 1.7.4, it is well established that HDL is able to suppress ICAM-1 and VCAM-1 expression in endothelial cell cultures. Further, there is recent clinical data that synthetic HDL is able to cause regression of atherosclerotic lesions in humans when given weekly by intravenous injections for just 5 weeks[216]. However, there is no data concerning HDL's effects in the hyperglycaemic or nephropathic environment.

In prior studies of HDL described in the literature, authors have used either "native HDL" (nHDL) or "reconstituted HDL" (rHDL). nHDL, which is derived from different subjects with minimal processing (centrifugation) and exhibits experiment to experiment variability depending on the donor[222]. In contrast, rHDL is a more highly purified form of HDL that shows little subject-to-subject variability, as well as being more potent in suppressing CAMs in endothelial cells[221].

Thus, the experiments described in this chapter are designed to examine whether rHDL is able to attenuate the adverse effects of high glucose and uraemic serum on endothelial CAM expression that was previously demonstrated in chapter 3.

6.2 Methods

6.2.1 CELL CULTURE CONDITIONS

6.2.1.1 Preparation of rHDL

Reconstituted HDLs containing ApoA-I and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC) were prepared by the cholate dialysis method described by Matz and Jonas[241]. ApoA-I HDL was isolated by ultracentrifugation (16 hours, 26 hours, then another 26 hours at 55000 rpm with a Ti 55.2 rotor), delipidated and subjected to anion-exchange chromatography on a column containing Q-Sepharose Fast Flow gel attached to a fast protein liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology, Uppsala, Sweden). After chromatography the apolipoprotein were dialysed extensively (3 x 5 litre) against 20 mM NH_4HCO_3, lyophilized, and stored at -20°C. When required, the apolipoprotein was reconstituted by dissolving in 10 mM Tris-HCl buffer (pH 8.2) containing 3 M guanidine-HCl and 0.01% EDTA-Na_2, followed by extensive dialysis (5 x 1 litre) against 10 mM Tris-buffered saline (TBS, pH 7.4) containing 150mM NaCl, 0.005% EDTA-Na_2, and 0.006% NaN_3.

Small unilamellar vesicles containing PLPC were prepared[242]. PLPC (12 mg) was placed in a large glass test tube to which chloroform–methanol (1:1 v/v, 500ml) was added. The PLPC was then dispersed as a thin film onto the walls of the tube before drying under nitrogen for 2 h at 40°C. After drying, endotoxin-free PBS (1 ml) was added and the solution was vortexed to obtain multilamellar vesicles

(MLVs). The MLVs were then sonicated at 4°C (Sonifier B12, Setting 1 to 2, Branson Sonic Power Company, Danbury, CT) with a microtip sonic probe in 3-min bursts followed by 1- to 2-min intervals (total of 30 min sonication, i.e., 10 x 3 min bursts). The resultant clear solution was then centrifuged in a microfuge (10 min, 15,000 rpm) to remove titanium fragments. Lipid-free ApoA-I was added and the mixture was left standing at room temperature for 2 hours. Any remaining lipid-free ApoA-I was removed by ultracentrifugation of the complexes with a single spin (16 h, 100 000 rpm) at a density of 1.25 g/ml. The resulting discoidal rHDL was dialysed against endotoxin-free PBS prior to addition to the cells.

6.2.1.2 Cells culture conditions

HAECs were cultured and exposed to various conditions. The conditions are summarised in table 6.

The concentration of serum used was 10%. Thus, for a total well volume of 1 ml, 100 µl of serum would be used. For the high glucose conditions, a 200 mmol/l solution of glucose was prepared. HAEC media was used for cell culture, which has a glucose concentration of 5 mmol/l. Thus, for a total well volume of 1ml, 100 µl of the high glucose concentrate was added to the media, resulting in a final concentration of 25 mmol/l glucose (HG, high glucose). The different conditions were used in various experiments as described in the experimental setup below.

