Expression and regulation of VCAM-1 and CD44 by cultured fibroblast-like synoviocytes

by

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A thesis submitted to The University of London for the degree of Doctor of Philosophy

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Acknowledgments

I wish to extend my deepest gratitude to my supervisor Dr IJsbrand Kramer for his guidance and encouragement throughout my studies. I am also very grateful to Dr Peter McIntyre (Novartis Institute for Medical Research, London) without whose help and assistance the molecular biology studies would not have been possible.

Many thanks also to Dr Victoria Chen, Dr Muriel Nobles and Auragun Wibulswas for their company and help during my studies. I would also like to thank Caroline Jones, Morven Brodie and Rowan Morris for excellent help with problems molecular biological, and for providing endless entertainment and humour. Also, a big thanks to Nick Hayes for his punctual remarks on the present perfect.

I am greatly indebted to Derek Davies of the Imperial Cancer Research Fund for his excellent help and assistance with flow cytometry studies. I would also like to thank Novartis Institute for Medical Research for allowing me to use their excellent facilities. The use of the facilities within the Departments of Pharmacology and Immunology is also gratefully acknowledged. Many thanks go out to Thérese Coquerelle and Dr Peter Dall (IGEN, Kernforschungzentrum, Karlsruhe, Germany) for their help with immunohistochemistry. Furthermore, I would like to thank Margaret Jones and Professor D Mason (Department of Cellular Science, John Radcliffe Hospital, Oxford) for their assistance with double-labelling immunofluorescence.

On a more personal level, I wish to thank Arshad, Caroline, Sonia, Sunjeev and Vikki for their friendship. And lastly, but most importantly, to my parents, Susan and Richard, and my sister Liz who have provided help and assistance throughout my entire higher education.

Abstract

The fibroblast-like synoviocyte (FLS) has been implicated in the destructive process associated with rheumatoid arthritis. In this thesis I demonstrate the expression and regulation of several adhesion molecules expressed on cultured FLSs obtained from inflamed synovium. Unlike fibroblasts from other areas of the body, FLSs constitutively express vascular cell adhesion molecule-1 (VCAM-1). VCAM-1 on RA FLSs is constitutively expressed at high levels during the first 2 weeks of culture. At later time points (4 weeks in culture), VCAM-1 expression declined to low basal levels. A number of strategies were employed to determine the exogenous factors that determine the initially high levels of VCAM-1 in FLSs. Of the extracellular matrix components examined only collagen type I enhanced VCAM-1 expression but this had only limited success. The pro-inflammatory cytokines, IL-1β (10 ng/ml) and TNF-α (10 ng/ml), were also tested and found to induce only transient increases in cell surface VCAM-1 expression. However, the chronic administration of IL-4 or IL-13 in combination with TNF-α resulted in elevated levels of VCAM-1 with prolonged expression Prolonged VCAM-1 expression was found to result in part from the capacity of IL-4 and IL-13 to stabilize VCAM-1 mRNA transcripts.

CD44 splice variants, isoforms of the CD44 receptor, that are implicated in the progression of a number of human tumours were also expressed by FLSs isolated from inflamed synovium. Immunohistochemistry, flow cytometry and RT-PCR analysis of CD44 splice variant expression revealed differential expression of a number of variant isoforms. Splice variant expression, at both the mRNA and cell surface protein level, was observed in a large percentage of RA FLSs, it is variable in those from OA synovium, and is absent in cells isolated from non-inflamed joints. However, RA FLSs showed a greater intensity of staining for variants v3, v5 and v7/8. VCAM-1-positive FLSs also demonstrated complex splice variant mRNA transcripts, comprising v3, v6, v7, v8, v9 and v10 in a variety of splicing combinations. These results indicate that the nature of CD44-splice variant expression is closely linked to the inflammatory state of the synovial joint. Moreover, expression of the CD44v7/8 epitope is associated with an increased cellular proliferation rate and could thus be functionally implicated in the hyperplasia observed in RA synovium.

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Abbreviations

b-FGF	Basic-fibroblast growth factor
bp	base pair
BK	Bradykinin
BSA	Bovine serum albumin
C5a	Complement fragments
CD	Clusters of differentiation
CD44	Cluster of differentiation antigen 44
CD44s/CD44H	Standard or hematopoietic form of CD44
CD44v	Variant isoforms of CD44
CD68	Cluster of differentiation antigen 68
cDNA	Complementary deoxyribonucleic acid
COX	Cyclo-oxygenase

CR	Complement receptor
Da	Dalton
DAF	Decay-accelerating factor
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagles medium
dNTP	Deoxynucleotide triphosphate
EBV	Epstein-Barr virus
ECM	Extracellular matrix
ECMR	Extracellular matrix receptor
EDTA	Ethylenediaminetetra-acetic acid
ELAM	Endothelial leukocyte adhesion molecule
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FBS	Foetal bovine serum
$Fc_\varepsilon RI$	High affinity receptor for IgE
FITC	Fluorescein isothiocyanate
FLS(s)	Fibroblast-like synoviocytes
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIT	Guanidinium isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
HA	Hyaluronic acid (also hyaluronan)
HLA-DR	Human leukocyte-associated antigen-DR
HPDS	Human plasma derived serum
HSP	Heat shock protein
HUVECs	Human umbilical vein endothelial cells
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IFN-γ	Interferon-γ
IL	Interleukin
INCAM-110	Inducible cell adhesion molecule-110 (CD106)
LAD	Leukocyte adhesion deficiency
LFA	Lymphocyte function associated antigen

LT	Leukotriene
LPAM	Leukocyte platelet adhesion molecule
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MFI	Mean fluorescence intensity
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MC	Mast cell
MC_{TC}	Tryptase-chymase-positive mast cell
MC_T	tryptase-positive mast cell
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMPs	Matrix metaloproteinases
MSE	Monocyte/macrophage specific esterase
NF-κB	Nuclear factor-κB
OA	Osteoarthritis
OD	Optical density
PAF	Platelet activating factor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PECAM-1	Platelet endothelial cell adhesion molecule-1
PG	Prostaglandin
PDBu	Phorbol-12, 13-dibutyrate
pT_2T_3	Human keratinocyte CD44 DNA consisting of only variant exons v3-v10
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RHAMM	Receptor for hyaluronan-mediated motility
RNA	Ribonucleic acid

RT-PCR	Reverse transcriptase polymerase chain reaction
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SSC	Standard saline-citrate buffer
SSPE	Saline-sodium phosphate-EDTA buffer
TE	Tris-EDTA buffer
TBE	Tris-borate-EDTA buffer
TCR	T cell receptor
TEMED	N, N, N´,N´ tetramethyl-ethylene diamine
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitors of metalloproteinase
T_m	Temperature of melting
TNF-α	Tumor necrosis factor-α
Tris	Tris [hydroxymethyl]-aminomethane
TSG-6	TNF-stimulated gene-6
UDPGD	Uridine diphosphoglucose dehydrogenase
VCAM-1	Vascular cell adhesion molcule-1 (CD106)
VCAM-6D	6 domain form of vascular cell adhesion molcule-1
VCAM-7D	7 domain form of vascular cell adhesion molcule-1
VLA	Very late antigen
v/v	volume per volume ratio
w/v	weight per volume ratio

Chapter 1

Introduction

Despite many decades of research into the study of rheumatoid arthritis (RA) we are still relatively ignorant as to its causes and propagation. It is now apparent that a number of inflammatory mediators and matrix-degrading enzymes are important in the propagation of RA. This chapter will be used to provide a general overview of the biology of the synovial joint, with particular emphasis on the changes which occur in the synovium during disease. In addition, it will also describe the importance of a variety of inflammatory mediators. Furthermore, it will also introduce the various families of adhesion molecules and their respective roles in inflammation.

1.1 Synovial joints

Of the three types of joint found in the human skeleton, synovial joints are the most commonly found and permit a wide range of movement (Gardner *et al.*, 1969). Synovial joints (Figure 1.1) are complex structures specialized to allow a wide range of movement with low frictional drag. They are characterized by the presence of a fluid-

filled cavity and surrounding connective tissue, or synovium. The joint space contains a small amount of synovial fluid which is highly viscous due to the presence of the glycosaminoglycan, hyaluronan. The synovium lines the whole of the joint capsule and is attached to bone at the cartilage-bone junction. Articular (hyaline) cartilage covers the bone ends and the joint is enclosed by a fibrous capsule. The combination of hyaline cartilage, viscous synovial fluid and deformability of the synovium allows friction-free movement of the synovial joint.

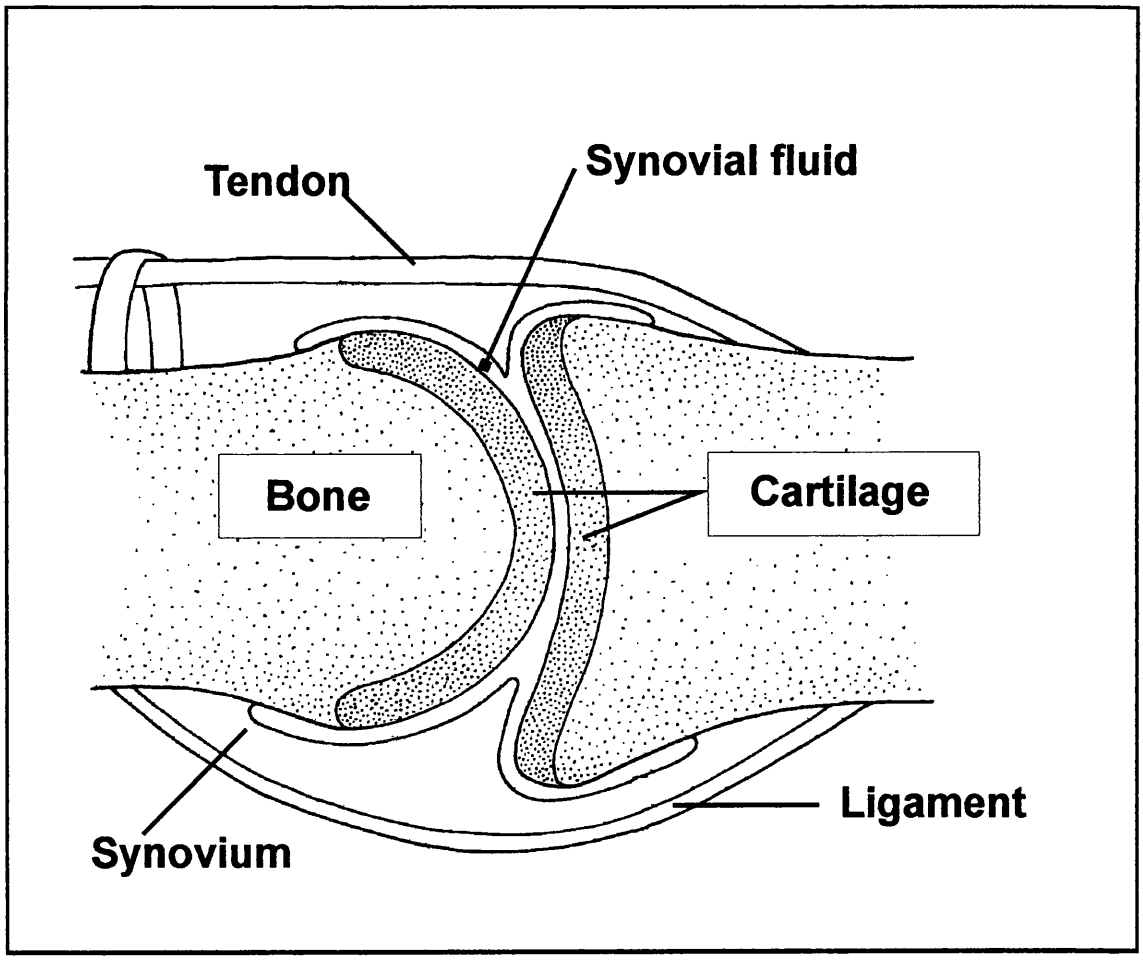


Figure 1.1 A schematic diagram of the synovial joint.

Synovial joints are formed between weeks 4 and 7 post-fertilization. The mesenchymal cells of the limb bud associate to form the blastema. Chondrification of the blastema occurs and glycosaminoglycans are deposited at the developing interzone. The primordial synovium forms as a vascularized mesenchymal layer at the edges of the interzone between the developing bones. Following cavitation at the centre of the interzone the joint space becomes lined with a synovial membrane (Henderson and Edwards, 1987a).

1.1.1 Articular (hyaline) cartilage

Articular cartilage is a specialized matrix that forms a smooth, glistening surface over the bone ends. Cartilage can be separated into four zones: superficial, intermediate, deep, and calcified (Harris, 1990). It is avascular and relatively acellular, with chondrocytes occupying approximately 0.1% of the cartilage volume. The chondrocyte is a highly specialized cell whose primary function is to produce and maintain the cartilage extracellular matrix. Chondrocytes within the intermediate zone contain well developed synthetic apparatus capable of synthesizing a range of matrix components such as the chondrocyte-specific collagen types II, IX, X and XI (Mendler *et al.*, 1989; Reichenberger *et al.*, 1991). They are also able to synthesize and secrete a variety of matrix-degrading enzymes (including cathepsins, collagenases and proteinases).

The cartilage matrix composition allows friction-free movement of opposing cartilage surfaces upon each other and the ability to tolerate the extremely high compressive loads which are generated on standing and walking (Afoke *et al.*, 1987). Cartilage is composed of several collagen types and high molecular weight aggregates of proteoglycan. The collagen fibres show differences in size, structure and

arrangement depending on the zone in which they are found (Muir, 1996). Aggrecan, a cartilage-specific proteoglycan, together with link protein, bind to hyaluronan (HA) to form high molecular weight aggregates which are trapped within the collagen meshwork (Nieduszynski *et al.*, 1980; Hardingham, 1979). These strongly anionic proteoglycan aggregates generate high osmotic pressures and the tangled collagen meshwork becomes enlarged due to the entry of water (70-80% of the wet weight) and electrolytes. The tension created in the collagen meshwork as a result of hydrodynamic swelling confers upon cartilage its ability to withstand large transient increases in load.

1.1.2 Synovium

Synovium is the soft connective tissue which lines the joint capsule of diarthrodial joints, tendon sheaths and bursae (Edwards, 1994). Synovium is highly variable with its structure depending upon the connective tissue which it covers. Synovium can be split into three main types: adipose, areolar and fibrous (Key, 1932). Adipose synovium is found covering the fatty pads which are present at the infrapatellar region. Fibrous synovium lines structures composed of fibrous tissue, such as ligaments and tendons. Areolar synovium is found as a distinct membrane that can either be arranged in folds, which disappear when stretched, or as distinct villi. This arrangement allows free movement over the underlying tissue.

Normal synovium can be separated into two distinct layers: the intimal and subintimal layers. The intima is the most superficial layer and is in direct contact with the synovial fluid. The synovial intima is a layer of overlapping cells that can be up to three cells deep. Within the intimal layer there are two distinct types of cell: type A cells (macrophage-like) and type B cells (fibroblast-like) (Barland *et al.*, 1962;

Wilkinson *et al.*, 1992). A rich capillary network can be found in close association with intimal cells (Wilkinson and Edwards, 1989). The extracellular matrix (ECM) surrounding the intimal cells contains large amounts of HA, but small amounts of collagen. In contrast to the intimal layer, the subintima (subsynovium) consists of a loose collagen fibre network and is relatively acellular with scattered fibroblasts, and occasionally lymphocytes, macrophages and mast cells.

Synovium functions as a deformable packing material, to provide a smooth non-adherent surface for friction-free movement of a joint. Intimal cells synthesize the molecules involved in lubrication of the opposing cartilage. Synovium is also thought to provide the avascular cartilage with its nutritional requirements with the degree of synovial vascularity affecting cartilage growth.

1.1.3 Synovial fluid

In normal individuals, the joint space contains a small volume of synovial fluid. The presence of synovial fluid within the joint is dependent on three factors: the entry of plasma diasylate due to subatmospheric pressure present within the cavity at rest; the addition of hyaluronan; and the presence of a continuous lining of synovium within the capsule (Edwards, 1994). Synovial fluid is essentially a dialysate of blood plasma which passes through the sub-synovial capillary endothelium and into the extracellular space where it combines with hyaluronan. Hyaluronan (hyaluronic acid; HA) is a high molecular weight anionic polysaccharide which is composed of repeating disaccharide units of β-1,4-glucuronate-β-1,3-N-acetylglucosamine. HA is synthesized by the fibroblast-like synoviocytes (type B) of the intimal layer and released into the synovial fluid at a concentration of 3.5 mg/ml. HA confers on the synovial fluid a highly viscous

consistency which provides the lubricating properties essential for ease of movement. In addition, HA acts as a molecular filter for plasma, preventing high molecular weight proteins, such as fibrinogen and IgM, from passing into the synovial fluid. Inflammation of the synovium results in a decrease in the concentration of hyaluronan in the synovial fluid and an increase in the quantities of high molecular weight proteins.

1.2 Intimal cells

The cellular distribution of the intimal layer can vary from a scattering of flattened cells through to tightly packed cuboidal cells several cells thick. Electron microscopy studies have demonstrated two distinct types of intimal cells, a macrophage-like cell (type A) and a fibroblast-like cell (type B) (Barland *et al.*, 1962; Graabeck, 1982).

The macrophage-like cells (type A) contain prominent, well-developed Golgi apparatus, abundant cytoplasmic vesicles and vacuoles, dense nuclear chromatin, and varying amounts of loosely arranged rough endoplasmic reticulum (Henderson and Edwards, 1987b). These ultrastructural features suggest that the type A cells are well equipped for phagocytosis and a degree of synthetic activity. They resemble tissue macrophages in their expression of CD14, CD68, Fc receptors, class II MHC (HLA-DR), and non-specific esterase activity (Hogg *et al.*, 1985; Bröker *et al.*, 1990; Zvaifler, 1995). In normal synovium 20-30% of the intimal cells are of the type A lineage.

Type B cells have a fibroblast-like morphology and are equipped for protein synthesis and secretion. They contain large amounts of closely packed rough endoplasmic reticulum, less prominent Golgi apparatus, prominent nucleoli and have an open nuclear chromatin pattern (Henderson and Edwards, 1987b). They are low in non-specific esterase activity but contain significant amounts of prolyl hydroxylase. They

also have a high content of the enzyme hyaluronan synthase (Worrall *et al.*, 1992). High activity of the enzyme uridine diphosphoglucose dehydrogenase (UDPGD), which converts UDP-glucose to UDP-glucoronate, has been demonstrated in type B cells thus distinguishing them from the deeper subintimal fibroblasts (Wilkinson *et al.*, 1992). UDP-glucoronate is one of the substrates used by the enzyme hyaluronan synthase in the production of HA. Recently, Edwards (1994) has proposed that the UDPGD-positive fibroblast-like cell may be regarded as the true synoviocyte since they possess a novel fibroblast phenotype. Type B cells also constitutively express the adhesion molecule VCAM-1 (Wilkinson *et al.*, 1993a). VCAM-1 binds the integrin $\alpha 4\beta 1$ and may be used in the retention of monocyte-like cells within the intimal layer.

It is now accepted that the type A cell is a bone marrow-derived macrophage which enters the synovium as a monocyte from the peripheral blood (Edwards and Willoughby, 1982). Studies using macrophage-deficient osteopetrotic mice have demonstrated that the intimal layer is devoid of macrophage-like cells (Naito *et al.*, 1991). Type B cells are generally regarded to be of local origin but the exact precursor cell is not known. The rate of replication and cellular turnover of type B cells is very slow (Henderson and Edwards, 1987b). Most evidence points to the type B cells originating from cell division deep within the subintima, and not being replaced by cell division within the intima.

1.3 Synovial joint diseases

With the advent of radiology at the start of this century, synovial joint diseases were shown to be of two distinct groups (Goldthwaite, 1897; Nichols and Richardson, 1909). The atrophic form was characterized by synovial inflammation accompanied by erosion

of the adjacent bone and cartilage. The second, or hypertrophic form, was found to result from focal loss of cartilage and hypertrophy of underlying bone and cartilage. The atrophic form was found to be polyarticular and demonstrated increased prevalence in younger patients, whereas the hypertrophic form was present in fewer joints and seemed to occur in older individuals (Goldthwaite, 1897). However, it was Garrod (1907) who first named the two conditions rheumatoid arthritis and osteoarthritis.

1.3.1 Osteoarthritis

Osteoarthritis (OA) is considered to be a non-inflammatory hypertrophic joint disease of unknown aetiology and pathogenesis. OA is not a single disease, but includes a heterogeneous group of conditions. It is characterized by symptoms of use-related joint pain, joint stiffness following inactivity, loss of joint movement, soft tissue swelling, formation of bony soft tissue swellings, and instability of the joint. OA is most commonly found in the joints of the knees, hips, hands and the apophyseal joints of the spine. OA is associated with a focal loss of articular cartilage, together with hypertrophy of the underlying subchondral bone and margin of the joint, cyst formation, and joint space narrowing. Inflammation of the synovium is common in OA and is considered to be a result of the primary joint destruction.

1.3.1.1 Epidemeology and risk factors

Osteoarthritis is the most common joint disorder, affecting approximately ten percent of the population of the UK. The prevalence of OA increases dramatically with age. In several recent studies it has been shown that the characteristic features of OA were found in the knees of 60% of men and 70% of women aged between 70-90 years, and in

the distal interphalangeal joints of 75% of women aged 60-70 years (Stankovic *et al.*, 1980; Van Saase *et al.*, 1989). There are marked gender differences with hand and knee OA showing an increased frequency in females compared to males. Geographical differences in the distribution of hip OA have also been reported (Cooper *et al.*, 1994). In addition, certain risk factors have been shown to increase the prevalence of OA. Two major groups of risk factors can be described: those which mark a generalized predisposition to OA, and secondly those which result in an altered joint loading. Factors which increase the susceptibility to OA include obesity, osteoporosis, heredity and hypermobility. Factors which alter the mechanical loading of joints include ligament damage, fracture, repetitive use of certain joints, and exercise.

1.3.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) describes a chronic inflammatory condition which affects the synovium, leading eventually to cartilage and bone erosion (Figure 1.2). RA is the most common and debilitating of the inflammatory joint diseases. The term rheumatoid arthritis was first used by Garrod in 1859, although reports of conditions similar to RA had been reported much earlier. Landré-Beauvais in 1800 described a condition which resulted in permanent swelling of the joints, deformities, and eventual disability of the patient (Short, 1974).

The clinical criteria for diagnosing rheumatoid arthritis were recently revised (Arnett *et al.*, 1988). These include: soft tissue swelling (arthritis) around three or more joints; morning stiffness in and around the joints; swelling of the hand joints (interphalangeal, metaphalangeal and wrist); symmetrical swelling; subcutaneous nodules; positive test for serum rheumatoid factor; and joint erosion. Any joint can be

affected although the joints of the hand, wrist, hip and knee seem to be the most commonly affected. Inflammation can also affect the synovial sheaths of the tendons and bursae. Persistent inflammation of the joint leads eventually to cartilage and bone erosion, tendon rupture and joint dislocation.

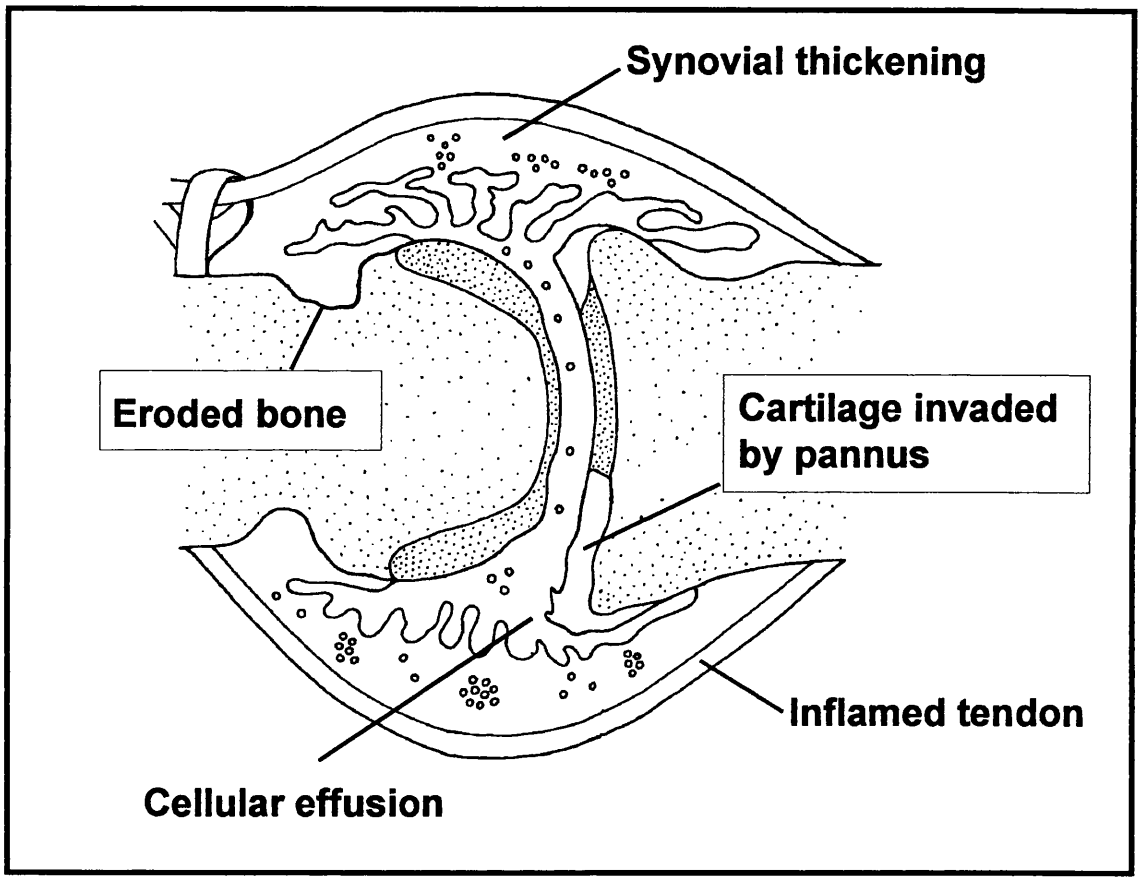


Figure 1.2 A schematic diagram of the rheumatoid joint.

Although RA mainly affects the joint, it is also a systemic condition with extra-articular manifestations occurring in other organs. Common extra-articular manifestations include anaemia, fever, susceptibility to infection, malaise, osteoporosis and weight loss. Systemic symptoms occur in patients suffering from the more active

forms of RA, with some 75% of rheumatoid patients having two or more extra-articular manifestations.

1.3.2.1 Epidemiology, aetiology and genetic factors

RA affects approximately 1% of the world-wide population. Several rural Chinese and African populations show significantly lower rates of prevalence (Spector, 1990). Increased prevalence rates of 5-6% have been identified in several native North American tribes (Alarcón, 1995). There is also an increased incidence in females (3:1) with periods of remission being observed in sufferers during pregnancy. Furthermore, the rate of incidence increases with age in both males and females (Hochberg, 1981).

Although much is known about the pathogenesis of RA, the causative agent(s) remains unknown. RA is a complex multifactorial disease, probably caused by a combination of environmental and genetic factors. Environmental stimuli, such as bacteria, mycoplasma and viruses, have been postulated to cause infection and a chronic persistent inflammatory response within the joint. Epstein-Barr virus (EBV) has long been a candidate causative agent for RA, with more than 80% of patient sera having antibodies present against EBV (Alspaugh et al., 1981). Serum antibodies against EBV recognise a peptide motif present in both the viral glycoprotein gp110 and the third hypervariable region of the DRβ chain of human leukocyte-associated (HLA) antigens-DR4 and HLA-DR1 (Roudier *et al.*, 1989). Other viruses, such as parvovirus B-19 and rubella virus, have been shown to be present in synovium and synovial fluid of some RA patients.

Heat shock proteins (HSPs) have also been implicated in RA. HSPs are a highly conserved group of proteins which cells produce in response to insult and injury.

Antibodies against mycobacterial HSP have been demonstrated at elevated levels in RA synovial fluid (Tsoulfa *et al.*, 1989). HSPs are found at increased levels in the cytoplasm of cells in synovial fluid, synovial lining, and cells at the cartilage-pannus junction of RA synovium (Karlsson-Parra *et al.*, 1990). Molecular mimicry may explain the role of HSPs in the aetiology of RA with an initial inflammatory response to bacterial HSP being prolonged by human HSP cross-reactivity (Kaufmann, 1990).

RA has long been regarded to have an autoimmune component to its aetiology. Two candidate groups of autoantibodies are the rheumatoid factors (RF) and the anti-collagen antibodies. Rheumatoid factors are autoantibodies of the IgM, IgG, and IgA classes that bind to the Fc region of IgG. RFs are the major class of autoantibody found in RA patients, being present in the serum of 80% of RA patients. IgG RF and IgM RF are produced by B cells in the germinal centres of RA synovium (Jasin, 1985). High titres of serum IgM RF are associated with articular forms of the disease and nodules, whereas both IgG RF and IgM RF are associated with severe extra-articular manifestations of RA (Tarkowski and Nilsson, 1983). Serum IgM RF has been found in patients who exhibit no early symptoms of RA (Walker *et al.*, 1986). Moreover, high titres of RF in non-RA patients has been shown to correlate with an increased risk of RA.

It has long been suggested that autoimmune reactions against collagen were central to the pathogenesis of RA. Injection of collagen type II has been shown to induce arthritis in mice and rats (Courtenay *et al.*, 1980; Trentham *et al.*, 1977). Collagen has a very slow turnover rate and so is not normally found in synovial fluid from non-inflamed joints. In RA, collagen breakdown products released into the synovial fluid may be antigenic and result in an autoimmune response. IgG antibodies

to collagen type II are detectable in a small percentage of patients with RA, whereas those to type IX are present in 44% of RA patients (Charriere *et al.*, 1988). Unlike RFs which can be present before the onset of the disease, antibodies to collagen are only present in established RA (Morgan *et al.*, 1989). RA synovial B cells have been shown to secrete antibodies to collagen type I and II (Tarkowski *et al.*, 1989). Collagen type II and III have been shown to activate peripheral T lymphocytes from RA patients, but were unable to activate T lymphocytes from normal patients (Trentham *et al.*, 1978).

Familial clustering of RA has indicated that there may be a genetic element to the disease. Genetic studies have shown that certain class II human leukocyte-associated (HLA) antigens confer greater susceptibility to RA. The HLA class II molecules are expressed on B lymphocytes, dendritic cells, macrophages and activated T lymphocytes where they are involved in antigen presentation. In North America and Western Europe expression of HLA-DR4 is associated with a 4-fold higher risk to RA, whereas HLA-DR1 shows increased association with RA in certain Asian, Hispanic and Israeli populations (Smith and Arnett, 1991). HLA-DR4 can be separated into five subtypes: Dw4, Dw10, Dw13, Dw14 and Dw15. The presence of Dw4, Dw14 and Dw15 confers increased susceptibility to RA. These various subtypes differ in the type of amino acid substitutions within the third hypervariable region of the DRβ chain. This region is referred to as the susceptibility epitope with the substitution at position 74 of glutamic acid (Dw13) for alanine (Dw14) dramatically increasing susceptibility to the disease. This epitope also determines the severity of the disease with an increased association of the DR4 epitope with the severe extra-articular forms of the disease.

1.3.2.2 Pathogenesis

The pathogenesis of RA has been described by Harris (1990) as a five stage disease. The first stage is where the immune system is exposed to antigen or other causative agent(s). Macrophages and dendritic cells, acting as antigen-presenting cells, present peptides derived from the causative agent to helper T cells. Possible causative agents include viruses, mycoplasma, collagens and immunoglobulins. The infection and accompanying immune response results in the inflammatory response being localized within the synovial joints. Why the inflammatory synovitis persists and becomes self-propagating is at present not known.

During the second and third stages, activated T cells stimulate the proliferation and differentiation of B cells into antibody secreting plasma cells, and neutrophils enter the synovial fluid. Angiogenesis in the synovial membrane is initiated to develop an extensive synovial capillary network. Pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α), are released from macrophages within the synovial membrane. These cytokines increase leukocyte attachment to, and migration through, the endothelium of the synovial postcapillary venules. Rheumatoid factors are found in patients with RA and may form complexes with other immunoglobulins and activate the complement cascade. Cytokines released from activated cells within the synovium also cause further cytokine release, cell proliferation, increased synthesis of prostaglandins and the release of matrix metalloproteinases (MMPs). Neutrophils migrate from the synovial capillaries, through the synovial membrane and into the synovial fluid in response to chemoattractants present in the synovial fluid. Activated neutrophils and fibroblasts release MMPs which degrade articular cartilage. Physical symptoms which are noticeable during the second and third stages include swelling of

the small joints of the hands and wrists, and morning stiffness due to an increase in the volume of synovial fluid. Stage four results in the irreversible destruction of the cartilage and subchondral bone. Proliferating fibroblasts and activated macrophages within the inflamed synovium form into pannus which invades cartilage, tendon and bone. Symptoms are similar to stages two and three but more severe with more pronounced swelling. During stage five severe erosion of subchondral bone and cartilage by the pannus results in narrowing of the joint space. Symptoms still include joint pain and swelling and can be accompanied by loss of movement, deformity and dislocation of the joint.

1.3.3 Changes in synovial structure in disease

Inflammation of the synovium and associated structures is a central feature of a number of joint diseases. In both OA and RA the synovium undergoes dramatic changes in structure and composition. Inflammation of the synovium (synovitis) is not thought to be a primary feature of OA, although most cases exhibit some degree of synovitis. The synovial inflammation seen in OA is believed to result from the release into the synovial fluid of cartilage fragments, collagen, and proteoglycans from eroding cartilage. There is some similarity in the degree of synovial inflammation between severe cases of OA and that of RA. Also, pannus-like tissue is commonly found on the surface of articular cartilage from OA patients, but the invasive nature of the pannus into the underlying cartilage is less severe.

In RA, the synovium loses its smooth, glistening pinkish-white appearance and instead becomes brown in colour, friable and engorged. The synovium increases greatly in mass with the formation of hypertrophied villi (Fassbender, 1994). The structure of

the synovium changes throughout the course of RA. Oedema formation occurs and the blood vessels of the synovium begin to proliferate during the early stages of the disease.

Increased cellularity of the synovium occurs by a combination of cellular infiltration by inflammatory cells, and hyperplasia of resident fibroblast-like synoviocytes. During the chronic phase of RA, hyperplasia of the intimal lining layer is striking, with the numbers of both intimal cell types being greatly increased. The subintimal layer shows a huge increase in cellularity, with B cells, T cells, macrophages and plasma cells being present. Lymphocytes can be found scattered throughout the subsynovium, often in close association with blood vessels, or in discrete lymphoid germinal centres. Electron microscopy studies by Kobayashi and Ziff (1973) have demonstrated the presence of three regions within the subintima: plasma cell-rich regions, lymphocyte-enriched regions, and a transitional region. T cells within the lymphocyte-enriched area are relatively quiescent but express markers indicating prior activation. Dendritic cells, often in association with T cells, can be found within the transitional region of the subintima. Multinuclear giant cells are also present within the RA synovium often being associated with the proliferating lining layer (Wilkinson *et al.*, 1993b). Extensive networks of blood vessels are present within the RA synovium; capillaries and post-capillary venules being found immediately below the intimal layer.

Fassbender has highlighted four stages which describe the changes which take place in the structure of synovium during the course of RA (Fassbender, 1994). The first stage or exudative stage, is where plasma exudation occurs and fibrin is deposited over the intimal layer, resulting in necrosis. During the subsequent proliferative stage fibroblast-like cells proliferate and migrate through the overlying fibrin meshwork, leading to regeneration of the intimal layer and the formation of villi. Proliferation of

the subintimal fibroblasts results in hypertrophy and hyperplasia of the subintima. This leads to a blurring of the boundary between the intimal and subintimal layers. The proliferating subintimal cells exhibit altered morphology and increased expression of the oncogenes *myb*, *myc*, *ras* and *fos*. Following the proliferative phase, the synovium enters a resting or transition phase. During this phase the proliferating cells of the stroma decrease in number and become quiescent. The intimal layer also decreases in thickness and reverts to its flattened single cell appearance. Lymphocytes and plasma cells enter the synovium, and new small diameter blood vessels are formed. The quiescent stage is characterized by synovial fibrosis, large numbers of lymphocytes and associated lymphoid aggregates, and the absence of plasma exudation and proliferation.

Pannus is a soft synovium-like tissue which can cover areas of the cartilage surface and bone. Two types of pannus are found in rheumatoid patients: lining layer pannus and vascular pannus (Maini and Feldmann, 1993). Lining layer pannus is thought to derive from the intimal layer of synovial cells which cover the bare areas of bone found at the cartilage edge. Lining layer pannus is relatively avascular and forms a distinct junction with the underlying cartilage. Underlying cartilage often shows signs of degradation, such as erosion of the matrix, a decrease in water content and a reduction in the number of chondrocytes. Vascular pannus is usually found overlying cartilage together with a multilayered zone of fibroblast-like cells. This type of pannus does not show any degradative activity but is thought to act as a fibrotic healing phase. An intermediate type of pannus, containing proliferating blood vessels and associated mononuclear cells, which infiltrates into cartilage has also been described (Kobayashi and Ziff, 1975). It is not known whether these varying forms of pannus are describing tissues at various stages of development. Pannus is primarily composed of proliferating

lining cells which have a highly invasive and destructive phenotype. These cells, recently termed 'pannocytes', express increased levels of oncogenes and mRNA encoding matrix-degrading metalloproteinases (Zvaifler and Firestein, 1994).

1.3.3.1 Dendritic cells

Dendritic cells are potent antigen-presenting cells that take up, sequester, and present antigen to T cells during immune responses (Steinman *et al.*, 1991). The identification of dendritic cells within inflamed tissue is of great interest as their presence is evidence for antigenic drive to the inflammatory process. They can be identified by characteristic ultrastructural features and show similar morphology to macrophages. These cells express class II MHC but unlike macrophages do not phagocytose and show reduced expression of a number of macrophage differentiation markers.

Dendritic cells are found in large numbers in synovial effusions from RA patients (de Vere Tyndall *et al.*, 1983; Zvaifler *et al.*, 1985). They comprise between 10-20% of the total mononuclear cell number in RA synovial fluid compared with 1-3% of peripheral blood mononuclear cells (Thomas *et al.*, 1994). However, they show similar functional activity with dendritic cells from both RA synovial fluid and peripheral blood demonstrating comparable allogeneic stimulation of peripheral blood T cells (Bergroth *et al.*, 1989). Thomas and co-workers (1994) have found that synovial fluid dendritic cells show a mature differentiated phenotype compared with peripheral blood dendritic cells. Dendritic cells ($CD33^{+}CD14^{dim}$) are enriched in RA synovium and synovial fluid compared with peripheral blood. Differentiation of peripheral blood dendritic cells, using granulocyte-macrophage colony stimulating factor (GM-CSF) and TNF-α in combination, was shown to stimulate the autologous mixed lymphocyte

reaction. Dendritic cells have also been identified in synovial tissue associated with lymphoid aggregates and high endothelial venules (Van Dinther-Janssen *et al.*, 1990). Moreover, studies have shown that dendritic cells found within the synovium are in close contact with activated T cells (Iguchi *et al.*, 1986).

1.3.3.2 Fibroblast-like cells: a transformed cell type

In situ, rheumatoid fibroblast-like synoviocytes (FLSs) possess an aggressive, highly-activated phenotype (Fassbender, 1983; Firestein, 1996). FLSs found within the intimal layer express a number of phenotypic markers, cytokines, inflammatory mediators, and matrix-degrading enzymes that are up-regulated during the course of synovial inflammation (Edwards and Wilkinson, 1996; Wilkinson *et al.*, 1992, 1993a; Bucala *et al.*, 1991; Wooley *et al.*, 1977; Firestein and Paine, 1992). Because of this fibroblast-like synoviocytes are now regarded to play a central role in the propagation of rheumatoid arthritis.

Intimal fibroblast-like synoviocytes found within RA synovium express elevated levels of the adhesion molecule VCAM-1 (Wilkinson *et al.*, 1993a; Morales-Ducret *et al.*, 1992). These cells are intimately associated with infiltrating leukocytes and resident macrophage-like synoviocytes expressing α4 integrins (α4β1 and α4β7), the counter-receptors for VCAM-1 (Wilkinson *et al.*, 1993a). VCAM-1 expression by intimal fibroblast-like synoviocytes may explain retention of α4 integrin-expressing cells within RA synovium. Lymphocytes and macrophages expressing high levels of α4β7 and α4β1 are found predominantly within the synovium, whereas neutrophils which are negative for α4 integrins are only found in the synovial fluid (Laffón *et al.*, 1991; El-

Gabalawy and Wilkins, 1993; Lazarovits and Karsh., 1993). VCAM-1 is also expressed on FLSs within the intimal layer of normal and OA synovium (Wilkinson *et al.*, 1993a; Morales-Ducret *et al.*, 1992). However, the level of constitutive expression correlates with the degree of inflammation of the synovium (Morales-Ducret *et al.*, 1992). Elevated VCAM-1 expression on RA FLSs is thought to be regulated by TNF-α (Morales-Ducret *et al.*, 1992).

The striking intimal hyperplasia so evident in rheumatoid arthritis is mediated, in part, by an increase in the number of resident fibroblast-like synoviocytes. FLSs express a number of cellular markers, such as the DNA polymerase cofactor PCNA (proliferating cell nuclear antigen), Ki-67 and nucleolar organiser, which are involved in cellular proliferation (Qu *et al.*, 1994; Morita *et al.*, 1997). A number of early response genes and oncogenes like egr-1, c-fos, myc and ras also show considerable up-regulation in RA fibroblast-like synoviocytes both *in situ* and *in vitro* (Müller-Ladner *et al.*, 1995). The products of these genes appear to function during proliferation and in the activation of genes that control the synthesis of matrix degrading enzymes. In addition to showing increased proliferation, fibroblast-like cells within the synovial intima exhibit minimal apoptosis despite demonstrating extensive DNA fragmentation and enhanced expression of the tumor suppressor gene p53 (Firestein *et al.*, 1995 and 1996; Matsumoto *et al.*, 1996). The combination of elevated proliferation and minimal apoptosis would allow aggressive fibroblast-like synoviocytes to accumulate within the synovium.

FLSs found within RA synovium are able to invade and destroy adjacent cartilage and bone (Geiler *et al.*, 1994; Müller-Ladner *et al.*, 1996). They do this by secreting a variety of matrix-degrading enzymes that are responsible for cartilage breakdown (Wooley *et al.*, 1977; Firestein and Paine, 1992; Geiler *et al.*, 1994). *In vitro*

studies demonstrate that fibroblast-like cells isolated from rheumatoid synovium are able to synthesize the matrix degrading enzymes collagenase, gelatinase (MMP-2) and stromolysin (Okada *et al.*, 1990; MacNaul *et al.*, 1990).

1.3.3.3 Macrophage-like cells

Mononuclear cell infiltration accounts for the huge increase in the number of macrophage-like cells found within the intimal layer of inflamed synovium. Hogg and co-workers (1985) have demonstrated differential distribution of monocytes and macrophages within the OA and RA synovium. In OA synovium, monocytes demonstrating a less mature phenotype are localized within the intimal layer, whereas macrophage-like cells are present in both the intimal and subintimal layers. It appears that monocytes enter the synovium and position themselves within the intimal layer, they start to mature and then migrate down into the deeper subintimal region. Large numbers of monocytes are found within the enlarged intimal layer of the RA synovium. These cells exhibit a phenotype similar to peripheral blood monocytes. Macrophage-like cells are less abundant and are scattered amongst the monocytes (Hogg *et al.*, 1985). A recent study has shown that synovial macrophage numbers correlate with the degree of articular destruction in RA patients (Mulherin *et al.*, 1996).

Macrophage-like cells exhibit an activated phenotype which is characterized by the expression of a number of markers (Koch *et al.*, 1991a). Immunohistochemical and *in situ* hybridization studies have shown that the macrophage-like cells within the intimal and subintimal layers show strong expression of HLA-DR, a class II MHC, which is involved in antigen presentation to CD4-positive T cells. Cultured macrophage-like cells express maximal levels of surface HLA-DR with interferon-γ

(IFN-γ) and GM-CSF, both potent inducers of HLA-DR expression, failing to increase levels further (Alvaro-Gracia *et al.*, 1991). *In situ* hybridization and double labelling studies have demonstrated that the CD14-positive cells within RA synovium are the greatest source of IL-1β and TNF-α (Brennan *et al.*, 1992; Wood *et al.*, 1992a). Moreover, IL-8, a cytokine with potent neutrophil chemoattractant properties, is expressed by subintimal perivascular macrophage-like cells and scattered intimal cells (Koch *et al.*, 1991b).

1.3.3.4 Lymphocytes

Lymphocyte activation is one of the first major amplification steps in the pathogenesis of RA (Harris, 1993). Clonal expansion of the T cell population following activation is able to drive B cell proliferation and differentiation to give antibody secreting plasma cells. Within RA synovium, large numbers of lymphocytes are distributed around blood vessels and germinal centres (Ziff, 1974). Electron microscopy studies have shown plasma-cell-rich regions, lymphocyte-enriched regions, and a transitional region within the RA subintima (Kobayashi and Ziff, 1973). The transitional zone is composed of a heterogeneous mixture of lymphocytes, undifferentiated blast cells, plasmablasts, and plasma cells.

B cell numbers are increased within RA synovium but still only comprise approximately 1-5% of synovial cells. They are organised into follicular structures found in the plasma-cell-rich and transitional zones within the subintimal layer of RA synovium. Plasma cells, which are differentiated B cells, are not co-localized with B cells but migrate away from the germinal centres following differentiation (Konttinen *et al.*, 1981). B cells isolated from RA synovial fluid and peripheral blood, exhibit an

altered phenotype compared to those from normal individuals. RA synovial B cells express the cell surface receptor CD5, normally found on T cells, and synthesize and secrete large amounts of rheumatoid factors and antibodies to type II collagen (Shirai *et al.*, 1991).

T cells are normally absent from the intimal layer being only present within the subintimal layer of RA synovium. They are arranged as either dense lymphoid aggregates or in a diffuse scattering pattern within the RA synovium. T cells make up approximately 30-50% of synovial cells with the majority of lymphocytes being of the CD4-positive helper cell phenotype. In RA, the CD4:CD8 ratio is considerably higher than in peripheral blood. T cell phenotypes are similar in both the early stages and late stages of disease. In addition, most of the CD4-positive T cells also express the memory cell marker CD45RO suggesting prior activation. T cells are also abundant in synovial fluid, but in contrast to those in synovium, the CD4-positive and CD8-positive cells are present in equal numbers (Forre *et al.*, 1982; Duclos *et al.*, 1982). The prevalence of CD8-positive T cells in RA synovial fluid may indicate the selective retention of the CD4-positive subset within the subintimal layer. The $\gamma\delta$ T cell subset, which are negative for both CD4 and CD8, are CD3-positive and are present in increased numbers in peripheral blood and synovial fluid from RA patients (Brennan *et al.*, 1988). These cells may play a role in the pathogenesis of RA since they can proliferate in response to a number of antigens (Holoshitz *et al.*, 1989).

1.3.3.5 Mast cells

Mast cells are found in increased numbers within the RA and OA synovium compared with normal synovium (Godfrey *et al.*, 1984; Castor, 1960). Human mast cells (MC) can be grouped according to their neutral protease composition, either containing both tryptase and chymase (MC_{TC}) or tryptase alone (MC_T). Both phenotypes have been identified in the RA synovium (Tetlow and Wooley, 1995; De Paulis *et al.*, 1996). Human synovial mast cells are found distributed at the pannus-cartilage-bone interface and around synovial blood vessels and are often degranulated (Bromley *et al.*, 1984; Godfrey *et al.*, 1984; Tetlow and Wooley, 1995; De Paulis *et al.*, 1996). Degranulation releases a range of newly generated and preformed pro-inflammatory mediators (Wasserman, 1984). Treatment of RA patients with disease-modifying anti-rheumatic drugs and non-steroidal anti-inflammatory drugs, respectively, has been shown to reduce the preformed mediator content and the number of mast cells present in the synovium (Bridges *et al.*, 1991). This has lead to speculation that synovial mast cells are involved in the pathogenesis of RA.

Mast cells express cell surface receptors ($Fc_\varepsilon RI$) which bind the Fc region of IgE with high affinity and specificity. Cross-linking of IgE with the $Fc_\varepsilon RI$ receptor results in mast cell activation and degranulation. Both anti-IgE and anti-$Fc_\varepsilon RI$ antibodies are found in the peripheral blood and synovial fluid of RA patients (Zuraw *et al.*, 1981; Gruber *et al.*, 1988a). Immunological activation of synovial mast cells results in histamine release together with the *de novo* synthesis of prostaglandin D_2 (PGD_2) and leukotriene C_4 (LTC_4) (De Paulis *et al.*, 1996). Mast cell tryptase has been shown to activate latent collagenase from rheumatoid synovial cells, increase IL-8 production, and to be mitogenic for fibroblasts (Gruber *et al.*, 1988b; De Paulis *et al.*, 1996; Ruoss et al.,

1991). Furthermore, synovial mast cell chymase is likely to play a central role in synovial inflammation since it is able to convert inactive IL-1β to its active form (Mizutani *et al.*, 1991).

1.3.3.6 Neutrophils

Neutrophils are conspicuous in their absence from within the RA synovium, being found only in small numbers at the cartilage-pannus junction (Bromley and Wooley, 1984; Mohr *et al.*, 1981). However, neutrophils are the predominant cell-type found in RA synovial fluid with numbers varying from 1000 to 100,000 neutrophils per mm^3 (Hollingsworth *et al.*, 1967). The differential distribution of neutrophils within the RA synovial joint can be explained by the absence of α4β1 integrin from the surface of neutrophils and the presence of elevated levels of chemoattractants, such as LTB_4 and TGF-β, within the synovial fluid. RA synovial fluid neutrophils exhibit signs of activation and show upregulation of FCγRI and the complement receptors CR1, CR3, and CR4 (Felzmann et al., 1991). They are able to phagocytose soluble immune complexes which can cause neutrophil degranulation and respiratory burst. Phagocytosis may provide the neutrophil with a mechanism for binding to cartilage: RA, but not normal, and OA, cartilage has been shown to have immune complexes attached to its surface (Ugai *et al.*, 1983). Neutrophils can therefore adhere to, and invade the cartilage surface. They are able to release matrix metalloproteinases which mediate cartilage and tendon destruction, and toxic oxygen metabolites which act as inhibitors of native antiproteinases found in synovial fluid (Weiss, 1989).

1.4 Inflammatory mediators in RA

1.4.1 Cytokines

The cytokines are a family of small polypeptide mediators, encompassing the chemokines, colony stimulating factors (CSF's), growth factors, interferons (IFN's), and interleukins (IL's). These agents are pleiotropic, regulating cellular function by a variety of autocrine and paracrine mechanisms. Cytokines mediate their effects by binding to specific high affinity, membrane-bound receptors. Members of the cytokine family play important roles in the maintenance of normal immune function. Cytokines also play a critical role in the initiation and maintenance of chronic inflammation and are thought to be central to the pathogenesis of rheumatoid arthritis.

In the RA joint, cytokines can be grouped according to the cell type from which they are released. T-cell cytokines, originally referred to as lymphokines, include IFN-γ, IL-2, IL-3, and IL-4. Macrophages and synovial fibroblasts produce a number of cytokines which include IL-1, IL-6, IL-8, GM-CSF and TNF-α. In contrast to T cell derived cytokines which are present in low concentrations within the RA synovial fluid, macrophage and fibroblast derived cytokines are found in abundance. This has led Firestein and Zvaifler (1990) to postulate a macrophage/fibroblast model to explain RA joint destruction. Analysis of synovial fluid and synovial tissue isolated from OA and RA patients has identified altered expression levels of a number of pro-inflammatory and anti-inflammatory cytokines. The pro-inflammatory cytokines GM-CSF, IL-1, IL-6, and TNF-α are expressed at increased levels in RA synovium compared with OA (Farahat *et al.*, 1993).

1.4.1.1 Interleukin-1

IL-1 is a potent pro-inflammatory cytokine synthesized mainly by monocytes and macrophages but also by a wide range of other cell types. Molecular cloning has identified two distinct forms of IL-1, IL-1α and IL-1β, both of which are synthesized as 31 kDa precursor peptides and then cleaved to give biologically active 17 kDa peptides (March *et al.*, 1985). *In situ* hybridization studies have identified the expression of immunoreactive IL-1 peptide by macrophage-like cells throughout the RA synovium, and by cells at the cartilage-pannus junction (Wood *et al.*, 1992a). IL-1 is a potent stimulator of PGE_2, collagenase, stromelysin, IL-6 and GM-CSF production by fibroblast-like synoviocytes (Dayer *et al.*, 1986; Unemori *et al.*, 1991a; Alvaro-Gracia *et al.*, 1991).

1.4.1.2 Tumour necrosis factor-α

Tumour necrosis factor (TNF) belongs to a family of ligands which activate a group of structurally related transmembrane receptors (Bazzoni and Beutler, 1996). TNF exists in two forms, TNF-α and TNF-β (lymphotoxin), which elicit similar biological responses. Both forms bind to the 55 kDa (p55) and 75 kDa (p75) TNF receptors (Brockhaus *et al.*, 1990; Bazzoni and Beutler, 1996).

Several studies have identified TNF-α messenger ribonucleic acid (mRNA) and TNF-α protein in RA synovial fluid and synovial tissue (Di Giovini *et al.*, 1988; Saxne *et al.*, 1988; Firestein *et al.*, 1990; Chu *et al.*, 1991). In contrast, TNF-β is not found in RA patient samples (Saxne *et al.*, 1988). TNF-α is found predominantly in the

macrophage-like cells within the RA synovial lining, and the cells at the cartilage-pannus junction (Chu *et al.*, 1991). However, both types of TNF receptor are expressed by macrophage-like and fibroblast-like cells present in the RA synovial lining layer (Deleuran *et al.*, 1992; Brennan *et al.*, 1992). In addition, other synovial cell types, including chondrocytes, express TNF receptors and are capable of being stimulated by TNF-α (Deleuran *et al.*, 1992). Both TNF-α and TNF receptors are found within the OA synovium but at much lower levels than in RA.

TNF-α is able to induce arthritis in a number of animal models, with transgenic mice overexpressing human TNF-α demonstrating a particularly destructive form of arthritis (Keffer *et al.*, 1991). Recent evidence suggests that TNF-α may be the key cytokine in the initiation and maintenance of the pro-inflammatory cytokine cascade in RA. However, it is not currently known what factor drives the release of TNF-α in the RA joint. When cultured synovial cells are treated with TNF-α they start to produce PGE_2 and collagenase, express a number of adhesion molecules, proliferate, and synthesize a range of cytokines (Dayer *et al.*, 1985; Unemori *et al.*, 1991a; Morales-Ducret *et al.*, 1992; Alvaro-Gracia *et al.*, 1990; Firestein *et al.*, 1990). Studies have demonstrated that anti-TNF-α antibodies are able to reduce IL-1, IL-6 and GM-CSF release by early synovial cell cultures (Brennan *et al.*, 1989; Haworth *et al.*, 1991). Moreover, human clinical trials have shown that treatment of RA patients with anti-TNF-α antibodies results in a dose-dependent improvement in the disease symptoms (Elliott *et al.*, 1994).

1.4.1.3 Interleukin-6

Unlike the macrophage-derived cytokines, IL-1 and TNF-α, IL-6 is predominantly synthesized by fibroblast-like synoviocytes (Wood *et al.*, 1992b). IL-6 affects conversion of B cells into antibody-secreting plasma cells, activation of T cells, and the production of acute phase proteins. IL-6 is present at elevated levels in RA synovial fluid (Houssiau *et al.*, 1988). *In situ* hybridization studies have demonstrated IL-6 mRNA expression in fibroblast-like cells present in the lining layer and pannus of RA synovium (Firestein et al., 1990; Wood *et al.*, 1992b). Both IL-1 and TNF-α are able to increase the constitutive secretion of IL-6 demonstrated by cultured RA synovial fibroblasts (Guerne *et al.*, 1989; Rosenbaum *et al.*, 1992).

1.4.1.4 Granulocyte-macrophage colony stimulating factor

GM-CSF is a pro-inflammatory cytokine which is found in the synovial fluid and synovial membrane of both OA and RA patients (Xu *et al.*, 1989; Alvaro-Gracia *et al.*, 1990; Farahat *et al.*, 1993). GM-CSF mRNA is expressed by macrophage-like cells in the RA synovium (Alvaro-Gracia *et al.*, 1991). RA synovial macrophage cultures transiently express GM-CSF, with expression being restored by IL-1 and TNF-α (Alvaro-Gracia *et al.*, 1991; Haworth *et al.*, 1991). Cultured RA synovial fibroblasts can release GM-CSF but only when stimulated with IL-1β or TNF-α. In addition, the combination of IL-1β and TNF-α has a synergistic effect on GM-CSF production (Alvaro-Gracia *et al.*, 1991). Given the above, and its ability to induce the expression of HLA-DR on monocytes it has been proposed that GM-CSF is involved in the pathogenesis of RA (Alvaro-Gracia *et al.*, 1989).

1.4.1.5 Anti-inflammatory cytokines

Members of this group of cytokines, such as IL-4, IL-10, and TGF-β, generally possess immunoregulatory and inhibitory properties. IL-4 has been shown to inhibit the release of IL-6 by RA synovium and RA synovial macrophages (Chomarat *et al.*, 1995a). Low level expression of IL-4 mRNA and IL-4 protein has been detected in RA synovium (Miossec *et al.* 1990; Isomäki *et al.*, 1996). Feldmann and co-workers postulate that the low levels of IL-4 and the absence of IL-4-secreting $CD4^+$ T helper-2 cells from the RA synovium may contribute to the pathogenesis of RA (Feldmann *et al.*, 1996). However, in other inflammatory diseases, such as asthma, IL-4 and the closely related cytokine IL-13 have pro-inflammatory effects.

IL-10 has been detected in sera, synovial fluid and synovial cell cultures taken from RA patients (Katsikis *et al.*, 1994; Llorente *et al.*, 1994; Cush *et al.*, 1995). Its expression has been localized to macrophages found in the synovial sublining layer and T cells within the subsynovium. Neutralization of IL-10 in RA synovial cell cultures results in the production of increased amounts of IL-1, IFN-γ and TNF-α (Katsikis *et al.*, 1994). Furthermore, addition of IL-10 to synovial cell cultures has been shown to inhibit TNF-α production, downregulate surface TNF receptor numbers, and induce the production of TNF inhibitors (Katsikis *et al.*, 1994; Joyce *et al.*, 1994).

Transforming growth factor-β (TGF-β) is found in both its latent and active forms in non-RA and RA synovial fluid, but non-RA samples have increased amounts of both (Brennan et al., 1990). It is constitutively secreted by synovial fluid macrophages and can be detected throughout the lining layer of RA synovium (Lafyatis *et al.*, 1989a; Wahl *et al.*, 1990; Miossec *et al.*, 1990). Cultured RA fibroblast-like

synovial cells constitutively express TGF-β at higher levels compared with those from normal synovium (Goddard *et al.*, 1992). TGF-β has a partial inhibitory effect on cultured RA synovial fibroblast growth and is able to inhibit T cell proliferation (Lafyatis *et al.*, 1989a; Wahl *et al.*, 1990). It may also function in tissue remodelling since TGF-β can stimulate the production of collagens and tissue inhibitors of metalloproteinases, and can inhibit collagenase synthesis (Wright et al., 1991; Lafyatis *et al.*, 1989a). The systemmic administration of TGF-β to mice with collagen-induced arthritis was shown to decrease the incidence and severity of joint inflammation (Kuruvilla *et al.*, 1991).

1.4.2 Eicosanoids

The eicosanoids are among the most important mediators and modulators of the inflammatory reaction. Upon cell activation with certain stimuli, such as bradykinin and C5a, membrane phospholipids are cleaved by phospholipase A_2 to give arachidonic acid. Arachidonic acid can be metabolized via either the cyclo-oxygenase (COX) or lipoxygenase (5-lipoxygenase) pathways to generate prostaglandins (PGD_2, PGE_2, PGF_{2_α}) and leukotrienes (LTB_4, LTC_4, LTD_4 and LTE_4,), respectively. PGE_2 causes vasodilatation of pre-capillary venules, erythema and oedema during the acute phase of inflammation. Leukotrienes can increase vascular permeability at post-capillary venules, and facilitate plasma leakage and oedema formation, with LTB_4 being also a potent chemoattractant for neutrophils.

The inducible form of cyclo-oxygenase, COX-2, is expressed at elevated levels in cultures from RA synovial tissue explants. In addition, early cultures of synovial fibroblasts show increased PGE_2 production. PGE_2 and LTB_4 are found at increased

levels in RA synovial fluid compared with those from OA and normal patients (Egg *et al.*, 1984; Klickstein *et al.*, 1980; Ahmadzadeh *et al.*, 1991). Eicosanoid production can be modulated by a number of cytokines with IL-1 and TNF-α causing increased PGE_2 release by synovial fibroblasts (Dayer *et al.*, 1986, 1985). In addition, PDGF acts synergistically with IL-1 to increase PGE_2 production (Kumkumian *et al.*, 1989). Prostaglandins may also function in neovascularisation of RA synovium with the recent finding that PGE_2 is able to induce vascular endothelial growth factor expression in cultured synovial fibroblasts (Ben-Av *et al.*, 1995).

1.4.3 Kinins

Kinins, such as bradykinin (BK), have been implicated in the pathogenesis of rheumatoid arthritis due to their ability to induce pain, vasodilation, and oedema (Proud and Kaplan, 1988; Bhoola *et al.*, 1992). Injection of bradykinin into the joint is able to produce an acute inflammatory reaction (Melmon *et al.*, 1967). A number of components of the kinin system are found at elevated levels in inflammatory joint diseases. Kinins are found at elevated levels in the synovial fluid from RA patients (Melmon *et al.*, 1967). Moreover, plasma kallikrein, a kinin-forming enzyme is found at elevated levels in RA synovial fluid compared with that from OA patients (Suzuki *et al.*, 1987). BK exerts its pro-inflammatory effects by stimulating the release of cytokines and prostaglandins. BK can stimulate IL-1β and TNF-α release from human monocytes and macrophages (Tiffany and Burch., 1989). In order to release PGE_2 in response to BK stimulation synovial fibroblasts first require pretreatment with IL-1β (Bathon *et al.*, 1989). IL-1β 'priming' of prostaglandin release by BK appears to work

by inducing COX-2 synthesis, phospholipase A_2 activation, and by increasing the number of kinin receptors (Crofford *et al.*, 1994; Gilman *et al.*, 1988; Bathon *et al.*, 1992). RA synovium exhibits increased numbers of kinin receptors compared to OA synovium which shows a negligible number of binding sites (Bathon *et al.*, 1992). This may be due to increased IL-1 production by macrophages in the RA joint.

1.5 Mediators of cartilage and bone destruction

Destruction of cartilage, subchondral bone and tendons is a common feature of advanced rheumatoid arthritis. The enzymes responsible for the breakdown of tissue matrix in these structures are the matrix metalloproteinases (MMPs). MMPs are secreted in a latent proenzyme form by a number of cell types within the RA joint. Proteases, such as trypsin and plasmin, cleave latent forms of MMPs to give active enzymes. Members of the MMP family include collagenase, gelatinase and stromelysin.

Collagenases are only able to digest collagen type I, II, III, VII and X when they are in their undenatured fibrillar form. Two types of collagenase have been identified in the RA joint: MMP-1 (fibroblast-type collagenase) is synthesized *de novo* as a latent proenzyme and released without storage by fibroblasts and macrophages, whereas MMP-8 (neutrophil collagenase) is found stored as a latent proenzyme in the specific granules of neutrophils. Collagenase mRNA and protein are mainly expressed by the synovial cells of the intima, with MMP-1 mRNA levels showing upregulation in RA compared with OA synovium (Okada *et al.*, 1990; Gravellese *et al.*, 1991; Firestein *et al.*, 1991). MMP-1 has been shown to degrade type I and III collagen found in abundance in synovium and tendons, whereas MMP-8 preferentially degrades type II collagen present in cartilage (Konttinen *et al.*, 1991; Hasty *et al.*, 1987). Synovial fluid

neutrophils are able to release increased levels of activated MMP-8 following interaction with articular cartilage (Sorsa *et al.*, 1992).

Denatured fibrillar collagen (types I, II and III), which has lost its triple helical structure, is susceptible to cleavage by gelatinase. Two forms of gelatinase are expressed by rheumatoid synovial fibroblasts: a 72-kDa form (type IV collagenase) and a 92-kD form (type V collagenase) (Unemori *et al.*, 1991b). The 72-kDa appears to be universally expressed by all fibroblasts with cells of the synovial lining demonstrating increased mRNA expression. Unlike fibroblasts from normal tissues early cultures of RA synovial fibroblasts constitutively express the 92-kDa gelatinase.

Stromelysin has a broader substrate specificity than either the collagenases or gelatinases, since it is able to cleave a number of proteoglycans. Several isoforms of stromelysin have been identified to date (Sirum and Brinckerhoff, 1989). Stromelysin (MMP-3) has been detected at the protein level in the intimal layer of the RA synovium (Okada *et al.*, 1992). In addition, several independent *in situ* hybridization studies have shown that stromelysin mRNA is found exclusively within the intimal layer (Firestein and Paine, 1992; Gravellese *et al.*, 1991). Moreover, cultured RA synovial fibroblasts have been shown to synthesize low levels of stromelysin (Firestein and Paine, 1992; MacNaul *et al.*, 1990). Unlike other matrix metalloproteinases stromelysin can activate latent MMP-1 (Suzuki *et al.*, 1990).

Natural metalloproteinase inhibitors, known as tissue inhibitors of metalloproteinases (TIMP), are also secreted by synovial fibroblasts. mRNA for these inhibitors can be found at comparable levels within the intimal layer of both OA and RA synovium, often being co-expressed with stromelysin in the fibroblast-like cells (MacNaul *et al.*, 1990; Firestein and Paine, 1992; Hembry et al., 1995).

1.6 Cell adhesion molecules

It is now known that the mechanisms of cell-cell and cell-extracellular matrix (ECM) attachment are mediated by a diverse group of molecules known as the cell adhesion molecules. Cell adhesion molecules play a central role in a wide array of biological processes including leukocyte migration, wound healing, embryogenesis, cell differentiation, immune recognition and tumour metastasis. The combination of structural determination, molecular biology and adhesion assays has shown that these cell membrane glycoproteins can be divided into a small number of families (Springer, 1990; Buck, 1992; Hynes, 1992; Lasky, 1995; Takeichi, 1995). The major adhesion molecule families are the cadherins, immunoglobulins, integrins and the selectins (Figure 1.3). Tables 1.1, 1.2, 1.3 and 1.4 show the individual members of each family and their respective ligands/counter receptors.

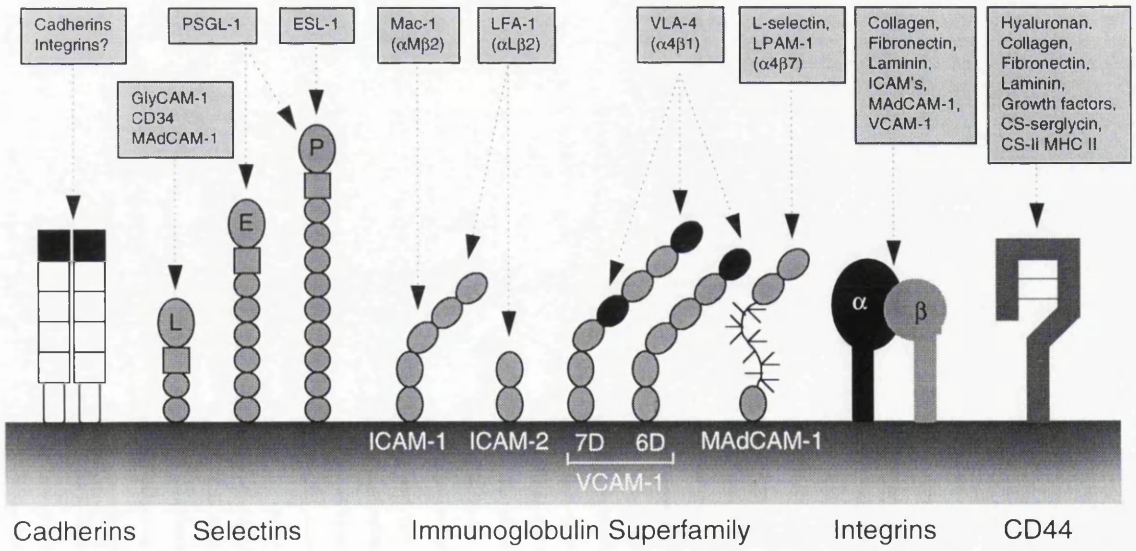


Figure 1.3 The major adhesion molecules and their counter-receptors and ligands (Adapted from Springer, 1995).

1.6.1 Cadherins

The cadherin family provide the adhesive contacts between cells which establish and maintain intercellular connections. They are calcium-dependent transmembrane proteins that bind to each other via homophillic interactions (Takeichi, 1991 and 1995). However, Cepek and co-workers (1994) have recently shown that E-cadherin can also bind αEβ7 an integrin expressed by intraepithelial T-cells. The most common cadherins are shown in Table 1.1. Cadherins are expressed by most adult tissues, with E-cadherin being expressed by epithelial tissues and N-cadherin by neural and muscle tissues. P-cadherin is expressed predominantly in placenta and epithelium, but can also show transient expression in a number of developing tissues. E-cadherin has been shown to be crucial for the tight association between cells in the epithelium. It is believed to act as a tumour suppressor with a loss of expression resulting in an increased invasiveness of transformed epithelial cells, and increased invasiveness and metastasis in certain human tumours (Birchmeier and Behrens., 1994; Takeichi., 1993).

Table 1.1 The cadherin family.

Name	CD nomenclature	Alternative name(s)	Ligands/counter-receptors
E-cadherin		uvomorulin	E-cadherin, αEβ7
N-cadherin		A-CAM	N-cadherin
P-cadherin			P-cadherin

1.6.2 Immunoglobulin superfamily

The immunoglobulin (Ig) superfamily (Table 1.2) is a large group of proteins which are involved in antigen recognition, neural development, leukocyte migration and signal

transduction. Members of this family share a variable number of common immunoglobulin domains. Each domain is composed of between 70 and 110 amino acids ordered into two parallel β sheets stabilized by disulphide bonds (Hunkapiller and Hood., 1989). The vast array of immunoglobulins appears to have evolved by duplication and divergence from a common ancestral immunoglobulin fold.

Table 1.2 The immunoglobulin superfamily.

Name	CD nomenclature	Alternative name(s)	Ligand/counter-receptor
ICAM-1	CD54		Mac-1 (αMβ2)
			LFA-1 (αLβ2)
ICAM-2	CD102		LFA-1 (αLβ2)
ICAM-3	CD50		LFA-1 (αLβ2)
LFA-3	CD58		CD2
N-CAM	CD56		N-CAM
			heparan sulphate
MAdCAM-1			LPAM-1 (α4β7)
			L-selectin
VCAM-1	CD106	INCAM-110	VLA-4 (α4β1)
			LPAM-1 (α4β7)
PECAM-1	CD31		PECAM-1
			αVβ3
	CD2	LFA-2	LFA-3
	CD4		MHC class II
	CD8		MHC class I

Intercellular adhesion molecule (ICAM) -1, ICAM-2 and vascular cell adhesion molecule-1 (VCAM-1) are involved in the adhesion of leukocytes to endothelial cells. ICAM-1 contains five immunoglobulin domains and is most closely related to ICAM-2

which contains two immunoglobulin domains (Staunton *et al.*, 1989). ICAM-1 is constitutively expressed on endothelial cells and can be induced by stimulation with IFN-γ, IL-1β and TNF-α (Dustin and Springer., 1988). ICAM-2 is constitutively expressed by endothelial cells, monocytes and lymphocytes, but unlike ICAM-1 expression it cannot be induced by inflammatory cytokines (de Fougerolles *et al.*, 1991). A third member of the ICAM family, ICAM-3, is expressed by lymphocytes but not endothelial cells (de Fougerolles *et al.*, 1991; de Fougerolles *et al.*, 1993). All three ICAM receptors bind the β2 integrin LFA-1, with ICAM-1 in addition binding Mac-1 (de Fougerolles *et al.*, 1994; Diamond *et al.*, 1990).

VCAM-1 is the counter-receptor for the integrins α4β1 and α4β7 (Chan *et al.*, 1992). The gene for VCAM-1 can be alternatively spliced to give distinct isoforms containing either six or seven immunoglobulin domains (Osborn *et al.*, 1989; Hession *et al.*, 1991). The 7-domain form is the most common and has an extra domain inserted between domains 3 and 4 of the 6D form (Hession *et al.*, 1991). VCAM-1 expression on endothelial cells can be induced by the cytokines IL-1, IL-4, IL-13 and TNF-α (Carlos *et al.*, 1990; Haraldsen *et al.*, 1996; Sironi *et al.*, 1994).

Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) contains a mucin-like region and three immunoglobulin domains, two of which share strong homology with ICAM-1 and VCAM-1 (Briskin *et al.*, 1993). MAdCAM-1 is a counter-receptor for both the integrin α4β7 and L-selectin (Berlin *et al.*, 1993; Berg *et al.*, 1993).

1.6.3 Integrins

The integrins are a family of heterodimeric membrane glycoproteins composed of non-covalently attached α and β subunits (Ruoslahti, 1991; Hynes, 1992). Integrins can be

divided into subfamilies, each with a common β subunit. There are at least 14 α subunits and 8 β subunits with most α subunits associating with more than one type of β subunit. Monoclonal antibodies against the various integrin α and β subunits have shown that they are expressed on a wide variety of cell types with most cells expressing several integrins. Integrins are involved in a wide range of cell-cell and cell-ECM interactions by their ability to bind members of the immunoglobulin superfamily, and components of basement membrane and extracellular matrix (Table 1.3). The integrins bind a wide range of counter-receptors/ligands in a divalent-cation dependent manner. Certain integrins bind to ECM components (collagen (Type I), fibronectin, fibrinogen, laminin and vitronectin) containing the RGD (arginine-glycine-aspartic acid) amino acid sequence (Hynes., 1992). Other integrins, such as α4β1, bind to the variable region (CS-1) of fibronectin which lacks an RGD site (Wayner *et al.*, 1989).

The β1 family of integrins is presently composed of ten members (Stewart *et al.*, 1995). α4β1 (VLA-4) can act both as a mediator of cell-cell and cell-ECM adhesion by binding VCAM-1 and fibronectin, respectively (Chan *et al.*, 1992; Wayner *et al.*, 1989). VCAM-1 and the CS-1 region of fibronectin attach to different binding sites in the VLA-4 receptor (Masumoto and Hemler., 1993). VLA-4 binds to domains 1 and 4 on the seven domain form of VCAM-1 molecule via two different mechanisms. Binding to domain 4 of VCAM-1 requires cell activation or high concentrations of manganese, whereas binding to domain 1 is an activation-independent event (Kilger *et al.*, 1995). VLA-4 is expressed on lymphocytes, monocytes and eosinophils but not neutrophils. VLA-4 has also been shown to mediate lymphocyte attachment and rolling on VCAM-1 (Alon *et al.*, 1995). Furthermore, migration of monocytes across IL-1β, TNF-α and

Table 1.3 The integrin family.

Chain composition	Name	CD nomenclature	Alternative name(s)	Ligand/counter-receptor
α1β1	VLA-1	CD49a/CD29		coll, lam
α2β1	VLA-2	CD49b/CD29	gpIa/IIa; ECMRII	coll, lam
α3β1	VLA-3	CD49c/CD29	ECMRI	fib, coll, lam
α4β1	VLA-4	CD49d/CD29		fib, VCAM-1
α5β1	VLA-5	CD49e/CD29	FNR; gpIc/IIa; ECMRVI	fib
α6β1	VLA-6	CD49f/CD29	gpIc/IIa	lam
αVβ1		CD51/CD29		fib
αLβ2	LFA-1	CD11a/CD18		ICAM-1, -2, -3
αMβ2	Mac-1	CD11b/CD18	CR3	ICAM-1, iC3b, fx, fib
αXβ2	p150,95	CD11c/CD18	CR4	fg, iC3b
αIIbβ3		CD41/CD61	gpIIb/IIIa	fib, fg, vWF
αVβ3		CD51/CD61	VNR	vit, fg, vWF
α4β7	LPAM-1			VCAM-1, fib, MAdCAM-1
α4βp	LPAM-2	CD49d/CD-		?
αVβ5		CD51/CD-		vit, fib
α6β4		CD49f/CD-		?

Abbreviations: ***coll*** : collagens; ***fib*** : fibronectin; ***fg*** : fibrinogen; ***fX*** : factor X; ***lam*** : laminin; ***iC3b*** : inactivated complement component C3; ***vit*** : vitronectin; ***vWF*** : von Willebrand factor; ***?*** : unknown.

lipopolysaccharide (LPS) stimulated endothelium is mediated by VLA-4 binding to either domains 1 or 4 of VCAM-1 (Chuluyan and Issekutz., 1993; Chuluyan *et al.*, 1995).

The β2 family of integrins are more commonly referred to as the 'leukocyte integrins' due to their expression by different leukocytes (Springer., 1995). The β2 chain can associate with four different α chains to give the integrins; lymphocyte function-associated antigen-1 (LFA-1; αLβ2), complement receptor-1 (Mac-1; αMβ2), p150/95 (αXβ2) and αDβ2 (Sanchez-Madrid et al., 1983; Danilenko *et al.*, 1995). LFA-1, Mac-1 and p150/95 mediate leukocyte binding to endothelial cells. β2 integrins on the surface of leukocytes are dynamically regulated by inflammatory mediators. LFA-1 is constitutively expressed on resting lymphocytes but its affinity for ICAM-1 is too weak to allow adhesion. Studies have shown that LFA-1 can be activated to bind ICAM-1 by chemoattractants or receptor cross-linking (Larson and Springer., 1990; Dustin and Springer., 1989; van Kooyk *et al.*, 1989). Mac-1 also requires activation by chemoattractants in order to bind to C3bi, fibrinogen, and ICAM-1 (Detmers *et al.*, 1990; Wright *et al.*, 1988; Smith *et al.*, 1989). Unlike with LFA-1, where cell surface expression levels are unaltered, activation causes a rapid increase in Mac-1 receptors at the cell surface by mobilization from intracellular stores (Miller *et al.*, 1987).

The central role played by the β2 integrins during transendothelial migration can be illustrated by patients suffering from a genetic condition known as leukocyte adhesion deficiency I (LAD-I) (Anderson and Springer., 1987). LAD-I is a recessive inherited genetic disorder caused by an amino acid substitution in a conserved region of the β2 subunit. This substitution prevents the β2 subunit associating with the α

subunits to form functional β2 integrins. Neutrophils in LAD-I patients are unable to cross endothelium and enter sites of inflammation, and as a result LAD-I patients suffer from massive bacterial infections.

1.6.4 Selectins

At sites of inflammation selectins mediate the initial attachment or tethering of moving leukocytes to the endothelium, and their subsequent rolling along its surface. The selectin family (Table 1.4) is composed of three cell surface glycoproteins that are expressed on endothelial cells (P- and E-selectin), leukocytes (L-selectin), and platelets (P-selectin) (Rosen and Bertozzi., 1994). L-selectin is also expressed by eosinophils, neutrophils and monocytes. The selectins are closely related in structure and all contain an amino-terminal C-type lectin, or carbohydrate recognition domain.

Table 1.4 The selectin family.

Name	CD nomenclature	Alternative name(s)	Ligands/counter-receptors
E-selectin	CD62E	ELAM-1	PSGL-1
L-selectin	CD62L	LECAM-1, LAM-1, $gp90^{MEL}$	GlyCAM-1, CD34, MAdCAM-1
P-selectin	CD62P	GMP-140; PADGEM	ESL-1, PSGL-1

Abbreviations: ***GlyCAM-1*** : glycosylation-dependent cell adhesion molecule-1; ***MAdCAM-1*** : mucosal addressin cell adhesion molecule-1; ***PSGL-1*** : P-selectin glycoprotein ligand-1; ***ESL-1*** : E-selectin ligand-1.

The C-type lectin domain of all the selectins binds a group of carbohydrate ligands, the sialylated and fucosylated tetrasaccharide antigens sialyl Lewis x (SLe^x) and sialyl Lewis a (SLe^a) (Lasky, 1995). The biological ligands for selectins are a group of O-glycosylated proteins known as the mucins (Shimizu and Shaw., 1993). Mucins include the HEV-associated ligands for L-selectin, glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) and the sialomucin CD34. Another L-selectin ligand MAdCAM-1 belongs to the immunoglobulin superfamily but contains a mucin-like domain.

Selectin expression at the cell surface is controlled by a number of mechanisms. E-selectin can be induced on vascular endothelium by the inflammatory cytokines IL-1β and TNF-α, or LPS (Bevilacqua *et al.*, 1989). E-selectin expression can be inhibited by TGF-β and IL-4, and the anti-inflammatory agent dexamethasone. In contrast, P-selectin is stored preformed in Weibel-Palade bodies of endothelial cells and the α-granules of platelets. Activation of the endothelium by histamine, thrombin and phorbol esters results in the rapid mobilization of P-selectin to the cell surface (Geng *et al.*, 1990). Unlike E- and P-selectin, L-selectin expression cannot be induced by inflammatory mediators and its mechanism of regulation involves decreasing cell surface expression by cleavage of the extracellular domain (Jutila *et al.*, 1989).

1.6.5 CD44 and other HA-binding proteins

Several proteins have been shown to bind the extracellular matrix component HA. These include the matrix proteins aggrecan, cartilage link protein, hyaluronectin and

versican, and the cell-surface receptors CD44 and receptor for hyaluronan-mediated motility (RHAMM).

CD44 is a broadly expressed family of cell surface glycoproteins which have been implicated in a range of adhesion dependent biological processes (Lesley *et al.*, 1993). CD44 is the major cell surface receptor for HA but can also bind other extracellular matrix components like collagen (type I and VI), fibronectin and laminin (Aruffo *et al.*, 1990; Lesley *et al.*, 1993). Alternative splicing of up to 10 variant exons (v1-v10) into CD44 mRNA, together with post-translational modification can generate a large number of variant CD44 isoforms (Dougherty *et al.*, 1991; Jackson *et al.*, 1992, 1995). The inclusion of variant exons can change the HA-binding capacity of CD44 and can affect the cell phenotype, with insertion of exon v6 increasing metastatic potential and growth of certain rat tumours (Stamenkovic *et al.*, 1991; Van der Voort *et al.*, 1995; Günthert *et al.*, 1991). The ability of CD44 to bind a number of cytokines may implicate CD44 in the presentation of chemokines to leukocytes at sites of inflammation (Bennett *et al.*, 1995a,b; Weber *et al.*, 1996; Tanaka *et al.*, 1993).

RHAMM was first identified in *ras*-transformed fibroblasts where increased cell motility, HA production and HA-binding were seen (Turley *et al.*, 1991). HA binding to RHAMM stimulates the locomotion of *ras*-transformed fibroblasts in a dose-dependent manner (Hall *et al.*, 1994). RHAMM has been shown to mediate the locomotion of astrocytes and microglia, neurite extension and the migration of smooth muscle cells following wounding (Turley *et al.*, 1994; Nagy *et al.*, 1995; Savani *et al.*, 1995). Moreover, overexpression of RHAMM in fibroblasts has been shown to cause anchorage-independent growth and the formation of metastatic tumours (Hall *et al.*, 1995).

1.7 Role of adhesion molecules in inflammation

Leukocytes patrol the body in search of inflammatory stimuli and foreign antigens found at sites of tissue injury or infection. Lymphocytes recirculate from blood, through tissue and into the lymphatic system, and finally back into blood in their quest to search out and destroy foreign antigens. Recirculation is a critical aspect of effective immune surveillance allowing specialized lymphocyte subsets access to specific lymphoid tissues (Springer, 1995; Butcher and Picker, 1996). In contrast, eosinophils, neutrophils and monocytes do not recirculate, but migrate through the endothelium activated in response to inflammatory stimuli, injury and infection. The various classes of adhesion molecules and chemoattractants provide leukocytes with a mechanism which allows them to bind activated endothelium and migrate to sites of inflammation. A three-step model has been proposed which describes the events involved during leukocyte binding to, rolling along, and migration through the endothelium.

The first step is mediated by selectins and involves reversible binding of leukocytes to the endothelium. This initial binding or tethering allows leukocytes to roll along the surface of the endothelium. All the selectins can mediate rolling of leukocytes along stimulated endothelium, but P-selectin appears to dominate during the initial stages. Recent studies have also shown that $\alpha4\beta1$ and $\alpha4\beta7$, via binding to VCAM-1 and MAdCAM-1 respectively, can mediate lymphocyte rolling on endothelium (Alon *et al.*, 1995; Berlin *et al.*, 1995). L-selectin and $\alpha4$ integrins are localized at the tips of lymphocyte microvilli where they provide the initial tethering (von Andrian *et al.*, 1995; Berlin *et al.*, 1995). The reversibility of leukocyte binding is mediated by proteolytic cleavage of L-selectin at a site close to the membrane.

The endothelium displays a broad range of adhesion molecules and chemoattractants in response to inflammatory mediators. Stimulation of endothelium with IL-1β and TNF-α induces E-selectin, ICAM-1 and VCAM-1 expression, and increases leukocyte binding. As leukocytes roll along the surface of the endothelium they become activated by chemoattractant molecules, such as CC chemokines, IL-8, platelet-activating factor, and N-formyl peptides. Recent evidence suggests that chemoattractants, such as macrophage inflammatory protein-1β (MIP-1β), are bound by endothelial CD44 and presented to rolling leukocytes (Tanaka *et al.*, 1993; Bennett *et al.*, 1995a). Chemoattractants bind to the serpentine family of G-protein-coupled receptors on leukocytes, resulting in cell activation and spreading (Bargatze and Butcher., 1993). Leukocyte activation causes a change in the conformation of integrins that results in increased affinity for ligand, and receptor clustering. N-formyl peptide and IL-8 are able to activate LFA-1 and Mac-1 on lymphocytes and neutrophils (Larson and Springer., 1990). Integrin activation allows leukocyte arrest and firm adhesion via integrin-immunoglobulin interactions. Tight adhesion and flattening of leukocytes allows transmigration through the endothelium. Transendothelial migration is mediated by LFA-1/ICAM-1, VLA-4/VCAM-1, CD44 and also by CD31 (PECAM-1). The type of interaction that is utilized may depend on the type of stimulus received by the endothelium. Transmigration of leukocytes is mainly mediated by the interaction of LFA-1 with ICAM-1 (Chuluyan and Issekutz, 1993; Oppenheimer-Marks *et al.*, 1991). However, when endothelium is stimulated with either IL-1β or TNF-α, the VLA-4/VCAM-1 interaction is utilized in monocyte migration (Chuluyan and Issekutz, 1993; Chuluyan *et al.*, 1995).

1.8 Adhesion molecules in the pathogenesis of RA

Infiltration of peripheral blood monocytes and lymphocytes into the synovial membranes, and accumulation of neutrophils in the synovial fluid is a key feature of RA. The entry and differential distribution of these various inflammatory cell types is now known to result from the interaction of various adhesion molecules up-regulated by the inflammatory reaction occuring in the joint. Immunohistochemical studies have demonstrated increased expression of a variety of cell adhesion molecules within RA synovium (Johnson *et al.*, 1993; Wilkinson *et al.*, 1993a; Haynes *et al.*, 1991) (Table 1.5). Adhesion molecules are also present in a soluble form in peripheral blood and synovial fluid of RA patients. Whether soluble adhesion molecules carry out any function or are just a result of receptor turnover due to increased tissue cellularity is not currently known.

In RA synovium, endothelial cells within post-capillary venules show altered morphology and begin to resemble lymphoid high endothelial venules (HEV). HEV are specialized postcapillary venules which, via their adhesion molecule repertoire, mediate peripheral blood leukocyte trafficking through lymphoid tissues. These changes in endothelial cell phenotype can be mimicked by exposure to pro-inflammatory cytokines (Munro *et al.*, 1989). The expression of adhesion molecules by inflamed synovial endothelial cells and leukocytes probably determines leukocyte access to the surrounding tissue. Endothelial cells in the rheumatoid synovium have been shown to express the adhesion molecules ICAM-1, ICAM-2, E-selectin and P-selectin (Szekanecz *et al.*, 1994; Morales-Ducret *et al.*, 1992; Koch *et al.*, 1991c; Johnson *et al.*, 1993). Lymphocytes are known to selectively migrate to inflamed synovium, and individual T

cell subsets are thought to acquire tissue specific adhesion molecule repertoires during the differentiation process from naive to memory cells.

Table 1.5 Adhesion molecule expression within inflamed synovium.

Adhesion molecule	Cell type	Expression	Reference
α4β1	M, L	↑ in RA	Laffón et al., 1991 El-Gabalawy and Wilkins, 1993
α4β7	L	Differentially expressed	Lazarovits and Karsh, 1993
CD31	EC	No up-regulation	Johnson *et al.*, 1993
	M	↑ in RA	
CD44	FLS, M	↑ in OA and RA	Haynes *et al.*, 1991 Johnson *et al.*, 1993
E-selectin	EC	↑ in RA	Fairburn *et al.*, 1993 Koch et al., 1991c
P-selectin	EC	No up-regulation	Johnson *et al.*, 1993
ICAM-1	EC, FLS	↑ in OA and RA	Fairburn *et al.*, 1993; Szekanecz *et al.*, 1994 Koch *et al.*, 1991c
	M	↑ in RA	
ICAM-2	EC	No up-regulation	Szekanecz *et al.*, 1994
ICAM-3	?M, L	-	El-Gabalawy *et al.*, 1994; Szekanecz *et al.*, 1994
VCAM-1	FLS	↑ in OA and RA	Morales-Ducret *et al.*, 1993
	EC	Low expression	Wilkinson *et al.*, 1993a

Abbreviations: **EC** : endothelial cell; **M** : macrophage; **L** : lymphocyte; **FLS** : fibroblast-like synoviocyte; ↑ :denotes increased expression in disease.

The differential distribution of α4 integrin-expressing cells between synovial tissue and synovial fluid has suggested an important role for these adhesion molecules

in the pathogenesis of RA. In contrast to neutrophils found in the synovial fluid, RA synovial tissue lymphocytes and macrophages express high levels of VLA-4 ($\alpha4\beta1$) (Laffón *et al.*, 1991; El-Gabalawy and Wilkins, 1993). Moreover, studies utilizing lymphocyte binding to frozen sections of RA synovium have indicated that $\alpha4$ integrins are involved in lymphocyte adhesion to synovial endothelium (Van Dinther-Janssen *et al.*, 1991). However, given the paucity of VCAM-1 expression by synovial endothelium the likely endothelial counter-receptor for $\alpha4\beta1$ appears to be the CS-1 isoform of fibronectin (Wilkinson *et al.*, 1993a; Elices *et al.*, 1994). The localization of CS-1 on the luminal surface of RA synovial endothelium may provide the preferred migratory mechanism of $\alpha4\beta1$-positive cells into the inflamed synovium (Elices *et al.*, 1994). $\alpha4\beta7$, an $\alpha4$ integrin known to participate in leukocyte rolling, has been shown to be differentially expressed by RA synovial lymphocytes (Lazarovits and Karsh., 1993). Lazarovits and Karsh found that 62% of RA synovial membrane T cells expressed elevated amounts of $\alpha4\beta7$ compared to synovial fluid and peripheral blood lymphocytes (4.7% and 9.1%, respectively). It is possible that the differential expression of $\alpha4\beta7$ may explain the selective localization of T cells in the RA synovium. However, it is not currently known whether MAdCAM-1, the main counter-receptor for $\alpha4\beta7$, is present on inflamed synovial endothelium. Mononuclear cell migration into human rheumatoid synovium can also occur via interactions with ICAM-1 (Jorgensen *et al.*, 1996a, b). In these studies labelled human mononuclear cells were injected into SCID mice engrafted with human RA synovium. Labelled cells were found to migrate into, and accumulate within the synovial grafts, with migration into the synovial graft being blocked by antibodies against ICAM-1 and its counter-receptor LFA-1.

Accumulating evidence suggests that CD44 may play a crucial role in the pathogenesis of RA since it is known to function as a lymphocyte homing receptor, stimulate monocyte IL-1 production, bind chemoattractants and is involved in leukocyte transendothelial migration (Mikecz *et al.*, 1995; Gruber *et al.*, 1992; Tanaka *et al.*, 1993; Streeter *et al.*, 1988). CD44 expression is up-regulated in RA synovium compared to normal synovium (Haynes *et al.*, 1991; Johnson *et al.*, 1993). Moreover, Mikecz and co-workers (1995) have shown using a murine model of RA that CD44 plays a crucial role in leukocyte migration into the inflamed synovium, with anti-CD44 antibody treatment leading to a reduction in joint oedema, leukocyte infiltration, and loss of leukocyte CD44 (Mikecz *et al.*, 1995).

1.9 Studying the pathology of RA: experimental animal models and *in vitro* studies

1.9.1 Experimental animal models of arthritis

Studies using experimental models of arthritis have allowed great inroads to be made in understanding the pathogenesis of rheumatoid arthritis. They also provide a useful system for the development and evaluation of novel therapeutic agents for use in the treatment of inflammatory joint disease.

The traditional animal models utilize arthritogenic compounds to induce acute and chronic inflammatory arthritis in susceptible strains of mice and rats. Of these, the most widely used models are streptococcal cell wall (SCW) arthritis in rats, adjuvant-induced arthritis in rats, collagen-induced arthritis (CIA), and antigen-induced arthritis (AIA) in mice (see Table 1.6) (Crofford and Wilder, 1997).

Table 1.6 Commonly used animal models of experimental arthritis

Model	Commonly used eliciting agent	Administration	Form of arthritis	Acute phase	Chronic phase
SCW arthritis	Cell wall fragments from *S pyogenes*	Intraperitoneal	Polyarthritis	Yes Within 72 hrs	Yes After 2-4 weeks
Adjuvant-induced	Heat-inactivated *M tuberculosis*	Intradermal	Polyarthritis	No	Yes After 10-12 days
Collagen-induced (CIA)	Native type II collagen (CII)	Intraperitoneal /Intradermal	Polyarthritis	No	Yes After 10-16 days
Antigen-induced (AIA)	Fibrin Ovalalbumin	Systemic immunization then intraarticular challenge	Monoarticular	Yes After 2 hours	Yes After 2 weeks

The arthritogenic agent is usually administered in a mineral oil emulsion by intradermal injection into the base of the tail, or by injection into the peritoneal cavity. In most cases, polyarthritis is induced in the distal peripheral joints, in particular the ankles, wrists and interphalangeal joints. SCW and AIA animals, in contrast to adjuvant-induced and collagen-induced models, develop biphasic arthritis (Crofford and Wilder, 1997). The acute phase, apparent within hours of injection, is characterized by swelling and erythema of the affected joints. Acute arthritis peaks after 1-3 days and thereafter gradually subsides. This is followed after 2-6 weeks by the chronic form of arthritis. All the commonly used experimental models exhibit chronic destructive forms of arthritis. As with human RA, the chronic phase is characterized by hyperplasia and hypertrophy of the synovium. Moreover, pannus formation leads to invasive destruction of articular cartilage and bone.

Experimental models show many other similarities and differences to human RA (Trentham, 1982; Wilder, 1988). For instance, SCW rats are deficient in IL-2, exhibit the characteristic waxing and waning of human disease, and show greater susceptibility to disease in female rats. In contrast, however, they do not show high serum levels of rheumatoid factor or the development of rheumatoid nodules. Whereas, AIA animals possess lymphoid follicles, a characteristic of RA synovium, and can also experience arthritic 'flare-ups' when challenged with small amounts of antigen. Although they have provided useful insights into possible disease mechanisms, the traditional models of experimental arthritis have never been fully able to reconstruct the disease seen in human RA. This is not entirely suprising given the differing time course in the onset of arthritis between experimental and rheumatoid arthritis: human RA evolves slowly over a number of years while chronic experimental arthritis is apparent 2-4 weeks after its induction.

Spontaneous arthritis can also develop in certain inbred strains of mice, such as MRL-*lpr/lpr*. Homozygous MRL-*lpr/lpr* mice possess a mutation in the Fas gene that can lead to a spontaneous symmetrical arthritis of the hind legs (O'Sullivan *et al.*, 1985). As with rheumatoid arthritis, these animals demonstrate invasive and destructive lesions within bone and articular cartilage. Moreover, MRL-*lpr/lpr* mice show increased levels of autoantibodies, immune complexes and rheumatoid factor. This type of arthritis is characterized by the absence of inflammatory cells from the arthritic synovium until after cartilage destruction has occurred. MRL-*lpr/lpr* also develop other autoimmune conditions, such as proliferative glomerulonephritis and polyarteritis. Although in some respects similar to rheumatoid arthritis, the presence of multisystem

autoimmune disease has meant that MRL-*lpr/lpr* mice are more commonly used as a model for systemic lupus erythematosus.