Abbreviation	Serum used	Final Glucose concentration	Final rHDL concentration
Ctrl NG	Control	5 mmol/l	-
Ctrl HG	Control	25 mmol/l	-
Ur NG	Uraemic	5 mmol/l	-
Ur HG	Uraemic	25 mmol/l	-
HDL Ctrl NG	Control	5 mmol/l	16 μmol/l
HDL Ctrl HG	Control	25 mmol/l	16 μmol/l
HDL Ur NG	Uraemic	5 mmol/l	16 μmol/l
HDL Ur HG	Uraemic	25 mmol/l	16 μmol/l

Table 6. Summary of conditions used for cell culture.

6.2.2 EXPERIMENTAL SETUP

Expression of ICAM-1 and VCAM-1 was measured at the level of mRNA and protein expression, using real-time RT-PCR and ELISA, respectively.

6.2.2.1 Real-time PCR

HAECs were grown to confluence in 6 well plates and used between passages 8 and 13. The conditions used were described in table 6. The final volume of media and conditions used was 1ml. Pre-incubation time in 16 μmol HDL was 20 hours, after which, the glucose/uraemia conditions were added, and then a further incubation continued for 4 hours. mRNA was extracted as described in the general methods in chapter 2. Real-time PCR in triplicate for each condition was used to assay mRNA expression. The serum within each group was pooled for both the

control and uraemic groups. All results were adjusted for the housekeeping gene, 18S RNA. There were a total of 3 independent experiments completed.

6.2.2.2 ELISA

HAECs were seeded in 96 well plates and used between passages 12 and 13. Of the conditions described in table 6, only Ctrl NG, Ur HG, HDL Ctrl NG and HDL Ur HG were used in order to confirm the mRNA findings. The final volume of media and conditions used was 100 µl/well. Incubation time was 24 hours, after which the cells were washed and ELISA for ICAM-1 and VCAM-1 protein was performed as described in chapter 2. Each condition was repeated in quadruplicate for both ICAM-1 and VCAM-1. The serum within each group was pooled for both the control and uraemic groups. There were a total of 3 independent experiments.

3.2.3 STATISTICAL ANALYSIS

Descriptive data are expressed as mean ± SE. Groups were compared by ANOVA with Fisher's procedure used to determine significance; p-values of <0.05 were assumed to represent statistical significance. Multiple data points for each condition (eg triplicates for real-time RT-PCR) were condensed to one mean value and used as one data point for further analysis. Statview 5.0.1 for Windows (SAS Institute Inc, Cary, NC, USA) was used for statistical analysis.

6.3 Results

6.3.1 mRNA RESULTS

Figure 31 summarises three independent experiments that examined the possible role of HDL treatment in inhibiting CAM expression in endothelial cells.

As can be predicted from the results of chapter 3, both ICAM-1 and VCAM-1 mRNA are increased significantly by high glucose, uraemia alone and uraemia together with high glucose. Again, the trend towards greater expression with the latter conditions was strong ($p=0.03$ for both genes of interest).

Adding HDL to the control normal glucose condition will depress ICAM-1 mRNA expression by 47% ($p<0.001$ by ANOVA) and VCAM-1 mRNA by 58%. Given this effect in control conditions, it is not surprising that the addition of HDL to the other conditions will significantly depress the expression both CAMs as compared to corresponding non-HDL condition (eg. Ur NG vs Ur NG + HDL).

However, the inhibitory effect of HDL was so strong that any of the conditions supplemented by HDL exhibited ICAM-1 and VCAM-1 mRNA levels that are significantly lower than that of control serum with normal glucose alone (p-values all <0.05). Taken as a whole (HDL+Ctrl NG, HDL+Ctrl HG, HDL+Ur NG, HDL+Ur HG) the average suppressive effect of HDL on ICAM-1 was 33%, and for VCAM-1 this figure was 54% below baseline without HDL.

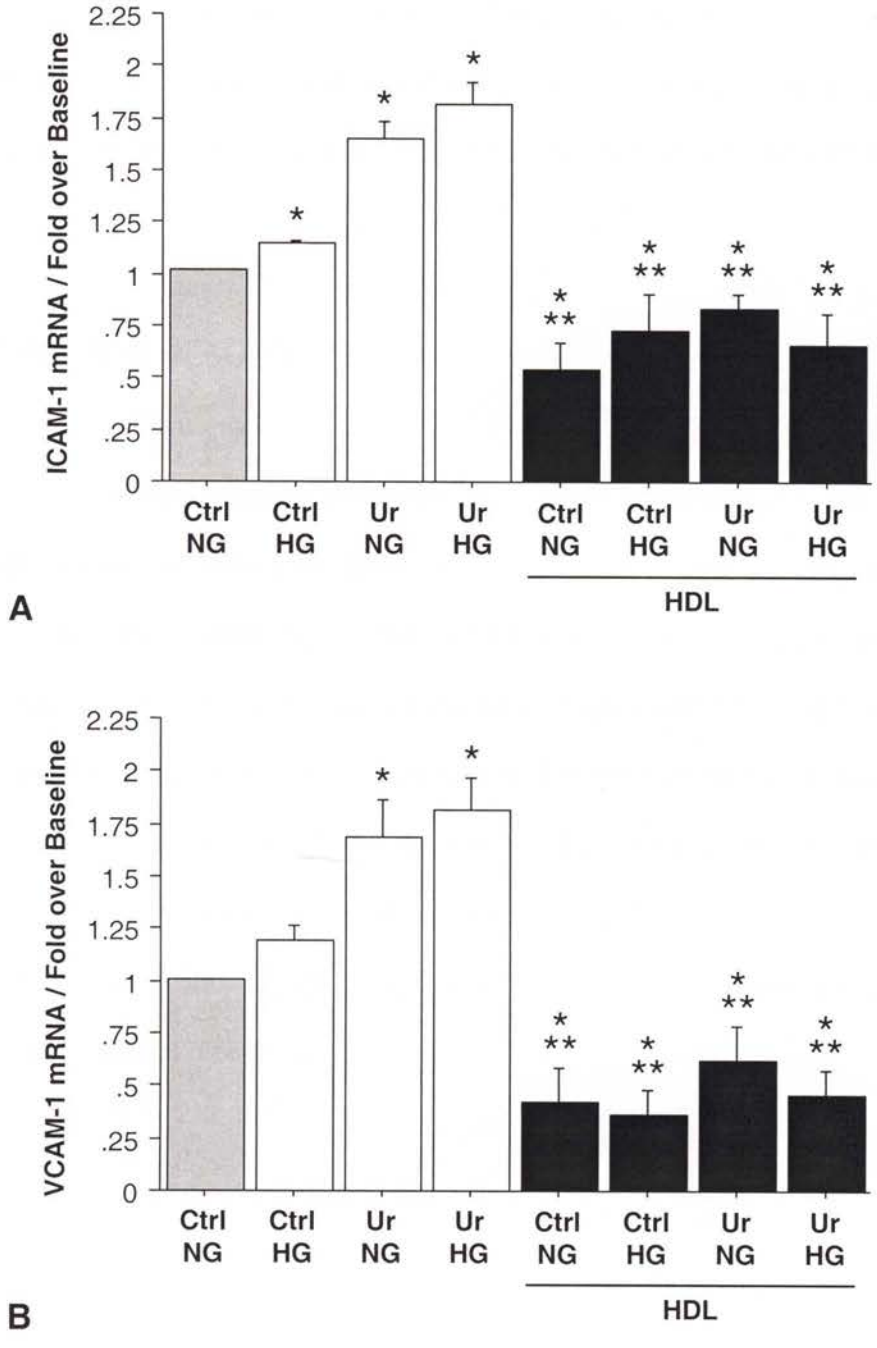
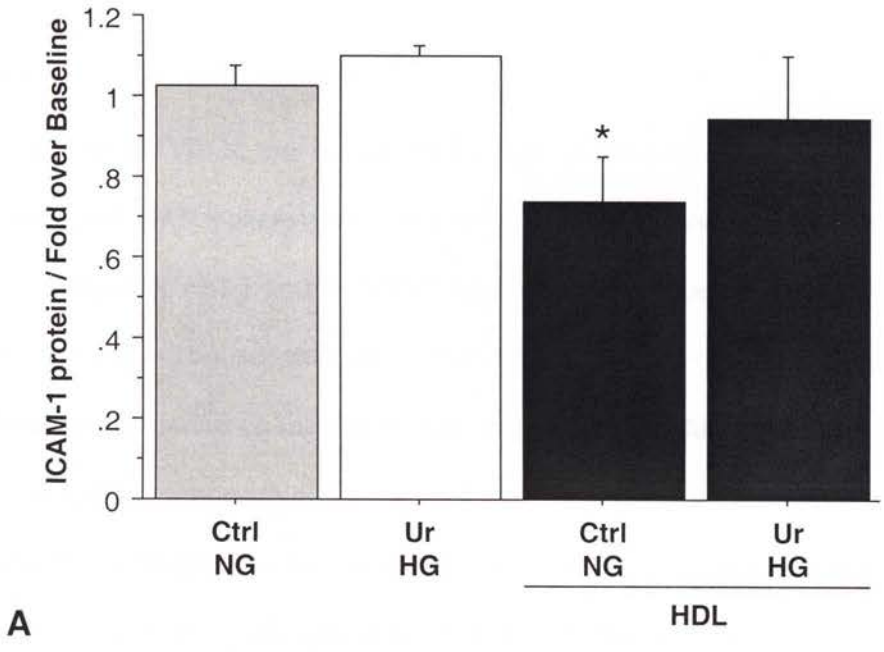