Recent advances in molecular biology have prompted the development of a wealth of new experimental models. The advent of transgenic animals, where an individual gene is overexpressed by all cell types, has enabled the roles of specific genes, like those for the major histocompatability molecules, to be studied (Rosloniec *et al.*, 1997; Keffer *et al.*, 1991; Iwakura *et al.*, 1991). The importance of TNF-α in arthritis was shown when TNF-α transgenic mice developed a highly destructive polyarticular form of chronic arthritis (Keffer *et al.*, 1991). Transgenic technology may also unearth interesting models that highlight the role of different cell types in arthritis. For instance, a highly destructive arthritis that is mediated by chondrocytes and fibroblast-like cells, whilst also being independent of lymphocytes, can be generated when TNF-α transgenic mice are backcrossed with mice susceptable to collagen-induced arthritis (Butler *et al.*, 1997).

The development of the severe combined immunodeficient (SCID) mouse has provided an excellent system for determining cellular involvement in the destructive process of RA. SCID mice lack functional T and B cells and so enable studies where engraftment of human synovial tissue or synovial cells are required (Geiler *et al.*, 1994; Mima *et al.*, 1995; Muller-Ladner *et al.*, 1996). Co-implantation of human cartilage explants with quiescent cultures of FLSs into SCID mice has shown that RA fibroblast-like cells possess an inherently invasive phenotype (Muller-Ladner *et al.*, 1996). The SCID mouse will no doubt provide an indispensible *in vivo* system for future probing of the mechanistics of rheumatoid arthritis.

1.9.2 *In vitro* studies with fibroblast-like synoviocytes

Interaction with leukocytes

Synovial fibroblasts can be removed from the synovial membrane by collagenase treatment and the cells can be kept in culture for a number of weeks. *In vitro* conditions have been used to study the consequences of synovial fibroblast-leukocyte interactions. These co-culture studies have given important information on the role of the fibroblast-like synoviocytes in the activation of monocytes and lymphocytes (Chomarat *et al.*, 1995b; Tsai *et al.*, 1996). In particular, the role of VCAM-1 could be studied in this context. However, data on VCAM-1 expression on cells in culture show remarkably lower levels than those found in freshly isolated cells, meaning that VCAM-1 must be lost in culture. The absence or reduced levels of macrophage cytokines like IL-1β and TNF-α in culture has long been regarded as the reason why VCAM-1 levels decline once cells are removed from the joint (Feldmann *et al.*, 1996; Firestein, 1996). Indeed, treatment of cultured fibroblast-like synoviocytes with either IL-1β or TNF-α results in up-regulation of VCAM-1 expression. In addition, these cytokines also increase the production of IL-6, PGE_2 and GM-CSF (Morales-Ducret *et al.*, 1992; Dayer *et al.*, 1986, 1985; Tan *et al.*, 1990; Alvaro-Gracia *et al.*, 1990). However, in all cases they only evoke a transient response. Surprisingly, none of these studies have examined long-term exposure to any of the cytokines. Thus, although VCAM-1 demonstrates constitutively elevated expression on intimal synovial cells *in situ*, treatment of cultured FLSs with IL-1β or TNF-α only increases expression for 24-48 hours (Morales-Ducret *et al.*, 1992). This transient induction of VCAM-1 by cultured fibroblast-like synoviocytes would suggest that other factors are likely to be responsible for the

phenotype as observed *in situ*. A possible candidate is IL-4 because this was shown, in the presence of TNF-α, to increase both the level and duration of VCAM-1 expression in endothelial cells (Iademarco *et al.*, 1995). In endothelial cells, IL-4 achieves this effect by reducing the degradation of VCAM-1 mRNA transcripts. It was also shown to potentiate TNF-α-induced VCAM-1 expression in fibroblast-like synoviocytes (Edwards *et al.*, 1997b). However, in the latter study neither the effect on the time course of expression or the mechanism of action was studied. Therefore, further understanding of the regulation of VCAM-1 expression in fibroblast-like synoviocytes, both at the mRNA and protein level, is clearly required.

Cell biology of the fibroblast-like synoviocyte

In vitro studies can also be used to identify factors, extrinsic or intrinsic, which maintain the aggressive, proliferative phenotype of fibroblast-like synoviocytes. Certain RA FLS characteristics like high proliferation rate, constitutively elevated production of IL-6 and the ability to invade cartilage matrix, are maintained even in the absence of cytokine treatment (Anastassiades *et al.*, 1978; Chen *et al.*, 1998; Miyazawa *et al.*, 1998; Müller-Ladner *et al.*, 1996). Sustained IL-6 production appears to result from constitutive activation of the IL-6 gene by the transcription factors NF-κB and CBF-1 (Miyazawa *et al.*, 1998). This may result from genetic transformation of fibroblast-like synoviocytes during the course of RA, since transformation of FLSs with SV40 virus enhances the expression of IL-6 mRNA transcripts (Miyazawa *et al.*, 1998; Shimozato *et al.*, 1996).

Increased proliferation and the capacity to invade cartilage also indicate some kind of cellular transformation (Firestein, 1996; Müller-Ladner *et al.*, 1996). Like certain tumor cells fibroblast-like synoviocytes can, in the presence of specific growth

factors, proliferate in an anchorage-independent manner, escape contact inhibition and form microfoci *in vitro* (Lafyatis *et al.*, 1989b). One mechanism by which the cells can maintain this transformed state is via somatic mutations in the p53 protein, through which they escape inhibition of DNA replication (Aupperle *et al.*, 1998: Firestein *et al.*, 1997). Altered proliferation and cell matrix interactions may also be a consequence of altered expression of certain adhesion molecules. For instance, it was shown in mouse pancreatic tumours that the inclusion of variant exons v4-v7 into CD44 resulted in metastatic behaviour of these cells (Günthert *et al.*, 1991). This finding created great interest in splice variants of CD44, in particular for their possible role in human tumour progression. Splice-variants are clearly implicated in cancer pathology but a direct correlation between their presence and changes in cell invasion or proliferation have not yet been shown (see for instance Koopman *et al.*, 1993; Driessens *et al.*, 1995; Dall *et al.*, 1996). Nevertheless, inclusion of exons v4-v7 increases the affinity for the main substrate hyaluronan, and also enables CD44 to bind to a larger range of glycosaminoglycans (Sleeman *et al.*, 1997). Splice variant expression has not yet been studied in fibroblast-like synoviocytes. Given the evidence that fibroblast-like synoviocytes are somehow transformed, changes in CD44-splice variant expression may also be apparent. These changes could have consequences for the behaviour of fibroblast-like synoviocytes.

1.10 Aims of the study

The aims of this thesis are as follows:

1. To attempt to restore constitutively elevated VCAM-1 expression by *in vitro* cultures of RA fibroblast-like synoviocytes.
2. To investigate CD44 splice variant expression in fibroblast-like synoviocytes from control, OA and RA tissues.
3. To study the functional implication of CD44 splice variant expression, with emphasis on proliferation.

1.11 Scientific approach

Aim 1

Fibroblast-like synoviocytes will be brought into *in vitro* culture and the time course of VCAM-1 expression determined. The role of various matrix components, like collagen, fibronectin and hyaluronan, will be tested on the expression of VCAM-1. Similar experiments will be performed with various combinations of the cytokines IL-4, IL-13, TNF-α, and IL-1β. VCAM-1 transcripts will be analyzed by a semi-quantitative RT-PCR protocol and cell surface protein expression will be quantified using a flow cytometry protocol.

Aim 2

The expression of CD44 splice variants will be analyzed in freshly isolated fibroblast-like synoviocytes using a double labelling immunocytochemistry protocol utilizing antibodies against VCAM-1, combined with various antibodies against CD44 splice

variants. In this way, the FLS can be identified through its VCAM-1 expression. Cells will also be cultured for various periods of time and CD44 splice variant expression will be measured both at the mRNA transcript level using RT-PCR, or at the protein level using immunocytochemistry. This should provide information about the level of expression with time. To address the question of what splicing combinations are expressed, VCAM-1-positive cells will be selected from primary cultures of fibroblast-like synoviocytes (day 14) using flow cytometry, and CD44 mRNA transcripts determined by Southern blotting using DNA probes against the variant CD44 exons.

Aim 3

This will briefly focus on the role of CD44 splice variants in the proliferation of FLSs. The total cell population will be separated on the basis of CD44 splice variant expression using a magnetic bead panning protocol. Cell proliferation rates of the various populations will be assessed. Total cell cultures will also be treated with exon-specific antibodies and the effect on ^{3}H-thymidine incorporation will be measured.

Chapter 2

Methods and materials

The basic methods utilized in this thesis are outlined below. At the start of each results chapter any additions or alterations to these methods are briefly explained.

2.1 Human cell lines and tissues

Human synovial tissue was kindly provided by the orthopaedic surgeons of University College London Hospital, London and the Whittington Hospital, London. Peripheral blood was obtained from healthy male AB donors. Human dermal fibroblasts and U937 cells were kindly provided by Dr. Patricia Perkis-McKay (Department of Dermatology, Royal London Hospital) and Dr. Peter Parker (Imperial Cancer Research Fund, Lincoln's Inn Fields, London), respectively.

2.2 Preparation of human plasma-derived serum (HPDS)

Peripheral human blood was obtained by venipuncture from healthy male AB donors. Fresh citrated blood was centrifuged (10,000 x g; 20 min; 4°C) and the supernatant (plasma) recovered. In order to test for platelet contamination, an aliquot of the plasma was added to an equal volume of Trypan blue (0.4% w/v) and any cells were counted in an improved Neubauer haemocytometer. If platelets were detected, the plasma was re-centrifuged (10,000 x g; 20 min; 4°C). $CaCl_2$ was added to the plasma to give a final

concentration of 4 mM. This was left to stand overnight at 4°C. Plasma was heated (54°C; 30 min) to inactivate the complement cascade, and centrifuged (10,000 x g; 20 min; 4°C) to remove any insoluble material. The supernatant (serum) was recovered, separated into aliquots and stored at -70°C prior to use. This plasma product is referred to as human plasma-derived serum (HPDS).

2.3 Isolation of fibroblast-like synoviocytes

Normal synovium was obtained from knee joints of patients undergoing amputation for sarcomata of the lower limb. Synovial tissue was taken from macroscopically normal sites at least 30 cm from the tumour. Diseased synovial tissue was collected from patients suffering from osteoarthritis (OA) and rheumatoid arthritis (RA) at the time of total hip, knee or shoulder joint replacement surgery. All tissues were processed within 1-2 hours following surgery.

Normal synovium was extensively washed with phosphate-buffered saline (PBS; w/o Ca^{2+} and Mg^{2+}) and treated with 2.5% trypsin plus EDTA for 1 hour at 37°C. The synovial surface was lightly scraped and the cell suspension grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with foetal bovine serum (FBS) (10% v/v) and penicillin-streptomycin solution (1% v/v).

Diseased synovium was dissected away from the surrounding joint capsule and washed extensively with PBS. The tissue was then chopped into small pieces (4-5 mm^2) and washed in DMEM to remove as much blood as possible. Any remaining fat and connective tissue was then dissected away. The synovium was finely chopped (1-2 mm^3) and digested in serum-free DMEM containing collagenase (2 mg/ml) and penicillin-streptomycin (1% v/v) for 1 hour at 37°C in a shaking incubator. The cell

suspension was sheared by repeatedly passing through a sterile syringe. The cell suspension was filtered through sterile nylon gauze (100 μm) to remove any undigested material. Synovial cells were recovered by cenrifugation (150 x g; 4 min; room temperature). The resultant cell pellet was washed three times in PBS containing bovine serum albumin (BSA) (1% w/v). The cell pellet obtained after the final wash was resuspended in DMEM containing FBS (10% v/v) and penicillin-streptomycin (1% v/v). The total number of cells isolated and the viability of the cells was determined by incubation of an aliquot of the cell suspension with an equal volume of Trypan blue (0.4% w/v). The viability of the cells was usually between 70-85%. The synovial cells were plated into large tissue culture flasks and incubated at 37°C for 18 hours in a humidified 5% CO_2 atmosphere. Non-adherent cells were removed by washing extensively with PBS. Adherent fibroblast-like synoviocytes (FLSs) were cultured as described below in section 2.4.

For double-labelled immunofluorescence studies, freshly dispersed synovial cells were plated on 8-well chamber slides (Nunc) at a concentration of 0.1×10^6 cells per well and incubated for 4 hours at 37°C. Non-adherent cells were washed away and the remaining adherent cells were fixed as described below in section 2.5.

2.4 Cell culture

Adherent cells were washed extensively in PBS and allowed to grow in fresh medium until nearly confluent. This usually took 2-3 days, after which the synovial cells were removed by trypsinization. In brief, adherent cells were washed with PBS and 2 ml of 2.5% trypsin-EDTA solution was added. This was rinsed over the surface of the cells

and then removed by aspiration. The trypsin-treated cells were inverted and incubated at 37°C for 5 min. DMEM supplemented with 10% v/v FBS (10 ml) was added to quench the enzyme reaction. FLSs were either seeded in a ratio of 1:3 for further growth, or were seeded into 6 well tissue culture plates at a density of 0.2×10^6 cells per well. These cells were incubated for 7 days (or longer time points) in DMEM containing FBS (10% v/v) or HPDS (10% v/v) and penicillin-streptomycin (1% v/v) at 37°C in a humidified 5% CO_2 atmosphere. Fresh medium was replaced every 5 days, but cells were not passaged during this period.

For cytokine stimulation experiments FLSs were used between passages 3 through 10 (where each passage was between 7-10 days). IL-1β, IL-4, IL-13 or TNF-α were added to fresh DMEM plus FBS (10% v/v) to give a final cytokine concentration of 10 ng/ml. FLSs were incubated with either medium only, medium supplemented with individual cytokines, or medium supplemented with specific cytokine combinations for the required time. For chronic cytokine treatment, medium alone or medium supplemented with cytokines was replaced every third or fourth day. For studies looking at mRNA stability, FLSs were cultured in medium only and medium supplemented with either IL-4, IL-13 or TNF-α alone, or in combination for either 16 hours or 10 days prior to the addition of actinomycin D (5 μg/ml).

For immunostaining, cells were plated on 4-well chamber slides at a concentration of 4×10^4 cells per well and incubated for 10 days at 37°C.

Human dermal fibroblasts were cultured under identical conditions as human FLSs. They were grown in DMEM containing FBS (10% v/v) and penicillin-streptomycin (1% v/v), with the medium being replaced every 5 days. When cells had

reached confluence they were removed by trypsin-EDTA treatment, split at a 1:3 ratio and recultured in fresh medium. Human dermal fibroblasts were used between passages 3 and 9, where each passage was between 5 and 7 days.

U937 cells were maintained in RPMI medium supplemented with FBS (10% v/v) and penicillin-streptomycin (1% v/v). U937 cells were passaged every 3rd day and reseeded at a density of 0.2×10^6 cells per ml of medium. U937 cells (0.1×10^6 cells) were cultured in DMEM containing FBS (10% v/v) alone or supplemented with hyaluronan (1 mg/ml), interferon-γ (200 Units/ml), or retinoic acid-vitamin D_3 (10^{-7} M) for 4 days. Alternatively, U937 cells were cultured for 4 days in conditioned medium (CM), recovered from primary cultures of RA synovial fibroblasts, supplemented with FBS (10% v/v). U937 cells were recovered by centrifugation and total RNA isolated as described in section 2.8.

2.5 Immunohistochemistry

Cells cultured on 4-well chamber slides were washed with PBS and fixed in ice-cold absolute methanol (4 min) followed by ice-cold acetone (1 min). Fixed cells were washed in PBS and incubated with FBS (10% v/v in PBS). After washing four times in PBS, cells were incubated (60 min; room temperature) with the appropriate primary antibody (either mouse anti-human monoclonal antibodies or rabbit anti-human antisera). Cells were washed twice with PBS, endogenous peroxidases were quenched with 3% hydrogen peroxide in methanol, and incubated (30 min; room temperature) with the secondary biotinylated antibody (either anti-mouse or anti-rabbit $F(ab')_2$). Visualization of the immunocomplex was performed with horseradish-peroxidase that had been coupled to biotin as a streptavidin-biotin-peroxidase complex. After

incubation with the streptavidin-biotin-peroxidase complex for 30 min the cells were incubated with 3,3-amino-9-ethylcarbazole for 10 min and the reaction stopped in H_2O. The cell nuclei were counterstained with hematoxylin, mounted and evaluated by microscopy.

Double-labelling immunofluorescence studies were performed on adherent synovial cells (4 hours post dispersion) to determine the cell type and intensity of staining of CD44 variant-positive cells. Cells were fixed as above and incubated (30 min; room temperature) with FBS (10% v/v) to block any non-specific binding. Cells were washed twice in PBS and incubated (45 min; room temperature) with primary antibody (mouse anti-human antibodies). Cells were washed twice with PBS and incubated (30 min; room temperature) with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG. They were then washed with PBS and incubated (45 min; room temperature) with biotin-labelled mouse anti-human VCAM-1. Cells were washed with PBS and incubated (30 min; room temperature) with a streptavidin-Texas Red conjugate. Cells were extensively washed with PBS, immersed in H_2O briefly, and then mounted in Fluoromount-G. Control stainings were performed by omitting the primary antibody to confirm that following blocking with 10% FBS no cross interference occurred. Microscopy was performed using a Zeiss microscope.

2.6 Fluorescence-activated cell sorting (FACS)

Following the required time in culture synovial fibroblasts were washed twice with ice-cold PBS. FLSs were harvested by incubation with PBS-4 mM EDTA (20 min; 4°C). Most of the cells rounded up following this treatment and could be removed by gentle agitation. Any cells which failed to detach were removed with gentle scraping. The cell

suspension was centrifuged (572 x g; 1.6 min) and the supernatant removed. The cells were resuspended in an appropriate volume of human AB serum (3% v/v), separated into aliquots, and incubated (20 min; 4°C). Cells were then washed in PBS containing BSA (1% w/v) and incubated (40 min; 4°C) with the respective primary antibody (10-25 μg/ml). Cells were washed twice with PBS-BSA (1% w/v) and incubated with phycoerythrin (PE)-conjugated Fab_2 anti-mouse IgG. Cell surface markers were measured using a FACScalibur flow cytometer (Becton Dickinson). Expression of these antigens is shown as mean fluorescence intensity (MFI) and/or % positive cell population per 5000 cells analysed.

For VCAM-1-selection studies, VCAM-1-positive cells were sorted using a FACSvantage flow cytometer (Becton Dickinson), centrifuged, and total RNA isolated as described in section 2.8 Total RNA isolation.

2.7 Cytokine analysis

Commercially available sandwich enzyme-linked immunosorbant assay (ELISA) kits were used to determine IL-1β and TNF-α levels. Supernatant samples were collected from cultures of RA FLSs at 6 days or 14 days post dispersion, and centrifuged (500 x g; 1 min) to remove any cellular debris. The supernatants were transferred to Eppendorf tubes and stored at -20°C. To ensure that supernatants under test met the concentration parameters of each kit, preliminary studies were performed with undiluted samples together with serial dilutions. If necessary, samples were diluted in order to fall within the concentration range of the standard curve. Cytokine standards were prepared by dissolution in an appropriate calibrator diluent and serial dilutions made. Samples under

test and dilution standards were aliquoted (200 µl/well) in duplicate into the cytokine microtiter plate and incubated (120 min; room temperature). Each well was washed three times with 400 µl of wash buffer and then incubated with 200 µl of cytokine conjugate for 60 min at room temperature. The wells were again washed three times with 400 µl of wash buffer and substrate solution (200 µl) added. This was left at room temperature for 20 min and then the reaction was stopped by the addition of 50 µl of stop reagent. The optical density of the solution in each well was recorded on a spectrophotometer at 450 nm with wavelength correction set at 540 nm. For each cytokine standard dilution series a standard curve was constructed of corrected absorbance versus [Cytokine] (pg/ml). From the absorbance value obtained for each sample the corresponding cytokine concentration could be obtained from the standard curve. To correct for sample dilution the concentration obtained from the standard curve was multiplied by the respective dilution factor.

2.8 Total RNA isolation

Total RNA was isolated from fresh synovium, cultured FLSs, cultured dermal fibroblasts, and U937 cells using the acid guanidinium-phenol extraction method described by Chomczynski and Sacchi (1987). Solutions for RNA isolation were made up using sterile diethyl pyrocarbonate (DEPC)-treated water. All procedures were performed on ice using RNAse-free reagents and materials.

RNA was isolated using a monophasic solution of phenol and guanidinium isothiocyanate (GIT). This was composed of 10 ml of GIT solution (4 M guanidinium isothiocyanate; 25 mM sodium citrate, pH 7.0; 0.5% w/v sarcosyl; 0.1 M 2-

mercaptoethanol), 10 ml of water-saturated phenol, and 1 ml of 2 M sodium acetate (pH 4.0). This solution (2 ml for < 10 x 10^6 cells) was added directly to the aspirated culture dish for adherent cells, or to the cell pellet for suspension cells. The cell lysate was sheared by repeatedly passing it through a sterile syringe fitted with a hypodermic needle (21 gauge). To remove cellular debris and high molecular weight DNA the lysate was centrifuged (12,000 x g; 10 min; 4°C). The supernatant was recovered and left to stand (5 min; room temperature). Chloroform (400 µl) was added to the supernatant and shaken thoroughly. The suspension was left to stand (20 min; 4°C) and then centrifuged (12,000 x g; 20 min; 4°C). The suspension was seen to separate into 3 phases: an aqueous upper phase containing the RNA; an interphase containing DNA; and a lower organic phase. The aqueous layer was recovered, taking care not to disturb the interphase, and propan-2-ol (100 µl) added. The solution was mixed and centrifuged (12,000 x g; 10 min; 4°C). The supernatant was recovered and 2 µl of glycogen (20 µg/ml) added. Propan-2-ol (900 µl) was added, mixed thoroughly, and left to stand (60 min; -20°C). The precipitated RNA was recovered by centrifugation (12,000 x g; 20 min; 4°C) and the supernatant removed. The RNA pellet was allowed to air dry (5 min; room temperature) and dissolved in GIT solution (400 µl). Propan-2-ol (400 µl) was added, mixed and left to stand (60 min; -20°C). The RNA was recovered by centrifugation (12,000 x g; 20 min; 4°C) and washed three times with 75% ethanol. RNA pellets were dissolved in DEPC-treated water (20 µl), heated (10 min; 60°C) to facilitate dissolution, and stored at -70°C until used.

The concentration and quality of each RNA sample was determined by measuring absorbance, and by electrophoresis on a 1.2% denaturing agarose gel. For

each sample, total RNA (3 µl) was mixed with DEPC-treated water (600 µl) and added to an RNAse-free quartz cuvette. The absorbance (A) was determined at 260 and 280 nm using a Beckman DU 50 spectrophotometer. RNA concentrations were calculated using the following calculation:

$$[RNA]\ (ng/\mu l) = A_{260} \times 40 \times 200$$

where A_{260} represents the absorbance at 260 nm, 40 is the extinction coefficient for RNA (1 mg/ml) at 260 nm, and 200 is the dilution factor (3 µl: 600 µl). RNA purity was estimated from the A_{260}/ A_{280} value. Values greater than 1.7 indicated that samples were free from contaminating DNA and proteins.

Electrophoresis on a 1.2% denaturing agarose gel was used to determine RNA quality. In brief, RNA samples (1 µg) were made up to 3 µl with DEPC-treated water, RNA sample loading buffer (6 µl) added, and the solution heated (10 min: 65°C). The solution was placed on ice prior to loading on a denaturing formaldehyde agarose gel (for composition see 2.19.1). The gel was set up in the electrophoresis tank (Pharmacia Biotech HE 99X Max) filled with 1× MEM. In order to determine the RNA band sizes a 0.24-9.5 Kb RNA ladder was also loaded. The samples were electrophoresed (65 V) until the lower bromophenol blue band had migrated two thirds of the length of the gel. RNA bands were visualized by UV transillumination (300 nm). The ribosomal RNA band 28S should be approximately twice as intense as the lower 18S band. RNA degradation was observed as an increase in staining intensity of the 18S band accompanied by smearing between these two bands. Only RNA showing no significant degradation was used for RT-PCR.

2.9 Reverse transcription of mRNA

Total RNA was reverse transcribed using the Moloney leukemia virus (M-LuLv) reverse transcriptase and an oligo (dT) primer to generate first strand cDNA. Each RNA (2 μg) was made up to 33 μl with DEPC-treated water and heated (5 min; 65°C). Both the RNA solution and the first-strand reaction tube (containing *Not* I-d$(T)_{18}$ 5'-d[AACTGGAAGAATTCGCGGCCGCAGGAAT_{18}]-3', dATP, dCTP, dGTP, dTTP, murine reverse transcriptase, RNAguard, and RNase/DNase-free BSA) were then incubated (5 min; 37°C). The RNA was added to the first-strand reaction tube and incubated (5 min; 37°C), mixed thoroughly, and the contents collected by brief centrifugation (8000 x g; 10 sec). The reaction was then incubated for 60 min at 37°C. The completed first-strand reaction mix was heated (5 min; 90°C) to inactivate the reverse transcriptase. The completed reverse transcription reactions were used immediately for PCR or stored at -20°C.

2.10 Primer design and synthesis

Primer sequences used in this study were obtained from previously published articles or were designed from published cDNA sequences using primer select software (DNA Star). For a detailed description of the criteria used to design primers see Appendix I.

Oligonucleotide primers were synthesized (40 nM scale) using an automatic DNA synthesizer (Applied Biosystems 391) according to the manufacturers instructions. Following synthesis primers were manually deprotected and removed from the solid-phase column support. To cleave and deprotect the oligonucleotide, ammonium hydroxide (0.5 ml) was injected into the synthesis column and left to stand (15-30 min;

room temperature). The ammonium hydroxide was recirculated occasionally and then transferred to a glass collection vial stoppered with a teflon-lined cap. The column was treated with ammonium hydroxide a further three times. Ammonium hydroxide (1 ml) was added to the crude oligonucleotide solution (2 ml) and heated (15 hours; 55°C). The crude oligonucleotide was recovered by centrifuging under vacuum and dissolved in sterile water (360 µl). 3 M sodium acetate (40 µl) was added, mixed, and left to stand (30 min; -70°C). The solution was centrifuged (10,000 x g; 10 min; 4°C) and the supernatant carefully removed. The oligonucleotide pellet was washed with 1 ml ethanol (80% v/v), centrifuged (10,000 x g; 10 min; 4°C) and then dried. The pellet was dissolved in 100 µl of Tris-EDTA buffer (TE) and the absorbance at 260 nm was measured using a LKB Biochrom Ultraspec II spectrophotometer.

2.11 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is an *in vitro* method which employs repeated cycles of amplification to copy a target segment of DNA (Mullis *et al.*, 1986). PCR products generated during earlier cycles are used as templates during subsequent amplification cycles, therefore resulting in an exponential synthesis of the target DNA fragment. The essential components for PCR are a single stranded DNA template, deoxynucleotide triphosphates (dNTPs), oligonucleotide primers, DNA polymerase, and a suitable Mg^{2+}-containing buffer. Taq DNA polymerase, isolated from the hot spring eubacterium *Thermus aquaticus* (*Taq*), or its genetically engineered counterparts (such as Amplitaq) are the most commonly used polymerases employed in PCR. Each cycle of amplification consists of three thermal steps: denaturation of double stranded DNA at

temperatures between 92°C and 96°C; annealing of oligonucleotide primers to their complementary sequences on template DNA at temperatures between 45°C and 72°C; and finally, primer extension at 72°C using a thermostable DNA polymerase. During the extension phase of each cycle the oligonucleotide primers are extended at their 3′ ends by successive additions of deoxynucleotide triphosphates (dNTPs) which are complementary to the template DNA.

The exponential increase in the production of target DNA will continue until any of the reaction components becomes limiting. As the cycle number increases accumulation of the PCR product occurs and the primer-template ratio decreases resulting in self-annealing of the DNA. When one or more reaction components become limiting, or self-annealing is commonplace, the amount of product formed begins to plateau and the amplification is no longer exponential. For RT-PCR to be used in a quantitative manner the amount of product generated needs to fall in the middle of the exponential rise. Therefore, the optimal number of amplification cycles and amount of template DNA used was determined empirically. In addition, a number of other reaction conditions were also be optimized to increase the specificity of amplification.

2.11.1 Optimization of PCR conditions

The optimal amplification conditions were determined empirically for oligonucleotide primer pairs specific for CD14, CD44, GAPDH, MSE-1, and VCAM-1 (see Table 2.1 for primer sequences and Appendix I for the criteria used in designing these primer pairs). PCR cycle number and annealing temperature optimization studies were carried out on 1 μl of cDNA which was equivalent to 100 ng of RNA. cDNA was

used at various dilutions in order to determine its effect on the generation of spurious by-products. For all subsequent studies cDNA was diluted 1 µl in 20 µl with 2 µl of the diluted sample being included in each PCR reaction tube. Primer concentration was varied in the range 0.1 µM to 1 µM in order to determine the optimal primer concentration.

Each PCR reaction contained 200 µM dNTPs, [α^{32}P] or [α^{33}P] dATP (2.5 µCi), 0.2 µM of sense primer, 0.2 µM of anti-sense primer, and cDNA (or water-only control) in a total volume of 25 µl. Hot start PCR, whereby amplitaq polymerase (1.25 units) diluted in 2.5 µl of 10× PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.001% (w/v) gelatin) was added to the reaction tube components which have been heated to 94 °C for 2 min, was carried out using a GeneAmp PCR system 9600 thermal cycler (Perkin Elmer). Following the initial denaturing step (94°C for 5 min), each cycle of amplification consisted of 30 sec at 94°C, 30 sec at a specified annealing temperature, and 1 min at 72°C. After the specified number of amplification cycles PCR reactions were 'finished-off' by including a final extension period at 72°C for 10 min. Primer-specific PCR products incorporating [α^{32}P] or [α^{33}P] -dATP were electrophoresed through a 6% polyacrylamide gel and quantified using a phosphorimager (Fuji BAS1000 BioImager). Band intensities are represented as total pixel units enclosed in the area containing the band.

A summary of the optimal PCR conditions for each primer pair are shown in Table 2.2. Furthermore, detailed experimental results showing optimization data can be found in Appendix II.

2.11.2 Semi-quantitative RT-PCR

Semi-quantitative PCR was carried out on cDNA (equivalent to 10 ng of RNA) using oligonucleotide primers specific for CD14, CD44, GAPDH, MSE-1, and VCAM-1. (see Table 2.1). Each PCR reaction contained amplitaq polymerase (1.25 units), 1× PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin), 200 μM deoxynucleotide triphosphates (dNTPs), [α^{32}P] or [α^{33}P] dATP (2.5 μCi), 0.2 μM of sense primer, 0.2 μM of anti-sense primer, and cDNA (or water control) in a total volume of 25 μl. For each primer pair a master mix containing all the above components except cDNA was made up. This was aliquoted into micro-PCR tubes containing cDNA to minimize contamination and variations between tubes. Several negative controls, such as no cDNA added and each primer alone, were also included in each PCR experiment. PCR was carried out using a GeneAmp PCR system 9600 thermal cycler (Perkin Elmer). Hot start PCR was performed for varying cycles of amplification (see Table 2.2). Following the initial denaturing step (94°C for 5 min), each cycle of amplification consisted of 30 sec at 94°C, 30 sec at an annealing temperature appropriate for the primer pair (see Table 2.2), and 1 min at 72°C. PCR reactions were 'finished-off' by including a final extension period at 72°C for 10 min.

Primer specific PCR products incorporating [α^{32}P] or [α^{33}P] -dATP were electrophoresed through a 6% polyacrylamide gel and quantified using a phosphorimager (Fuji BAS1000 BioImager).

PCR products required for Southern blot analysis were generated in a similar way except 30 cycles (94°C for 30 sec; 62°C for 30 sec; 72°C for 1 min) of

amplification were performed in the absence of [α^{32}P]- or [α^{33}P]-dATP from the reaction mix.

2.12 Detection and measurement of PCR products

2.12.1 Polyacrylamide gel analysis

The composition of the buffers and 6% polyacrylamide gels used in this study are described in sections 2.18.2 and 2.19.3. The gels (0.75 mm thick) were poured and arranged in the electrophoresis apparatus (Biorad Miniprotean) according to manufacturers instructions. The electrophoresis tank was filled with 1× Tris-borate-EDTA (TBE) running buffer. DNA samples were mixed with orange-blue dye in the ratio of 5:1 and 10 µl loaded onto the gel. The samples were allowed to run slowly into the gel (20 V; 50 min) and then electrophoresed at 35 V until the lower blue band migrated off. The gel was carefully removed from the glass plates and the DNA fixed by incubating (20 min; room temperature) in a solution of acetic acid (10% v/v) and methanol (40% v/v). After washing in distilled water the gels were placed on filter paper and dried (45 min; 80°C) using a gel dryer (BioRad 583). The gels were exposed to a phosphorscreen for 4-16 hours (depending on isotope incorporated) and analysed using a phosphorimager (Fuji BAS1000 BioImager).

2.12.2 Southern blot analysis of PCR products

Following amplification, PCR products and ^{32}P-labelled 1 Kb DNA ladder (see 2.13) were separated by electrophoresis on a 1.5% agarose-TBE gel (see 2.19.2). The DNA products were visualized by UV transillumination and photographed. The PCR

products were transferred onto maximum strength nylon membrane (Schleicher and Schuell) by rapid downward capillary transfer under alkaline conditions. In brief, the gel was incubated (30 min; room temperature) in 500 ml of denaturing buffer. This step was repeated and the gel was then washed in transfer buffer for 15 min. The nylon transfer membrane was washed in distilled water for 15 min and the transfer stack was set up according to manufacturers instructions. The transfer was left (60-90 min) and then carefully disassembled. The nylon membrane (blot) was washed with 1× neutralizing buffer (5 min; room temperature) and then baked (60 min; 80°C). Blots were stored at 4°C.

Two different protocols for hybridization were used depending on whether the blot was to be probed with radioactively-labelled oligonucleotides or DNA probes. If the blot was to be probed with oligonucleotides they were first prehybridized with 15 ml of oligonucleotide prehybridization solution (6× standard saline-citrate (SSC) buffer, 5× Denhardt's reagent, 0.5% sodium dodecyl sulphate (SDS) and 100 μg/ml Herring sperm DNA) for 90 min at 60°C. This was completely removed and 15 ml of hybridization solution (6× SSC, 0.5% SDS and 100 μg/ml Herring sperm DNA) added. The radioactive oligonucleotide probe was synthesized (see 2.14) added to this and left to hybridize overnight. The membrane was briefly rinsed in 6× SSC/ 0.5% SDS at room temperature and then at a calculated temperature (oligo T_m-5°C) for 10 min. The blots were placed against a phosphorscreen and scanned using a phosphorimager (Fuji BAS1000 BioImager).

For hybridization with radioactive DNA fragments the membrane was prehybridized with hybridization solution (6× saline-sodium phosphate-EDTA (SSPE)

buffer, 50% formamide, 5× Denhardt's reagent, 0.5% SDS and 100 μg/ml Herring sperm DNA) for 2 hours at 42°C. The membrane was sequentially hybridized with ^{32}P-labelled CD44 variant exon-specific probes. These ^{32}P-labelled probes were synthesized using variant exon DNA templates and exon-specific primers (see table 2.3) with a Megaprime DNA labelling kit. The labelled probes were hybridized with the membrane at 42°C overnight and then washed with 4× SSPE/ 0.1% SDS for 10 min, 1× SSPE/ 0.1% SDS for 20 min, 0.1× SSPE/ 0.1% SDS for 20 min, respectively, at 50°C. The membrane was exposed to a phosphorscreen for 3 hours and analysed using a phosphorimager (Molecular Dynamics Storm II).

2.13 Radioactive labelling of 1 Kb DNA ladder by T4 DNA polymerase

T4 DNA polymerase is able to catalyse the synthesis of DNA (5′→3′), but also possesses 3′→5′ exonuclease activity. A 1 Kb DNA ladder was labelled by partial exonucleolytic degradation of the 3′-ends followed by resynthesis. The DNA ladder (10 μg) was incubated (2 min; 37°C) in a total reaction volume of 20 μl containing 1× T4 DNA polymerase reaction buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, 50 μg/ml BSA) and T4 DNA polymerase (24 units) to degrade the 3′-ends. The reaction was stopped by cooling on ice. The degraded 3′-ends were resynthesized by the addition of 5× T4 DNA polymerase reaction buffer (3 μl), 2 mM dNTP mix (dCTP, dGTP, and dTTP) (15 μl), and [α^{32}P] or [α^{33}P] dATP (10 pmol), and incubating at 37°C for 2 min. 5 μl of dATP (2 mM) was then added, incubated (2 min; 37°C), and the reaction stopped by the addition of 0.5 M EDTA (2.5 μl). The labelled ladder was

separated from unincorporated nucleotides by passing through a G50 sephadex (NICK) column. The cpm incorporated into the ladder were counted (Bioscan QC 2000). The ladder was loaded onto a gel (agarose or polyacrylamide) at 1-5 $\times$ 10^6 cpm per lane.

2.14 3′ [α^{32}P] dCTP tailing of oligonucleotides with terminal transferase

Terminal transferase was used to add radioactively labelled homopolymer tails to the 3′-ends of oligonucleotides (see table 2.4). Oligonucleotides (10 pmol) were end-labelled in a total reaction volume of 20 µl containing [α^{32}P] dCTP (10 pmol), 1× reaction buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, BSA (25 µg/µl), cobalt chloride (0.75 mM), and terminal transferase (25 units). This was incubated (20 min; 37°C) and the reaction stopped by the addition of 2.5 µl of EDTA (0.5 M). The end-labelled oligonucleotides (probes) were separated from unincorporated nucleotide by passing through a NICK column. The purified probes were used immediately by adding to hybridization flasks (Hybaid) containing the blot and 15 ml of oligonucleotide hybridization solution.

2.15 [α^{32}P] dATP labelling of CD44 variant exon DNA templates

Short fragments of variant exon-specific CD44 DNA were prepared by amplification of a variant CD44 template DNA (pT_2T_3) using exon-specific primers (see table 2.3). pT_2T_3 DNA was kindly provided by Dr Peter Dall (IGEN, Kernforschungzentrum, Karlsruhe, Germany). The pT_2T_3 template DNA consisted of sequence for only the

variant exons v3-v10. pT_2T_3 DNA (5 ng) was amplified in a total volume of 100 µl containing 5 units of amplitaq polymerase, 1× PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin), 200 µM dNTPs, 0.4 µM of sense primer, and 0.4 µM of anti-sense primer. Following the initial denaturing step (94°C for 5 min), 30 cycles (each cycle consisted of 30 sec at 94°C, 30 sec at 58°C, and 1 min at 72°C) of amplification were performed. PCR reactions were 'finished-off' by including a final extension period at 72°C for 10 min. The products were electrophoresed on a 1.5% agarose gel and product sizes checked. For each variant exon product the band was carefully excised from the gel, weighed and transferred to a 1.5 ml Eppendorf tube. DNA was extracted from the agarose gel slices and purified using a Jetsorb DNA extraction kit (AMS Biotechnology) according to manufacturers instructions. The concentration of each variant exon DNA was determined and an aliquot electrophoresed on a 1.5% agarose gel to check purification.

Each variant exon DNA template was labelled with [α^{32}P] dATP using a Megaprime DNA labelling kit (Amersham). Denatured variant exon DNA template (25 ng) was labelled in a 50 µl reaction containing DNA polymerase I Klenow fragment (2 Units), 1× reaction buffer, dNTPs, 0.4 µM of sense primer, 0.4 µM of antisense primer, and [α^{32}P] dATP (50 µCi). This was incubated at 37°C for 10 min and the reaction stopped by the addition of 5 µl of EDTA (0.2 M). The ^{32}P-labelled DNA fragments (probes) were separated from unincorporated nucleotide by passing through a NICK column. The purified probe was mixed with an equal volume of hybridization solution and denatured by heating (10 min; 70°C). Labelled DNA probe (1×10^6 cpm/ml) was added to pre-hybridized blots and allowed to hybridize for 16 hours at 42°C.

2.16 Cytokines and reagents used

Recombinant human (rh) IL-1β, rhIL-4, rhIL-13 and rhTNF-α were obtained in lyophilized form and stored at -70 °C. IL-1β (5 μg) was dissolved in 5 ml of sterile PBS containing BSA (50 μg/ml) to give a stock solution of 1 μg/ml. The cytokines IL-4 (10 μg), IL-13 (10 μg) and TNF-α (10 μg), respectively, were each solubilized in 1 ml of sterile PBS containing BSA (50 μg/ml) to give stock solutions of 10 μg/ml. These stock solutions were aliquoted into sterile microcentrifuge tubes and stored at -20°C until used. Endotoxin levels were less than 0.1 pg per 1 μg of cytokine. Both retinoic acid and vitamin D_3 were dissolved in DMSO to give 10^{-3} M stock solutions and stored at -20°C until used.

2.17 Statistical analysis

All values given are the means ± standard error (S.E.) of the mean for the number (n) of experiments performed. Statistical analyses were performed using analysis of variance (ANOVA). *P* values less than 0.05 were considered as significant. Where statistical significance was found Student's paired *t* tests were performed to identify differences between groups.

2.18 Buffers for electrophoresis

Stock solutions of electrophoresis buffers are shown below. All buffers were made up in double distilled water unless mentioned otherwise.

2.18.1 10× MEM (composition/ 1000 ml)

MOPS	220 mM (46 g/l)
EDTA (0.5 M; pH 8.0)	10 mM (20 ml/l)
Sodium acetate (3 M; pH 5.2)	51 mM (17 ml/l)

This buffer was diluted to a working strength of 1× with DEPC water.

2.18.2 5× TBE (composition/ 1000 ml)

Tris-Base	445 mM (54 g/l)
Boric acid	445 mM (27.5 g/l)
Na_2EDTA (0.5 M; pH 8.0)	10 mM (20 ml/l)

This buffer was used at a working strength of 0.5× for agarose gel electrophoresis and 1× for polyacrylamide gel electrophoresis.

2.19 Composition of electrophoresis gels

2.19.1 1.2% Denaturing formaldehyde-agarose gel (composition/ 30 ml gel)

Agarose	0.24 g
Formaldehyde	5.4 ml
10× MEM	3.0 ml

2.19.2 1.5 % Agarose-TBE gel (composition/ 250 ml gel)

Agarose	3.75 g
5× TBE	25 ml
Ethidium bromide	0.5 µg/ml

2.19.3 6% Polyacrylamide gel (ml/ 30 ml)

Acrylamide (30%)	6 ml
5× TBE	6 ml
Water	18 ml
Ammonium persulphate (10% w/v)	300 µl
TEMED	30 µl

2.20 Buffers for Southern blotting

2.20.1 Denaturing buffer

NaCl	3 M (175.5 g/l)
NaOH	400 mM (16.0 g/l)

2.20.2 Transfer buffer

NaCl	3 M (175.5 g/l)
NaOH	8 mM (0.32 g/l)
Sarcosyl	2 mM (0.63 g/l)

2.20.3 5× Neutralizing buffer (pH 6.8)

Na_2HPO_4	558 mM (79.25 g/l)
$NaH_2PO_4.H_2O$	437 mM (60.25 g/l)

2.21 Buffers and hybridization solutions

2.21.1 20× SSC (Standard saline-citrate buffer) pH 7.0

NaCl	3 M (175.3 g/l)
Sodium citrate	0.3 M (88.4 g/l)

2.21.2 20× SSPE (Saline sodium phosphate EDTA buffer) pH 7.4

NaCl	3 M (175.3 g/l)
Sodium citrate	0.3 M (88.4 g/l)
EDTA	0.02 M (7.4 g/l)

2.21.3 Oligonucleotide hybridization solution (composition/ 100 ml)

20× SSC	30 ml
SDS (10% w/v)	0.5% v/v (5 ml)
ss Herring sperm DNA	100 µg/ml

2.21.4 Oligonucleotide pre-hybridization solution (composition/ 100 ml)

Same composition as the hybridization solution but is supplemented with 5× Denhardt's solution.

2.21.5 DNA probe hybridization solution (composition/ 100 ml)

20× SSPE	30 ml
Formamide	50% v/v (50 ml)
SDS (10% w/v)	0.5% v/v (5 ml)
ss Herring sperm DNA	100 µg/ml

2.22 Materials

The reagents and materials used in this study are shown below.

2.22.1 Materials for tissue culture

Actinomycin D	Sigma
Collagenase (Type II)	Worthington, USA
Chamber slides (8-well)	Nunc
DMEM	Gibco BRL
FBS	Gibco BRL
PBS	Gibco BRL
Penicillin-streptomycin solution	Gibco BRL
RPMI	Gibco BRL
Trypsin-EDTA	Gibco BRL
Tissue culture flasks	Nunc

2.22.2 Materials for FACS and immunohistochemistry

3,3-amino-9-ethylcarbozole	Sigma
Biotin-labelled mouse ant-human VCAM-1	Southern Biotechnology Associates Inc., AL
FITC-conjugated goat anti-mouse IgG	Southern Biotechnology Associates Inc., AL
Fluoromount-G	Southern Biotechnology Associates Inc., AL
Haematoxylin	Sigma
Human AB serum	ICN
Mouse anti-human CD14	Dako
Mouse anti-human CD44s	Becton Dickinson
Mouse anti-human CD44 v3	R&D Systems
Mouse anti-human CD44 v5	BioWhittaker
Mouse anti-human CD44 v6	R&D Systems
Mouse anti-human CD44 v7/8	BioWhittaker
Mouse anti-human CD68	Dako
Mouse anti-human VCAM-1	Becton Dickinson
Normal goat serum	ICN
PE-conjugated Fab_2 anti-mouse IgG	Sigma
Streptavidin-Texas Red conjugate	Jackson ImmunoResearch Laboratories, Inc., PA

2.22.3 Materials for total RNA isolation

Chloroform	Sigma
DEPC	Sigma
Glycogen	Boehringer Mannheim
Guanidinium isothiocyanate	Gibco BRL

Jetsorb DNA extraction kit	AMS Biotechnology
2-mercaptoethanol	Sigma
Phenol	Gibco BRL
Propan-2-ol	Sigma
RNA sample loading buffer	Sigma
0.24-9.5 Kb RNA ladder	Gibco BRL
Sodium acetate	Sigma
Sodium citrate	Sigma
Sarcosyl (Lauryl sarcosine)	Sigma

2.22.4 Materials used for RT-PCR

[α^{32}P] dATP	Amersham
[α^{33}P] dATP	Amersham
Amplitaq DNA polymerase	Perkin Elmer
10× PCR buffer	Perkin Elmer
dATP	Perkin Elmer
dCTP	Perkin Elmer
dGTP	Perkin Elmer
dTTP	Perkin Elmer
PCR microtubes	Perkin Elmer
T primed first strand cDNA kit	Pharmacia Biotech

2.22.5 Materials used in radioactive labelling

[α^{32}P] dATP	Amersham
[α^{32}P] dCTP	Amersham
Cobalt chloride	Boehringer Mannheim
1 Kb DNA ladder	Gibco BRL
Megaprime DNA labelling kit	Amersham
NICK columns	Pharmacia Biotech
T4 DNA polymerase	New England Biolabs
T4 DNA polymerase reaction buffer	New England Biolabs
Terminal transferase	Boehringer Mannheim
Terminal transferase reaction buffer	Boehringer Mannheim

2.22.6 Materials used in PCR product detection

Acetic acid	BDH chemicals
Agarose	Gibco BRL
Acrylamide/Bisacrylamide	Bio-Rad
Ammonium persulphate	Bio-Rad
Denhardt's reagent	Sigma
Formamide	Sigma
Herring sperm DNA	Sigma

Orange-blue dye	Promega
Methanol	BDH chemicals
Nytran nylon membrane	Schleicher and Schuell
TEMED	Sigma
Turboblotter rapid transfer system	Schleicher and Schuell
SDS	Sigma

2.22.7 Materials for buffers

Boric acid	Sigma
EDTA	Sigma
MOPS	Sigma
Na_2HPO_4	BDH chemicals
$NaH_2PO_4.H_2O$	BDH chemicals
Sodium acetate	Sigma
Sodium chloride	Sigma
Sodium hydroxide	Sigma
Tris-Base	Sigma
Tris-HCl	Sigma

2.22.8 Other materials

Acetone	BDH chemicals
Actinomycin D	Sigma
Ammonium hydroxide	Applied Biosystems
BSA	ICN
Calcium chloride	BDH chemicals
Dynabeads (M-450)	Dynal
DNA collection vials and caps	Wheaton
ELISA cytokine assay kits	R&D Systems
rhIFN-γ	R&D Systems
rhIL-1β	R&D Systems
rhIL-4	R&D Systems
rhIL-13	R&D Systems
Nylon gauze	Cadisch Precision Meshes
rhTNF-α	R&D Systems
Primer synthesis reagents and columns	Applied Biosystems
Retinoic acid	Calbiochem Novabiochem
Tris-EDTA (TE) buffer	Sigma
Vitamin D_3	Calbiochem Novabiochem

Table 2.1 Oligonucleotide sequences of PCR primers

Marker	Primer name	Strand	Sequence (5′→3′)	Position
CD14	14.533	Sense	CGT GGG CGA CAG GGC GTT CTT	533-553
	14.1038	Antisense	TTC CCG TCC AGT GTC AGG TTA TCC	1038-1061
CD44s	44.513	Sense	CAG ACC TGC CCA ATG CCT TTG ATG GAC C	513-540
	44.934	Antisense	CAA AGC CAA GGC CAA GAG GGA TGC C	934-958
GAP-DH	gap.67	Sense	AAG GTG AAG GTC GGA GTC AAC	67-88
	gap.406	Antisense	GGC AGA GAT GAT GAC CCT TTT GGC	406-429
MSE-1	mse.46	Sense	AAT GCC ACC TCG TAC CCT CCT ATG	46-69
	mse.469	Antisense	GAC ACT TTC TCC TCC CGC TGA CTC	469-492
VCAM-1	vcam.751	Sense	CAA GTC TAC ATA TCA CCC AAG	751-771
	vcam.1361	Antisense	GGA ACC TTG CAG CTT ACA GTG	1361-1382

All sequences are listed from 5′ to 3′. Primers were designed using Primer Select (DNA Star) using the following published sequences: CD14 (Simmons *et al.*, 1989; Genbank accession number M86511); and MSE-1 (Zschunke *et al.*, 1991; Genbank accession number X52973). VCAM-1 primers were modified from those published by Hession *et al.*, 1991.