Figure 31. The effect of HDL on CAM mRNA in endothelial cells. **(A)** ICAM-1 mRNA and **(B)** VCAM-1 mRNA, measured by real-time RT-PCR. p-values for increasing trend Ctrl NG → Ur HG **(A)** 0.0296 **(B)** 0.0255. Bars represent means ±SE. *p<0.05 vs Ctrl NG, **p<0.05 vs corresponding non-HDL condition (for example, Ur NG vs HDL + Ur NG).

Within the HDL conditions, uraemia still increased ICAM-1 and VCAM-1 expression more than control serum with either normal or high glucose. There was a trend towards lower CAM expression with Ur HG+HDL than Ur NG+HDL.

6.3.2 PROTEIN RESULTS

Three independent protein ELISA experiments were performed to confirm the mRNA results, as shown in figure 32. In a result similar to the mRNA experiments, HDL suppressed CAM expression in uraemic high glucose conditions to below control normal glucose levels without HDL for both ICAM-1 (8% below baseline, $p=0.6$ by ANOVA) and VCAM-1 (19% below baseline, $p=0.01$), albeit non-significantly for the former adhesion molecule. The addition of HDL to control normal glucose did decrease ICAM-1 expression significantly (by 42%, $p<0.05$) and VCAM-1 expression slightly (4%) as compared to the equivalent non-HDL condition.



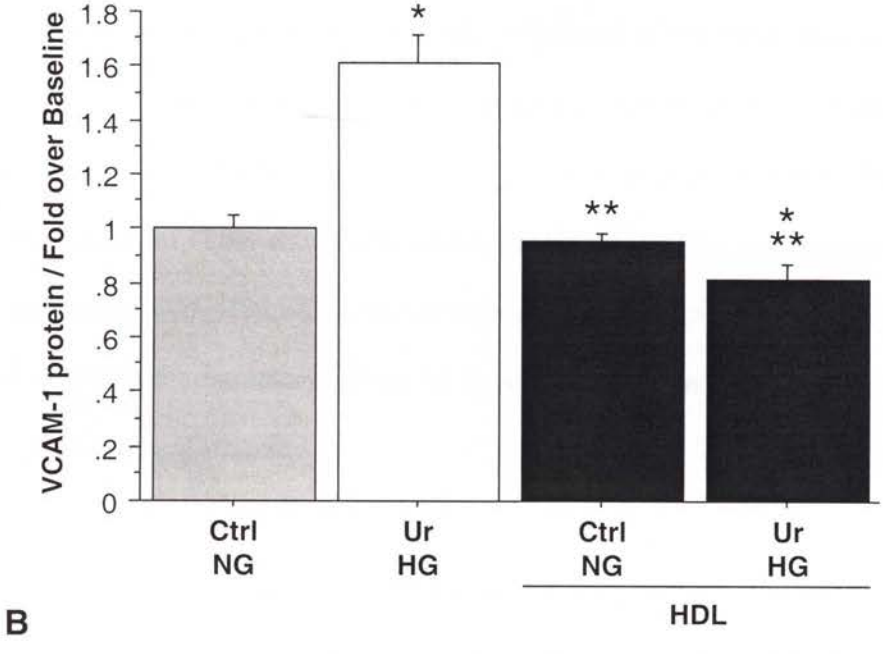


Figure 32. The effect of HDL on CAM protein in endothelial cells. **(A)** ICAM-1 protein and **(B)** VCAM-1 protein, as measured by ELISA. Bars represent means ±SE. *p<0.05 vs Ctrl NG, **p<0.05 vs Ur HG.