Table 2.2 Summary of optimal PCR conditions for oligonucleotide primers

Primer	Cycle number	Annealing temperature (°C)	cDNA (µl)
CD14	30	64	1/20
CD44	30	64	1/20
MSE-1	34	62-64	1/20
GAPDH	30	64-68	1/20
VCAM-1	30	62	1/20

Table 2.3 Oligonucleotide sequences of primers used in the synthesis of CD44 variant exon DNA probes

Marker	Primer name	Strand	Sequence (5′→3′)	Position
CD44 v3	v3.24	Sense	GCA GGC TGG GAG CCA AAT GAA GAA AAT G	24-51
	v3.82	Antisense	ATC TTC ATC ATC ATC AAT GCC TGA TCC AG	82-110
CD44 v4	v4.128	Sense	TTC AAC CAC ACC ACG GGC CTT TGA C	128-154
	v4.213	Antisense	AGT CAT CCT TGT GGT TGT CTG AAG TAG	213-239
CD44 v5	v5.243	Sense	GTA GAC AGA AAT GGC ACC ACT GCT TAT G	243-270
	v5.324	Antisense	TGT GCT TGT AGA ATG TGG GGT CTC TTC	324-356
CD44 v6	v6.357	Sense	ATC CAG GCA ACT CCT AGT AGT ACA AC	357-382
	v6.453	Antisense	TGT CCC TGT TGT CGA ATG GGA GTC	453-482
CD44 v7	v7.489	Sense	GCC TCA GCT CAT ACC AAC CAT CCA ATG	489-515
	v7.581	Antisense	CCT TCT TCC TGC TTG ATG ACC TCG TC	581-614
CD44 v8	v8.621	Sense	ATG GAC TCC AGT CAT AGT ACA ACG C	621-645
	v8.689	Antisense	GTT GTC ATT GAA AGA GGT CCT GTC	689-718
CD44 v9	v9.722	Sense	GCA GAG TAA TTC TCA GAG CTT CTC	722-745
	v9.773	Antisense	TTG ATG TCA GAG TAG AAG TTG TTG G	773-808
CD44 v10	v10.812	Sense	TAG GAA TGA TGT CAC AGG TGG AAG	812-835
	v10.985	Antisense	TGA TAA GGA ACG ATT GAC ATT AGA G	985-1013

All sequences are listed from 5′ to 3′. CD44 variant exon specific primers were modified from those published in Dall *et al.*, 1995.

Table 2.4 Oligonucleotides used as hybridization probes in the detection of VCAM-1 domains

Marker	Primer name	Strand	Sequence (5′→3′)	Position
VCAM 6 Domain	3-4	Antisense	CTA GGG AAT GCT TGA ACA AT	1018-1027
VCAM 7 Domain	3b	Antisense	CAA TCC GGG GTC CAG GGG AGA TCT CAA CAG	1040-1069

All sequences are listed from 5′ to 3′. (Hession *et al.*, 1991).

Chapter 3

VCAM-1 expression by cultured fibroblast-like synoviocytes

3.1 Introduction

Rheumatoid arthritis is a chronic inflammatory condition which is characterized in part by hyperplasia of the synovial lining (Gay *et al.*, 1993). Hyperplasia of the synovial lining results from proliferation of the resident fibroblast-like synoviocytes together with invasion of blood-borne inflammatory cells. These inflammatory cells migrate through the synovial high endothelial venules and partition themselves within the synovium and synovial fluid. The recruitment and retention of these cells is mediated by cell adhesion molecules (Haskard *et al.*, 1995). Adhesion molecules are now regarded to play a crucial role in the pathogenesis of RA due to their up-regulation in response to certain pro-inflammatory cytokines and their ability to act as co-stimulatory receptors in the activation of T cells (Liao and Haynes, 1995). One adhesion molecule that shows prominent up-regulation in RA synovium is vascular cell adhesion molecule-1 (VCAM-1; CD106; INCAM-110) (Morales-Ducret *et al.*, 1992; Wilkinson *et al.*, 1993a; Kriegsmann *et al.*, 1995).

VCAM-1, a member of the immunoglobulin (Ig) superfamily of adhesion molecules, was originally identified as a 90-110 kDa glycoprotein induced on human umbilical vein endothelial cells (HUVECs) following treatment with inflammatory cytokines (Osborn *et al.*, 1989; Rice and Bevilacqua, 1989). It is now known that two

isoforms of VCAM-1 (VCAM-6D and VCAM-7D) arise by alternative splicing of the VCAM-1 gene (Cybulsky *et al.*, 1991a,b; Hession *et al.*, 1991). The seven domain form (VCAM-7D) differs from the six domain form (VCAM-6D) by the insertion of an extra Ig-like domain (termed AS-1 or domain 4) (Cybulsky *et al.*, 1991b; Hession *et al.*, 1991). Although both VCAM-6D and VCAM-7D forms are found in cytokine-treated HUVECs, the 7D form predominates (Cybulsky *et al.*, 1991b; Hession *et al.*, 1991).

VCAM-1 is expressed on fibroblast-like synoviocytes found within the intimal layer of normal, osteoarthritis and RA synovium (Wilkinson *et al.*, 1993a). Moreover, the intensity of VCAM-1 expression correlates with the degree of synovial inflammation (Morales-Ducret *et al.*, 1992; Wilkinson *et al.*, 1993a). Adhesive interactions between VCAM-1 and its ligands, the integrins $\alpha4\beta1$ and $\alpha4\beta7$, may explain the retention of $\alpha4\beta1$-positive mononuclear cells and $\alpha4\beta7$-positive lymphocytes within synovium, and the loss of $\alpha4$-negative cells to the synovial fluid (Elices *et al.*, 1990; Chan *et al.*, 1992; Morales-Ducret *et al.*, 1992; Lazarovits and Karsh, 1993). RA FLSs are also able to induce the release of a number of pro-inflammatory cytokines and metalloproteinases from both T cells and monocytes following their co-culture (Bombara *et al.*, 1993; Blue *et al.*, 1993; Chen *et al.*, 1998). A specific role for VCAM-1 in the induction of metalloproteinase expression has been clearly demonstrated (Romanic and Madri, 1994). Engagement of endothelial cell VCAM-1 with $\alpha4\beta1$ induces production of active 72 kDa gelatinase by $CD4^+$ T lymphocytes. Gelatinase expression is thought to facilitate lymphocyte transendothelial migration and invasion of underlying tissues. These results, together with those recently published by Müller-Ladner et al. (1996) demonstrating that VCAM-1-positive FLSs possess an invasive and destructive phenotype, would suggest that fibroblast-like synoviocytes are involved in the

pathogenesis and perpetuation of RA with VCAM-1 possibly playing a central role (Romanic and Madri, 1994; Müller-Ladner *et al.*, 1996).

Although elevated levels of VCAM-1 *in situ* are generally interpreted as a consequence of high levels of TNF-α and IL-1β in the joint, *in vitro* these factors only cause transiently elevated levels of expression (Morales-Ducret *et al.*, 1992; Marlor *et al.*, 1992). This study will examine the effects of human plasma on the expression levels of VCAM-1. Serum, routinely used in cell cultures, is an inflammatory medium *par excellence* and thus may have long-term regulatory effects on the expression of certain adhesion molecules. In addition, many reports on the expression of VCAM-1 *in vitro* indicate that basal levels of expression are very low, much lower than those observed *in situ*. Furthermore, it is not known whether other factors, such as extracellular matrix components, regulate VCAM-1 expression on fibroblast-like synoviocytes. The possible involvement of extracellular matrix components in maintaining VCAM-1 expression has been given credence with the recent discovery that both fibronectin and low molecular weight fragments of hyaluronan can activate NF-κB, a transcription factor involved in the induction of VCAM-1 in endothelial cells (Qwarnström *et al.*, 1994; Noble *et al.*, 1996; Iademarco *et al.*, 1992; Neish *et al.*, 1992). Importantly, low molecular weight hyaluronan fragments are commonly found in the synovial fluid of RA patients (Sunblad, 1965; Dahl and Husby, 1985). Therefore, in this chapter I will explore the role of extracellular matrix components in the regulation of VCAM-1 expression with special emphasis on their long-term effects.

3.2 Methods and Materials

All methods and materials used in this chapter are as earlier described in chapter 2. Any additions to these methods are described below or are included in the legends to figures. Freshly dispersed adherent synoviocytes were cultured for 2 to 3 days or until confluent. These cells were passaged and cultured for a further 7 days. Unless otherwise stated cell surface marker expression on fibroblast-like synoviocytes was determined after 7 days. For cytokine-induced VCAM-1 expression studies fibroblast-like synoviocytes were used between passage 3 and 10, which corresponded with 3-12 weeks in culture. Dermal fibroblasts and normal fibroblast-like synoviocytes were isolated from non-inflamed skin and synovium, respectively, and used between passage 3 and 6.

3.2.1 Preparation of low molecular weight (LMW) hyaluronan

LMW hyaluronan (HA) was prepared as desribed previously by Noble et al. (1996). Hyaluronan was dissolved in phosphate-buffered saline to give a final concentration of 5 mg/ml. HA solutions were sonicated on ice using a Heat Systems ultrasonic processor XL sonicator set at the microtip level. Sonication was repeated for varying periods of time. Sonicated HA samples were electrophoresed on a 0.7% agarose gel to determine the size of the products. In brief, 5-10 μg of HA sample was mixed with 5x sample buffer in the ratio of 5:1 and loaded on a 0.7% agarose-TAE gel. A 1Kb DNA marker was also included on the gel to allow the determination of fragment size. Samples were electrophoresed at 50V until the DNA marker had run approximately 2/3 the length of the gel. Electrophoresed markers and HA fragments were visualized by overnight incubation of the agarose gel in 50% ethanol containing 0.005% (w/v) "Stains-all" (3,3'-

destain by slow shaking in water. The gel was photographed under normal illumination using a BioRad gel documentation sytem. As can be seen from Figure 3.1, sonication of HA polymer for 4 to 5 minutes produced HA fragments (LMW-HA) of approximately 1 x 10^6 Da. LMW-HA was added to DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin to give a final concentration of 1 mg/ml.

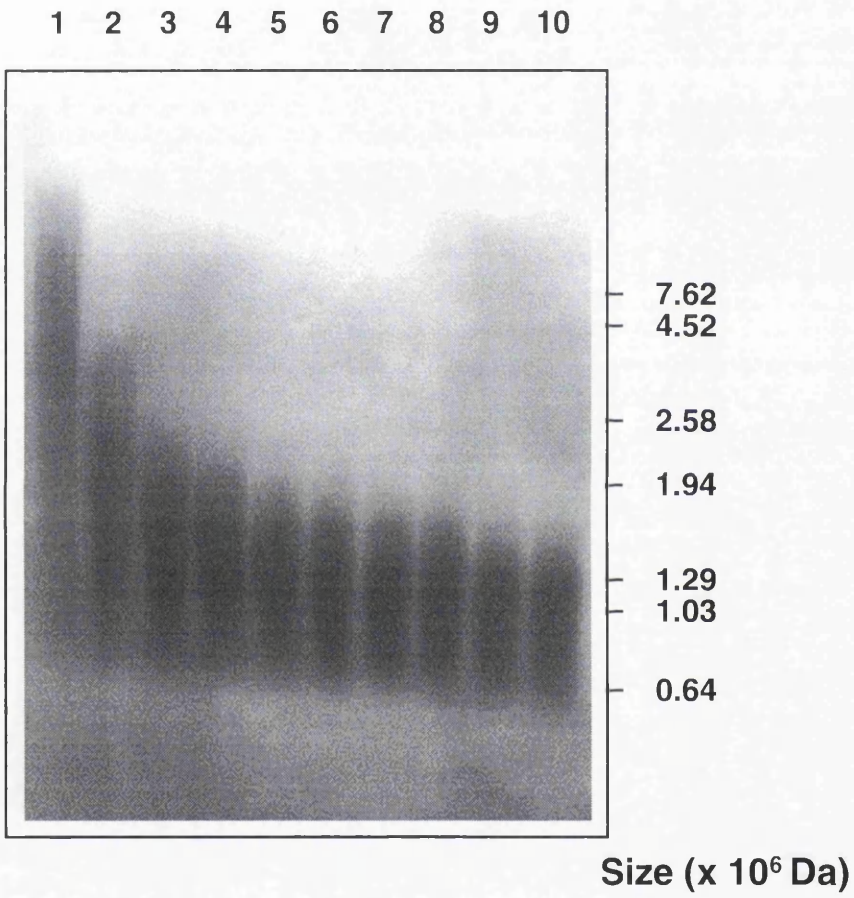


Figure 3.1 The effect of sonication on hyaluronan size. Hyaluronan polymer (5 mg/ml) was sonicated at the microtip level for various lengths of time and the products electrophoresed on a 0.7% agarose gel. Sonication was performed for 0 sec (lane 1), 15 seconds (lane 2), 45 seconds (lane 3), 1 min (lane 4), 1.5 min (lane 5), 2 min (lane 6), 2.5 min (lane 7), 3 min (lane 8), 4 min (lane 9), and 5 min (lane 10).

3.3 Results

3.3.1 A gradual loss of highly elevated VCAM-1 expression during *in vitro* culture

Flow cytometric analysis showed that RA fibroblast-like synoviocytes exhibit moderateVCAM-1 expression (Figure 3.2). This is in stark contrast to primary cultures of both normal fibroblast-like synoviocytes (isolated from non-inflamed synovium) and dermal fibroblasts, which under identical culture conditions demonstrate minimal VCAM-1 expression. In order to determine how VCAM-1 expression in RA fibroblast-like synoviocytes changes with time in culture, cell surface expression of VCAM-1 was followed in primary cultures of fibroblast-like synoviocytes over a period of 6 weeks. To assess the level of monocyte contamination in these cultures, CD14 expression was also determined. Upon removal from the rheumatoid synovium, fibroblast-like synoviocytes demonstrate high levels of cell surface VCAM-1-expression for a period of 2 weeks. With increasing time in culture however, this level gradually decreases to a low but constitutive level of expression (Figure 3.3(A)). With respect to VCAM-1 mRNA, a similar pattern was observed (See Chapter 5, Figure 5.2). Elevated levels of both the 6- and 7-domain forms of VCAM-1 mRNA were seen in the first week of culture, with a gradual loss over the remaining time in culture until only the 7-domain form was detectable. Southern blot analysis using domain-specific oligonucleotide probes was used to confirm the identity of 6D and 7D VCAM-1 PCR products (Figure 3.6). CD14 levels were found to be low throughout the entire 6 week culture period (Figure 3.3(B)). However, CD14 and VCAM-1 expression appeared to decline in a similar fashion indicating that monocyte/macrophage contamination of primary cultures of fibroblast-like synoviocytes may determine the level of VCAM-1 expression.

3.3.2 Contaminating monocytes do not alter VCAM-1 expression on fibroblast-like synoviocytes

To assess the role of monocyte contamination, VCAM-1 expression was next followed in cultures with the continuous presence of contaminating synovial monocytes. To do this contaminating cells were co-cultured with adherent fibroblast-like synoviocytes (Figure 3.4). Again, fibroblast-like synoviocytes exhibited a similar rundown of VCAM-1 expression. As can be seen in Figure 3.4(A), the amount of monocyte contamination does not appear to determine VCAM-1 expression. Moreover, fibroblast-like synoviocytes that were cultured with high levels of contaminating CD14-positive cells express lower levels of VCAM-1 than cells which were washed to reduce monocyte contamination. Furthermore, some fibroblast-like synoviocyte cultures showed no expression of VCAM-1 (low VCAM-1-expressing cells). Again, VCAM-1 expression in these cells was unaltered by the presence of contaminating monocytes (Figure 3.4(B)).

3.3.3 Initial high VCAM-1 expression on fibroblast-like synoviocytes is independent of IL-1β or TNF-α

The results described earlier showed that both cell surface VCAM-1 and VCAM-1 mRNA levels are initially high and then decline with increasing time in culture. To test whether the presence of IL-1β or TNF-α could be responsible for the initially high expression of VCAM-1, the levels of these two cytokines in supernatants recovered from one and two week-old cultures of FLSs obtained from RA patients was determined. As shown in Table 3.1, neither IL-1β nor TNF-α could be detected in the culture media (termed RA-conditioned medium). To determine whether VCAM-1

expression on primary fibroblast-like synoviocytes was caused by other inflammatory stimuli present in the early passage culture medium, FLSs where VCAM-1 expression had run down were cultured in early-passage conditioned medium. The conditioned media had no effect on VCAM-1 expression (Figure 3.5). The addition of TNF-α (10 ng/ml) was used as a positive control to show the induction of VCAM-1 expression. We also assessed possible synergy between conditioned medium and TNF-α, but found that the combination of these significantly reduced VCAM-1 expression on FLSs ($p=0.0187$). By 72 hours, however, this difference was no longer apparent even though VCAM-1 expression was significantly higher in those cultures treated with TNF-α compared to either normal medium or RA-conditioned medium alone.

3.3.4 Plasma does not alter VCAM-1 expression

As mentioned previously, the use of serum as a supplement of the culture medium could mean a sustained down-regulatory effect on adhesion molecule expression. It should be kept in mind that plasma rather than serum perfuses ordinary tissues. This has led me to study VCAM-1 expression on cells cultured in human plasma. Under plasma culture conditions it was found that both cell surface VCAM-1 and VCAM-1 mRNA expression was not significantly different on fibroblast-like synoviocytes (Figure 3.2, 3.6). Plasma and serum were also tested at various concentrations, in the range of 1 to 10% (v/v), but were found to have no significant effect on VCAM-1 expression after two weeks in culture (Figure 3.7(A)). Moreover, culture of fibroblast-like synoviocytes for longer periods of time with plasma-supplemented media had no significant effect on VCAM-1 expression (Figure 3.2).

3.3.5 Low molecular weight HA cannot up-regulate VCAM-1 expression

An abundant extracellular matrix component present in the synovial membrane is the glycosaminoglycan hyaluronan (HA). Initially, I tested the effect of polymerized hyaluronan (1mg/ml) in combination with either FBS or HPDS on the expression of VCAM-1. As can be seen in Figure 3.8, this form of HA had no effect on VCAM-1 expression at the time point tested. However, since low molecular weight forms of hyaluronan are present in the chronic inflamed joint I also tested their effect on VCAM-1 expression. In this experiment, both the low molecular weight form and polymerized forms of HA were tested over a shorter time period. FLSs were treated with low molecular weight HA (1 mg/ml) for either 1, 3, or 7 days and VCAM-1 expression determined. As can be seen from Figure 3.9, low molecular weight HA had no effect on VCAM-1 expression at any of the time points tested.. TNF-α was included in these experiments as a positive control.

3.3.6 Type I collagen up-regulates VCAM-1 expression on fibroblast-like synoviocytes but does not prevents its decline

Lastly, the effect of extracellular matrix components on the level and duration of expression of VCAM-1 was examined. I initially compared cells grown on ordinary tissue-culture plastic with those grown on a special material designed for culturing primary cells (Primaria) but no found difference (Figure 3.10(A)). Again, the use of plasma had no effect on VCAM-1 expression. Next, various extracellular matrix components were tested as shown in Figure 3.11(A). Cells grown for 1 week on collagen type I matrices, but not on fibronectin and laminin, with 10% (v/v) FBS demonstrated significantly higher levels of VCAM-1 expression than those grown on

uncoated tissue culture plastic. Collagen type I was unable to arrest the decline in VCAM-1 expression seen with increased time in culture with no difference observed between the control cells at 6 weeks of culture (Figure 3.11(B)).

Figure 3.2 Flow cytometric analysis of VCAM-1 on dermal fibroblasts, normal non-inflamed fibroblast-like synoviocytes, and RA fibroblast-like synoviocytes. Cells were grown with 10% (v/v) FBS and used between passage 3 and 6. Adherent cells were removed by incubation with 4 mM EDTA. The cell suspension was washed and incubated with saturating amounts of anti-VCAM-1 or IgG1, followed by an incubation with phycoerythrin-conjugated anti-mouse IgG. Samples were analysed using a FACStar flow cytometer. The shaded histograms represent cells incubated with phycoerythrin-conjugated anti-mouse IgG only. Unshaded histograms represent cells stained with both first antibody and phycoerythrin-conjugated anti-mouse IgG. Results shown are representative of four separate experiments.

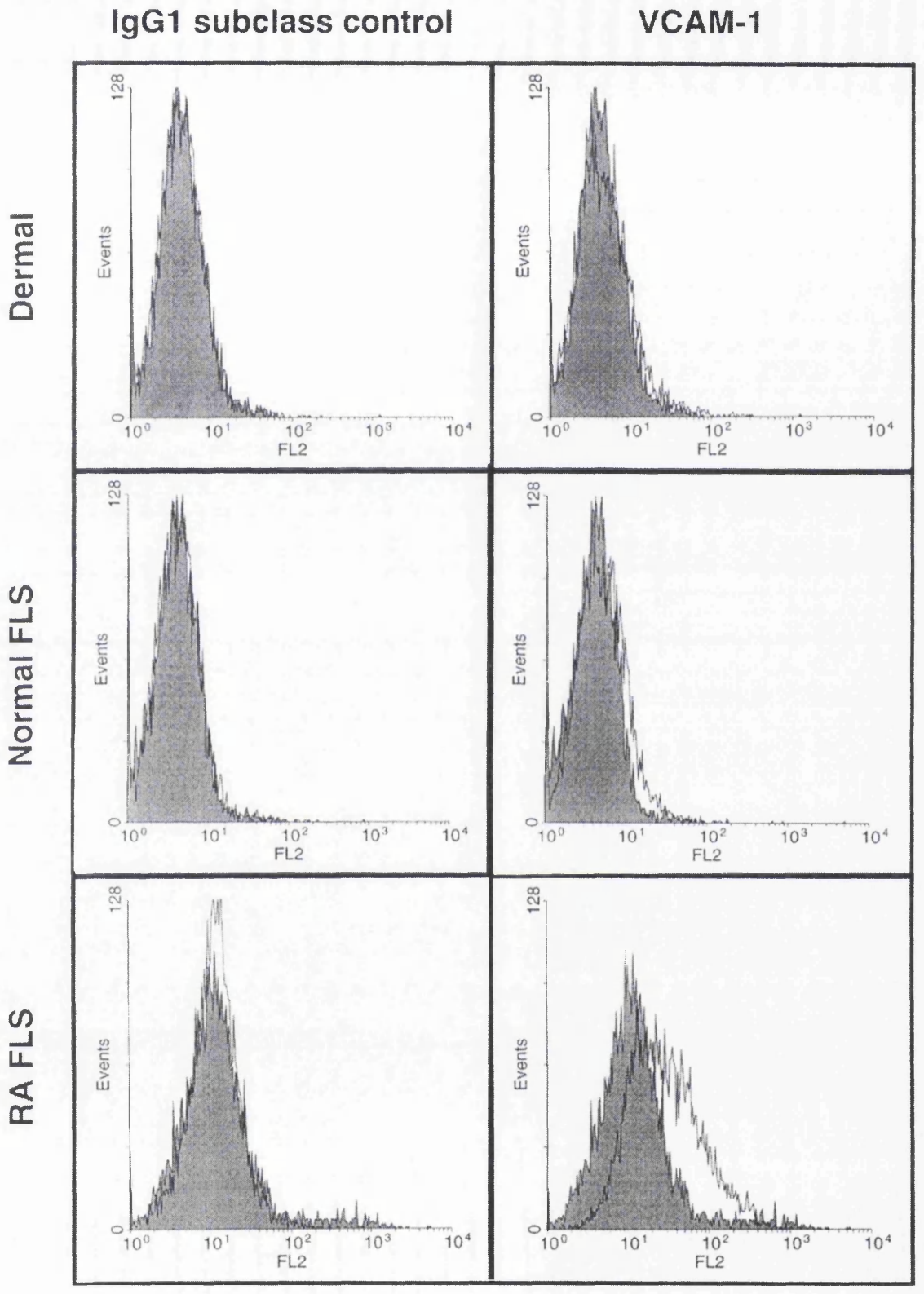

IgG1 subclass control
VCAM-1
Dermal
Normal FLS
RA FLS
Events
FL2
128
0
10^0
10^1
10^2
10^3
10^4

Figure 3.2

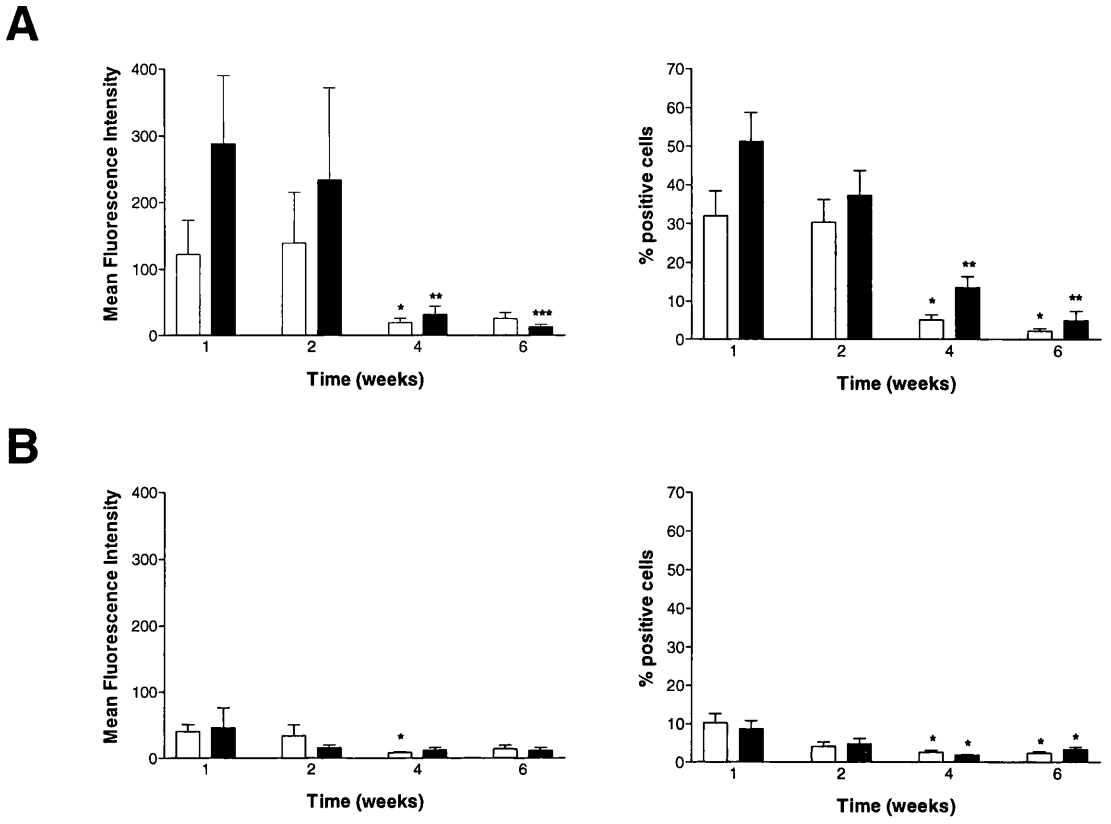
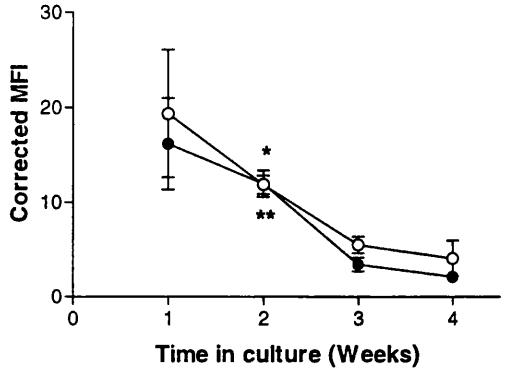
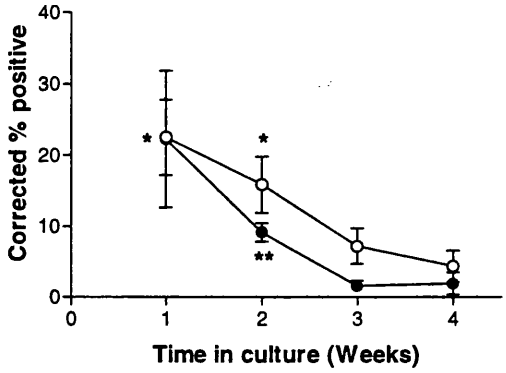


Figure 3.3 Time course of VCAM-1 and CD14 expression on cultured rheumatoid fibroblast-like synoviocytes. Fibroblast-like synoviocytes were isolated as described earlier (See methods and Materials) and cultured with 10% (v/v) FBS (open bars) or 10% (v/v) HPDS (solid bars) for 1, 2, 4, or 6 weeks. Medium was replenished every 5 days. At the respective time points, cells were harvested by incubation with 4 mM EDTA. The suspension of fibroblast-like synoviocytes was washed and incubated with saturating amounts of anti-VCAM-1 (**A**), or anti-CD14 (**B**), followed by an incubation with phycoerythrin-conjugated anti-mouse IgG. Samples were analysed using a FACStar. Results are expressed as both corrected MFI and corrected % positive cells. Data is presented as the mean ± SEM of four or five experiments. * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared with the results at week 1).

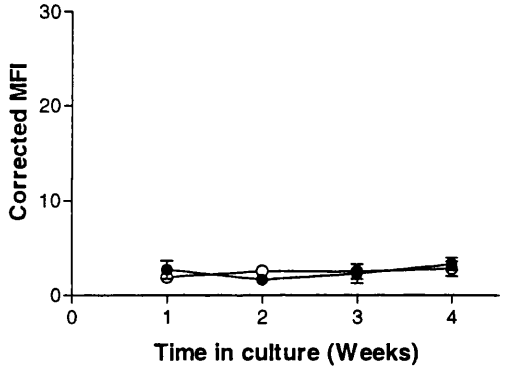
Figure 3.4 The effect of monocyte contamination on VCAM-1 expression by cultured fibroblast-like synoviocytes. Freshly isolated synoviocytes were prepared as described earlier (see Methods and Materials) and cultured with 10% (v/v) FBS. After 18 hours, adherent cells were either washed (open circles) with PBS to remove contaminating cells, or further cultured with contaminating cells (closed circles). Medium was replenished every 5 days. At medium change, contaminating cells were recovered by centrifugation and added back to the respective culture. At the respective time points, cells were harvested by incubation with 4 mM EDTA. The suspension of fibroblast-like synoviocytes was washed and incubated with saturating amounts of anti-VCAM-1 (**A** and **B**) or anti-CD14 (**C**), followed by an incubation with phycoerythrin-conjugated anti-mouse IgG. Samples were analysed using a FACStar and the results expressed as corrected MFI and corrected % positive cells. Data is presented as the mean ± SEM of four or six experiments. * indicates $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$ (compared with results at week 4).

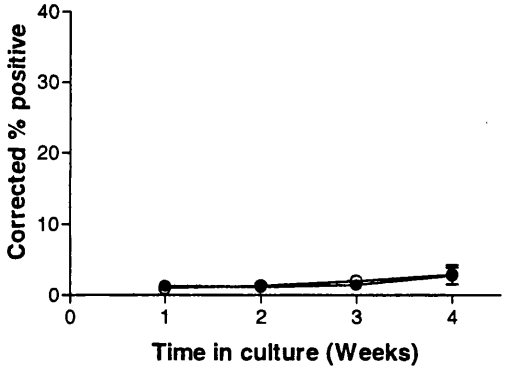
A. High VCAM-1-expressing cells



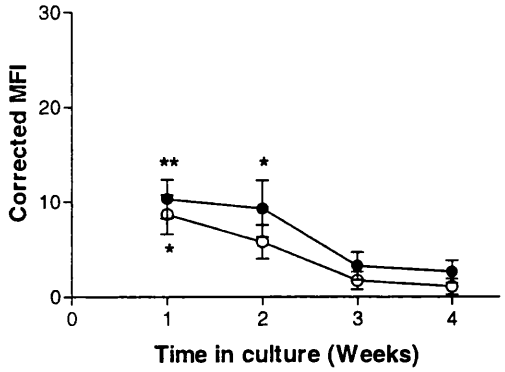


B. Low VCAM-1-expressing cells





C. CD14 contamination



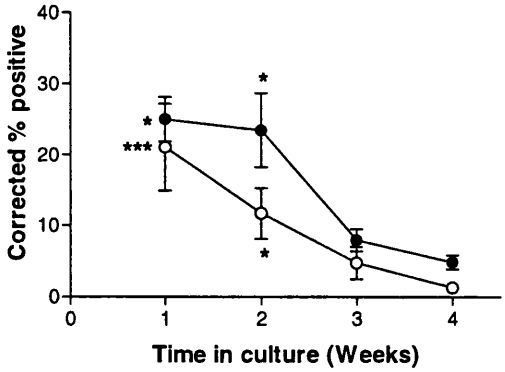


Figure 3.4

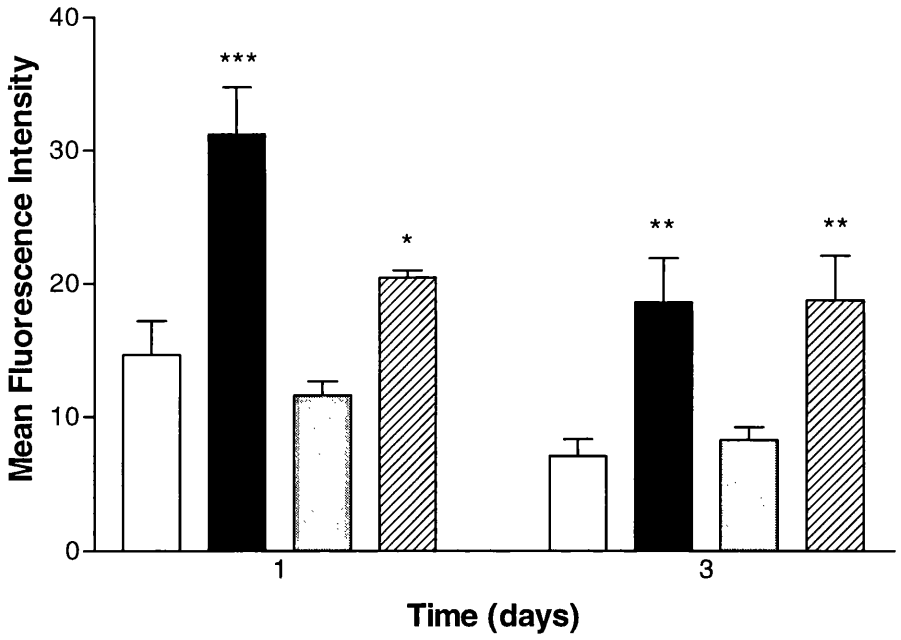
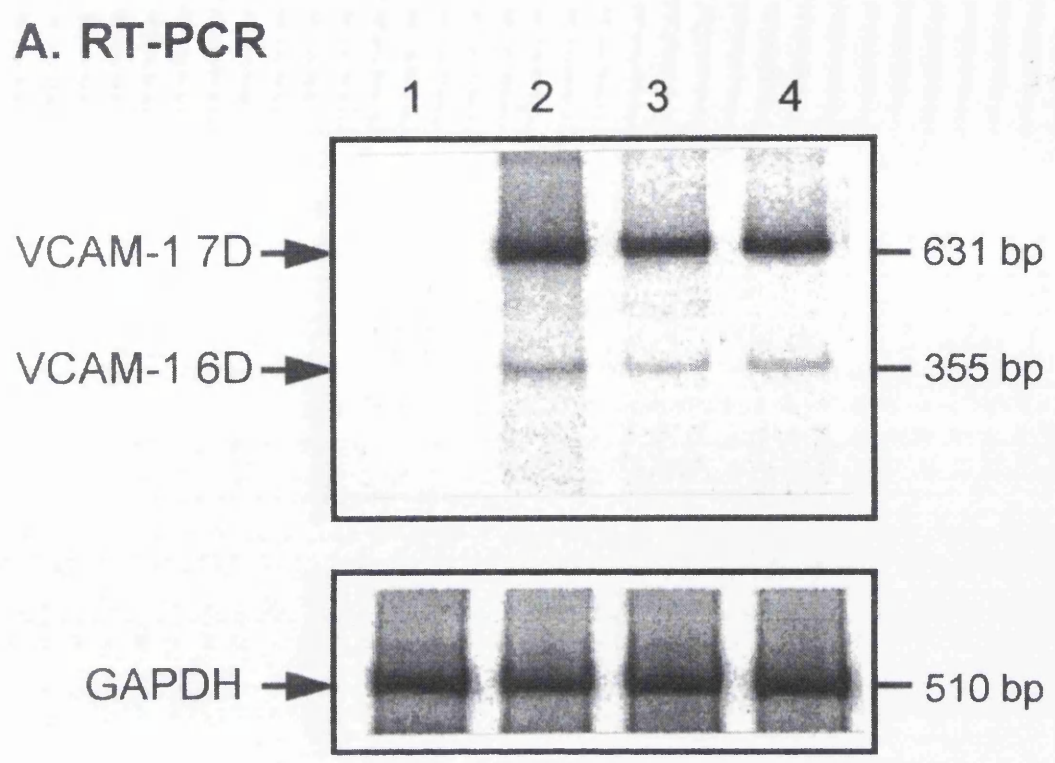


Figure 3.5 RA-conditioned medium does not up-regulate VCAM-1 expression on fibroblast-like synoviocytes. Fibroblast-like synoviocytes were used between passages 3 through 6. Cell surface VCAM-1 was determined by FACS after incubation with either medium only (open bars), medium plus TNF-α (10 ng/ml) (solid bars), conditioned medium only (stippled bars), or conditioned medium plus TNF-α (10 ng/ml) (hatched bars) for 1 and 3 days. Conditioned medium describes supernatants recovered from primary RA fibroblast-like synoviocytes. Subsequently, cells were harvested by incubating with 4 mM EDTA. The suspension of fibroblast-like synoviocytes was washed and incubated with saturating amounts of anti-VCAM-1, followed by an incubation with phycoerythrin-conjugated anti-mouse IgG. Samples were analysed using a FACStar and the results expressed as corrected MFI. Data is presented as the mean ± SEM of six experiments. * indicates $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$ (compared with medium only at the respective time point).

Figure 3.6 Primary fibroblast-like synoviocytes express mRNA for both VCAM-1 6D and VCAM-1 7D. Total RNA was isolated from normal fibroblast-like synoviocytes after culture for 3 weeks in 10% (v/v) FBS. RA fibroblast-like synoviocytes were prepared from RA synovium as described earlier (see Methods and Materials) and cultured with 10% (v/v) FBS or 10% (v/v) HPDS for 14 days. For Day 0 studies, total RNA was isolated following enzymatic disaggregation of synovium. **(A)** RT-PCR analysis of VCAM-1 mRNA and GAPDH mRNA expression in total RNA isolated from normal fibroblast-like synoviocytes (lane 1), RA Day 0 (lane 2), and RA fibroblast-like synoviocytes cultured for 14 days with 10% FBS (lane 3) or 10% HPDS (lane 4). Total RNA was isolated and reverse transcribed (see Methods and Materials). First strand cDNA was amplified by PCR using specific VCAM-1 primers (vcam.751 and vcam.1361) or GAPDH primers (Gap.67 and Gap.406) in the presence of [α^{32}P]- or [α^{33}P]-dATP. PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid, and dried under vacumn. Gels were exposed to phosphorimager plates for 24 hours and scanned using a Fuji Bas 1000 phosphorimager. **(B)** Southern blot analysis of VCAM-1 PCR products. Total RNA was isolated from normal fibroblast-like synoviocytes (lane 1), RA Day 0 (lane 2), and RA fibroblast-like synoviocytes cultured for 14 days with 10% FBS (lane 3) or 10% HPDS (lane 4), and reverse transcribed (see Methods and Materials). First strand cDNA was amplified by PCR using specific VCAM-1 primers (vcam.751 and vcam.1361). PCR-products were electrophoresed on a 1.5% agarose gel and transferred onto a nytran membrane. VCAM-1 7D PCR-products were identified by hybridization to a ^{32}P-labelled antisense oligonucleotide probe (Oligo 3b) (Top panel). The blot was stripped and reprobed using an antisense ^{32}P-labelled oligonucleotide (Oligo 3-4) which binds to regions encoded in domains 3 and 4 of the 6D form. After each hybridization the blots were exposed to phosphorimager plates for 24 hours and scanned using a Fuji Bas 1000 phosphorimager. Results shown are representative of three experiments performed.


A. RT-PCR
1
2
3
4
VCAM-1 7D
VCAM-1 6D
631 bp
355 bp
GAPDH
510 bp

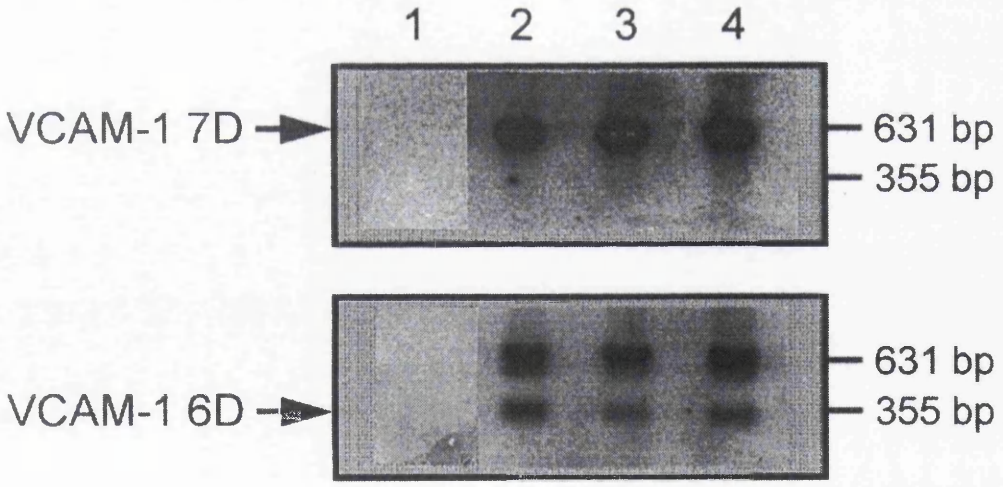

B. Southern blot
1
2
3
4
VCAM-1 7D
631 bp
355 bp
VCAM-1 6D
631 bp
355 bp

Figure 3.6

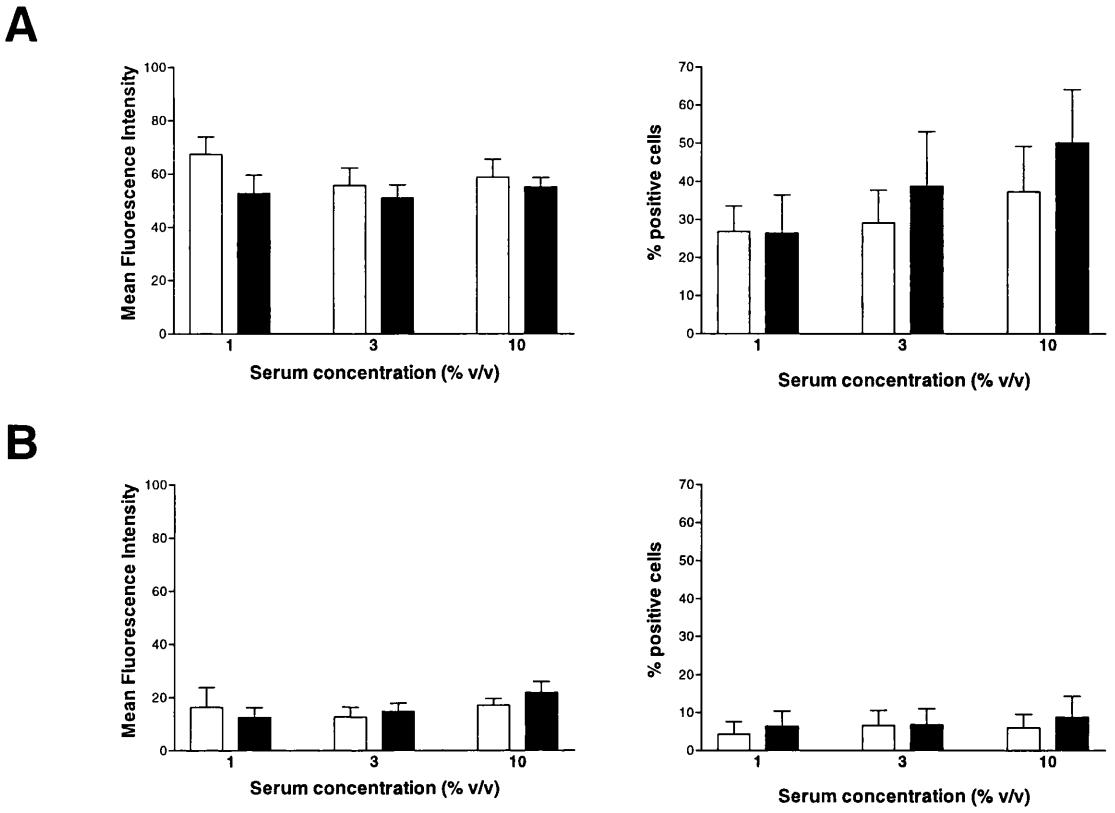


Figure 3.7 Serum concentration has no effect on the expression of cell surface VCAM-1 and CD14 by rheumatoid fibroblast-like synoviocytes. Fibroblast-like synoviocytes were isolated as described earlier (see Methods and Materials) and cultured with 10% (v/v) FBS (open bars) or 10% (v/v) HPDS (solid bars) for 14 days. Subsequently, cells were recovered by incubation with 4 mM EDTA. The suspension of fibroblast-like synoviocytes was washed and incubated with saturating amounts of anti-VCAM-1 (**A**), or anti-CD14 (**B**), followed by an incubation with phycoerythrin-conjugated anti-mouse IgG. Samples were analysed using a FACStar flow cytometer. Results are expressed as both corrected MFI and corrected % positive cells. Data is presented as the mean ± SEM of six experiments.

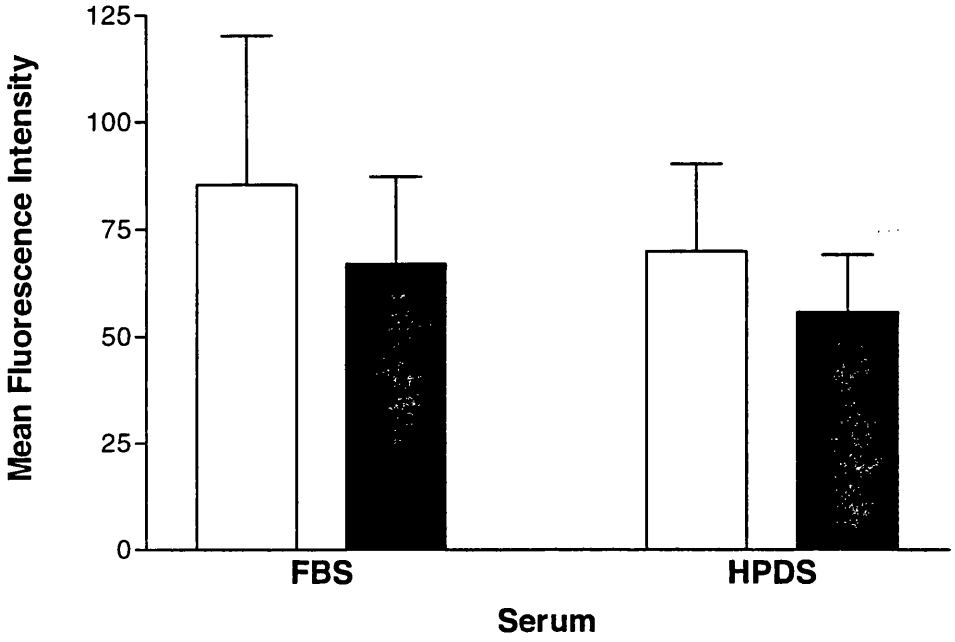


Figure 3.8 The effect of hyaluronic acid (HA) on VCAM-1 expression by fibroblast-like synoviocytes. Fibroblast-like synoviocytes were isolated as described earlier (see Methods and Materials) and cultured for 7 days with 10% (v/v) FBS or 10% (v/v) HPDS. Cells were treated with medium only (open bars) or medium plus HA (1 mg/ml w/v) (solid bars). Subsequently, cells were harvested by incubating with 4 mM EDTA. The suspension of fibroblast-like synoviocytes was washed and incubated with saturating amounts of anti-VCAM-1, followed by an incubation with phycoerythrin-conjugated anti-mouse IgG. Samples were analysed using a FACStar and the results expressed as corrected MFI. Data is presented as the mean ± SEM of six experiments.

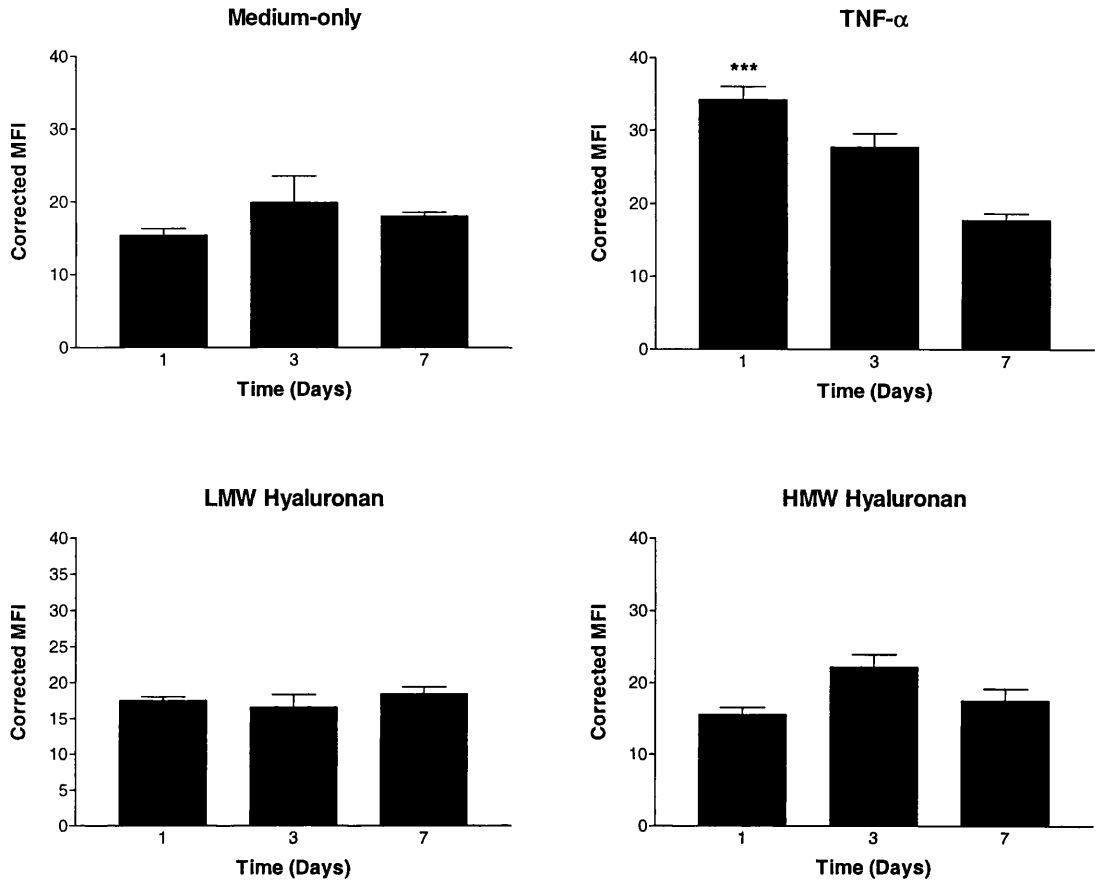


Figure 3.9 Low molecular weight hyaluronan has no effect on VCAM-1 expression by fibroblast-like synoviocytes. Long-term passages of fibroblast-like synoviocytes were cultured for up to 7 days with either medium-only, medium plus TNF-α (10 ng/ml), medium plus LMW hyaluronan (1 mg/ml w/v), or medium plus HMW hyaluronan (1 mg/ml w/v). Cells were harvested at the respective time point and cell surface VCAM-1 determined. Samples were analysed using a FACStar and the results expressed as corrected MFI. Data is presented as the mean ± SEM of four experiments. *** indicates $p < 0.001$ (compared with medium only at the respective time point).

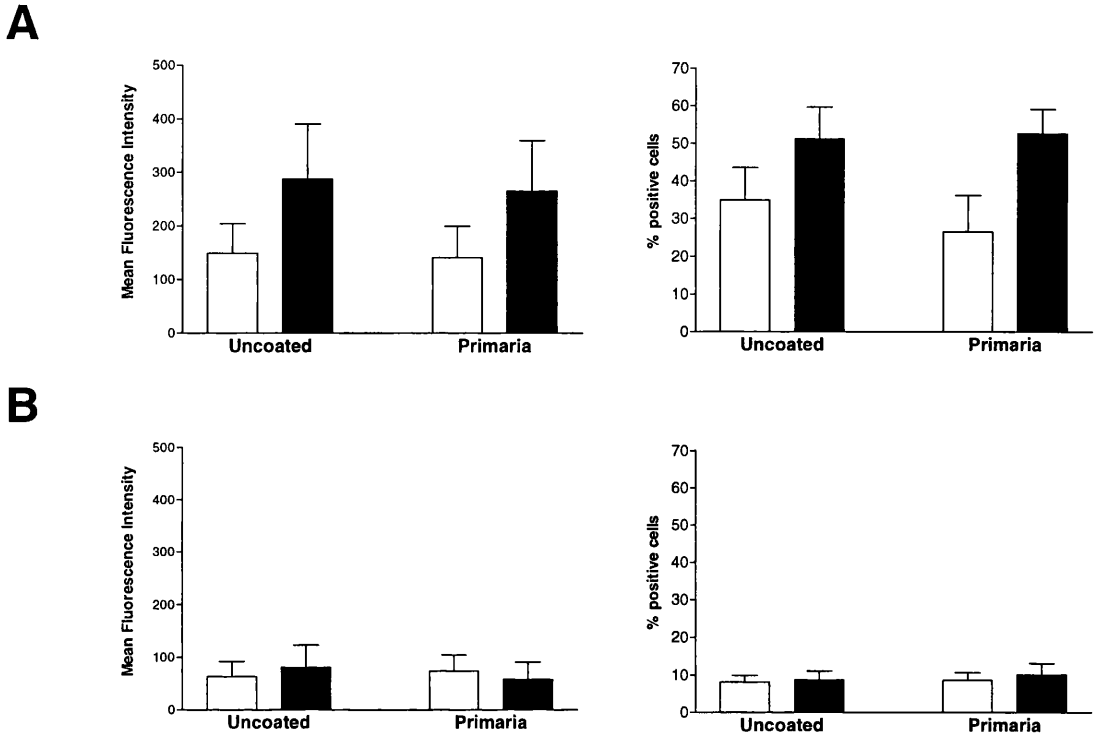


Figure 3.10 Cell surface VCAM-1 and CD14 expression on rheumatoid fibroblast-like synoviocytes cultured on different types of tissue culture plastic. Fibroblast-like synoviocytes were isolated as described (see Methods and Materials) and grown on normal tissue culture plastic dishes (Uncoated) or commercially available primary culture dishes (Primaria). Following culture with 10% (v/v) FBS (open bars) or 10% (v/v) HPDS (solid bars) for 7 days, cells were removed by incubation with 4 mM EDTA. The suspension of fibroblast-like synoviocytes was washed and incubated with saturating amounts of anti-VCAM-1 (**A**), or anti-CD14 (**B**), followed by an incubation with phycoerythrin-conjugated anti-mouse IgG. Samples were analysed using a FACStar flow cytometer and the results expressed as either corrected MFI or as corrected % positve. Data is presented as the mean ± SEM of four or five experiments.

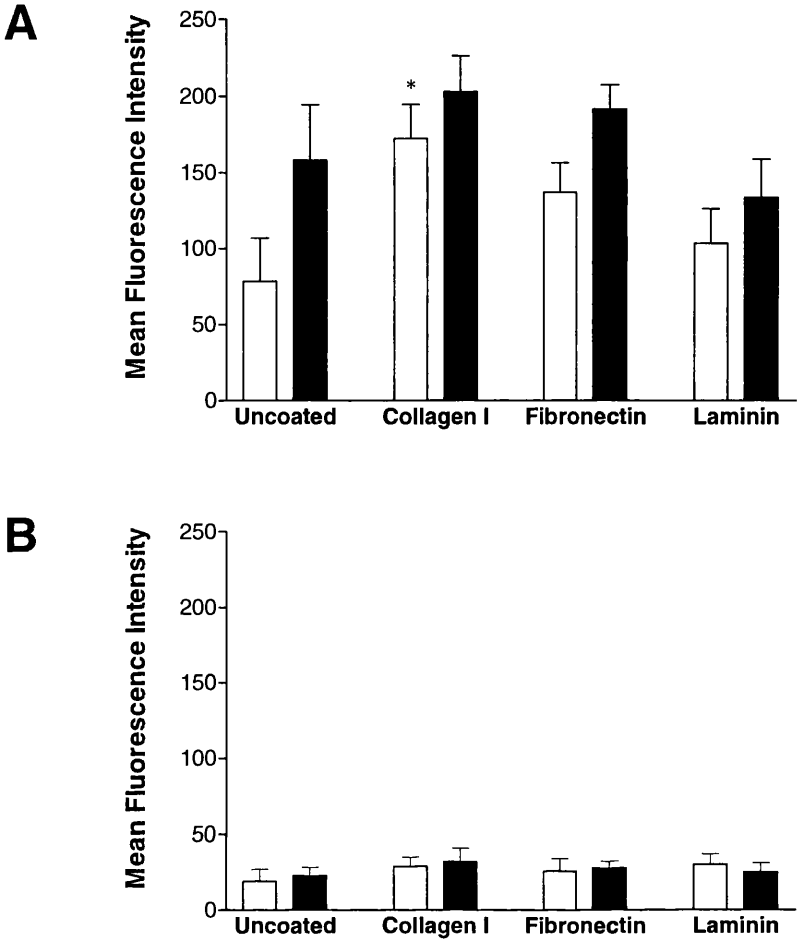


Figure 3.11 The effect of extracellular matrix on VCAM-1 expression by rheumatoid fibroblast-like synoviocytes. Fibroblast-like synoviocytes were isolated as described earlier (see Methods and Materials) and grown on normal tissue culture dishes (Uncoated) or dishes precoated with collagen type I, fibronectin, or laminin, with 10% (v/v) FBS (open bars) or 10% (v/v) HPDS (solid bars). Cells were harvested after 1 (**A**) and 6 (**B**) weeks by incubation with 4 mM EDTA. The suspension of fibroblast-like synoviocytes was washed and incubated with saturating amounts of anti-VCAM-1, followed by an incubation with phycoerythrin-conjugated anti-mouse IgG. Samples were analysed using a FACStar and the results expressed as corrected MFI. Data is presented as the mean ± SEM of four experiments. * indicates $p < 0.05$.

Table 3.1 Levels of IL-1β and TNF-α in supernatants recovered from primary cultures of RA fibroblast-like synoviocytes

	IL-1β (pg/ml)	TNF-α (pg/ml)
Day 6 (HPDS)	2.1	1.4
Day 14 (HPDS)	1.0	0
Day 14 (FBS)	1.5	0

Cytokine levels were measured by ELISA according to manufacturers instructions (see Methods and Materials). Supernatants were recovered from primary cultures of RA fibroblast-like synoviocytes after 6 days and 14 days in culture. Cells were grown with either 10% (v/v) FBS or 10% (v/v) HPDS.

3.4. Discussion

The present study has shown that primary cultures of fibroblast-like synoviocytes maintain their cell surface VCAM-1 expression for a period of 2 weeks, after which expression levels steadily decline, with low levels of expression being observed after 4 weeks of culture. This observation was paralleled by a similar decline in the expression of VCAM-1 mRNA transcripts. These results support data from earlier published studies that observed low basal levels of cell surface VCAM-1 expression on long-term cultures of fibroblast-like synoviocytes (Morales-Ducret *et al.*, 1992; Marlor *et al.*, 1992). In these studies fibroblast-like synoviocytes were used after passage 3 which in our experiments corresponded to three or four weeks in culture.

Several studies have demonstrated that VCAM-1 expression can be briefly up-regulated by the addition of pro-inflammatory cytokines or by co-culturing with monocytes or lymphocytes (Morales-Ducret *et al.*, 1992; Marlor *et al.*, 1992; Bombara *et al.*, 1993; Blue *et al.*, 1993). However, these studies have not addressed how these factors affect VCAM-1 expression levels in the long term. Thus, to date it remains unknown as to what causes the prolonged elevated expression of VCAM-1 observed *in situ* and during the first few weeks of *in vitro* culture. This study has shown that elevated cell surface expression of VCAM-1 by fibroblast-like synoviocytes in culture occurs in the absence of relevant levels of IL-1β and TNF-α. Furthermore, conditioned medium recovered from early passages of RA FLSs, when VCAM-1 is still high, cannot elevate the rundown levels of VCAM-1 apparent in late passage synoviocytes. This would suggest that factors present in conditioned medium are not responsible for maintaining VCAM-1 expression. Moreover, it would also imply that fibroblast-like synoviocytes are not secreting mediators that regulate VCAM-1 expression on these

cells.

The observed loss of VCAM-1 expression may result from disrupted contact with the extracellular matrix that occurs upon disaggregation and placement in *in vitro* culture. However, of the extracellular matrix factors tested, only the presence of type I collagen significantly elevated the levels of VCAM-1 expression but again it was unable to prevent its decline. In addition, hyaluronan fragments that are of a similar size (approximately 1 x 10^6 Da) to those found in RA synovial fluid were also found to have no effect on VCAM-1 expression (Dahl and Husby, 1985). This low molecular weight form of hyaluronan does possess biological activity since other studies have shown it to be a potent inducer of inflammatory gene expression in macrophages (McKee *et al.*, 1996, 1997). It is possible that the pro-inflammatory actions of low molecular weight HA are cell-type specific and only mediate the induction of inflammatory genes in macrophage-like cells. *In vitro* cultures of RA fibroblast-like synoviocytes synthesize increased amounts of very small HA fragments (50,000 Da). It is, however, unlikely that these have any affect on VCAM-1 expression since RA-conditioned medium, in which these fragments are present, was unable to up-regulate VCAM-1 expression. It is possible that other extracellular matrix components in conjuction with other soluble factors are capable of regulating VCAM-1 expression.

This study has also further demonstrated that RA fibroblast-like synoviocytes predominantly express the 7 domain form of VCAM-1. Messenger RNA transcripts encoding both VCAM-1 6D and VCAM-1 7D were identified in total RNA isolated from primary fibroblast-like synoviocytes and freshly dispersed synovial cells. The expression of mRNA transcripts for both domains were found to gradually diminish with increasing time in culture. The expression of VCAM-1 6D mRNA reduced to

almost undetectable levels in long-term FLS cultures. In contrast, although VCAM-1 7D mRNA also declined with time in culture, a basal level of VCAM-1 expression was still detectable in these cells after 8 weeks. The results presented here, together with those previously published, indicate that VCAM-1 expression in fibroblast-like synoviocytes is under both constitutive and inducible forms of control. Importantly, fibroblast-like synoviocytes are the only cell type currently known to possess both forms of VCAM-1 expression. Endothelial cells and smooth muscle cells whilst demonstrating cytokine-inducible VCAM-1 expression exhibit no constitutive VCAM-1 expression (Iademarco *et al.*, 1995; Barks *et al.*, 1997). In contrast, skeletal muscle cells demonstrate moderate levels of constitutive VCAM-1 expression which cannot be up-regulated by cytokines (Iademarco *et al.*, 1993). These differences in expression between endothelial cells and skeletal muscle cells are regulated at the promoter level of the VCAM-1 gene. Skeletal muscle cells, in contrast to endothelial cells, possess a position-specific enhancer within the VCAM-1 promoter which when bound to a specific nuclear protein is able to override up-stream silencer elements and NF-κB, and thus maintain VCAM-1 expression (Iademarco et al., 1993). The expression of this nuclear protein may determine constitutive VCAM-1 expression in RA fibroblast-like synoviocytes. A distinctive RA cell type, termed the pannocyte, has recently been identified and found to maintain high levels of VCAM-1 expression throughout their time in *in vitro* culture (Zvaifler *et al.*, 1997). A detailed analysis of the VCAM-1 promoter in fibroblast-like synoviocytes and pannocytes is therefore required to determine the endogenous transcription factors utilized in the control of VCAM-1 expression in these cells.

The observation that contaminating mononuclear cells were unable to increase

VCAM-1 expression was rather interesting. More so because the co-culture of fibroblast-like synoviocytes with either peripheral blood monocytes or activated T lymphocytes has been previously shown to up-regulate VCAM-1 expression (Blue *et al.*, 1993; Bombara *et al.*, 1993). These studies identified TNF-α release from mononuclear leukocytes as the mediator responsible for increased VCAM-1 expression. However, TNF-α levels were minimal in the supernatants recovered from primary cultures of RA fibroblast-like synoviocytes. This could explain the inability of the contaminating leukocytes to affect the level of VCAM-1 expression. Although TNF-α levels were low in RA-conditioned medium, it is still possible that this cytokine is responsible for the VCAM-1 expression observed *in situ*. Moreover, other cytokines acting in concert with TNF-α could regulate VCAM-1 on fibroblast-like synoviocytes. Indeed, Blue et al. (1993) have identified IL-4, a cytokine secreted from mast cells and T lymphocytes, as enhancing monocyte-induced VCAM-1 expression. The role of inflammatory cytokines in the regulation of fibroblast-like synoviocyte VCAM-1 expression will be explored in Chapter 4. The local release and complex interplay of TNF-α and other cytokines within the RA synovium may explain the elevated levels of VCAM-1 expression observed on fibroblast-like synoviocytes *in situ* and during the first few weeks of *in vitro* culture.

In conclusion, elevated levels of VCAM-1 on fibroblast-like synoviocytes were maintained for the first 2 weeks of *in vitro* culture following their removal from the RA synovial membrane. Of the factors tested, all related to the extracellular environment of the fibroblast-like synoviocyte *in situ*, none were able to maintain elevated levels of expression of VCAM-1 and could therefore not be considered instrumental in the regulation of constitutively high levels of VCAM-1 observed in the synovial membrane.

Chapter 4

Sustained elevated levels of VCAM-1 in cultured fibroblast-like synoviocytes can be achieved by TNF-α in combination with either IL-4 or IL-13 through increased mRNA stability

4.1 Introduction

Vascular cell adhesion molecule-1 was first identified on human umbilical vein endothelial cells after treatment with the inflammatory cytokine IL-1β (Osborn *et al.*, 1989). Subsequent studies have shown that a variety of other cytokines can also induce VCAM-1 expression (Bochner *et al.*, 1995; Haraldsen *et al.*, 1996; Iademarco *et al.*, 1995; Lechleitner *et al.*, 1998). Moreover, certain cytokines can also up-regulate VCAM-1 expression in fibroblast-like synoviocytes and smooth muscle cells (Morales-Ducret *et al.*, 1992; Barks *et al.*, 1997). The combination of IL-4 and TNF-α synergistically increases VCAM-1 expression on endothelial cells, vascular smooth muscle cells, and fibroblast-like synoviocytes (Iademarco *et al.*, 1995; Barks *et al.*, 1997; Edwards *et al.*, 1997b). However, the mechanisms by which they increase expression differs markedly between cell types (Iademarco *et al.*, 1995; Barks *et al.*, 1997). In vascular smooth muscle cells, both IL-4 and TNF-α increase transcription of the VCAM-1 gene (Barks *et al.*, 1997). In contrast, in endothelial cells TNF-α activates VCAM-1 transcription whereas IL-4 mediates stabilization of mRNA transcripts

(Iademarco *et al.*, 1995). In addition, IL-4 also reduces the threshold concentration of TNF-α required to induce VCAM-1 transcription. In fibroblast-like synoviocytes however, the mechanism of action of IL-4 remains to be studied. The cytokine IL-13 has also been shown to synergistically up-regulate VCAM-1 expression on endothelial cells and vascular smooth muscle cells (Barks *et al.*, 1997). Interestingly, IL-13 is found at elevated levels in the synovium and synovial fluid of RA patients (Isomäki *et al.*, 1996).

Upon removal from the rheumatoid synovium, fibroblast-like synoviocytes demonstrate high levels of cell surface VCAM-1 for a period of two weeks (See Chapter 3). With increasing time in culture VCAM-1 levels were found to gradually decrease to a low constitutive level of expression. A similar pattern of expression was also observed for VCAM-1 mRNA (See Chapter 5; Figure 5.2). These data could mean that once in *in vitro* culture, the observed reduction in VCAM-1 expression may be a consequence of the loss of a number of inflammatory cytokines, in particular the loss of IL-1β and TNF-α. However, the effects of these cytokines have only been studied in short-term experiments and none of the reports have addressed the question of long-term cytokine exposure and its consequences. This study will investigate whether or not cytokine-dependent mechanisms can account for the sustained (long-term) elevated expression of VCAM-1 initially exhibited by fibroblast-like synoviocytes isolated from RA synovium. Emphasis was put on studying the possible role of IL-4 and IL-13 in combination with TNF-α.

4.2 Methods and Materials

All methods and materials used in this chapter are as earlier described in chapter 2. Any additions to these methods are included in the legends to figures. Cultured fibroblast-like synoviocytes were used between passage 3 and 10, which corresponded with 3-12 weeks in culture.

4.3 Results

4.3.1 Treatment of fibroblast-like synoviocytes with IL-1β and TNF-α up-regulates VCAM-1 expression

As described earlier, IL-1β and TNF-α could not be detected in the culture medium recovered from primary cultures of RA fibroblast-like cells. It was therefore reasoned that the addition of these cytokines could possibly restore the constitutively elevated levels of VCAM-1. In order to test this hypothesis, IL-1β or TNF-α, either alone or in combination, were added at a final concentration of 10 ng/ml to passaged fibroblast-like synoviocytes and VCAM-1 expression determined.

As can be seen from the time course shown in Figure 4.1, VCAM-1 expression on fibroblast-like synoviocytes was induced after exposure to a single dose of either IL-1β or TNF-α. Cell surface VCAM-1 levels were maximal after 24 hours and returned to control levels by 72 hours, following treatment with either cytokine. TNF-α was found to be more potent than IL-1β. In addition, treatment of fibroblast-like synoviocytes with a combination of IL-1β and TNF-α resulted in an up-regulation of VCAM-1 expression

that was equivalent in magnitude to that observed with TNF-α alone (Figure 4.2). This implies that both TNF-α and IL-1β are acting via similar signalling pathways.

VCAM-1 7D mRNA expression was also determined after the addition of IL-1β or TNF-α at 1, 3, or 7 days (Figure 4.3). Steady state levels of VCAM-1 7D mRNA were identified in all the control (Untreated) cultures tested. Upon TNF-α treatment, VCAM-1 7D mRNA levels were elevated at 24 hours, but had returned to levels observed in untreated cells after 3 days. In contrast, treatment with IL-1β had no effect on the expression of VCAM-1 7D mRNA at any of the time points studied. It is possible that VCAM-1 7D mRNA expression was up-regulated during the first 12 hours following IL-1β stimulation, but had fallen to control levels by 24 hours.

4.3.2 Chronic treatment of primary fibroblast-like synoviocytes with IL-1β or TNF-α induces only a transient expression of VCAM-1

The effect of repeated cytokine additions (also referred to as chronic treatment) was next assessed. Here, IL-1β and TNF-α were added at day 0 and re-added after each third-day medium change. Cell surface VCAM-1 was determined on day 2, 5, 8 and 11. Surprisingly, as can be seen from Figure 4.4, VCAM-1 expression was maximal on day 2, but then declined and returned to baseline at day 8. VCAM-1 expression was maintained at baseline levels for the remaining time in culture. The addition of TNF-α alone, therefore, could not explain the prolonged levels of VCAM-1 observed in fresh cultures of fibroblast-like synoviocytes.

4.3.3 A synergistic increase in VCAM-1 expression can be observed with TNF-α in combination with IL-4

The effect of IL-4, either alone or in combination with TNF-α, was initially tested in a single-addition experiment and VCAM-1 expression measured after 72 hours. This time point was chosen because TNF-α-induced VCAM-1 expression had by then returned to baseline (MFI 15.0). As can be seen in Figure 4.5, a single treatment of IL-4 alone resulted in elevated VCAM-1 expression (MFI of 37.5). However, the combination of IL-4 with TNF-α resulted in a synergistic increase (MFI of 67.77). Higher doses of IL-4 (50 ng/ml) either alone or in combination with TNF-α (10 ng/ml) were not able to further increase VCAM-1 expression (Figure 4.6).

4.3.4 TNF-α in combination with either IL-4 or IL-13 can greatly prolong the expression of VCAM-1

IL-13 has a number of similar biological actions which are mediated via its binding to the IL-4 receptor (Bochner *et al.*, 1995; Jahnsen *et al.*, 1997; Kotowicz *et al.*, 1996). The effect of IL-4 and, therefore, IL-13 on the time course of VCAM-1 expression was next tested. Firstly, the effect of a single application of IL-4 or IL-13 either alone or in combination with TNF-α was determined. The addition of a single dose of IL-4 (10 ng/ml) resulted in an immediate up-regulation of VCAM-1 expression which peaked at day 3 and had returned to baseline levels by day 7 (Figure 4.7(A)). The combination of IL-4 and TNF-α, however, gave a synergistic increase in VCAM-1 expression, which peaked at day 7 and had returned to baseline by day 14. Similar data were obtained with

IL-13, except that VCAM-1 expression levels were lower compared to IL-4 (Figure 4.7(B)).

The effect of chronic treatment with TNF-α (10 ng/ml) in combination with IL-4 or IL-13 (each at 10 ng/ml) was next assessed over a long-term incubation. Cytokines were added at day 0 and re-added after each fourth-day medium change. Cell surface expression of VCAM-1 was determined on day 4, 8, 16 and 20. As can be seen in Figure 4.8, chronic treatment with TNF-α was unable to prolong the transient expression of VCAM-1. Both IL-4 and IL-13 alone gave a significant up-regulation compared to control and, moreover, induce a higher level of expression than TNF-α alone. The combination of either IL-4 or IL-13 with TNF-α not only resulted in a synergistic increase in VCAM-1 expression but it also prolonged elevated expression for over 16 days. Again, IL-4 was found to be more potent than IL-13.

4.3.5 Elevated VCAM-1 expression can be achieved by pathophysiological concentrations of IL-13 in combination with TNF-α

VCAM-1 expression levels induced by different concentrations of IL-13 in the presence of TNF-α (10 ng/ml) were also assessed by immunohistochemistry. As can be seen in Figure 4.9, highly elevated levels of VCAM-1 could be observed with as little as 1.0 ng/ml of IL-13 in combination with TNF-α (10 ng/ml). These concentrations are within the range detected in the diseased joint (Isomäki *et al.*, 1996). It is therefore pertinent to conclude that pathophysiological concentrations of IL-13 could be instrumental in maintaining high levels of VCAM-1 in the rheumatoid synovium.

4.3.6 Induction and prolonged expression of VCAM-1 mRNA by TNF-α in combination with either IL-4 or IL-13

Treatment of fibroblast-like synoviocytes with either IL-4 or IL-13 up-regulates VCAM-1 mRNA expression (Figure 4.10 (A)). Again the combination of these cytokines with TNF-α resulted in a dramatic up-regulation of expression. As can be seen from Figure 4.10 (B), normalizing VCAM-1 band intensity to that of GAPDH enables a semiquantitative assessment of VCAM-1 mRNA expression. Induction of VCAM-1 mRNA was also analysed over a short time course (Figure 4.11). VCAM-1 mRNA transcripts were found to be expressed at low levels in untreated cells (medium-only). IL-4 and IL-13 were both able to induce VCAM-1 mRNA expression that exhibited slower kinetics of induction compared to TNF-α. The combination of IL-4 and TNF-α gave a dramatic increase in both the level and duration of expression of VCAM-1. Moderate synergy was found with IL-13 in combination with TNF-α.

4.3.7 IL-4 and IL-13 stabilize VCAM-1 mRNA transcripts

To study mRNA stability, its degradation was studied in the presence of actinomycin D (an inhibitor of transcription). Fibroblast-like synoviocytes were cultured with IL-4, IL-13 or TNF-α alone, or in combinations for 16 hours. Actinomycin D (5 μg/ml) was added after 16 hours and total RNA isolated thereafter at 0, 2, 4 and 10 hours. Semiquantitative RT-PCR was carried out (Figure 4.12(A)). VCAM-1 mRNA transcripts induced by TNF-α and IL-4 exhibited half-lives of 2 and 6 hours, respectively. When TNF-α and IL-4 were combined the half-life was extended to over

10 hours, i.e. no reduction in the level of VCAM-1 mRNA expression could be detected. Similar results were also obtained with IL-13. The effectiveness of these cytokines in stabilizing mRNA after 10 days of chronic treatment was also assessed. Elevated levels of VCAM-1 mRNA were still observed in cells treated with the combination of IL-4 and TNF-α, but not with TNF-α alone. The stability of the transcripts was still increased at day 10 in cells treated with the combination of IL-4 and TNF-α (Figure 4.12(B) and (C)), albeit at a lower level. In summary, IL-4 and IL-13 may play a prominent part in the maintenance of sustained cell surface expression of VCAM-1 by stabilizing VCAM-1 mRNA transcripts.

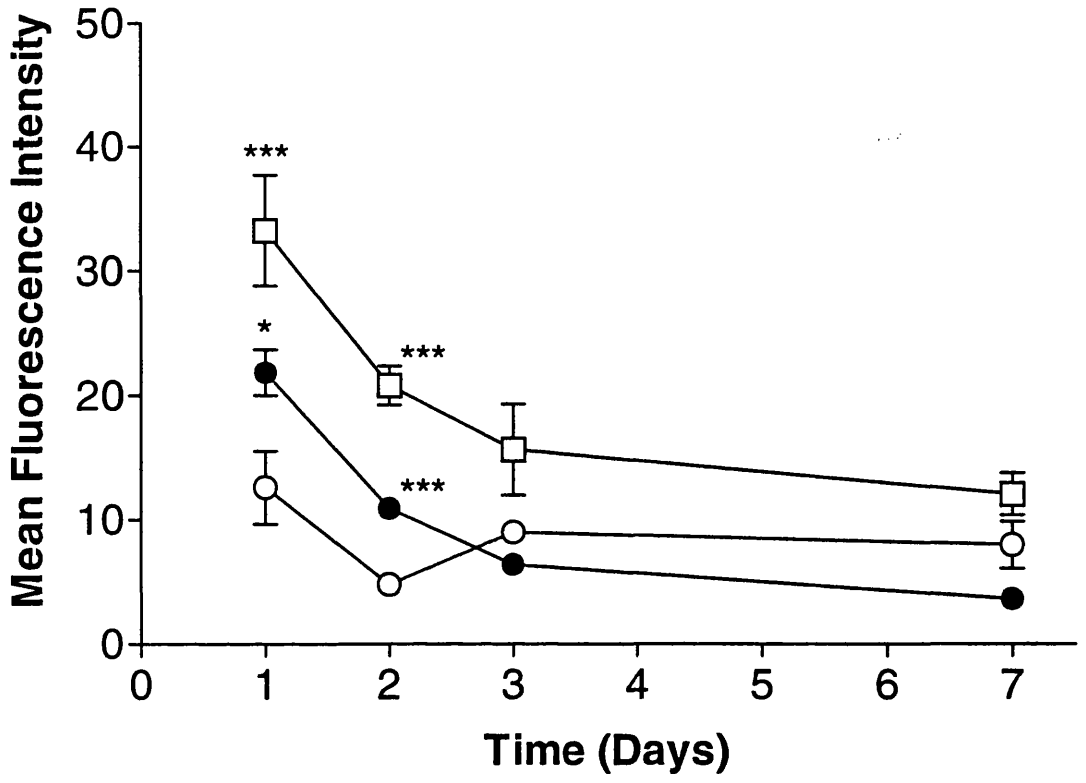


Figure 4.1 IL-1β and TNF-α induce transient VCAM-1 expression on fibroblast-like synoviocytes. Cells were treated with either medium only (open circles), or medium plus IL-1β (10 ng/ml) (closed circles), or medium plus TNF-α (10 ng/ml) (open squares) for 1, 2, 3, and 7 days. Cell surface VCAM-1 expression was determined by flow cytometry using mAb BBA-5 as primary antibody. Samples were analysed using a FACScan and the results expressed as corrected MFI. Data are presented as the mean ± SEM of six experiments. * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared with medium only at the respective time point).

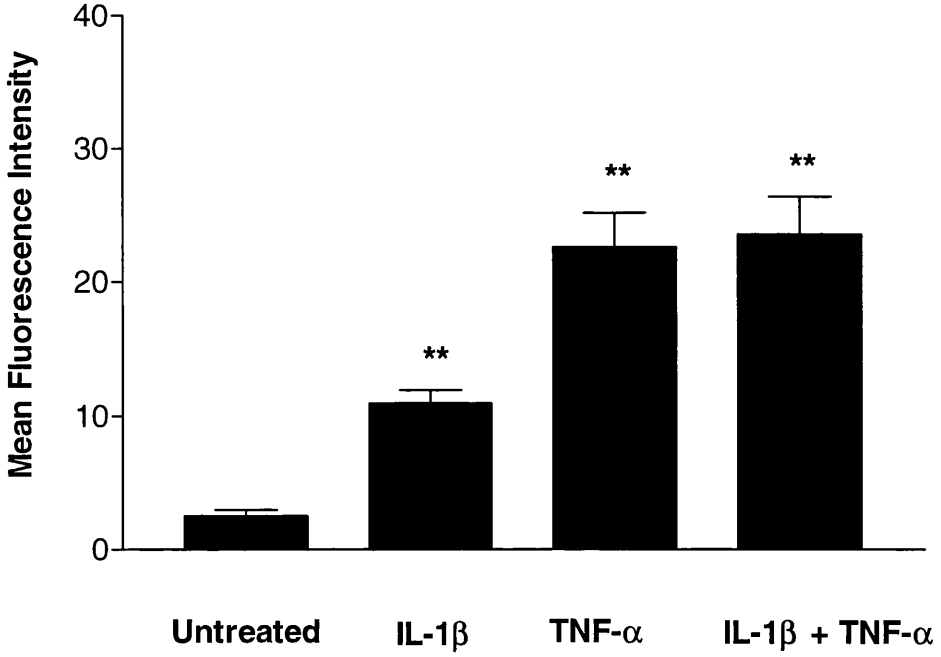


Figure 4.2 IL-1β and TNF-α do not synergize to potentiate VCAM-1 expression on fibroblast-like synoviocytes. Cells were treated with either medium only (Untreated), or medium plus IL-1β (10 ng/ml), medium plus TNF-α (10 ng/ml), or medium plus IL-1β and TNF-α (both at 10 ng/ml) for 48 hours. Cell surface VCAM-1 expression was determined by flow cytometry using mAb BBA-5 as primary antibody. Samples were analysed using a FACScan and the results expressed as corrected MFI. Data are presented as the mean ± SEM of three experiments. ** indicates $p < 0.01$ (compared with medium only). Note, the difference between IL-1β and IL-1β plus TNF-α was significant ($p < 0.05$), whereas the difference between TNF-α and IL-1β plus TNF-α was not.

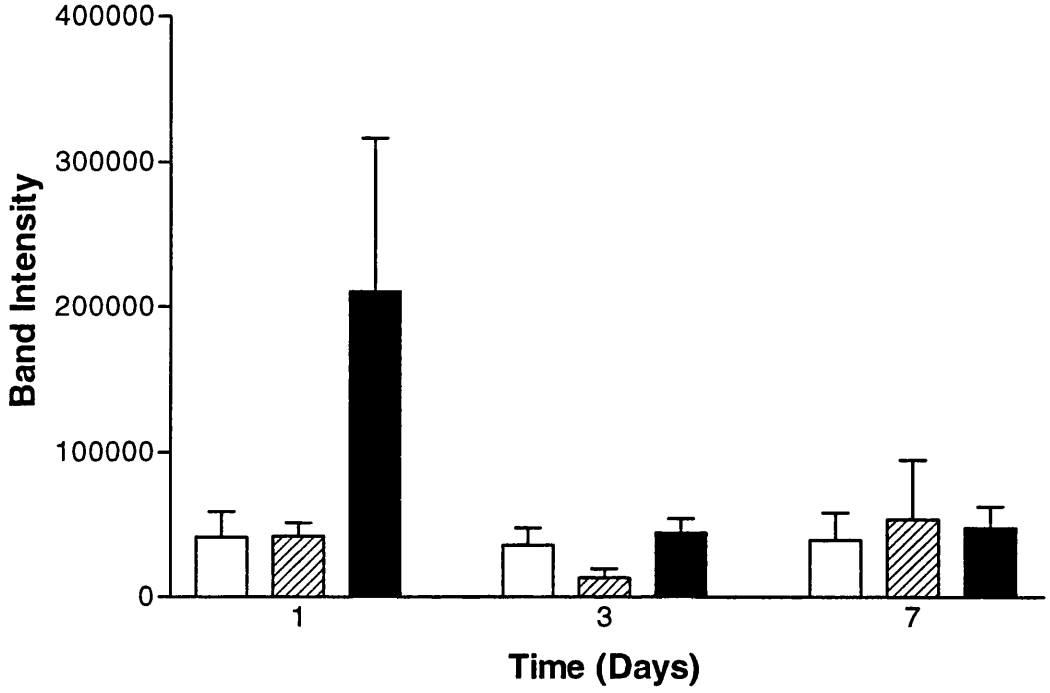


Figure 4.3 Time course of cytokine-induced VCAM-1 7D mRNA expression by fibroblast-like synoviocytes. Fibroblast-like synoviocytes (passage 3 through 6) were treated with medium only (open bars), medium plus IL-1β (10 ng/ml) (hatched bars), or medium plus TNF-α (10 ng/ml) (closed bars) at 1, 3 and 7 days prior to RNA isolation. Total RNA was isolated and reverse transcribed (see Methods and Materials). First strand cDNA was amplified by PCR using specific VCAM-1 primers (vcam.751 and vcam.1361). PCR-products were electrophoresed on a 1.5% agarose gel and transferred onto a nytran membrane. VCAM-1 7D PCR-products were identified by hybridization to a ^{32}P-labelled probe (Oligo 3b). Blots were exposed to phosphorimager plates for 24 hours and scanned using a Storm II phosphorimager. Data is presented as the mean band intensity ± SEM of three experiments.

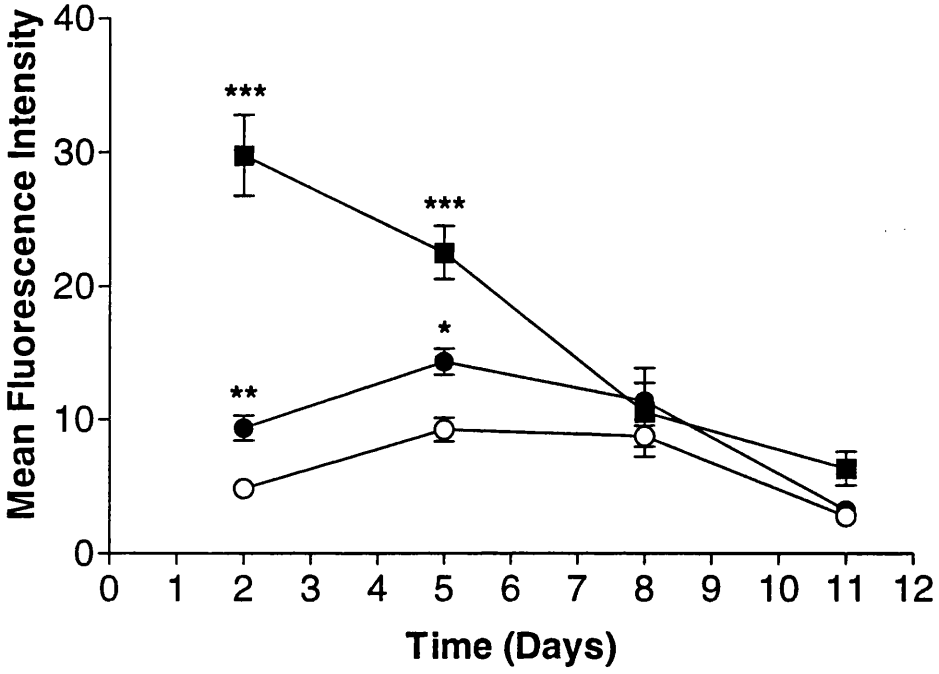


Figure 4.4 Chronic-cytokine treatment with TNF-α and IL-1β prolongs transient VCAM-1 expression on fibroblast-like synoviocytes. Medium plus IL-1β (10 ng/ml) (closed circles), medium plus TNF-α (10 ng/ml) (closed squares) or medium alone (open circles) were added at day 0 and re-added at day 3, 6, and 9 over a 12 day period. Cell surface VCAM-1 expression was determined 48 hours after each cytokine addition using flow cytometry and mAb BBA-5 as primary antibody. Samples were analysed using a FACScan and the results expressed as corrected MFI. Data are presented as the mean ± SEM of six experiments. * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared with medium only control at the respective time point).

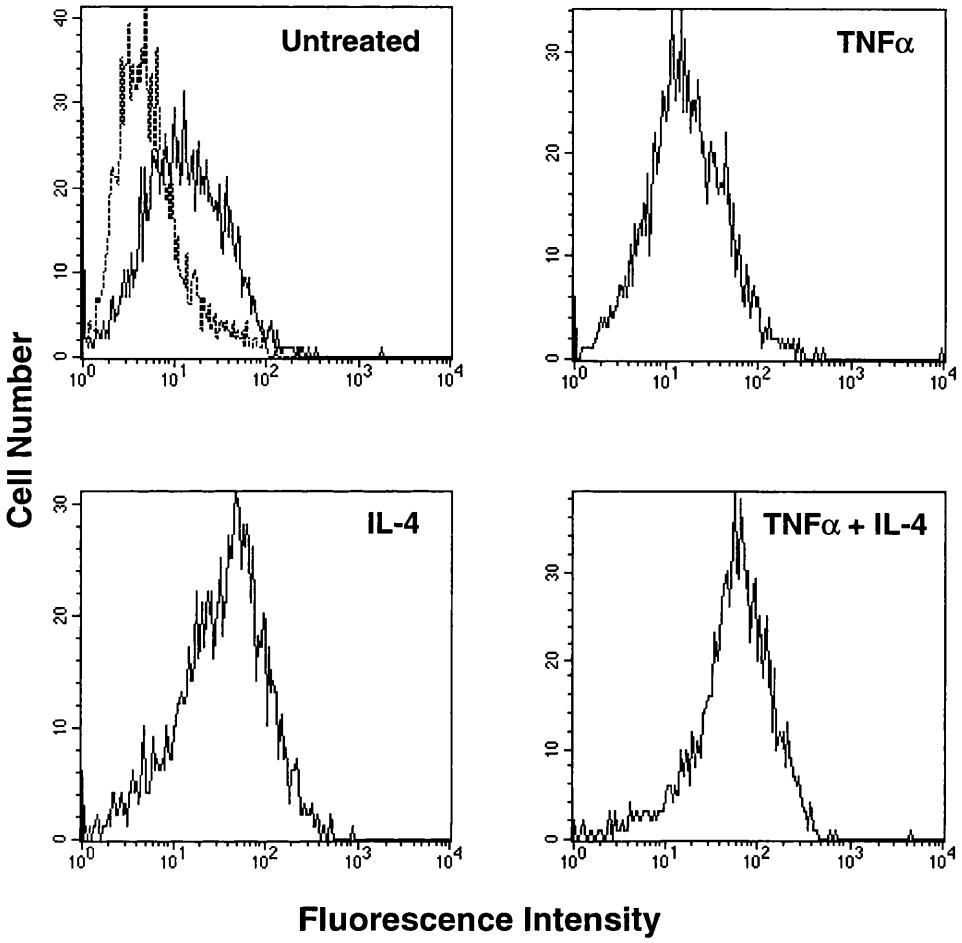


Figure 4.5 Flow-cytometric analysis of VCAM-1 on RA fibroblast-like synoviocytes treated with TNF-α and IL-4. Cells were cultured with either medium only (Untreated), TNF-α (10 ng/ml), IL-4 (10 ng/ml) or TNF-α plus IL-4 for 3 days. Flow cytometry for cell surface VCAM-1 was carried out using mAb BBA-5 as primary antibody. Samples were analysed using a FACScan flow cytometer. The dashed histogram (in untreated cells) represents staining with PE-conjugated anti-mouse IgG only. Solid line histograms represent cells stained with anti-VCAM-1 and PE-conjugated anti-mouse IgG. Results shown are representative of four experiments.

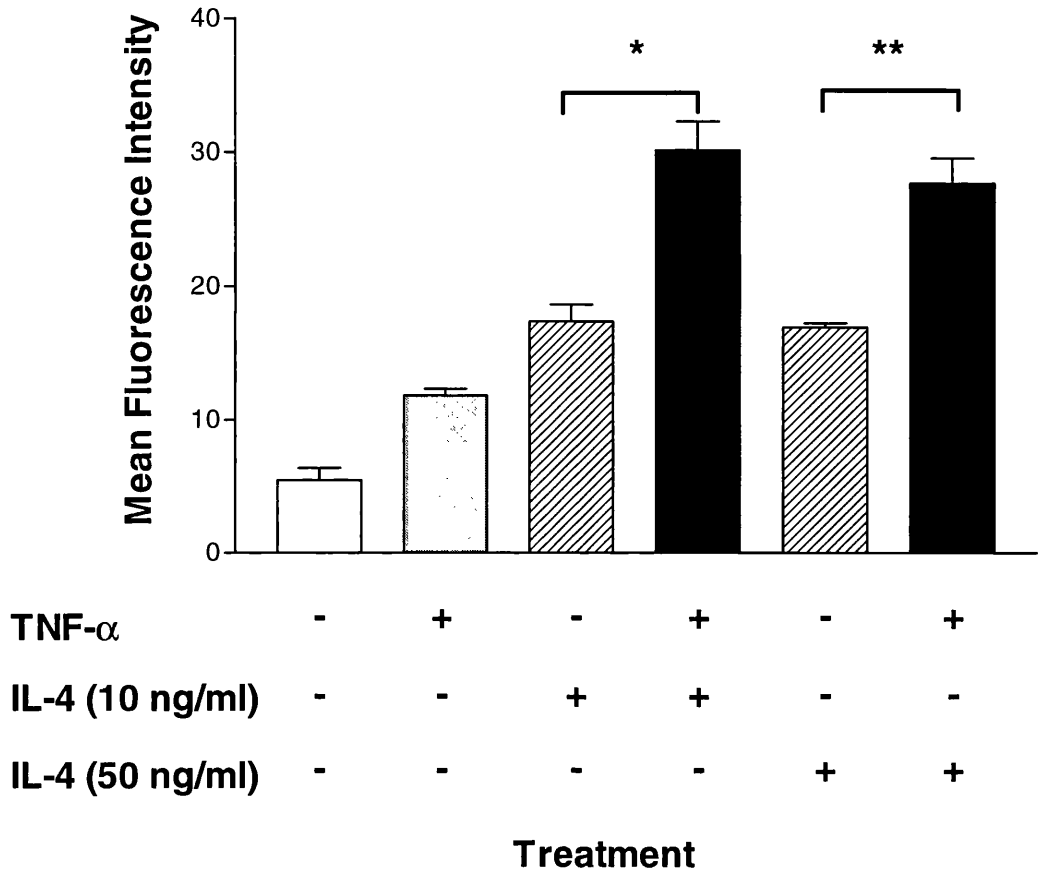


Figure 4.6 The effect of high doses of IL-4 on VCAM-1 expression by fibroblast-like synoviocytes. Cells were cultured with either medium-only, or IL-4 either alone, or in combination with TNF-α (10 ng/ml) for 48 hours. Cell surface VCAM-1 levels were determined by flow cytometry with mAb BBA-5. Samples were analysed using a FACScan flow cytometer. Results are expressed as corrected MFI. Data are presented as the mean ± SEM of 4 experiments. * indicates $p < 0.05$, ** $p < 0.01$. All treatments were significantly different from medium-only.