6.4 Discussion

HAECs, like HUVECs, are stimulated by high glucose and uraemic serum to increase CAM mRNA expression. These results clearly demonstrate the ability of HDL to reduce ICAM-1 and VCAM-1 expression when added to the media of endothelial cells. This suppression of CAM mRNA levels occurs regardless of whether the cells were co-incubated with normal glucose, high glucose, control serum or uraemic serum conditions. In each case, HDL was able to decrease CAM expression significantly lower than in the corresponding conditions without HDL (eg Ur NG + HDL is significantly lower than Ur NG alone).

Perhaps the most remarkable aspect was the magnitude of the effect: even for the most stimulatory conditions (uraemia ± hyperglycaemia), HDL was able to downregulate CAM mRNA expression to levels significantly below that of baseline conditions. This result was confirmed by the ELISA experiments that were performed with HDL. This demonstrates the potency with which HDL is able to inhibit the stimulatory effects of hyperglycaemia and glucose on CAM expression in the vasculature.

In addition, it should be noted that without HDL, HAECs behave like HUVECs in response to glucose and uraemic serum. Both ICAM-1 and VCAM-1 mRNA expression is increased when HAECs are exposed to (in ascending order) hyperglycaemia alone, uraemic serum alone and uraemic serum with high glucose.

6.4.1 CORRELATION WITH CURRENT LITERATURE

This is the first study to look at the effect of HDL on endothelial CAM expression when combined with hyperglycaemia or uraemic serum, and thus cannot be compared to other studies on that basis. However, there are many other groups who have examined the potential for HDL to decrease cytokine-induced CAMs expression.

On a simplistic level, the results presented in this chapter accord with the vast majority of reports of suppression of ICAM-1[218-221] and VCAM-1[218, 220-226, 230] expression in endothelial cells by HDL.

On further analysis of the known literature, however, it is noted that there two groups which did not find that HDL inhibited endothelial CAM expression. It has been suggested by Stannard *et al*[232] that VCAM-1 is not downregulated by nHDL, and by Zhang *et al*[225] that both ICAM-1 and VCAM-1 fail to be suppressed by nHDL in endothelial cells. The most obvious difference between these two studies and all other studies outlined above is the use of nHDL instead on rHDL. Indeed, when one of the groups used rHDL in their cells, VCAM-1[225] was successfully suppressed (though ICAM-1 still remained resistant to downregulation by rHDL). nHDL has not only been observed to show heterogeneity between donors[222], but has also been found to be 100 times less potent at decreasing CAM expression than rHDL[221]. The results in this chapter supports the notion that rHDL is a

powerful inhibitor of ICAM-1 and VCAM-1 expression, and is in agreement with the vast majority of prior work in this field.

6.4.2 CLINICAL IMPLICATIONS

Previous animal studies[213-215] and a recent human study[216] that have demonstrated HDL given directly *in vivo* has the potential to greatly, and in the case of the human study very quickly, cause regression of atherosclerotic lesions. In addition, clinical studies show that sICAM-1[206, 207] and sVCAM-1[201, 207] are negatively correlated with plasma HDL levels. However, it is unclear whether the pro-inflammatory influence of the diabetic nephropathic environment can modify the atheroprotective effect of HDL.

While this is not a clinical intervention study, these results provide evidence that even under the most adverse condition (uraemia with hyperglycaemia), HDL is still able to inhibit both endothelial ICAM-1 and VCAM-1 mRNA and protein expression to levels significantly below that of control conditions. The clear implication is that direct administration of HDL to diabetic patients with nephropathy could represent a novel and potent treatment in preventing CV disease in this very high risk population.