Figure 4.7 Time course of VCAM-1 expression in fibroblast-like synoviocytes treated with a single application of IL-4 (A) and IL-13 (B). Cells were cultured with either medium only (open circles), TNF-α (10 ng/ml) (closed circles), IL-4 (10 ng/ml) (open squares), IL-4 plus TNF-α (both at 10 ng/ml) (closed squares), IL-13 (10 ng/ml) (open triangles), or IL-13 plus TNF-α (both at 10 ng/ml) (closed triangles). Cell surface VCAM-1 expression was analysed at day 1, 3, 7, and 14 by flow cytometry using mAb BBA-5. Samples were analysed using a FACScan and the results expressed as corrected MFI. Data are presented as the mean ± SEM of four or five experiments. ** indicates $p < 0.01$, *** $p < 0.001$ (compared with medium only at the respective time point).

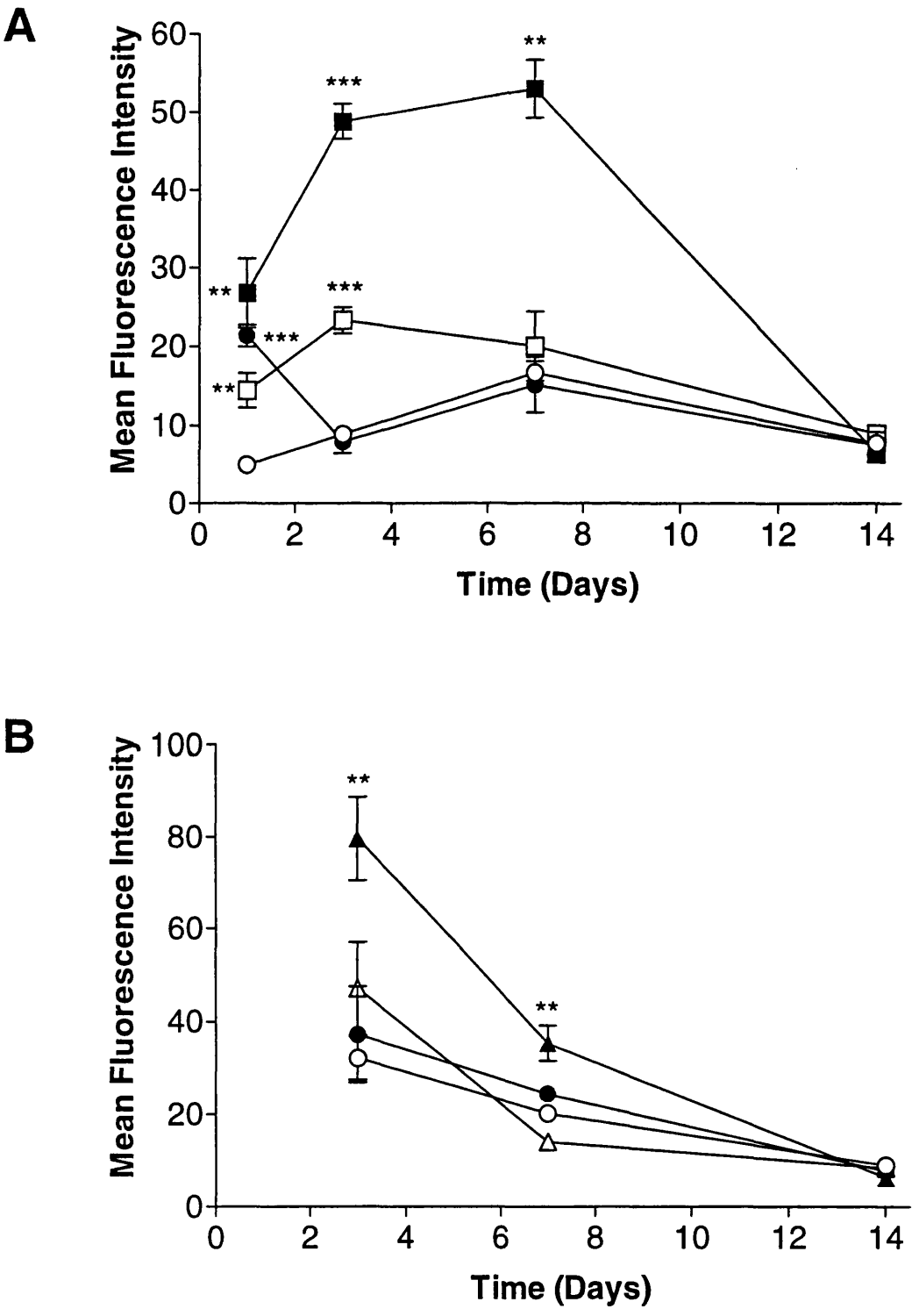

A
Mean Fluorescence Intensity
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Time (Days)
**
***
**
***
***
**
B
Mean Fluorescence Intensity
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (Days)
**
**

Figure 4.7

Figure 4.8 Chronic treatment with TNF-α in combination with either IL-4 (A) or IL-13 (B) can maintain elevated VCAM-1 expression on fibroblast-like synoviocytes for up to 16 days. Cells were treated with either medium only (open circles), TNF-α (10 ng/ml) (closed circles), IL-4 (10 ng/ml) (open squares) or IL-4 plus TNF-α (both at 10 ng/ml) (closed squares), IL-13 (10 ng/ml) (open triangles) or IL-13 plus TNF-α (both at 10 ng/ml) (closed triangles) at day 0. Cytokines were re-added at day 4, 8, 12 and 16 over a 20 day period. Cell surface VCAM-1 expression was measured 4 days after cytokine application in a flow cytometry protocol with the aid of mAb BBA-5. Results are expressed as corrected MFI. Data are presented as the mean ± SEM of four experiments. * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (In the top panel comparison is between IL-4 alone and IL-4 plus TNF-α and in the bottom panel IL-13 alone is compared with IL-13 plus TNF-α). In addition, for both IL-4 and IL-13 alone, VCAM-1 expression was significantly different from medium only control ($p < 0.05$) at the time points day 4 and day 8.

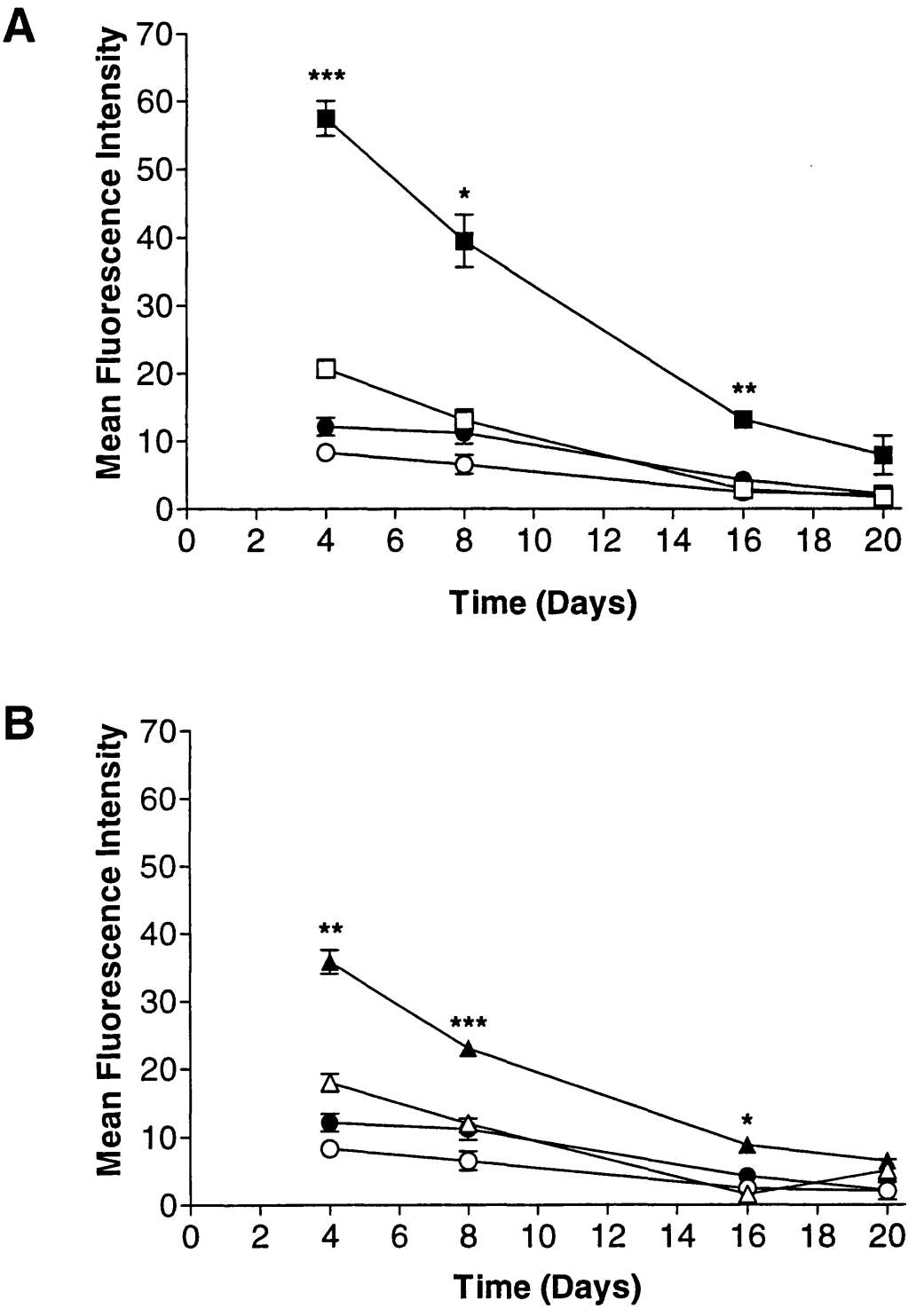

A
Mean Fluorescence Intensity
70
60
50
40
30
20
10
0
***
*
**
0 2 4 6 8 10 12 14 16 18 20
Time (Days)
B
Mean Fluorescence Intensity
70
60
50
40
30
20
10
0
**
***
*
0 2 4 6 8 10 12 14 16 18 20
Time (Days)

Figure 4.8

Figure 4.9 Immunohistochemical staining demonstrating that pathophysiological concentrations of IL-13 can synergize with TNF-α to enhance VCAM-1 expression on fibroblast-like synoviocytes. Cultured FLSs were treated with IL-13 at 0.1, 1 and 10 ng/ml as indicated and TNF-α (10 ng/ml) for 4 days. FLSs were fixed with methanol and acetone, and then stained with mouse anti-human VCAM-1 mAb (BBA-5) and visualized using the streptavidin-biotin-peroxidase method. The results shown are representative of four separate experiments.

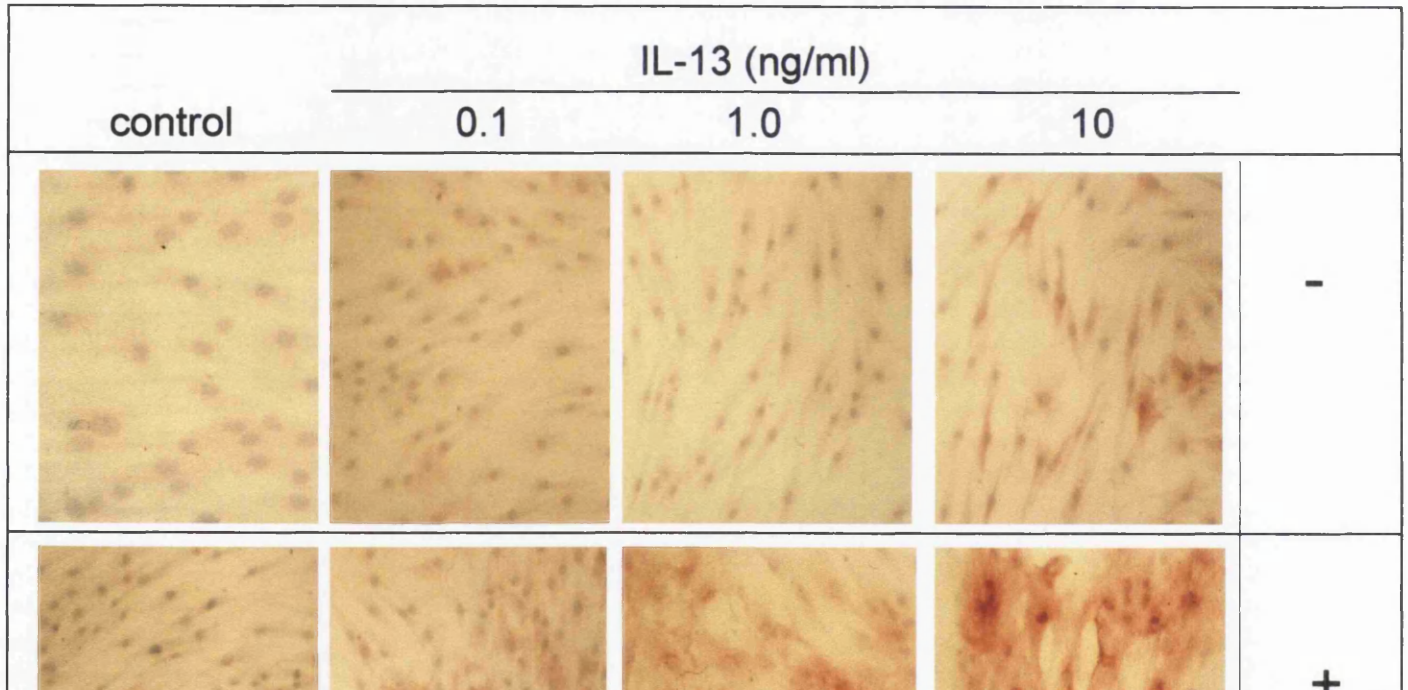

IL-13 (ng/ml)
control
0.1
1.0
10
-
+
TNF-α
VCAM-1 expression in fibroblast-like synoviocytes

Figure 4.9

Figure 4.10 Induction of VCAM-1 mRNA transcripts in fibroblast-like synoviocytes by combinations of cytokines. Cells were cultured with either medium only (Untreated), IL-4 (10 ng/ml), IL-13 (10 ng/ml) or TNF-α (10 ng/ml) either alone or in combination with IL-4 or IL-13. Total RNA was isolated at the indicated time periods and semiquantitative RT-PCR performed using VCAM-1-specific and GAPDH-specific primers (see Methods and Materials). PCR products were electrophoresed on an 8% polyacrylamide gel and visualized using a phosphorimager. Similar amounts of amplified GAPDH PCR product indicates similar levels of GAPDH mRNA in each sample. Note, control PCR reactions (i.e. no cDNA) were run in the control lane and indicate the absence of DNA contamination from PCR reagents. The normalized results of three separate PCR experiments are shown in panel B. Normalized data are expressed as the ratio of each VCAM-1 band intensity to the respective GAPDH band intensity. The results shown above are representative of three separate experiments.

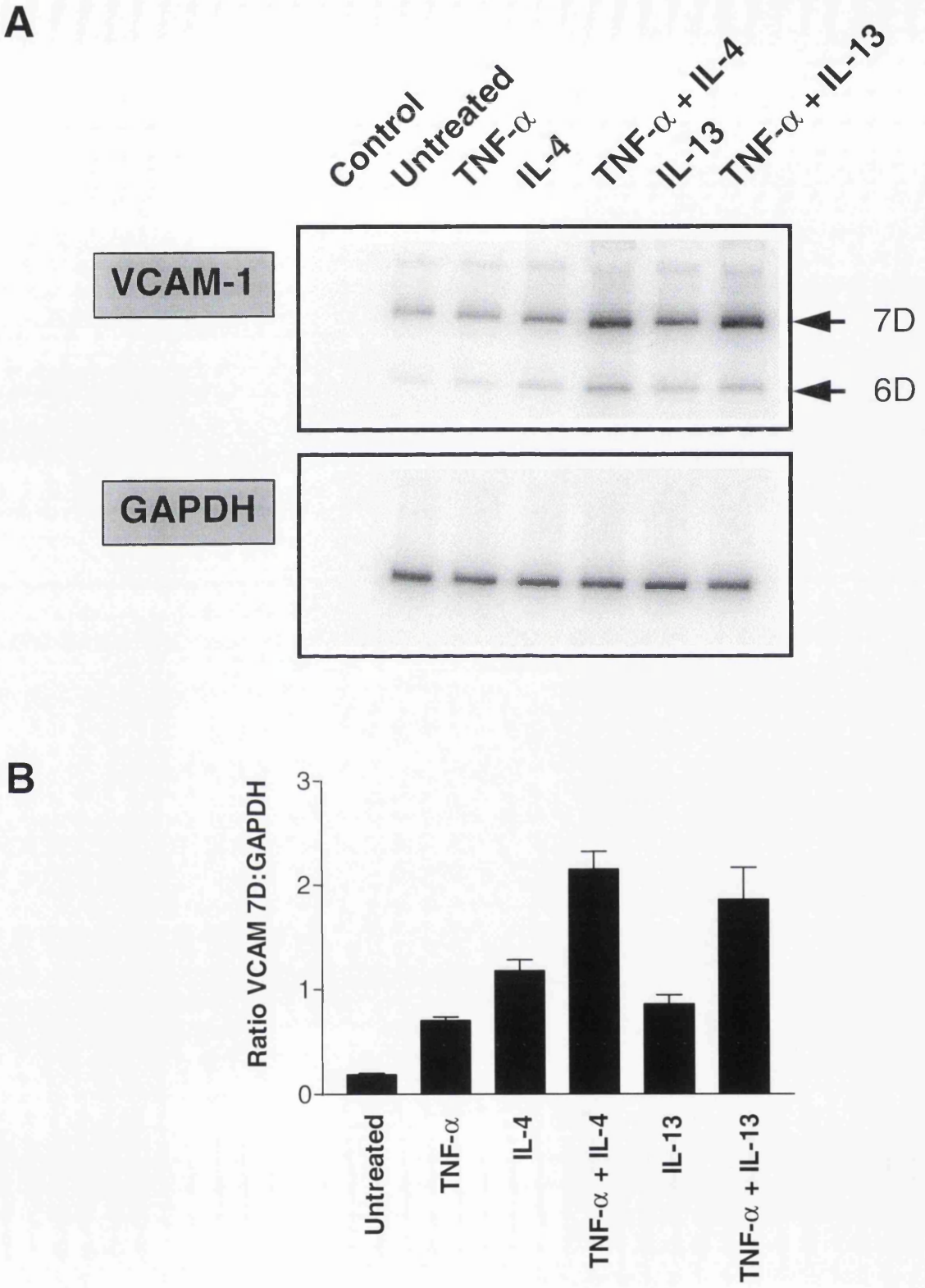

A
Control
Untreated
TNF-α
IL-4
TNF-α + IL-4
IL-13
TNF-α + IL-13
VCAM-1
7D
6D
GAPDH
B
Ratio VCAM 7D:GAPDH
3
2
1
0
Untreated
TNF-α
IL-4
TNF-α + IL-4
IL-13
TNF-α + IL-13

Figure 4.10

Figure 4.11 Time course of induction of VCAM-1 mRNA transcripts in fibroblast-like synoviocytes by combinations of cytokines. Cells were cultured with either medium only (Untreated), IL-4 (10 ng/ml), IL-13 (10 ng/ml) or TNF-α (10 ng/ml) either alone or in combination with IL-4 or IL-13. Total RNA was isolated at the time periods shown and semiquantitative RT-PCR performed using VCAM-1-specific and GAPDH-specific primers (see Methods and Materials). PCR products were electrophoresed on an 8% polyacrylamide gel and visualized using a phosphorimager. Similar amounts of amplified GAPDH PCR product indicates similar levels of GAPDH mRNA in each sample. The results shown above are representative of three experiments.

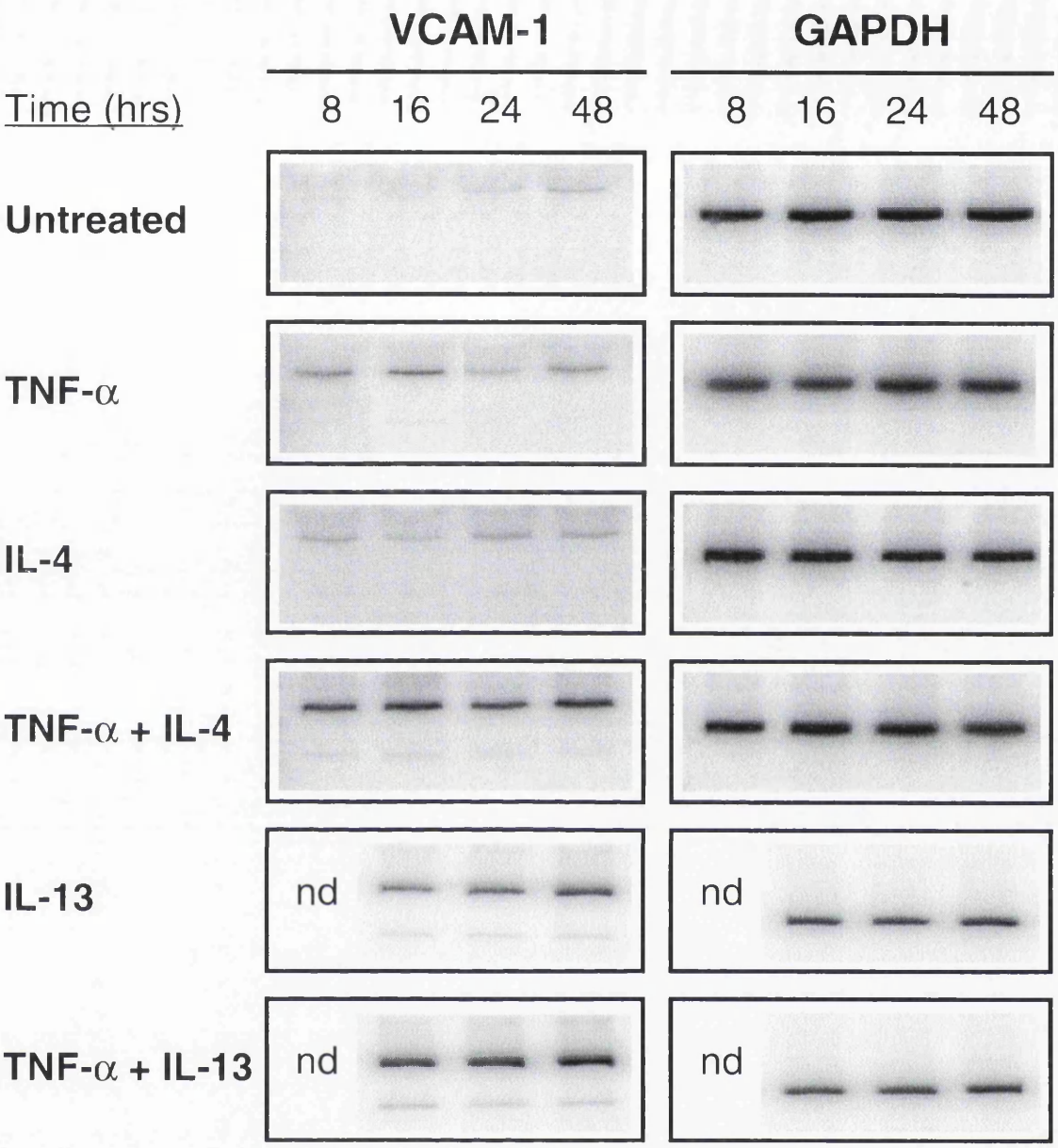

VCAM-1
GAPDH
Time (hrs)
8 16 24 48
8 16 24 48
Untreated
TNF-α
IL-4
TNF-α + IL-4
IL-13
nd
nd
TNF-α + IL-13
nd
nd

Figure 4.11

Figure 4.12 VCAM-1 mRNA stability at 16 hours and 10 days after treatment of fibroblast-like synoviocytes with various combinations of cytokines. Cells were cultured with medium-only (Untreated), TNF-α (10 ng/ml), IL-4 (10 ng/ml) or IL-13 (10 ng/ml) and, in addition, were treated with TNF-α (10 ng/ml) in combination with IL-4 or IL-13. Cytokines were re-added at day 5 and 10. At 16 hr (panel A) or 10 days (panel B) after the start of the culture, Actinomycin-D (5 μg/ml) was added and mRNA was isolated at 0, 2, 4 or 10 hours thereafter. To measure the amount of mRNA transcripts, semiquantitative RT-PCR was performed with VCAM-1-specific and GAPDH-specific primers. The normalized results of three separate PCR experiments are shown in panel (C). Normalized data are expressed as the ratio of each VCAM-1 band intensity to the respective GAPDH band intensity. Symbols represent: medium-only (open circles); TNF-α (closed circles); IL-4 (open squares): TNF-α + IL-4 (closed squares).

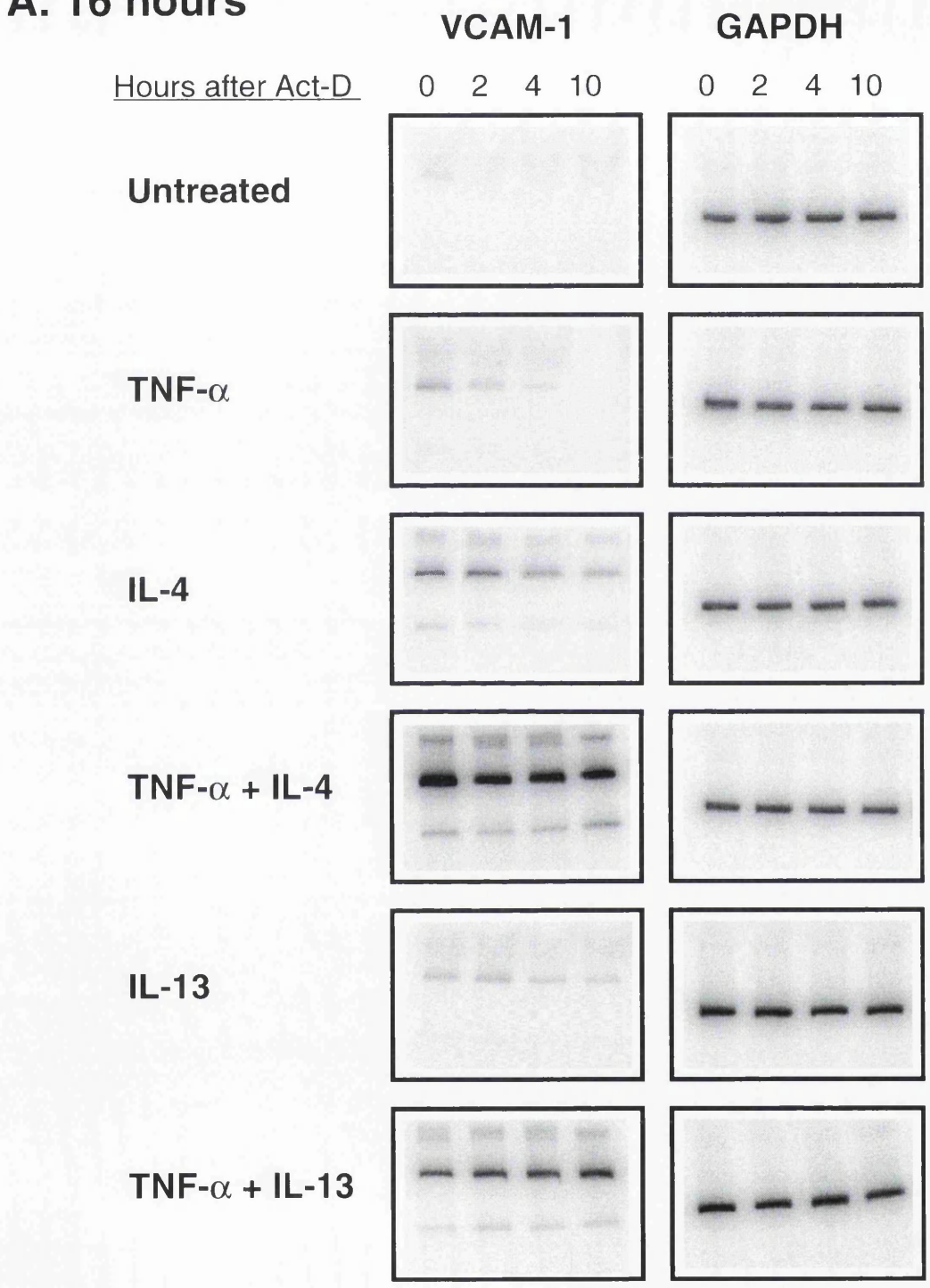

A. 16 hours
VCAM-1
GAPDH
Hours after Act-D
0 2 4 10
0 2 4 10
Untreated
TNF-α
IL-4
TNF-α + IL-4
IL-13
TNF-α + IL-13

Figure 4.12

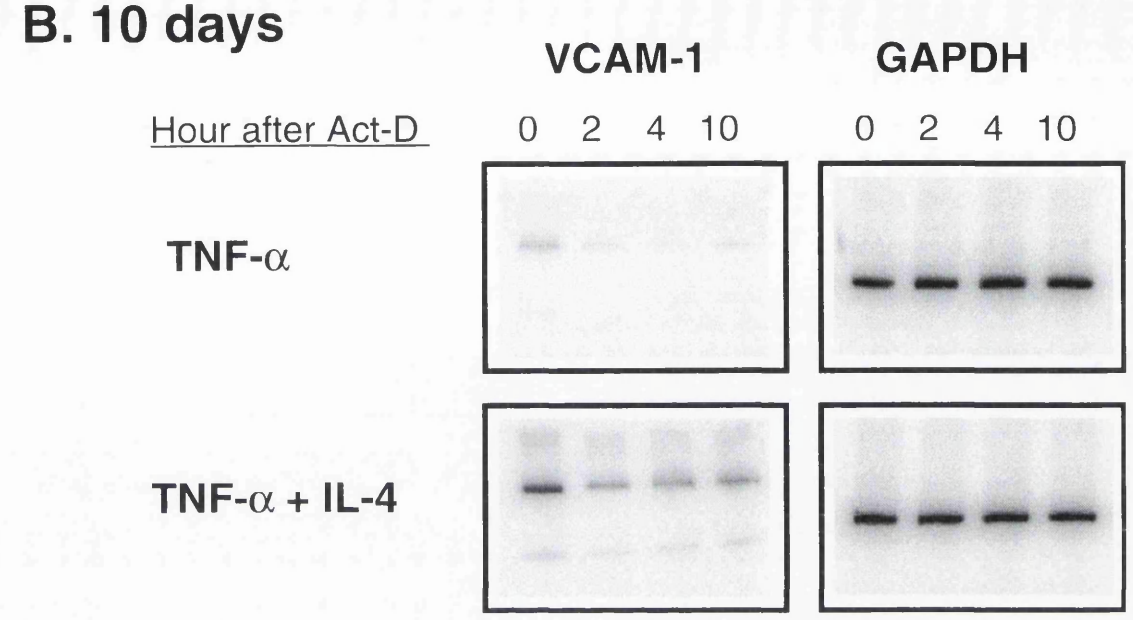

B. 10 days
VCAM-1
GAPDH
Hour after Act-D
0 2 4 10
0 2 4 10
TNF-α
TNF-α + IL-4

Figure 4.12

C

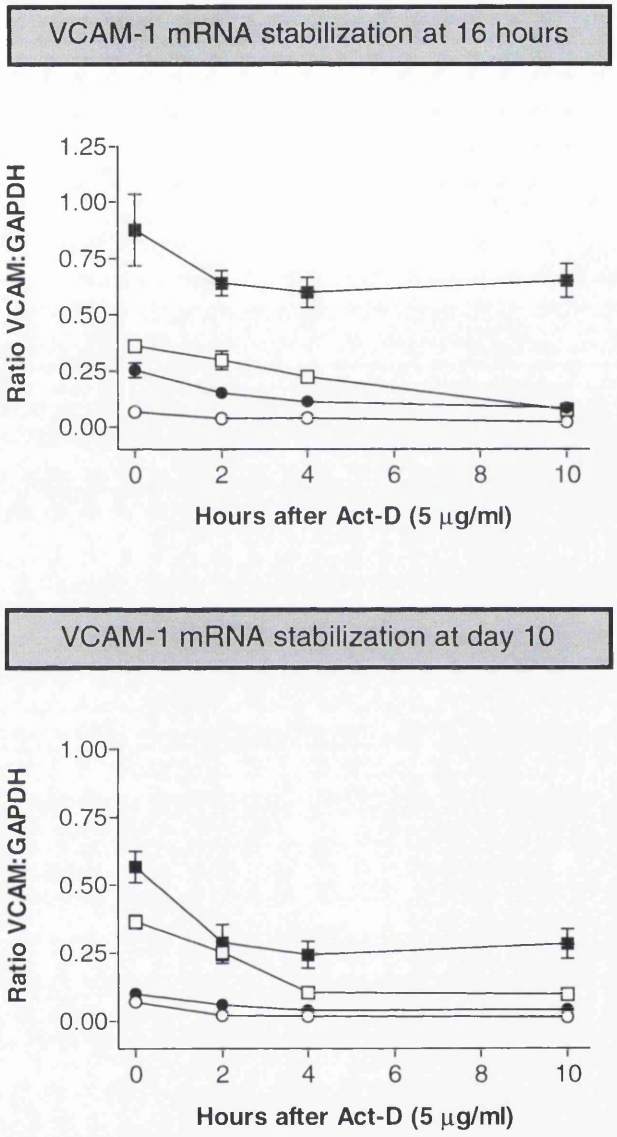

VCAM-1 mRNA stabilization at 16 hours
Ratio VCAM:GAPDH
1.25
1.00
0.75
0.50
0.25
0.00
0
2
4
6
8
10
Hours after Act-D (5 μg/ml)
VCAM-1 mRNA stabilization at day 10
Ratio VCAM:GAPDH
1.00
0.75
0.50
0.25
0.00
0
2
4
6
8
10
Hours after Act-D (5 μg/ml)

Figure 4.12

4.4. Discussion

Elevated expression of VCAM-1 by fibroblast-like synoviocytes is generally regarded to be mediated by the cytokine-rich milieu present within the RA synovium (Morales-Ducret *et al.*, 1992). In recent years, particular emphasis has been placed on the putative role of IL-1β and TNF-α. This is because VCAM-1 expression on RA fibroblast-like synoviocytes can be transiently restored by the addition of these cytokines (Marlor *et al.*, 1992; Morales-Ducret *et al.*, 1992). Moreover, treatment with anti-TNF-α antibody appears to decrease VCAM-1 expression in synovial biopsies taken from RA patients (Feldmann *et al.*, 1996).

The current study demonstrates that sustained elevated expression of VCAM-1 on cultured fibroblast-like synoviocytes cannot be obtained by the presence of TNF-α or IL-1β alone. Even prolonged treatment, where cytokines are re-added every three or four days, only results in an initial transient expression that, once returned to baseline, remains low throughout the remaining time in culture. This pattern of regulation greatly resembles that observed in vascular endothelial cells where transient VCAM-1 expression results from rapid post-induction transcriptional repression of NF-κB (Read *et al.*, 1994, 1996). This repressive mechanism limits the duration of VCAM-1 mRNA transcript expression, thereby preventing their prolonged expression. It is possible that fibroblast-like synoviocytes control TNF-α-mediated VCAM-1 expression by a similar regulatory mechanism. Transcriptional mechanisms such as this are essential in both the initiation, and also the subsequent termination of the local inflammatory response.

Our results show that IL-4 (and IL-13) provides a potent mechanism for prolonging VCAM-1 expression on RA FLSs. The combination of IL-4 and TNF-α was

most effective not only in increasing the level of VCAM-1 to levels that were measured on cells in their first two weeks of culture, but also in maintaining the high levels of expression for a period of over sixteen days. Furthermore, this study shows that the contribution of IL-4 and IL-13 to the sustained elevated levels could in part be explained by their capacity to dramatically prolong the half-life of VCAM-1 mRNA. Stable transcripts were still observed at day 10 of incubation, albeit at a lower level of protection. This finding indicates that indeed the preservation of mRNA could be one mechanism by which elevated levels of VCAM-1 protein expression can be achieved in fibroblast-like synoviocytes. Post-transcriptional mechanisms that prevent the degradation of mRNA transcripts are also used by other inflammatory genes. For instance, the up-regulation of cyclo-oxygenase-2 (COX-2) by pro-inflammatory cytokines is a consequence of both transcriptional activation and increased COX-2 mRNA stability (Crofford *et al.*, 1997b; Ristimäki *et al.*, 1994). Post-transcriptional mechanisms which prolong the expression of inflammatory genes, such as VCAM-1 and COX-2, may go someway to explaining the chronic nature of rheumatoid arthritis and other inflammatory diseases.

Interestingly, treatment of fibroblast-like synoviocytes with IL-4 alone also results in a rapid up-regulation of VCAM-1. This could result from the stabilization of constitutively expressed VCAM-1 mRNA transcripts, thereby leading to their accumulation. If this is so then VCAM-1 mRNA must be undergoing rapid turnover in untreated fibroblast-like synoviocytes. Alternatively, IL-4 (or IL-13) may also activate the transcription of the VCAM-1 gene in a similar fashion to that seen in smooth muscle cells (Barks *et al.*, 1997). Moreover, both IL-4 and IL-13 are able to activate transcription through the STAT pathway (Lugli *et al.*, 1997). The present study,

however, has not addressed this possibility. Attempts have been made to study this using a nuclear run-on assay. However, the number of cells required (approximately 20 x 10^6 cells per treatment) for these studies could not be achieved. This aspect therefore remains to be determined.

There is evidence to suggest that the synergistic increase in VCAM-1 expression may also occur in part by co-operative transcriptional activation. For example, TNF-α, in addition to activating the transcription of the VCAM-1 gene, is also able to up-regulate the expression of IL-4 receptors and to enhance IL-4-mediated STAT activation (Lugli *et al.*, 1997). Thus ensuring that TNF-α potentiates its own effect on VCAM-1 transcription by up-regulating IL-4 receptors involved in VCAM-1 mRNA stabilization.

A recent study by Isomäki and colleagues (1996) has identified the presence of IL-4 and IL-13 in the synovial fluid and synovium of RA patients. The observation that pathophysiological concentrations of IL-13 augment VCAM-1 expression, in particular in the presence of TNF-α, indicates that these cytokines may be instrumental in the regulation of VCAM-1 expression in the rheumatoid joint. This possibility is perhaps best illustrated by the finding that VCAM-1 expression (both mRNA and cell surface protein) by fibroblast-like synoviocytes slowly declines when these cells are removed from the RA synovial joint and placed in culture. If expression of VCAM-1 in the synovial joint was solely regulated by elevated levels of TNF-α and IL-1β, then, when cells are placed in culture, we would expect a rather more rapid decline in the levels of expression.

This study also further highlights the differing mechanisms of transcriptional control of the VCAM-1 gene in different cell types (Iademarco *et al.*, 1995; Barks *et al.*, 1997). VCAM-1 regulation in fibroblast-like synoviocytes is different to that observed

in HUVECs and smooth muscle cells for the following findings. Firstly, fibroblast-like synoviocytes transiently up-regulate VCAM-1 mRNA expression in response to TNF-α (as with HUVECs but in contrast to smooth muscle cells). Secondly, IL-4 rapidly induces VCAM-1 mRNA followed by cell surface expression (as with smooth muscle cells but in contrast to HUVECs). What they all have in common, however, is that all three cell types show a synergistic increase in VCAM-1 expression following treatment with IL-4 in combination with TNF-α (Barks *et al.*, 1997). As mentioned earlier, a distinctive cell type has recently been identified within the rheumatoid pannus that constitutively express VCAM-1 (Zvaifler *et al.*, 1997). These cells, termed pannocytes, maintain high levels of VCAM-1 throughout long-term *in vitro* culture. Future studies will no doubt show whether cytokines can regulate pannocyte VCAM-1 expression and how this compares with the other cell types mentioned above.

In rheumatoid arthritis, cytokine-dependent modulation of VCAM-1 on fibroblast-like synoviocytes may function in retaining α4 integrin-expressing cells within the synovial intima (Morales-Ducret *et al.*, 1992). The interaction of VCAM-1 with α4-integrins can stimulate the activation of leukocytes, and the release of other inflammatory cytokines and matrix-degrading enzymes (Damle and Aruffo, 1991; Bombara *et al.*, 1993; Romanic and Madri, 1994). Given recent experimental evidence, VCAM-1 on fibroblast-like synoviocytes may also act to signal, via its interaction with α4 integrins, the proliferation of fibroblast-like synoviocytes (Lazaar *et al.*, 1994).

In conclusion, these data have shown that IL-4 or IL-13 either alone or in combination with TNF-α results in elevated levels of VCAM-1 expression in fibroblast-like synoviocytes. Moreover, the chronic administration of these cytokine combinations can

achieve a sustained elevated level of VCAM-1 expression. This sustained expression can be explained in part by the capacity of IL-4 and IL-13 to stabilize VCAM-1 mRNA. Thus, VCAM-1 mRNA stability appears to be a major factor in the sustained up-regulation of VCAM-1 expression in fibroblast-like synoviocytes. These cytokines which are all present in varying amounts in the synovial fluid and synovium of the RA joint may explain sustained VCAM-1 expression as demonstrated by RA FLSs both *in situ* and during initial *in vitro* culture.

Chapter 5

Expression of CD44v7/8 enhances proliferation of fibroblast-like synoviocytes

5.1 Introduction

Hyperplasia of the synovial membrane is a characteristic of rheumatoid arthritis (Gay *et al.*, 1993). This results from an increase in cellularity of the intimal layer due to invasion of inflammatory cells into synovium, and a local proliferation of resident type B (fibroblast-like) synoviocytes (Hogg *et al.*, 1985; Lalor *et al.*, 1987; Qu *et al.*, 1994). Fibroblast-like synoviocytes found within the intimal layer and at sites of cartilage and bone erosion show signs of transformation (Fassbender, 1983; Firestein, 1996; Müller-Ladner *et al.*, 1996). This transformation allows an increasing number of highly aggressive synoviocytes to accumulate within RA synovium (Wooley *et al.*, 1977; Firestein and Paine, 1992). The 'transformed' fibroblast-like cells demonstrate minimal apoptosis, are able to invade bone and cartilage, and express a number of cell proliferation markers, such as Ki-67, Myc and PCNA (Aupperle *et al.*, 1998; Müller-Ladner *et al.*, 1996; Qu *et al.*, 1994; Firestein *et al.*, 1995). It is likely that transformed fibroblast-like synoviocytes express other markers implicated in tumour progression.

One such marker could be the expression of splice variants of CD44, an adhesion molecule implicated in the progression of a variety of human cancers (Koopman *et al.*, 1993; Mulder *et al.*, 1994; Kaufmann *et al.*, 1995). In humans, CD44 is encoded by a single gene comprised of 20 exons (see Appendix III, Figure A.2)

(Screaton *et al.*, 1992, 1993). Alternative exon splicing of the CD44 gene can occur by insertion of the variant (v) exons (v2 to v10) in a variety of combinations at a distinct site within the extracellular domain (Screaton *et al.*, 1992, 1993; Tölg *et al.*, 1993). Alternative RNA splicing combined with post-translational modification, via glycosylation, chondroitin sulphate and heparan sulphate attachment, has the potential of generating a vast array of CD44 isoforms (see Appendix III, Figures A.2 and A.3) (Brown *et al.*, 1991; Dougherty *et al.*, 1991; Lesley *et al.*, 1993; Jackson *et al.*, 1992, 1995). The standard (CD44s) or hemopoietic (CD44H) form of CD44, where all variant exons are excised, is widely expressed by most cell types (Lesley *et al.*, 1993; Brown *et al.*, 1991; Culty *et al.*, 1990; Camp *et al.*, 1991). Splice variant isoforms of CD44, in contrast, show a more restricted expression pattern. They are primarily expressed by epithelial cells, but can also be found on astrocytes, activated leukocytes and keratinocytes (Heider *et al.*, 1993; Mackay *et al.*, 1994; Haegel *et al.*, 1993; Levesque and Haynes, 1996; Brown *et al.*, 1991; Kugelman *et al.*, 1992).

Insertion of variant exons affects the functional properties of CD44. For instance, they can alter the affinity of the receptor for its main ligand hyaluronan (Stamenkovic *et al.*, 1991; Sleeman *et al.*, 1996; Aruffo *et al.*, 1990; Miyake *et al.*, 1990). Certain CD44 isoforms can also bind growth factors, such as basic-fibroblast growth factor (Tanaka *et al.*, 1993; Bennett *et al.*, 1995a; Jackson *et al.*, 1995). The importance of CD44 variants in growth factor-presentation has recently been demonstrated in developing rat embryonic limbs where it was shown that CD44v3-v10-mediated binding of fibroblast-growth factor-8 (FGF-8) was instrumental in the activation of proliferation of adjacent mesenchymal cells (Sherman *et al.*, 1998). Changes in CD44 splice variant expression have also been shown to have pronounced

effects on cell function. For instance, they are implicated in tumour cell growth and metastasis, lymphocyte activation and extravasation, clearance of apoptotic neutrophils, and matrix invasion (Günthert *et al.*, 1991; Arch *et al.*, 1992; Moll *et al.*, 1996; Mikecz *et al.*, 1995; DeGrendele *et al.*, 1997; Hart *et al.*, 1997; Lamb *et al.*, 1997).

A number of studies have investigated CD44 expression in inflamed and normal synovium, but there appears to be some controversy as to whether CD44 levels are up-regulated in disease (Haynes *et al.*, 1991; Johnson *et al.*, 1993; Henderson *et al.*, 1994). With regards to individual cell types, cultured RA fibroblast-like synoviocytes have been demonstrated to show reduced expression of CD44 compared with those from normal synovium (Henderson *et al.*, 1994). However, expression of variant exons of CD44 has not been studied in fibroblast-like synoviocytes. In light of this, a study was set out, employing a combination of immunohistochemistry, RT-PCR and flow cytometry techniques, to determine variant-exon expression in these cells. A comparison is made between fibroblast-like synoviocytes obtained from normal, OA or RA tissue specimens. CD44 splice variants are prominent in cells from RA tissue, less so in cells obtained from OA and absent in normal tissues. Expression of CD44v7/8 is linked to an increased cellular proliferation rate and could thus be functionally implicated in hyperplasia of the synovial membrane in RA.

5.2 Methods and materials

All methods and materials are as described in chapter 2. Additional experimental procedures are described below.

5.2.1 Selection of CD44v7/8-expressing cells

Cell suspensions were obtained from cultures of fibroblast-like synoviocytes by treatment with Sigma dissociation solution at 37°C for 15 minutes. All further steps were performed at 4°C. Cell suspensions (5 x 10^5 cells) were incubated with extensively dialyzed (azide-free) anti-CD44v7/8 (10 μg/ml) for 1 hour. Dynabeads (M-450) coated with anti-mouse IgG were added to the cell suspension, according to manufacturers instructions, and incubated for 20 mins. Cells recognised by the antibody were removed from the suspension by a magnet, to give a positively selected population. The positively selected population was treated with trypsin-EDTA solution (4 min at 37°C) to remove antibody coupled to beads. Both the positively and negatively selected cell populations were washed and subsequently cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin for 4 days. The effectiveness of selection was assessed by immunohistochemistry prior to further experimentation.

5.2.2 Cell proliferation assays

For cell counting, fibroblast-like cell cultures were prepared in 24-well plates at a density of 3 x 10^3 cells/well and incubated at 37°C. After 4 days, cells were harvested by treatment with trypsin-EDTA, 4 min at 37°C. The cells were resuspended in DMEM supplemented with 10% (v/v) FBS and subsequently diluted in Isoton II buffer for

counting in a ZB1 Coulter counter (100 μm orifice, with a 50 "lower" and 100 "upper" threshold). Cell numbers are expressed as cells per well.

For ^{3}H-thymidine incorporation studies, fibroblast-like cells were prepared in 96-well plates at a density of 2 x 10^{3} cells/well in 100μl of medium. Cells were incubated for 4 hours at 37°C and then antibodies against CD44v3, CD44v6, CD44v7/8, and VCAM-1. The latter was used as a negative control. Antibodies were added at a final concentration of 10 μg/ml. Cells were cultured for a further 3 days after which 0.5 μCi/well ^{3}H-thymidine was added. After another 16 hours, cells were washed in PBS and fixed in 10% (w/v) TCA. DNA was solubilized in 0.25M NaOH for 1 hour and incorporated radioactivity was counted using a Beckman scintillation counter. ^{3}H-thymidine incorporation was performed in quadruplicate for each sample. Values are given as the mean ± standard error.

5.3 Results

5.3.1 Freshly dispersed VCAM-1-positive synovial cells from both OA and RA patients express CD44 splice variants

Immediately following cell dispersion from synovial tissue, cells from OA and RA patients were allowed to adhere to chamber slides for four hours at 37°C after which non-adherent cells were removed by extensive washing. In a double staining protocol, cells stained positive for both VCAM-1 and CD44v3 or CD44v7/8 (Figure 5.1). Not all VCAM-1-positive cells are variant CD44-positive, indicating that the VCAM-1-positive fibroblast-like synoviocytes are not a homogeneous population. The majority of cells stain positive for VCAM-1 which means that this marker is co-expressed with the CD44 splice variants. CD68-positive synovial cells were also found to express CD44v7/8 (Figure 5.1C). Equivalent double staining studies with synovial cells isolated from non-inflamed synovium were unable to be performed because of the low numbers of cells isolated by enzymatic disaggregation.

5.3.2 Freshly dispersed and cultured synovial cells display a complex pattern of CD44 splice variant expression at the mRNA level

Variant CD44 mRNA expression by the total population of freshly dispersed (day 0) and cultured synovial cells was assessed by RT-PCR. As can be seen from figure 5.2, variant CD44 mRNA transcripts could not be detected in RNA isolated from fibroblast-like synoviocytes isolated from non-inflamed synovium. However, in tissues derived from diseased joints a variety of CD44 splice variant bands were observed in addition to CD44s (Figure 5.2). To determine whether or not variant CD44 transcript expression

altered significantly throughout cell culture, the expression of the major CD44 variants was determined over a period of 8 weeks. Furthermore, the expression of VCAM-1 mRNA transcripts was also investigated. Unlike VCAM-1 expression which showed a steady decline with time, splice variant CD44 mRNA transcripts were present at fairly similar levels throughout their time in culture (Figure 5.2). This indicates to us that CD44 splice variant mRNA is constitutively expressed by cultured fibroblast-like synoviocytes derived from inflamed synovium. This can be seen in the top panel of figure 5.2 if the major high molecular weight bands in the 500-1000 bp range at week 0 are compared with those at week 8.

The complex pattern of splice variant expression observed in freshly dispersed OA and RA synovial cells was confirmed by Southern blotting of PCR products and analysed using exon-specific probes (Figure 5.3). Complex splice variant transcript patterns containing every variant exon were obtained from both freshly dispersed OA and RA synovial cells. This highly complex pattern most probably represents a combination of splice variant transcripts from a number of synovial cell types present within the inflamed synovium.

To illustrate further the differences in the amount of mRNA encoding the variant CD44 isoforms, various numbers of RT-PCR cycles were run (Figure 5.4). These experiments demonstrate again the absence of variant CD44 transcripts in fibroblast-like synoviocytes isolated from non-inflamed synovium. They also show that the level of expression of variant CD44 transcripts varied considerably between OA patients whereas with RA patients it was consistently high. The variability between OA samples can be clearly seen with the two extreme samples shown.

5.3.3 Immunohistochemical analysis of variant CD44 isoform expression in cultured synovial cells

In a parallel study, cell surface CD44 splice variant expression by 10 day cultured cells isolated from non-inflamed, OA, and RA synovium was assessed. After 10 days in culture, the majority of synovial cells express VCAM-1 and a few cells stained positive for the monocyte marker CD14 (Figure 5.5). In agreement with the above RT-PCR studies, fibroblast-like synoviocytes derived from non-inflamed synovium showed minimal reactivity for variant CD44 isoforms. Both OA and RA fibroblast-like synovial cells demonstrated immunoreactivity for both CD44v5 and CD44v7/8. However, with OA fibroblasts the staining varied from weak to intense, whereas for RA fibroblasts the staining was always found to be intense (Figure 5.5 and Table 5.1). Cells derived from OA patients showed variation in expression, either as lower expression per cell or as fewer positive cells. The observation that the majority of cells expressed both VCAM-1 and CD44v5 or CD44v7/8 indicates that these two adhesion molecules are co-expressed. However, as with freshly dispersed cells it appears that not all of the VCAM-1-positive cells also express variant CD44 isoforms. Flow cytometric analysis of VCAM-1, CD14, CD44 and CD44v7/8 was performed to more accurately determine expression levels. These experiments confirmed that CD44v7/8 was expressed at much lower levels than total CD44 (Figure 5.6 and Table 5.1). These data are in agreement with RT-PCR data that show the CD44H isoform is the predominant form of CD44 expressed in these cells. CD44v7/8 expression could not be detected on cells from OA patients by flow cytometry, despite positive immunohistochemistry and Southern blot data. CD44 expression by cells from non-inflamed tissue was not studied because of the low numbers of cells obtained from these tissues.

5.3.4 Selected VCAM-1-positive fibroblast-like synoviocytes express various splicing combinations of CD44

To analyse exactly which combinations of CD44 variant exons are included, RT-PCR was performed with total RNA isolated from VCAM-1-selected cells after 10 days in culture. VCAM-1-positive fibroblast-like synoviocytes were selected by flow cytometry (Figure 5.6 and 5.7). This selection procedure was used to isolate a homogeneous population of VCAM-1-positive fibroblast-like synoviocytes which was free from contamination by other synovial cell types. As an estimate for enrichment of the selected VCAM-1-positive synovial cells, expression of the monocyte/macrophage markers CD14 and MSE-1 was assessed. As can be seen from figure 5.7, the VCAM-1-positive cell fraction showed minimal expression of mRNA transcripts encoding CD14 and MSE-1 but was enriched for transcripts encoding VCAM-1.

Southern blot analysis was performed to determine splicing combinations of individual CD44-mRNA transcripts. VCAM-1-positive synovial fibroblasts from all RA patients abundantly express splice variants, whereas OA patients showed a more diverse pattern of expression (Figure 5.8). Two of the OA patients studied expressed very few splice variants in VCAM-1-positive cells, whereas others showed similar patterns of expression as fibroblast-like synoviocytes isolated from RA patients. Surprisingly, in contrast to immunohistochemical data, mRNA transcripts expressing exon v5 could not be detected. Typical exons included v3, v6, v7, v8, v9, and v10 in splicing combinations as summarized in figure 5.9. RT-PCR products corresponding to incompletely processed mRNA have been excluded from this diagram. Southern blot analysis could not detect CD44 variants in fibroblast-like synoviocytes from normal synovial joints (n=2) (Data not shown).

5.3.5 Fibroblast-like synoviocyte proliferation is inhibited by antibodies against CD44v7/8

During the course of these studies, it was observed that the percentage of cells positive for CD44v7/8 increased with prolonged time in culture. This observation suggested that cells expressing CD44v7/8 either have a proliferation advantage or that cells gradually switch on CD44 splice variants. This switch could for instance be a consequence of the cell culture conditions. However, the latter observations seemed unlikely because cells obtained from non-inflamed synovial specimen never switched to expressing CD44 splice variants. To test the first possibility, various cell proliferation assays were performed. The population of fibroblast-like synoviocytes was enriched for CD44v7/8-expressing cells using a magnetic bead panning protocol. Proliferation rates of both positively and negatively selected populations were compared. This enrichment protocol gave an almost 100% CD44v7/8-positive population whereas the negatively selected cells expressed roughly 20% of CD44v7/8-bearing cells (Figure 5.10(A)). As can be seen in Figure 5.10(B), fibroblast-like synoviocyte cultures that were enriched for CD44v7/8 demonstrated a 3.2-fold increase in cell numbers compared with a 2.0-fold increase for the negatively selected population.

Studies were next performed to test the functional implication of CD44v7/8-expression in enhanced proliferation. ^{3}H-thymidine incorporation was determined in the presence of anti-CD44v3, anti-CD44v6, anti-CD44v7/8, and anti-VCAM-1. Only the addition of anti-CDv7/8 resulted in an inhibition of proliferation as measured by a reduction in incorporation of ^{3}H-thymidine (Figure 5.11A). Anti-CD44v3, anti-CD44v6 or anti-VCAM-1 antibodies did not have any effect on fibroblast-like synoviocyte proliferation. Similar data were obtained in cell proliferation studies (Figure 5.11B).

We take these findings to mean that expression of the CD44v7/8 confers upon the cells a higher proliferation rate.

In summary, these data indicate that VCAM-1-positive fibroblast-like synoviocytes from diseased synovium express CD44v7/8 isoforms that are functionally involved in the proliferation of these cells. These variants are absent from synovial cells isolated from non-inflamed synovium.

Figure 5.1 Double immunofluorescence staining of freshly dispersed synovial cells. Cells were isolated from arthritic synovium by collagenase treatment and allowed to adhere to chamber slides for 4 hours. After fixing they were double stained for anti-CD44v3 (**A**) or anti-CD44v7/8 (**B**) (second antibody FITC-conjugated) (*green*) and biotinylated anti-VCAM-1 (streptavidin-Texas red) (*red*). A similar protocol was performed with anti-CD44v7/8 (second antibody FITC-conjugated) and anti-CD68 (**C**) (second antibody Texas red-conjugated). Double-positive cells appear yellow.

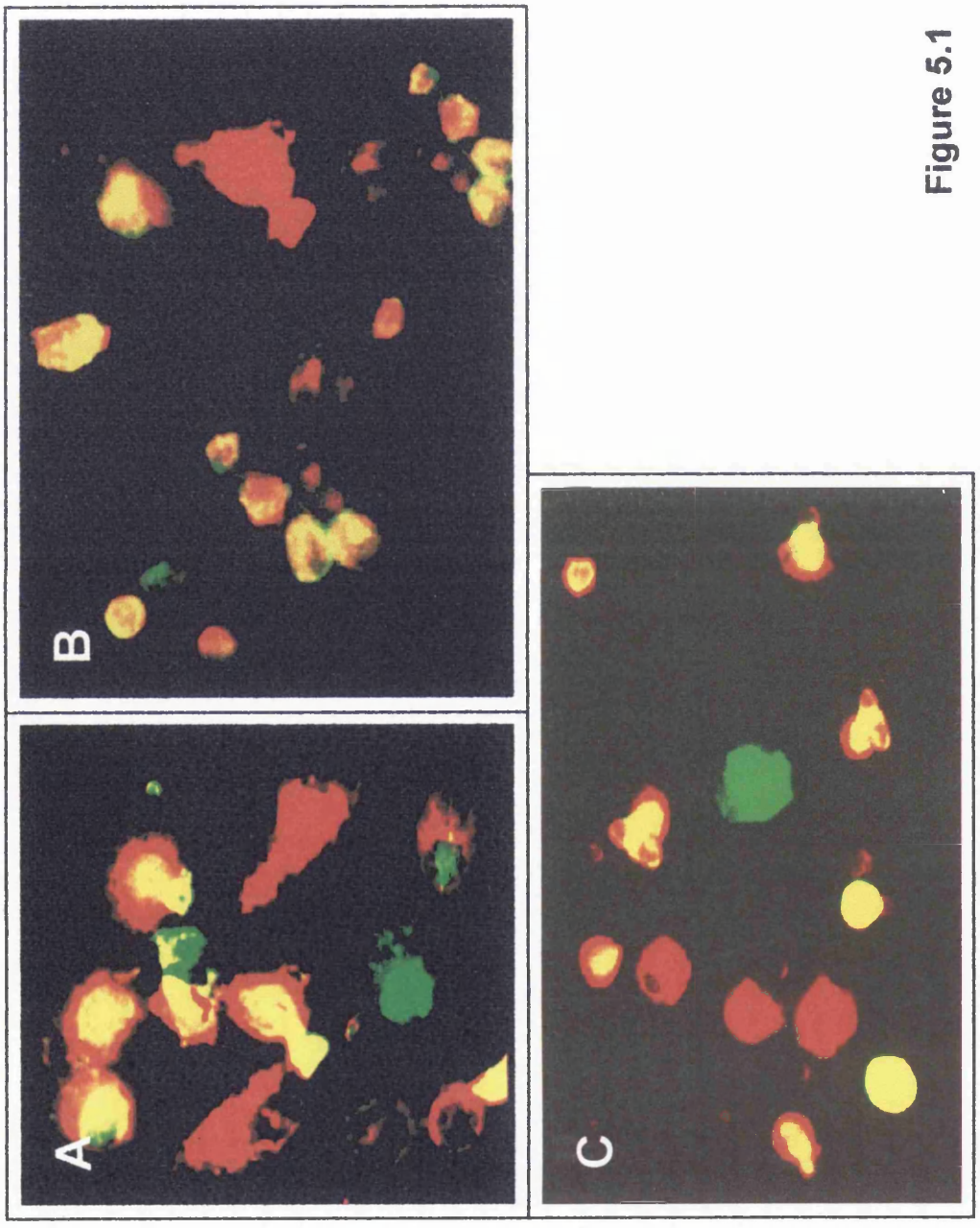


Figure 5.1

Figure 5.2 RT-PCR analysis of CD44 and VCAM-1 mRNA expression at different time points of fibroblast-like synoviocyte culture. Cells were isolated from non-inflamed (normal) and rheumatoid arthritic synovium by collagenase treatment and cultured for various times as indicated. Total RNA was isolated and reverse transcribed (see Methods and Materials). Hot start PCR was performed with first strand cDNA (equivalent to 10 ng of total RNA) using specific CD44 primers (44.513 and 44.934), VCAM-1 primers (vcam.751 and vcam.1361) or GAPDH primers (Gap.67 and Gap.406). The CD44 primers were upstream and downstream of the variant exon insertion point. [α^{33}P]-dATP was added to the PCR reaction mix (see Methods and Materials) to allow direct quantitation of the amount of PCR product formed. PCR was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer). The number of amplification cycles was varied with each cycle consisting of 30 sec at 94°C, 30 sec at an annealing temperature of 58°C, and 30 sec at 72°C. The number of amplification cycles was increased from 30 cycles to 40 cycles for the amplification of VCAM-1 transcripts in normal cDNA samples. PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid, and dried under vacuum. Gels were exposed to phosphorimager plates for 6-48 hours and scanned using a Fuji Bas 1000 phosphorimager. Normal VCAM-1 gels were exposed for longer periods compared to RA VCAM-1 gels. Note the presence of high molecular weight CD44 splice variants (500-1000 bp) in RNA isolated from rheumatoid tissue at all culture time points. Week 0 represents freshly dispersed synovial cells. Note also the immediate loss of the VCAM-6D isoform and the gradual loss of the VCAM-7D isoform in culture. Results shown are representative of three separate PCR experiments performed.

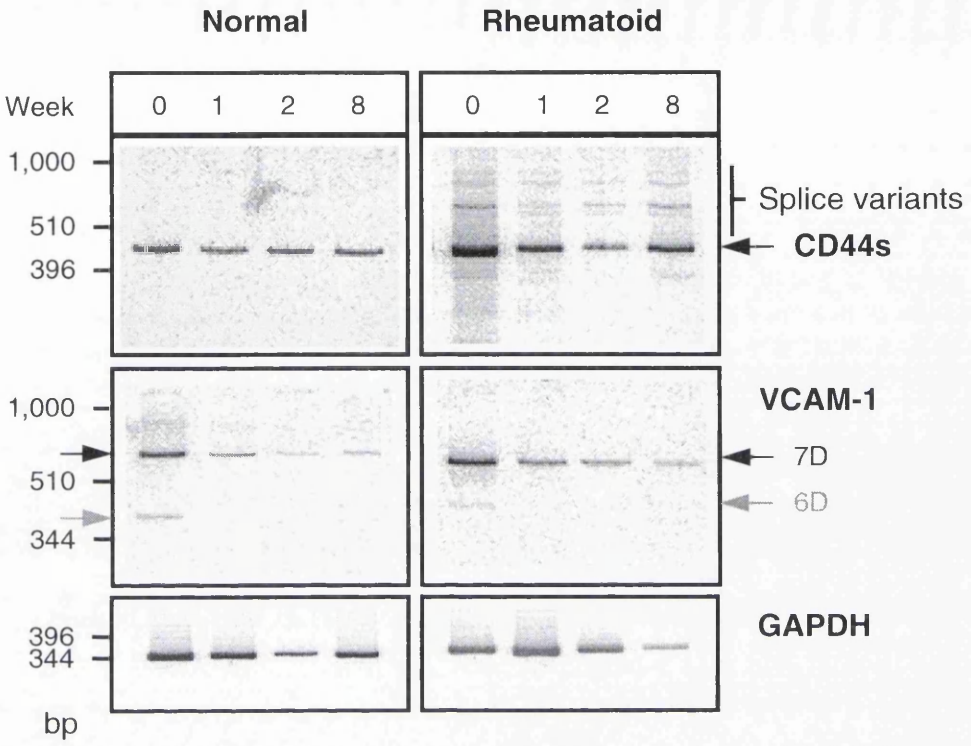

Normal
Rheumatoid
Week
0 1 2 8
0 1 2 8
1,000
510
396
Splice variants
CD44s
1,000
510
344
VCAM-1
7D
6D
396
344
GAPDH
bp

Figure 5.2

Figure 5.3 Southern blot analysis of CD44 PCR products from freshly dispersed RA (**A**) and OA (**B**) synovial cells (Day 0). The upper panels in each case are composites of the Southern blots. The lower panels are schematic representations of the Southern blots, showing the major bands (thick lines) and minor bands (thin lines). Total RNA was isolated and reverse transcribed (see Methods and Materials). Hot start PCR was performed with first strand cDNA (equivalent to 10 ng of total RNA) using specific CD44 primers (44.513 and 44.934). The CD44 primers were upstream and downstream of the variant exon insertion point. PCR-products were electrophoresed on a 1.5% agarose gel and transferred onto a nytran membrane. Variant exon DNA templates were labelled with [α^{32}P] dATP using a Megaprime DNA labelling kit (Amersham). Labelled variant exon-specific probes were added to pre-hybridized blots and allowed to hybridize for 16 hours at 42°C. After each hybridization the blots were exposed to phosphorimager plates for 24 hours and scanned using a Storm II phosphorimager. The blot was stripped and consecutively reprobed with the other exon probes. Results shown are representative of three experiments performed.

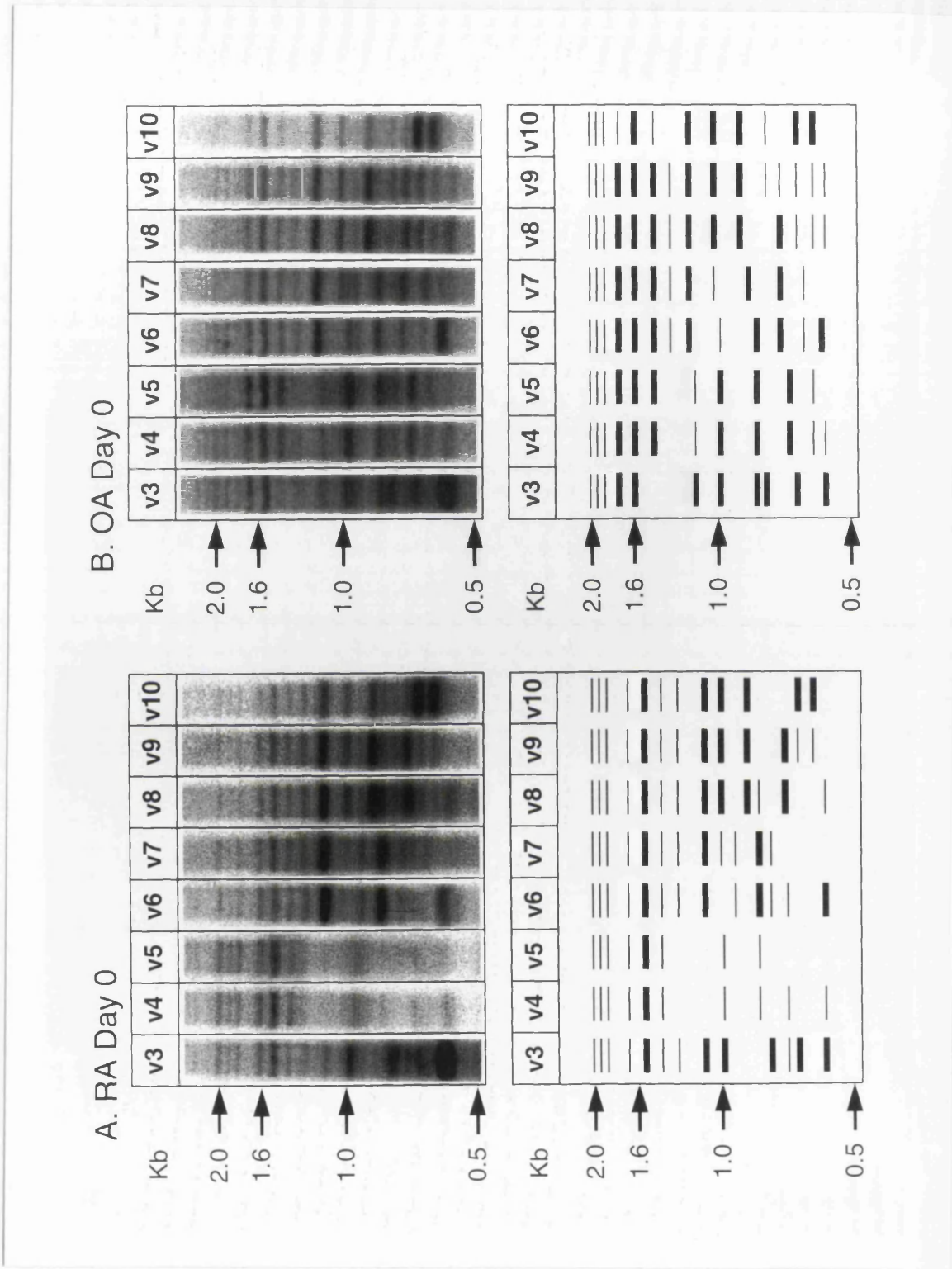


Figure 5.3

Figure 5.4 The effect of cycle number on the amplification of splice variant cDNA. Total RNA was isolated from freshly dispersed non-inflamed (normal), osteoarthritic (Osteo), and rheumatoid arthritic (Rheumatoid) synovium and reverse transcribed (see Methods and Materials). Hot start PCR was performed with first strand cDNA (equivalent to 10 ng of total RNA) using specific CD44 primers (44.513 and 44.934) or GAPDH primers (Gap.67 and Gap.406). The CD44 primers were upstream and downstream of the variant exon insertion point. [α^{33}P]-dATP was added to the PCR reaction mix (see Methods and Materials) to allow direct quantitation of the amount of PCR product formed. PCR was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer). The number of amplification cycles was varied between 25 and 40 cycles. Each cycle consisted of 30 sec at 94°C, 30 sec at an annealing temperature of 62°C, and 30 sec at 72°C. Following amplification, PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid and dried under vacuum. Gels were exposed to phosphorimager plates for 4-24 hours and scanned using a Fuji Bas 1000 phosphorimager. Note the absence of CD44 splice variants in cells derived from normal non-inflamed synovium and the variability in the expression of cells derived from osteoarthritic synovium. Results shown are representative of two separate PCR experiments performed.

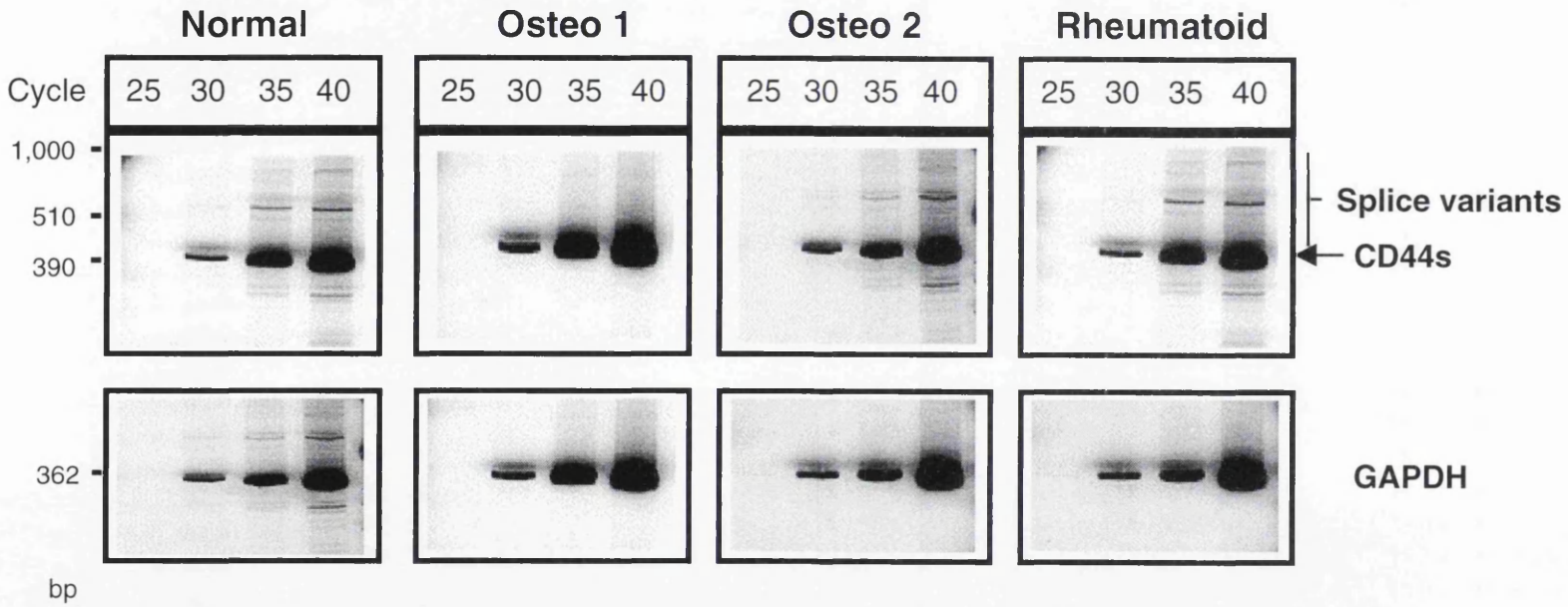

Normal
Osteo 1
Osteo 2
Rheumatoid
Cycle
25 30 35 40
1,000
510
390
362
bp
Splice variants
CD44s
GAPDH

Figure 5.4

Figure 5.5 Immunofluorescence staining of fibroblast-like synoviocytes cultured for 10 days. Top row, immunofluorescence staining for CD14 and VCAM-1 in fibroblast-like synoviocytes cultured for 10 days. Middle and bottom rows, immunohistochemical staining for CD44v7/8 in synovial cells isolated from non-inflamed (normal) synovium (**A**), osteoarthritic (OA) (**B** and **C**) and rheumatoid arthritic (**D**) cultured for ten days.

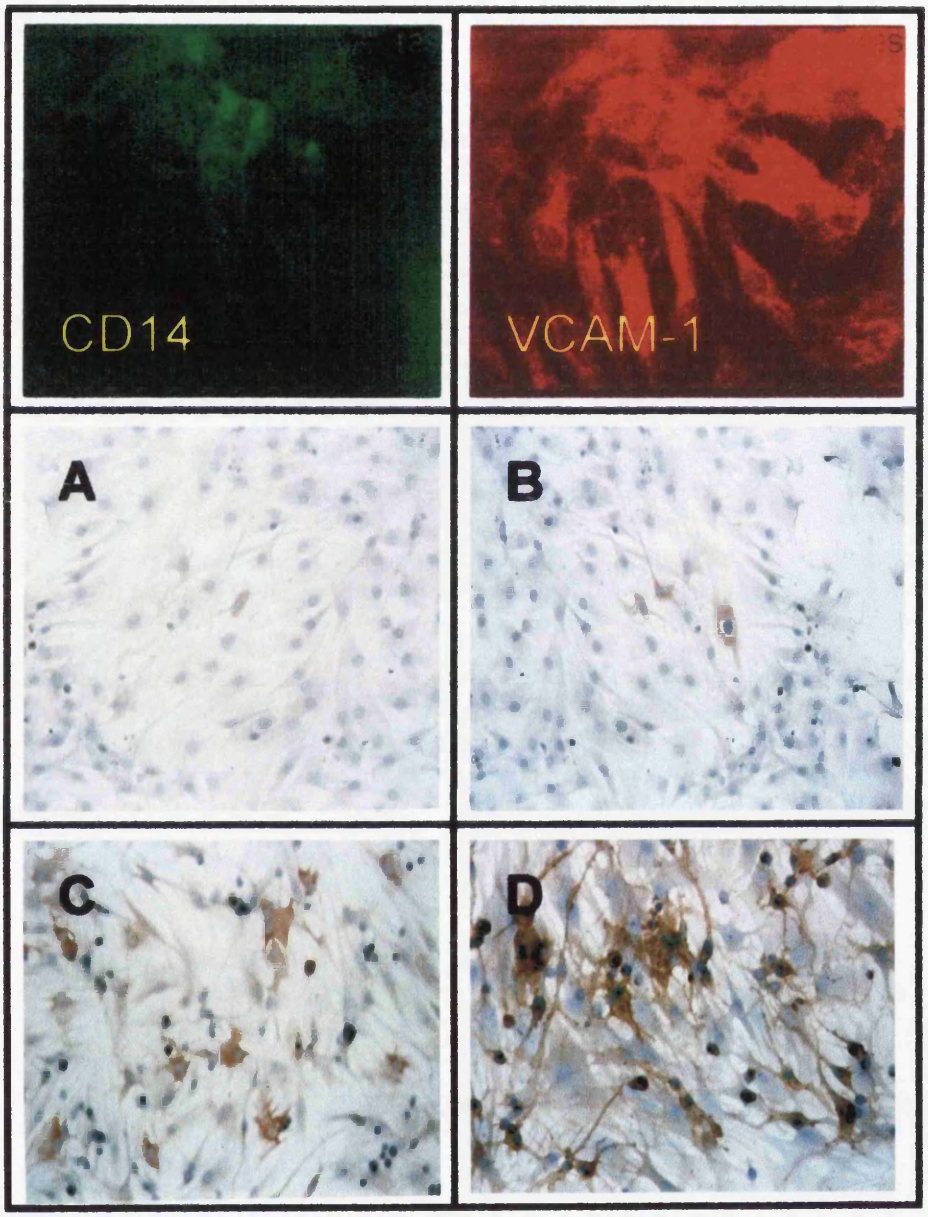


Figure 5.5

Figure 5.6 Flow cytometric analysis of cell surface VCAM-1, CD14, CD44 and CD44v7/8 expression on RA fibroblast-like synoviocytes cultured for 10 days. Fibroblast-like synoviocytes were isolated as described earlier in Chapter 2. Following culture with 10% (v/v) FBS for 10 days, cells were removed by incubation with 4 mM EDTA. The suspension of fibroblast-like synoviocytes was washed and incubated with saturating amounts of anti-VCAM-1, anti-CD14, anti-CD44, or anti-CD44v7/8, followed by an incubation with phycoerythrin-conjugated anti-mouse IgG. Samples were analysed using a FACSvantage. The dashed line histograms represent cells incubated with phycoerythrin-conjugated anti-mouse IgG only. Solid line histograms represent cells stained with anti-VCAM-1, anti-CD14, anti-CD44, or anti-CD44v7/8 antibodies, respectively, and phycoerythrin-conjugated anti-mouse IgG. Results shown are representative of four separate experiments performed.

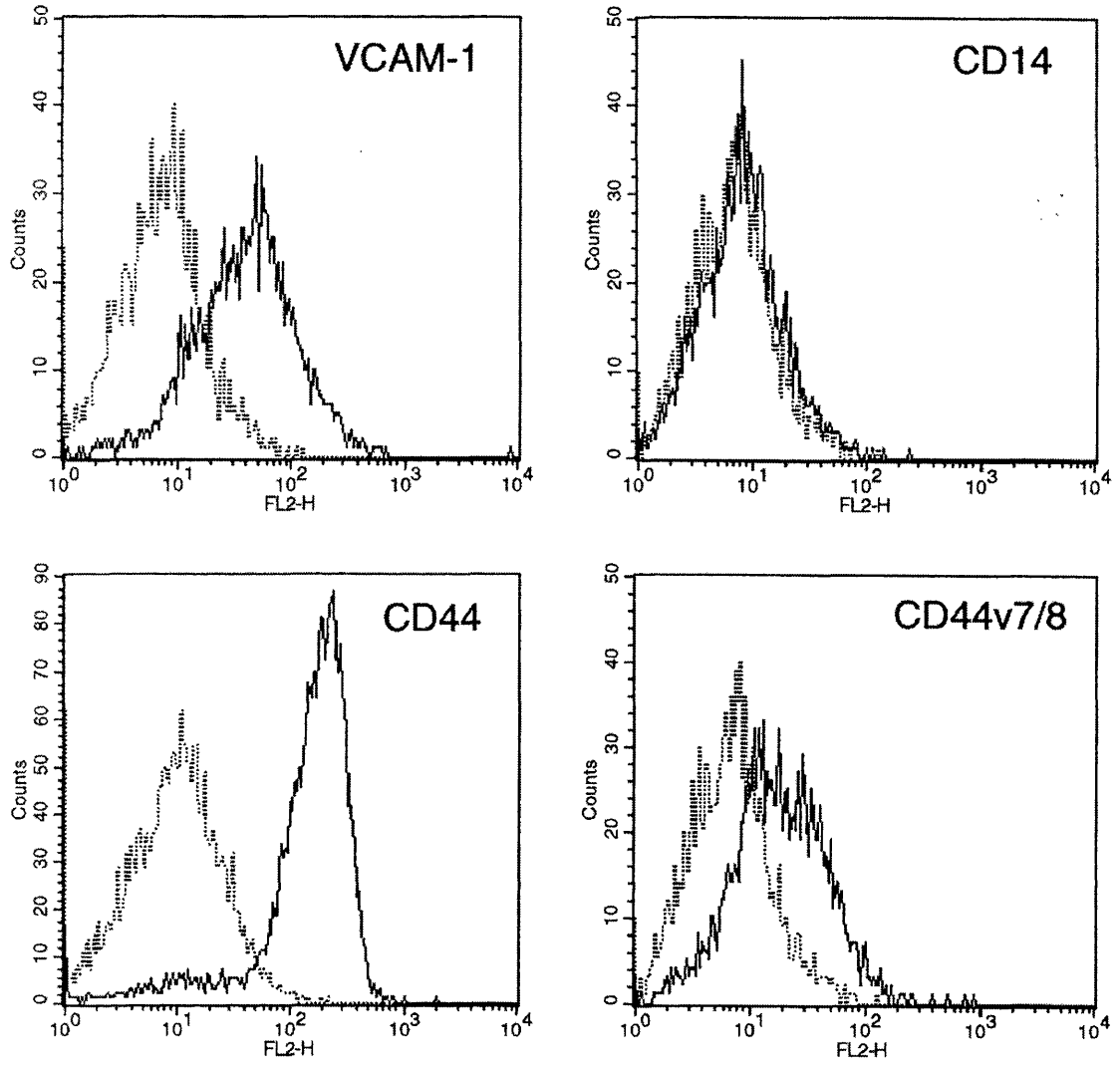


Figure 5.6

Figure 5.7 RT-PCR analysis of VCAM-1-selected synovial cells using CD14, CD44, GAPDH, MSE-1, and VCAM-1-specific oligonucleotide primers. Fibroblast-like synoviocytes were isolated from rheumatoid synovium (see Methods and Materials). Total RNA was isolated either immediately (Day 0) or cells were cultured for 10 days and stained for VCAM-1. With the aid of flow cytometry the total population of fibroblast-like synoviocytes was separated into low and high VCAM-1-expressing cells. Total RNA was isolated from freshly dispersed RA synovium and reverse transcribed (see methods and materials). Hot start PCR was performed with first strand cDNA (equivalent to 10 ng of total RNA) and the respective oligonucleotide primer pair (see Table 2.1). [α^{33}P]-dATP was added to the PCR reaction mix (see Methods and Materials) to allow direct quantitation of the amount of PCR product. PCR was carried out for 30 cycles in a GeneAmp 9600 thermal cycler (Perkin Elmer). Each cycle consisted of 30 sec at 94°C, 30 sec at an annealing temperature of 62°C, and 30 sec at 72°C. Following amplification, PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid and dried under vacuum. Gels were exposed to phosphorimager plates for 4-24 hours and scanned using a Fuji Bas 1000 phosphorimager. The numbers underneath each lane of the autoradiograph represent GAPDH-normalized expression of the various PCR products. Note the enrichment for VCAM-1 and the depletion of CD14 and MSE-1 in the VCAM-1-positive (+ve) population. Results shown are representative of three separate PCR experiments performed.

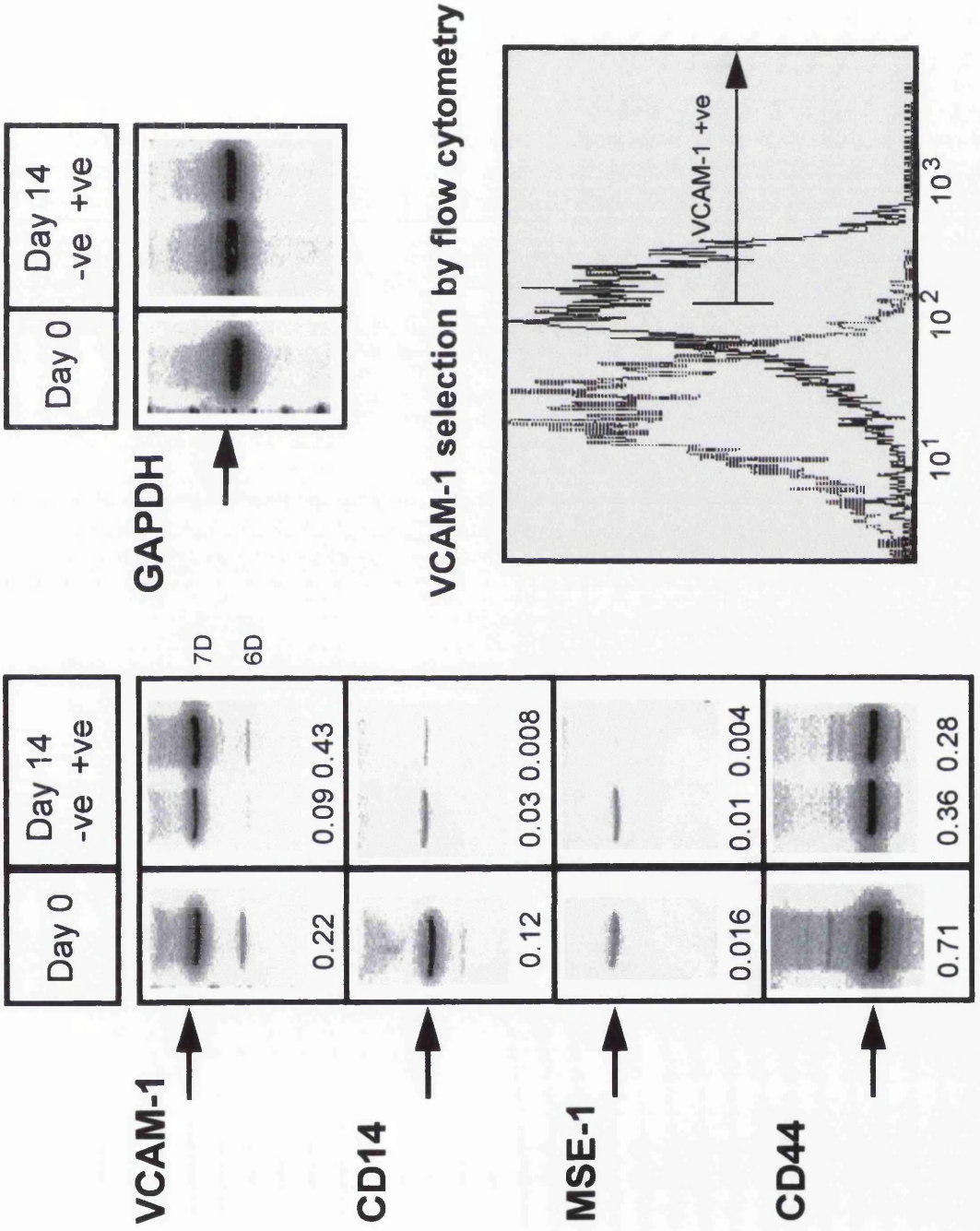

Day 0
Day 14 -ve +ve
GAPDH
VCAM-1 selection by flow cytometry
VCAM-1 +ve
10^1
10^2
10^3
7D
6D
VCAM-1
CD14
MSE-1
CD44
0.22
0.09 0.43
0.12
0.03 0.008
0.016
0.01 0.004
0.71
0.36 0.28

Figure 5.7

Figure 5.8 Southern blot analysis of CD44 variant exon expression in VCAM-1-positive fibroblast-like synoviocytes. Fibroblast-like synoviocytes were isolated from inflamed synovium and cultured for 10 days as described in Methods and Materials. VCAM-1-positive fibroblast-like synoviocytes were isolated by flow cytometry (Figure 5.7). Fibroblast-like synoviocytes expressing low (-ve) and high (+ve) cell surface VCAM-1 were recovered from osteoarthritic (Osteo) and rheumatoid arthritic (Rheumatoid) synovium. Total RNA was isolated and reverse transcribed (see Methods and Materials). Hot start PCR was performed with first strand cDNA (equivalent to 10 ng of total RNA) using specific CD44 primers (44.513 and 44.934). The CD44 primers were upstream and downstream of the variant exon insertion point. PCR-products were electrophoresed on a 1.5% agarose gel and transferred onto a nytran membrane. Variant exon DNA templates were labelled with [α^{32}P] dATP using a Megaprime DNA labelling kit (Amersham). Labelled variant exon-specific probes were added to pre-hybridized blots and allowed to hybridize for 16 hours at 42°C. After each hybridization the blots were exposed to phosphorimager plates for 24 hours and scanned using a Storm II phosphorimager. The blot was stripped and consecutively reprobed with the exon probes. Results shown are representative of three experiments performed.

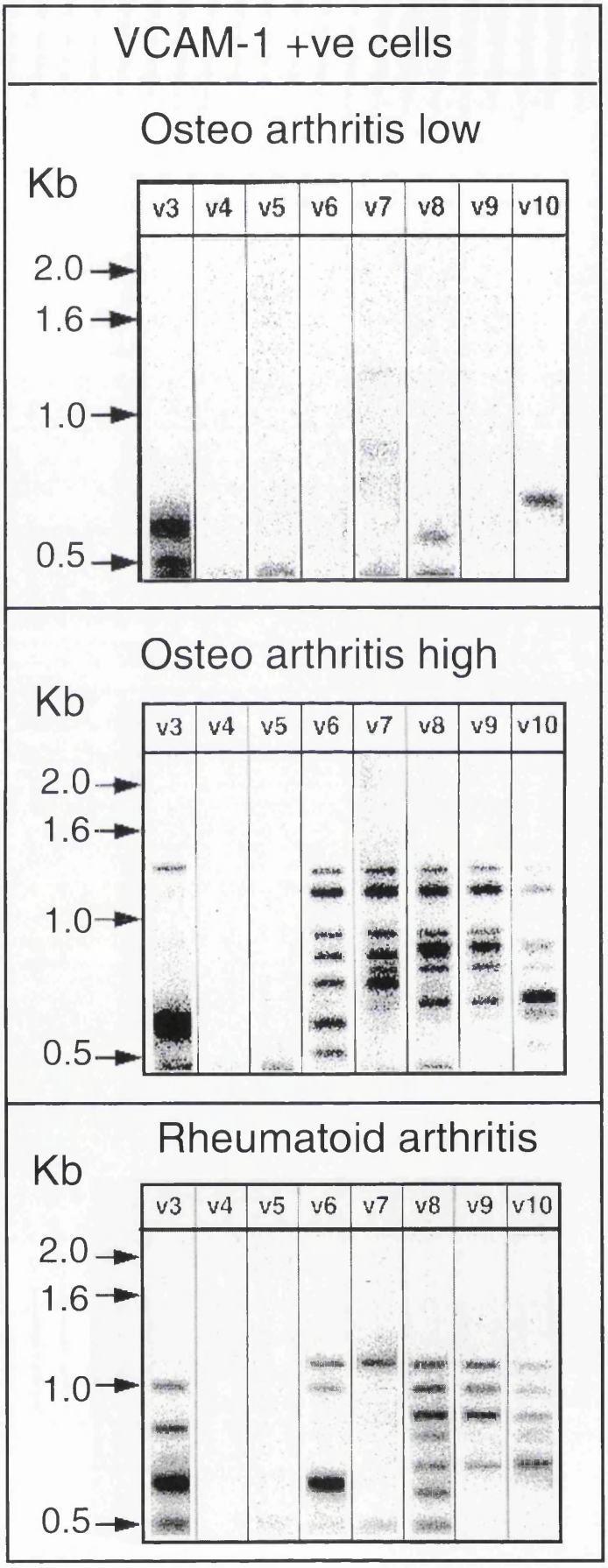

VCAM-1 +ve cells
Osteo arthritis low
Kb
v3
v4
v5
v6
v7
v8
v9
v10
2.0
1.6
1.0
0.5
Osteo arthritis high
Kb
v3
v4
v5
v6
v7
v8
v9
v10
2.0
1.6
1.0
0.5
Rheumatoid arthritis
Kb
v3
v4
v5
v6
v7
v8
v9
v10
2.0
1.6
1.0
0.5

Figure 5.8

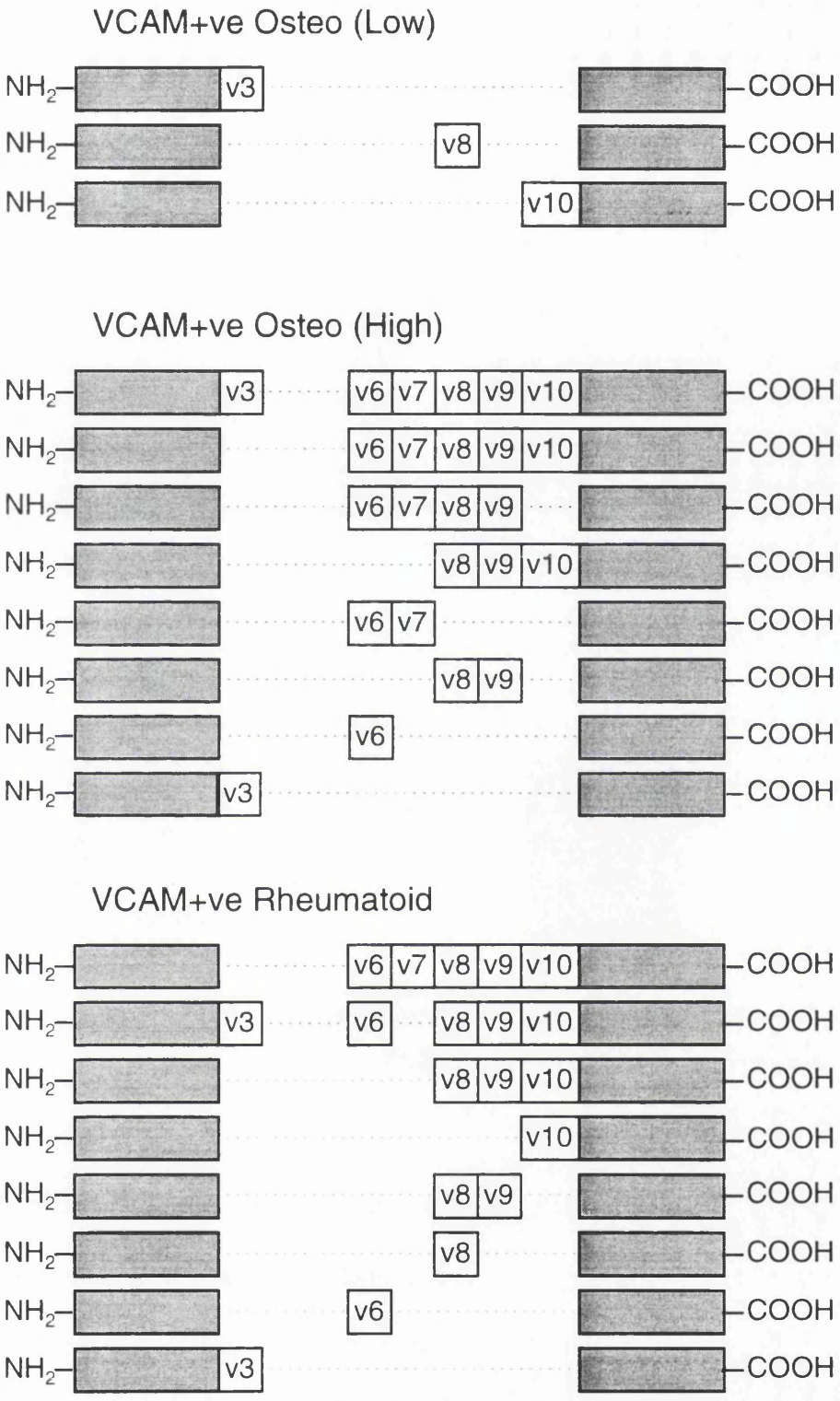


Figure 5.9 Schematic representation of the possible CD44 splice variants present in the Southern blots shown in figure 5.8. Note the VCAM-1-positive (+ve) synovial cells from RA and OA patient samples express mRNA encoding for a broad range of variant exon-containing CD44 molecules.