6.4.3 STUDY LIMITATIONS

Even though each mRNA condition was done in triplicate and three separate experiments done, and similarly the protein conditions performed in quadruplicate in three separate experiments, there was still some experimental error evident in the results. This was most obvious in the case of VCAM-1 protein ELISA in HDL Ctrl NG, where expression was very similar to that of Ctrl NG without HDL (figure 32B). This was due entirely to one ELISA experiment where the HDL condition demonstrated higher VCAM-1 protein levels than that control. This was clearly an aberrant result as it was discordant with all other mRNA and protein results in all other experiments. While it would have been desirable to repeat some to decrease experimental error, and perhaps increase statistical significance, there were only limited quantities of uraemic serum available because the HDL experiments were amongst the last ones to be performed for this thesis. As it is, the magnitude of HDL's effect was such that the results seemed to be clear.

The review of the literature reveals that two types of HDL are commonly used in cell culture experiments, rHDL and nHDL. The choice of using the former over the latter was taken mainly because nHDL is known to exhibit varying degrees of potency depending on the donor from which the HDL was purified[222]. In addition, rHDL seems to be more effective in reducing CAM expression than nHDL in endothelial cells[221, 225, 232]. For these reasons, rHDL was the more reasonable choice.

Another possible criticism of these experiments is that the endothelial cells used (HAECs) were different to those used in chapters 3 and 4 (HUVECs). However, it should be noted that in the mRNA experiments, an identical pattern of CAM stimulation with glucose and uraemic serum was demonstrated in HAECs for the non-HDL conditions (see figure 31) as for similar conditions in HUVECs (see chapter 3). Further, the literature indicates that HDL suppresses CAM expression in HUVECs more than HAECs[225]. Thus, had HUVECs been used for these experiments, it is possible that even more significant suppressive results may have been obtained. However, HAECs were chosen because it is a more physiologically relevant cell type for this study of intervention for CV disease.

Current literature provides evidence that the downregulation of CAMs by HDL in HUVECs leads directly to decrease leukocyte adhesion[227, 219, 221, 225] and it would seem reasonable that the model described in this chapter would show the same result. Adhesion experiments to support the mRNA and protein data will form the basis of ongoing experiments.

Similarly, while not specifically tested here, it could be postulated that because uraemia and hyperglycaemia are upregulating CAM expression via an NF-κB dependent pathway (as described in chapter 4), HDL antagonises the glucose/uraemia effect by the same pathway. There are studies that support this proposition by demonstrating that the inhibition of CAM expression by HDL occurs via blockade of NF-κB activation[231, 226] and ongoing work will investigate this hypothesis.

Finally, while the experiments in this chapter focus on the direct administration of HDL to cells, there are also new compounds that are emerging as potent agents in upregulating HDL levels *in vivo*. One such group of agents is the cholesteryl ester transfer protein (CETP) inhibitors[243-245] and future work could involve the study into the effects of these agents in diabetic subjects with nephropathy.

6.5 Conclusion

The results in this chapter demonstrate that rHDL is able to effectively abrogate the stimulatory effect of hyperglycaemia and uraemic serum on the expression of ICAM-1 and VCAM-1 in endothelial cells. The magnitude of this effect is such that the addition of HDL to uraemia plus hyperglycaemia renders the CAM expression to levels significantly below even that of control serum with normal glucose without HDL. This takes place at the mRNA, and to a lesser extent at the protein level. The clear implication of these experiments is that direct administration of HDL to diabetic patients with nephropathy could represent a novel and potent treatment in preventing CV disease in this very high risk population.

Chapter 7

Summary, Conclusions and Future Directions

The main aim of this thesis has been to explore the effect of high glucose and increasing degrees of diabetic nephropathy (alone and in combination) on the expression and regulation of ICAM-1 and VCAM-1 in endothelial cells.

Chapter 3 demonstrated that high glucose significantly increases ICAM-1 and VCAM-1 expression in endothelial cells at the mRNA and protein level via a partially osmotic mechanism. While uraemic serum stimulates the expression of both CAMs by the greatest magnitude (much more than high glucose alone), the intermediate degrees of diabetic nephropathy also upregulate the CAMs, and there is a strong trend to a stepwise increase in CAM expression with increasing kidney disease. Combining high glucose with uraemic serum results in the highest levels of both ICAM-1 and VCAM-1 (with glucose acting via a non-osmotic pathway). The increased molecular expression of the CAMs also resulted in a functional increase in monocyte adherence to endothelial cells.

Chapter 4 showed that the regulation of ICAM-1 and VCAM-1 in high glucose and nephropathic conditions occurs primarily at the level of the promoter regions of each gene. NF-κB was found to be the principle nuclear binding factor involved, though there was evidence of support from other factors such as AP-1. There was no post-transcriptional control of ICAM-1 mRNA evident, though there was some decrease to VCAM-1 mRNA stability in hyperglycaemia. This

effect was relatively small, and was overcome by NF-κB activation and the subsequent increase in VCAM-1 gene transcription.

Chapter 5 established that the *in vitro* measures of ICAM-1 and VCAM-1 expression derived from the treatment of endothelial cell cultures correlate well with the clinical measurement of sICAM-1 and sVCAM-1 levels within individual subjects, thus allowing direct comparison of cell culture studies with clinical studies which measure only soluble CAM concentrations. The levels of both the soluble and *in vitro* measures of CAMs were associated with several known risk factors of cardiovascular disease including HbA_{1c}, spot urine albumin, serum creatinine and prior history of CV disease. This emphasised the strength of CAM levels as markers of CV disease.

Finally, chapter 6 highlighted the ability of HDL to greatly diminish uraemia and hyperglycaemia stimulated expression of ICAM-1 and VCAM-1 in endothelial cells, an effect that was so strong that the level of CAMs in the most adverse condition (uraemia + high glucose) was reduced to below that of control conditions without HDL.

While most of the findings are novel, those which are not are well supported by previous publications found in the literature. Figure 33 summarises the major findings and the pathways of action that are described in this thesis.

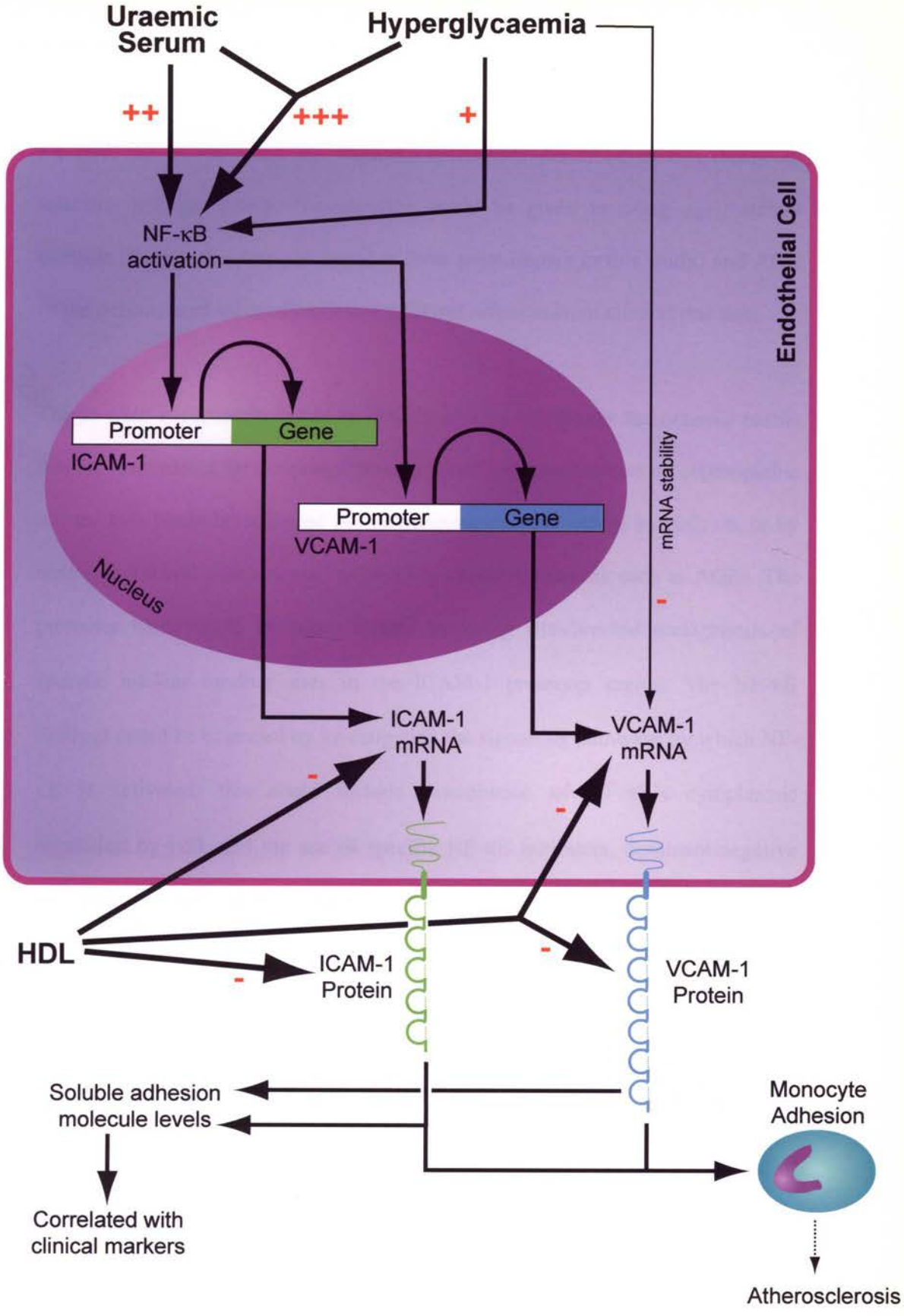


Figure 33. Schematic representation of the major findings of this thesis.

There are a number of limitations that have been discussed in the respective chapters, but these serve to point the way to future work. Firstly, a larger study population would provide further support and possibly increase statistical power for both the *in vitro* and the clinical experiments described in this thesis. In selecting a larger group, consideration could be given to using age-matched controls (though this was not found to have great impact in this study) and ACR as the definition of microalbuminuria to better reflect current clinical practice.

The *in vitro* experiments could be extended to try to identify the uraemic toxins that are responsible for the pro-inflammatory effect demonstrated by nephropathic serum. This could be achieved by fractionating uraemic serum by dialysis, or by testing individual components known to be raised in uraemia such as AGEs. The promoter work could be taken further by using site-directed mutagenesis of specific nuclear binding sites in the ICAM-1 promoter region. The NF-κB findings could be extended by investigating the signalling pathways by which NF-κB is activated: this could include examination of NF-κB's cytoplasmic regulation by IκB, and the use of specific NF-κB inhibitors, dominant-negative transfection or new siRNA technology.

While the HDL experiments indicate the remarkable potential in its use as a treatment for atherosclerosis, the molecular mechanisms and regulation of HDL's effect form obvious targets for future research. These experiments could also be extended to *in vivo* studies of direct HDL administration to diabetic subjects with nephropathy.

Finally, while ICAM-1 and VCAM-1 are critical in the initiation of atherosclerosis, there are many pathological processes that follow adhesion. Future work could include the investigation of the effect of glucose and uraemia on other endpoints in the atherosclerotic pathway, such as foam cell formation, smooth muscle proliferation and plaque rupture.

In summary, however, the experiments in this thesis demonstrate that the massively increased risk of atherosclerosis and CV disease in diabetic patients with nephropathy can be explained in part by the upregulation of ICAM-1 and VCAM-1 on endothelial cells under the influence of both hyperglycaemia and the nephropathic environment. Further, while each of these conditions has an adverse effect on the endothelium, the combination of the two conditions is even more pro-inflammatory. The increase in CAM expression occurs at multiple levels and is under the regulation of the respective promoter regions and nuclear binding factors, such as NF-κB. These findings are also very clinically relevant, as demonstrated by the strong correlation of CAM expression with clinically established markers of CV disease and the profoundly favourable effect of HDL in endothelial cells.

It can thus be concluded that both ICAM-1 and VCAM-1 have pivotal roles in the underlying pathogenesis, as clinical markers and as targets of treatment of atherosclerosis and macrovascular disease in the very high risk population of diabetic patients with kidney disease.

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