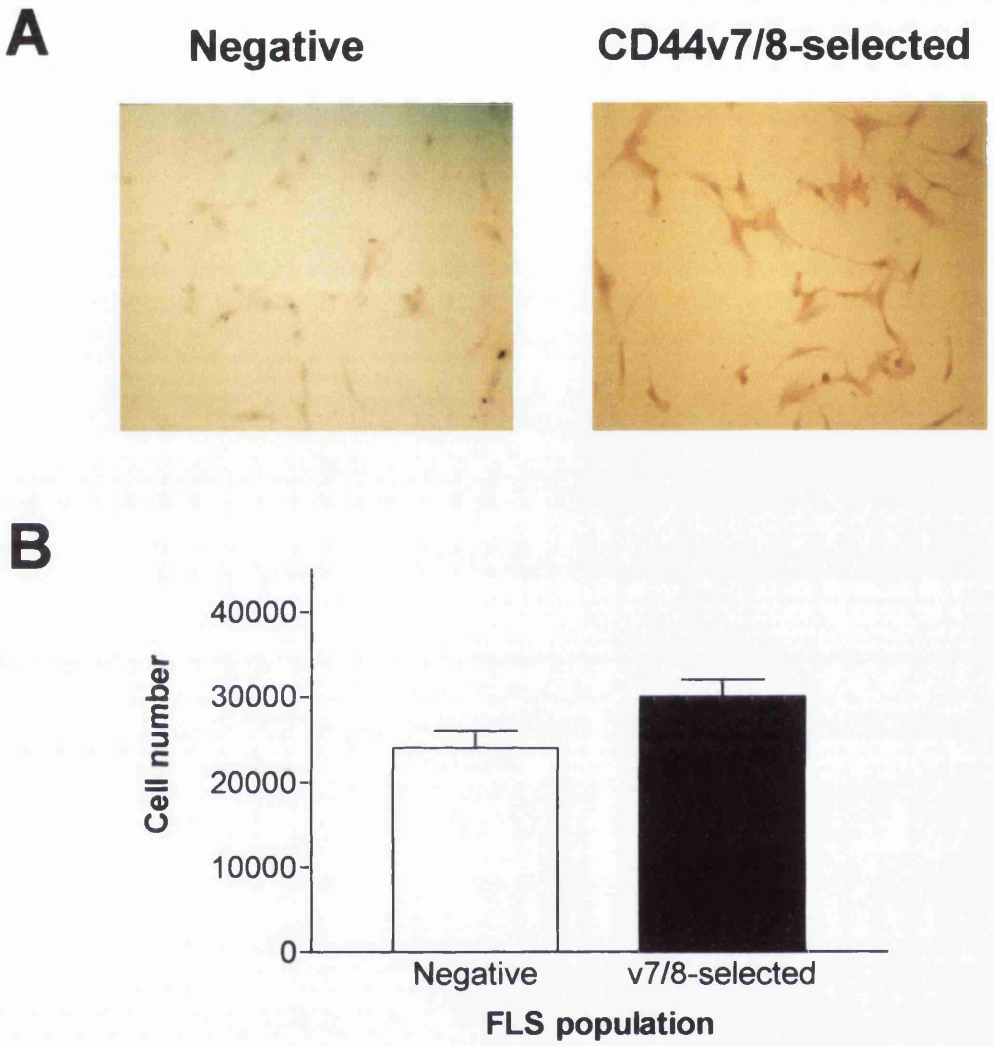


Figure 5.10 CD44v7/8-selected cells demonstrate an increased rate of proliferation. (**A**). Immunohistochemical staining of CD44v7/8-positive cells selected using magnetic beads. The negative population represents cells that did not attach to magnetic beads. (**B**). Cell numbers in negatively-selected and CD44v7/8-selected fibroblast-like synoviocytes following 4 days in culture. Cell numbers were determined using a ZB1 Coulter counter with the orifice set to 100 μm. Data is presented as the mean ± SEM of four experiments.

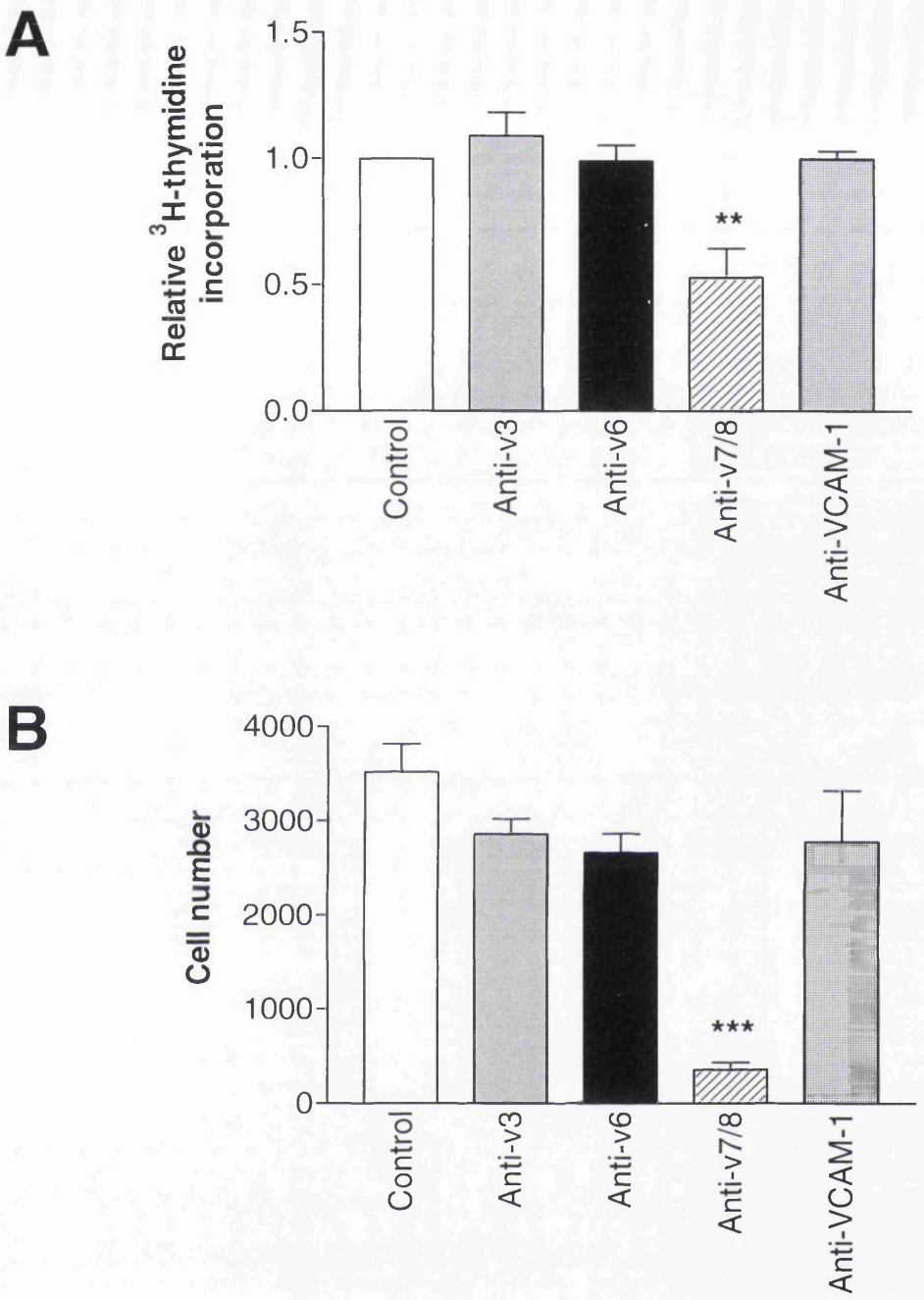


Figure 5.11 Anti-CD44v7/8 inhibits ^{3}H-thymidine incorporation and cell proliferation of fibroblast-like synoviocytes. Cells were incubated with or without anti-CD44v3, anti-CD44v6, anti-CD44v7/8, or anti-VCAM-1 antibodies. (**A**) After three days of culture ^{3}H-thymidine was added and its incorporation was measured after a further 16 hours in culture. Each sample was assayed in quadruplicate. (**B**) After four days of culture, cell numbers were counted using a Coulter counter. Data is presented as the mean ± SEM of four separate experiments. ** indicate $p < 0.01$, *** $p < 0.005$ compared with control (i.e. no antibody) treatment.

Table 5.1 Immunohistochemical detection of CD44 and CD44 splice variant expression by synovial fibroblasts from normal, OA and RA patients

Patient	SFF2 (Total CD44)	VFF8 (v5)	VFF17 (v7/8)
Normal 1	+++	+/–	–
Normal 2	+++	–	–
OA 1	+++	++	–
OA 2	+++	+/–	++
OA 3	+++	+	+
OA 4	+++	+	+
OA 5	+++	+	+
RA 1	+++	++	++
RA 2	+++	++	++
RA 3	+++	+	+
RA 4	+++	+/–	++
RA 5	+++	+	++

where – = no detectable expression; + = weak expression; ++ = moderate expression; +++ = strong expression.

5.4 Discussion

Fibroblast-like synoviocytes derived from diseased tissues express CD44 splice variants. Southern blot analysis has demonstrated that various splicing combinations exist, comprising exons v3, v4, v6, v7, v8, v9, and v10. CD44 splice variant expression is always observed in a large percentage of the cells in cultures of RA synovial cells, it is variable in those from OA synovium, and absent in cells isolated from non-inflamed joints. This pattern in some way resembles that of monocyte infiltration of the synovial membrane, high in RA, variable in OA and very little in non-inflamed joints. As such, CD44 splice variant expression may be a marker for the severity of inflammation (Morales-Ducret *et al.*, 1992; Hogg *et al.*, 1985). Interestingly, in these studies it was shown that monocytes first appear within the intimal layer of the synovium, associated with the lining fibroblast-like synoviocytes, where they mature towards macrophages. They subsequently migrate into other areas of the synovium (Hogg *et al.*, 1985). It is postulated that fibroblast-like synoviocytes may play an important role in the retention and maturation of monocytes within the intimal layer, a process that may involve adhesion molecules such as CD44. Hale et al. (1995) demonstrated that CD44v9-positive macrophages are indeed found within the intimal layer and a recent study has shown that CD44v8-10 can bind to both CD44v8-10 and CD44H (Droll *et al.*, 1995). These observations may indicate a role for CD44 splice variants in retention of monocytes and this aspect warrants further study.

This report gives evidence for one important implication of CD44v7/8 expression, namely it conveys a proliferative advantage upon fibroblast-like synoviocytes compared to similar cells that do not express this splice-variant and are obtained from the same synovial specimen. This proliferative advantage could explain

why an increase in the number of v7/8-positive fibroblast-like synoviocytes was observed with increasing time in culture. Enrichment of v7/8-positive cells could, in theory, also be explained by a slow induction of variant expression during culture (Weiss *et al.*, 1997). However, this seems unlikely because cells obtained from non-inflamed joints fail to express these splice variants even after long-term culture. Expression of the CD44v7/8 epitope may explain, at least in part, hyperplasia of the synovial membrane in rheumatoid arthritis. A similar proliferation advantage could play a role in uterine cervical carcinogenesis where it was found that the extent of squamous intra-epithelial lesions correlated with an increased number of cells expressing CD44v7/8 (Dall *et al.*, 1996). It remains to be determined at what stage of rheumatoid arthritis CD44 splice variants appear.

With respect to rheumatoid arthritis, I believe that hyperplasia has a role in the development of a chronic erosive environment within the synovial joint, irrespective of the role of the immune system therein (Zvaifler and Firestein, 1994). I base this idea not only on the findings that the degree of synovial hyperplasia correlates with the degree of joint erosion and that H2-c-fos transgenic mice develop destructive arthritis, but also on our recent finding that fibroblast-like synoviocytes produce excessive amounts of IL-6 (Rooney *et al.*, 1988; Shiozawa *et al.*, 1992; Chen *et al.*, 1998). Importantly, this production took place in the absence of inflammatory mediators like TNF-α and IL-1β and could be elevated by the presence of synovial fluid or hyaluronan. High levels of IL-6, as found in the synovial joint in rheumatoid arthritis, could thus be a mere consequence of elevated numbers of fibroblast-like synoviocytes. High levels of IL-6 are instrumental in the reduction of cartilage production (Nietfield *et al.*, 1990). In addition, in an *in vitro* study with fibroblast-like synoviocytes, IL-6 was shown to be

involved in cartilage breakdown (Scott *et al.*, 1997). Given these considerations our present observation, that antibodies against the CD44v7/8 epitope inhibit fibroblast-like synoviocyte proliferation, makes this epitope a potential target for pharmacological intervention in the treatment of rheumatoid arthritis.

The mechanism by which the CD44v7/8 epitope increases cell proliferation is unclear at the moment. Sleeman et al. (1997) showed that inclusion of exons v6 and v7, as part of a CD44v4-v7 splicing combination, not only resulted in a stronger binding to hyaluronan but also enabled CD44 to bind to a larger range of glycosaminoglycans. Hyaluronan binding of fibroblast-like synoviocytes has been tested and showed that cells from RA patients had an enhanced CD44-dependent binding capacity compared to cells from non-diseased joints and moreover, CD44v7/8-enriched cells had an enhanced CD44-dependent binding capacity compared to negatively selected cells (Data not shown). Unfortunately CD44v7/8 expression levels were too low to study further glycosaminoglycan binding in a CPC (Cetylpyridinium chloride) precipitation protocol (Sleeman *et al.*, 1997). The proliferative advantage may be explained by an altered outside-in signalling through either an increased binding to hyaluronan or binding to other glycosaminoglycans. Outside-in signalling has great implications for cell cycle progression as shown in studies with the integrin family of adhesion molecules (Assoian, 1997; Giancotti, 1997). Focal adhesion complexes play an important part in this outside-in signalling. To date CD44 has not been shown to be present in these complexes, but it is found in association with members of the ezrin-radixin-moesin (ERM) family of proteins and components of the cytoskeleton (Bourguignon *et al.*, 1992; Lokeshwar *et al.*, 1996, Tsukita *et al.*, 1994). This, together with the finding that CD44 can signal through association with tyrosine protein kinases make it likely that

CD44 contributes to the outside-in signalling and thus may effect cell proliferation (Bourguignon *et al.*, 1997; Ilangumaran *et al.*, 1998; Taher *et al.*, 1996). I have cloned the CD44-splicing combinations from rheumatoid arthritic tissues that will allow me to further study their biochemical and functional implications.

The RA synovium has been attributed several characteristics which are similar to those of invasive tumours (Gay *et al.*, 1989; Firestein, 1996). It is therefore of particular interest that among the CD44 variants expressed by fibroblast-like synoviocytes are those containing the variant exon v6, a variant which has been implicated in tumour metastasis (Günthert *et al.*, 1991). RA Fibroblast-like synoviocytes also possess invasive behaviour since they are able to invade and destroy human cartilage (Müller-Ladner *et al.*, 1996; Scott *et al.*, 1997). Interestingly, part of the invasive behaviour observed has now been shown to be mediated by CD44 (Scott *et al.*, 1997). Studies are currently in hand to determine whether any of the CD44 splice variants expressed by fibroblast-like synoviocytes are involved in invasion.

In conclusion, this study has shown that VCAM-1-positive fibroblast-like synoviocytes, isolated from both OA and RA synovium, express a number of CD44 splice variants. These CD44 proteins include those containing the v7/8 epitope. In contrast, fibroblast-like cells obtained from non-inflamed synovium did not express these variant forms. These v7/8-containing CD44 isoforms demonstrate an essential role in fibroblast-like synoviocyte proliferation, and thus may explain hyperplasia of the RA synovium.

Chapter 6

General discussion

It is generally regarded that resident fibroblast-like synoviocytes have a role in the orchestration of destruction of the synovial joint that is observed in rheumatoid arthritis (Shiozawa *et al.*, 1992; Müller-Ladner *et al.*, 1996). Fibroblast-like synoviocytes may be involved in two ways. This could be indirectly through the activation or maturation of infiltrating leukocytes, with the consequent induction of inflammation followed by local tissue destruction. Alternatively, it may also occur directly through an enhanced release of cytokines and proteases that results from their transformed state, thereby resulting in hyperplasia and invasion of surrounding tissue. However, it is clear that relatively little is known about the biology of the fibroblast-like synoviocyte. These cells are identified by their location within the synovial membrane, their production of the glycosaminoglycan hyaluronan, and the constitutive expression of VCAM-1 (Pitsillides *et al.*, 1993; Wilkinson *et al.*, 1993a). How they influence the infiltrating leukocytes and what exactly happens to these cells when a chronic inflammatory state develops within the synovial joint remains thus far largely unknown. This study highlights two aspects that relate to the above. Firstly, it contributes to the understanding of the regulation of VCAM-1 expression in fibroblast-like synoviocytes, this being relevant for interactions with leukocytes. And secondly, it describes the expression of CD44 splice variants that are intimately associated with tumour proliferation and metastasis. In this discussion I will address these two aspects.

Regulation of expression of VCAM-1

A role for IL-4 and IL-13

VCAM-1 expression by fibroblast-like synoviocytes appears to be under dual control. Constitutive or basal low level VCAM-1 expression appears to result from constitutive production of VCAM-1 mRNA, coupled with a high level of degradation. In addition, there is an inducible component, mediated by pro-inflammatory cytokines like TNF-α and IL-1β (Morales-Ducret *et al.*, 1992; Marlor *et al.*, 1992). However, inducible VCAM-1 expression is very short lived. Importantly, in this thesis I have shown that the presence of either IL-4 or IL-13 dramatically prolongs the half-life of VCAM-1 mRNA in these cells, thereby resulting in a prolonged elevated expression even in the absence of other inflammatory cytokines. In fact, IL-4 and IL-13 are much more potent mediators of VCAM-1 expression when compared with the induction mediated by TNF-α or IL-1β. This, together with the synergy between these two cytokines and TNF-α, strengthens the point that IL-4 or IL-13 could make a significant contribution to elevated levels of VCAM-1 expression observed in the synovial membrane.

Although both IL-4 and IL-13 are present in the RA synovial membrane, it is IL-13 that has been consistently identified at elevated levels within the synovial fluid and synovium of RA patients (Isomäki *et al.*, 1996). IL-13 is a Th2 cytokine that can also be produced by mast cells and activated Th0 and Th1 cells. This may explain the high levels of IL-13 in RA synovial fluid. Mast cells are found in elevated numbers at the cartilage-pannus junction of the arthritic joint (Bradding *et al.*, 1995; Jaffe *et al.*, 1996; Bromley *et al.*, 1984). Moreover, many of the mast cells found at the cartilage-pannus junction show signs of activation, namely degranulation, which may imply that they play an essential role in the pathogenesis of RA (Tetlow and Wooley, 1995). In

addition, studies have demonstrated that mast cells of the MC_{TC} phenotype, a phenotype which has been identified in the RA synovium, preferentially express IL-4 and IL-13 (Bradding *et al.*, 1995; Jaffe *et al.*, 1996). Immunological activation of human lung mast cells with anti-IgE has been shown to result in the induction of mRNA and protein for both IL-4 and IL-13 (Okayama *et al.*, 1995; Jaffe *et al.*, 1996). This may be of particular interest since a proportion of RA patients show elevated levels of serum IgE antibodies. Given the effects of IL-4 and IL-13 on expression of VCAM-1, its role in the interaction of fibroblast-like synoviocytes with leukocytes needs to be explored further.

VCAM-1 expression and transformation of fibroblast-like synoviocytes

As indicated in the previous section, fibroblast-like synoviocytes obtained from RA synovium exhibit both constitutive and cytokine-inducible VCAM-1 expression. With these cells, constitutive VCAM-1 expression is apparent in the absence of inflammatory cytokines and declines slowly with time in culture. In contrast, VCAM-1 expression on pannocytes, a name given to fibroblast-like cells that have invaded bone and cartilage, is maintained at elevated levels throughout long-term culture (Zvaifler *et al.*, 1997). Unfortunately, in their study Zvaifler and colleagues (1997) did not address whether cytokines are able to regulate VCAM-1 expression in these cells. Nevertheless, if we assume that pannocytes reflect fibroblast-like synoviocytes at a different stage of development, then an interesting picture starts to emerge. The gradual change from mainly inducible to constitutive expression may be characteristic of a gradual change of phenotype towards a more "aggressive cell type". As such the disease may deteriorate in a fashion similar to that seen with certain tumours, which gradually change from

benign to malignant with the constitutive expression of VCAM-1 being a marker for that process. It would be of interest to find out which genes are involved in this gradual change, and in particular, like in tumour progression, are these changes mutation dependent or are they cytokine-induced?

The role of VCAM-1 in the pathogenesis of rheumatoid arthritis?

All the efforts to characterize VCAM-1 expression in the fibroblast-like synoviocytes are based on the assumption that this molecule plays an essential role in the orchestration of the inflammatory (immune) response. Apart from the comparison with the role of VCAM-1 in binding monocytes and eosinophils to and their subsequent transmigration through vascular endothelial cells, there are indications that it may play a similar role in the synovial membrane. It has been proposed that the VCAM-1-positive cells within the intimal layer may initiate the first contacts with infiltrating monocytes which enter the synovium in response to inflammatory stimuli. Once here, monocytes differentiate and mature into macrophages and then subsequently move into the lower subsynovial layers (Hogg *et al.*, 1985). In an *in vivo* study using synovial tissue engrafted into SCID mice, a similar role has been identified for ICAM-1 (Jorgensen *et al.*, 1996a).

The intimal fibroblast-like synoviocyte has also been suggested to function as a follicular dendritic cell with the recent discovery that VCAM-1 and decay-accelerating factor are co-expressed by this cell type (Edwards *et al.*, 1997b). Moreover, VCAM-1 expression by follicular dendritic cells has been found to mediate binding of human B cells (Freedman *et al.*, 1990). Of particular interest is the study by Chen et al. (1997) where it was shown that fibroblast-like synoviocytes constitutively release elevated

levels of IL-6. The presence of adherent monocytes greatly increased IL-6 expression which may explain the high levels of IL-6 observed in the RA joint. Preliminary data indicate roles for VLA-4 (the counter receptor of VCAM-1) and CD14 in the inducing-capacity of monocytes. Given the essential role of IL-6 in B-cell maturation and immunoglobulin synthesis, the synovial membrane thus appears to be an ideal environment for excessive antibody production.

Functional implications for CD44 splice variant expression

Cell transformation

This study demonstrates the expression of CD44 splice variants in diseased synovial tissue. Not only that, the data also clearly demonstrate that inclusion of exon v7 and v8 conveys a proliferative advantage on these cells. It has long been known that fibroblast-like synoviocytes obtained from severely inflamed tissues have a higher proliferation rate than cells obtained from non-inflamed or non-diseased joints (Anastassiades *et al.*, 1978). I now provide evidence that expression of certain CD44-splicing combinations may explain in part this proliferative advantage. As such, expression of CD44-splice variants indicates that fibroblast-like synoviocytes in RA have indeed progressed towards a transformed phenotype. It is interesting to note that CD44-plays a pivotal role in phenotypic changes. For instance, matrix invasion by c-fos transformed cells is entirely dependent on CD44 expression with CD44 being recruited to pseudopodial extensions (Lamb *et al.*, 1997). CD44 has also recently been found to mediate the adhesive interaction between RA fibroblast-like cells and cartilage in an *in vitro* model of cartilage degradation (Scott *et al.*, 1997). The role of CD44 and its splice variants in the phenomenon of matrix invasion by fibroblast-like synoviocytes is currently being

tested in the laboratory of Dr. IJsbrand Kramer.

Rescue from apoptosis?

CD44 receptors expressed by fibroblast-like synoviocytes may also have a role in the induction of controlled cell death, apoptosis. Henke and co-workers (1996) have shown that ligation of the CD44 receptor using an anti-CD44 antibody induces apoptosis in human lung fibroblasts. Moreover, CD44 shedding and detachment from a HA substratum precede DNA fragmentation in colon carcinoma cells undergoing apoptosis in response to ligation of CD95 (APO-1/Fas) receptors (Gunthert *et al.*, 1996). RA fibroblast-like synoviocytes possess a number of characteristics which are indicative of increased apoptosis. For instance, they demonstrate extensive DNA fragmentation both *in situ* or when cultured *in vitro* with either TNF-α or anti-Fas antibody (Firestein *et al.*, 1995). Strangely enough, the observed cell death is much lower than would be expected from the level of DNA fragmentation. This has led Firestein and colleagues to hypothesize that the increased number of RA fibroblast-like synoviocytes in the synovial membrane may be due to defective apoptosis resulting from a somatic mutation in p53 (Firestein *et al.*, 1997; Aupperle *et al.*, 1998). It is also possible that the extent of DNA strand breaks observed in fibroblast-like synoviocytes *in situ* may be due to elevated levels of TNF-α or the abundance of low molecular weight HA found in the RA joint. However, certain rescue factors must be present in the RA synovium which prevent fibroblast-like synoviocytes from progressing down the apoptotic pathway. Not only somatic mutations in p53 but also possibly rescue via signals emanating from adhesion molecules, such as CD44 splice variants, might play a role in this rescue action.

T-cell activation?

CD44 splice variants are able to participate in normal immune function with anti-v6 antibodies inhibiting T cell activation (Arch *et al.*, 1992). Furthermore, transgenic mice constitutively expressing CD44v4-v7 are able to mount accelerated immune responses to antigenic stimuli (Moll *et al.*, 1996). It remains to be studied at what site CD44 splice variants mediate their role, that is at the site of antigen presenting cells, lymphocytes or both. A recent study by Weiss et al. (1998) showed that antibodies against CD44 epitopes inhibit the emigration of Langerhans cells from the epidermis and prevent binding of these cells to the T cell zones of lymph nodes. As a consequence, masking epitopes CD44v6 and v9 severely inhibits their capacity to induce a delayed type hypersensitivity reaction to a skin hapten *in vivo*. Assuming that fibroblast-like synoviocytes play a role in T-cell activation *in situ*, since they are able to co-stimulate T cell activation *in vitro*, it is possible that their CD44 splice variants may function in this.

Models to study the phenotypic changes of CD44 splice variant expressing cells

I have recently cloned a number of CD44 splice variants from RA synovium which will be used to study further the functional implications of variant isoforms. Ectopic expression of these splice variants in an appropriate fibroblast cell line will be used to investigate splice variant involvement in cell proliferation, matrix invasion, and the interaction with leukocytes. In addition, a mouse model would be highly beneficial for studying tissue destruction. Co-implantation of fibroblasts expressing various CD44-splicing combinations with human cartilage into SCID mice might be an appropriate

model. This model has already proved its usefulness in studying the consequence of cellular transformation in the destruction of cartilage and for studying pharmacological intervention (Müller-Ladner *et al.*, 1996; Sakai *et al.*, 1998).

Further characterization of fibroblast-like synoviocytes is needed

The determination of a number of extra fibroblast-like synoviocyte-specific markers would prove invaluable for further characterization of the fibroblast-like synoviocyte phenotype. One way of doing this would be by screening fibroblast-like synoviocyte mRNA using a human cDNA expression array. Human cDNA expression arrays consist of a number of human cDNAs immobilized to a nylon membrane. One commercially available human cDNA expression array allows the simultaneous screening of nearly 600 human genes. These include genes for stress response elements, apoptosis markers, oncogenes, cell adhesion receptors, cytokines, and growth factors. However, these expression arrays are not able to identify novel differentially expressed genes. This screening method could be used to determine differences in gene expression patterns between RA fibroblast-like synoviocytes compared with dermal fibroblasts, or cytokine-treated fibroblast-like synoviocytes compared to untreated cells. It is possible that by screening human genes in this manner we may identify fibsroblast-like synoviocyte markers that may contribute to the pathogenesis of RA.

In conclusion, it is hoped that the work presented in this study will stimulate and aid further experimentation into the characterization of the fibroblast-like synoviocyte phenotype. Moreover, further studies are required to dissect the individual components utilized by fibroblast-like synoviocytes in the pathogenesis of rheumatoid arthritis.

Specifically and importantly, the mechanisms underlying CD44v7/8-mediated proliferation of fibroblast-like synoviocytes will need to be investigated. The determination of these mechanisms may allow for the development of novel therapies used in the treatment of RA.

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Appendix

I. Criteria for primer design

Good primer design is critical for efficient and specific DNA amplification by PCR. A number of factors, such as base composition, repetitive motifs, primer length, and primer concentration, are able to influence mispriming and secondary structure formation. Using Primer select software primer pairs were designed to be 17-25 bases in length, have a guanine-cytosine (G+C) content of approximately 50-60%, and have matching melting temperatures (T_ms) in the range of 65-75°C. In addition, the primers used showed minimal complementarity at the 3′-end to avoid primer-dimer formation, and also exhibited minimal secondary structure. Primer-dimers can be amplified during the course of the PCR and result in a decreased yield of the required product. Furthermore, primers were designed to span an intron so that amplification of genomic DNA cannot occur.

Primer select chooses primer pairs by calculating the DNA duplex stability based on pairwise interacting nearest neighbour analysis. The programme generates suitable primer pairs which conform to the criteria shown overleaf and yield products in the ideal size range (usually 0.3-0.8 Kb). These primer pairs are scored according to product size, primer T_m match, product T_m difference and internal stability.

Initial conditions

Primer concentration	0.2 μM
Salt concentration	50 mM
Temperature for Delta-G calculations	25°C

Primer characteristics

Primer length	Min = 17 bp
	Max = 24 bp
3′ Pentamer stability	8.5 -Kc/M
Unique 3′ sequence of	7 bp
Accept dimer duplexing of	2 bp
Accept hairpin duplexing of	2 bp
Ignore duplexing of	8 bp from 3′ end
Ambiguous residues	0 bp

Mispriming

Average stability cut-off [-G/Primer length]	0.76
Allow a 1 bp mismatch within 7 bp of 3′	
Accept dimers of	3 bp or less
Accept hairpins of	3 bp or less

II. Optimization of the conditions used for semi-quantitative RT-PCR

With PCR, one of the major problems associated with the amplification of target DNA is the generation and amplification of spurious DNA fragments. The amplification of spurious products can occur by either mispriming to non-specific sequences or by primer-dimerization. Amplification of by-products has the effect of reducing PCR efficiency by introducing competition with target DNA for the various reaction components. Moreover, amplification of primer-dimers can drastically reduce the yield of the target product since oligonucleotide primers are usually present in excess. Thus, for PCR to be used for any analytical or diagnostic purposes it is first necessary to thoroughly optimize the reaction conditions for amplification. Optimization maximizes the efficiency, sensitivity, and specificity of the PCR. In theory, any one of the PCR components, either chemical or physical, can be optimized. However, in practice only a few components are routinely optimized. In this study, specificity has been maximized by optimizing the cycle number, annealing temperature, primer concentration, and the amount of template cDNA used. In addition, the use of 'hot start' PCR has further reduced mispriming and primer-dimerization and thereby increased the specificity of amplification (Erlich *et al.*, 1991; D'Aquila *et al.*, 1991). 'Hot start' PCR involves adding one of the reaction components, here DNA polymerase diluted in reaction buffer, to the other reaction components that are at a temperature of 94°C.

The optimization data shown in this appendix is summarized in Table 2.2. All methods and materials used are as described in chapter 2. Preliminary PCR studies using 35 cycles of amplification showed that total RNA isolated from freshly dispersed RA synovium contained variable expression levels of CD14, CD44, MSE-1, and

VCAM-1 mRNA transcripts (data not shown). Amplification using CD14, CD44, GAPDH, MSE-1 and VCAM-1 primer pairs was shown to give major products with the expected sizes of 529 base pairs (bp), 446 bp, 363 bp, 447 bp and 631 bp respectively.

A.2.1 The effect of cycle number on the amount of PCR product formed

In order to study changes in expression levels of mRNA transcripts the number of amplification cycles which failed to result in saturation of the DNA polymerase or depletion of PCR reactants was determined. The optimal cycle number for amplification of target cDNA was determined for each primer set. This was achieved by altering the cycle number from 20 to 40 cycles whilst keeping the temperature of annealing constant. As can be seen from Figure A.1(A), amplification of CD14 mRNA increased in an exponential manner between 28 and 40 cycles. The amplification of CD14 failed to plateau at the maximum cycle number studied. Similar results were found with the amplification of MSE-1 where the amount of PCR product was found to increase exponentially between 30 and 40 cycles (Figure A.4(A)). The amount of CD44 and GAPDH product increased up to 35 cycles and started to plateau at 40 cycles (Figure A.2(A) and Figure A.3(A)). Amplification of the VCAM-1 product (631 bp) revealed an exponential increase between 26 and 30 cycles after which it began to plateau (Figure A.5(A)). A second VCAM-1 product (355 bp) was apparent after 30 cycles of amplification (Figure A.5(B)). This product was much less abundant than the 631 bp product and failed to reach a plateau after 40 cycles. Southern blot analysis revealed that the additional VCAM-1 product was not due to mispriming but resulted from amplification of an alternatively spliced VCAM mRNA (Figure A.5(B)).

Representative examples showing the effect of cycle number on the amplification of target DNA fragments are shown in Figure A.6.

A.2.2 The effect of annealing temperature on the amount of PCR product formed

During the annealing phase of PCR, primers screen the template cDNA in order to find the complementary binding site. The use of an annealing temperature which is too low will increase the probability that primers hybridize to a binding site which shows partial complementarity. Optimizing the annealing temperature therefore ensured that amplification of the target DNA fragment was occurring under stringent conditions. Increasing the annealing temperature increases the association/dissociation kinetics of the primers and thus increases specificity. In addition, raising the annealing temperature will also increase the sensitivity of PCR.

PCR was carried out using annealing temperatures which varied between 54°C and 72°C. This was done to reduce the amplification of non-specific products during the first few cycles and so ensure increased specificity, and thus maximal amplification of the desired product. For each primer pair a cycle number was chosen so that the amount of PCR product formed was in the exponential phase of amplification (see Table 2.2). As can be seen from Figures A.1(B), A.2(B), A.3(B), and A.4(B), increasing the annealing temperature above 64°C resulted in a steady decrease in the amount of PCR product formed. An annealing temperature of 64°C was chosen for CD14, CD44, GAPDH, and MSE-1 primers, since this gave a strongly amplified product in each case. For VCAM-1 primers 62°C was chosen because the major VCAM-1 product (631 bp) demonstrated a dramatic decline in amplification as the annealing temperature was

raised above 62°C (Figure A.5(B)). Representative examples showing the effect of annealing temperature on amplification are shown in Figure A.7.

A.2.3 Decreasing the amount of cDNA in the PCR minimizes amplification of spurious products

As would be expected reducing the amount of cDNA to be amplified was shown to affect the amount of PCR product synthesized (Figure A.8). However, increasing the volume of cDNA (or RNA equivalents) to be amplified from 1 µl (equivalent to 100 ng of RNA) to 2 µl (equivalent to 200 ng of RNA) reduced the amount of PCR product formed. For most of the primer pairs studied some high molecular weight by-products were apparent which did not disappear with increasing annealing temperatures. A more effective way of reducing the amplification of these spurious by-products and thus increase the efficiency of amplification was found by reducing the amount of cDNA in the PCR reaction (Figure A.9). The higher molecular weight products which are apparent with CD44 primers were due to the amplification of splice variant forms of CD44 mRNA and not spurious by-products (See Chapter 5).

A.2.4 The effect of primer concentration on the amount of PCR product formed

Increasing the primer concentrations between 0.1 µM and 1.0 µM was found to affect the amplification of the PCR product (data not shown). Primer concentrations above 0.2 µM were found to result in the production of spurious by-products. When primer concentrations were used at 0.5 µM and above the formation of non-specific high

molecular weight products was observed. High primer concentrations can cause mispriming to non-specific template sequences and also an increase the probability of primer-dimer formation, both of which can reduce the efficiency of amplification and thereby decreasing the yield of target product. Optimal primer concentrations were found to be approximately 0.2-0.4 μM, with 0.2 μM being used for all subsequent PCR studies.

A.2.5 CD14 and MSE-1 mRNA transcripts can be detected in differentiated U937 cells

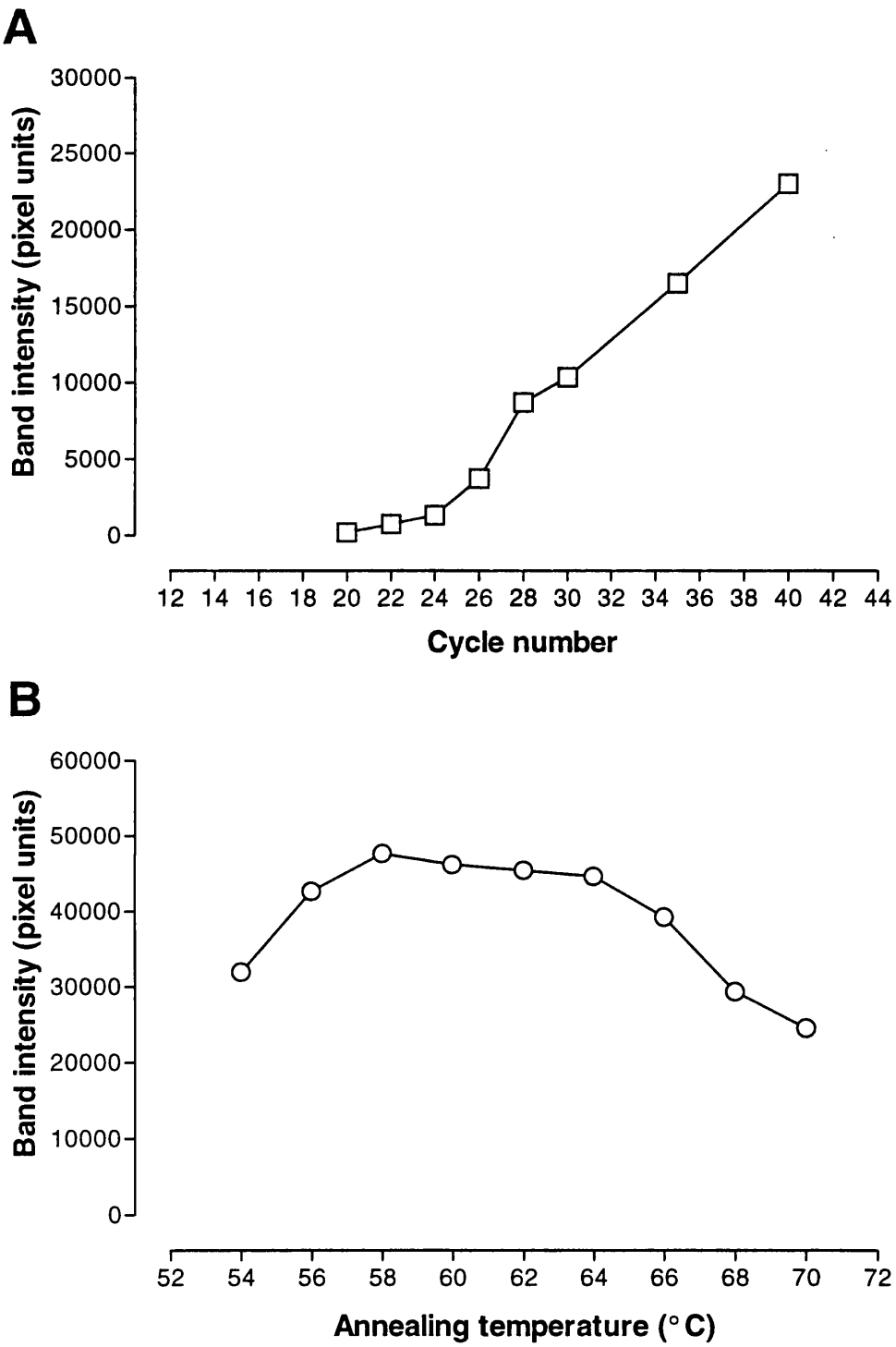
Initial studies showed that CD14 and MSE-1 products could be detected in PDBu-treated U937 cells (data not shown). In order to determine whether CD14 and MSE-1 primers could give quantitative results it was decided to test whether differentiation of the U937 cells resulted in observable changes in the expression levels of CD14 and MSE-1 mRNA transcripts. Studies by other members of the laboratory have demonstrated that hyaluronan (HA), interferon-γ (IFN-γ), and retinoic acid plus vitamin D_3 (Ret-D_3) are able to induce differentiation of U937 cells. Incubation of U937 cells with the above reagents results in their differentiation along a monocyte-macrophage pathway. FACS analysis has shown that this change in cellular phenotype is accompanied by up-regulation of a number of cell surface monocyte markers, such as CD14 (data not shown). As can be seen in Figure A.10, both CD14 and MSE-1 mRNA transcripts are induced following differentiation of U937 cells. CD14 and MSE-1 products were not observed in U937 cells cultured with FBS only, but showed substantial increases in expression levels following treatment with either RA-

conditioned medium or IFN-γ. Unlike MSE-1 mRNA, CD14 mRNA was abundantly expressed by U937 cells differentiated with Ret-D_3.

A.2.6 Cultured dermal fibroblasts do not express CD14, MSE-1, or VCAM-1 mRNA transcripts

A further experiment was carried out to test the integrity of the above primer pairs in the optimized semi-quantitative PCR. This involved screening mRNA isolated from human dermal fibroblasts for CD14, CD44, MSE-1, and VCAM-1 transcripts. Studies performed in the laboratory (see chapter 3 and 5) have shown that human dermal fibroblasts express CD44 but not CD14 or VCAM-1. Semi-quantitative PCR demonstrated that CD14, MSE-1, and VCAM-1 mRNA transcripts were not expressed in human dermal fibroblasts (data not shown). In contrast, CD44 and GAPDH mRNA were present in abundance.

Figure A.1 The effect of cycle number and annealing temperature on the amplification of cDNA using CD14-specific oligonucleotide primers. Total RNA was isolated from differentiated U937 cells and reverse transcribed (see Methods and Materials). Hot start PCR was performed with first strand cDNA (equivalent to 100 ng of total RNA) and specific CD14 primers (14.533 and 14.1038) (see Table 2.1). [α^{33}P]-dATP was added to the PCR reaction mix (see Methods and Materials) to allow direct quantification of the amount of PCR product. PCR was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer). The number of amplification cycles was varied with each cycle consisting of 30 sec at 94°C, 30 sec at an annealing temperature of 58°C, and 30 sec at 72°C (**A**). The annealing temperature of each cycle (30 sec at 94°C, 30 sec at an annealing temperature, and 30 sec at 72°C) was altered over a wide range using a cycle number in the linear phase of amplification (**B**). Following amplification, PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid and dried under vacuum. Gels were exposed to phosphorimager plates for 4-24 hours and scanned using a Fuji Bas 1000 phosphorimager. Band intensity (pixel units) represents the total pixel volumes generated by analysis of the area enclosing the bands using BAS 1000 software. Results shown are representative of three separate PCR experiments performed.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Cycle number
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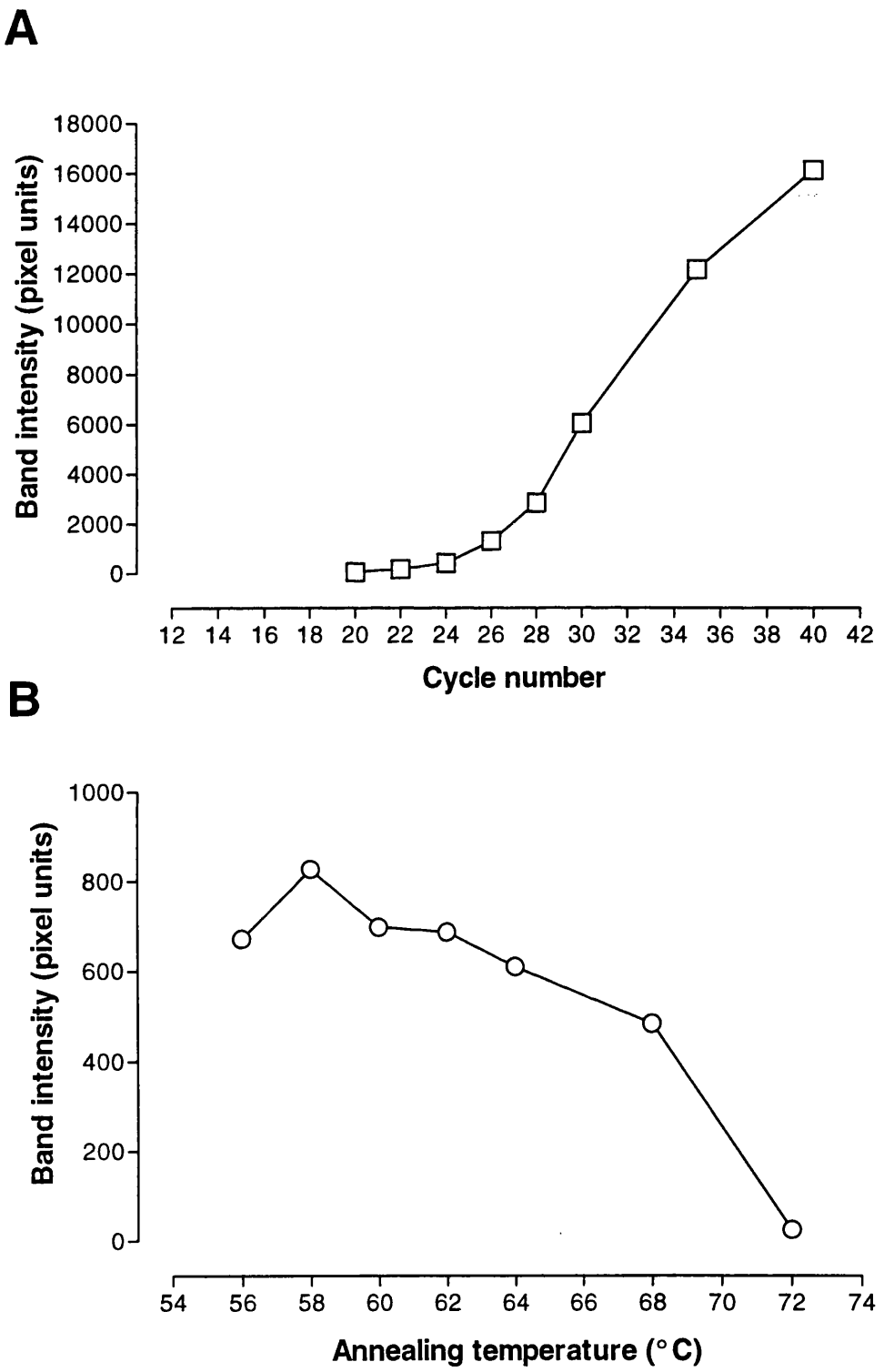
50000
40000
30000
20000
10000
0
52 54 56 58 60 62 64 66 68 70 72
Annealing temperature (° C)

Figure A.1

Figure A.2 The effect of cycle number and annealing temperature on the amplification of cDNA using CD44-specific oligonucleotide primers. Total RNA was isolated from dermal fibroblasts and reverse transcribed (see Methods and Materials). Hot start PCR was performed with first strand cDNA (equivalent to 100 ng of total RNA) and specific CD44 primers (44.513 and 44.934) (see Table 2.1). These primers were upstream and downstream of the variant exon insertion point. [α^{33}P]-dATP was added to the PCR reaction mix (see Methods and Materials) to allow direct quantification of the amount of PCR product. PCR was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer). The number of amplification cycles was varied with each cycle consisting of 30 sec at 94°C, 30 sec at an annealing temperature of 58°C, and 30 sec at 72°C (**A**). The annealing temperature of each cycle (30 sec at 94°C, 30 sec at an annealing temperature, and 30 sec at 72°C) was altered over a wide range using a cycle number in the linear phase of amplification (**B**). Following amplification, PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid and dried under vacuum. Gels were exposed to phosphorimager plates for 4-6 hours and scanned using a Fuji Bas 1000 phosphorimager. Band intensity (pixel units) represents the total pixel volumes generated by analysis of the area enclosing the bands using BAS 1000 software. Results shown are representative of three separate PCR experiments performed.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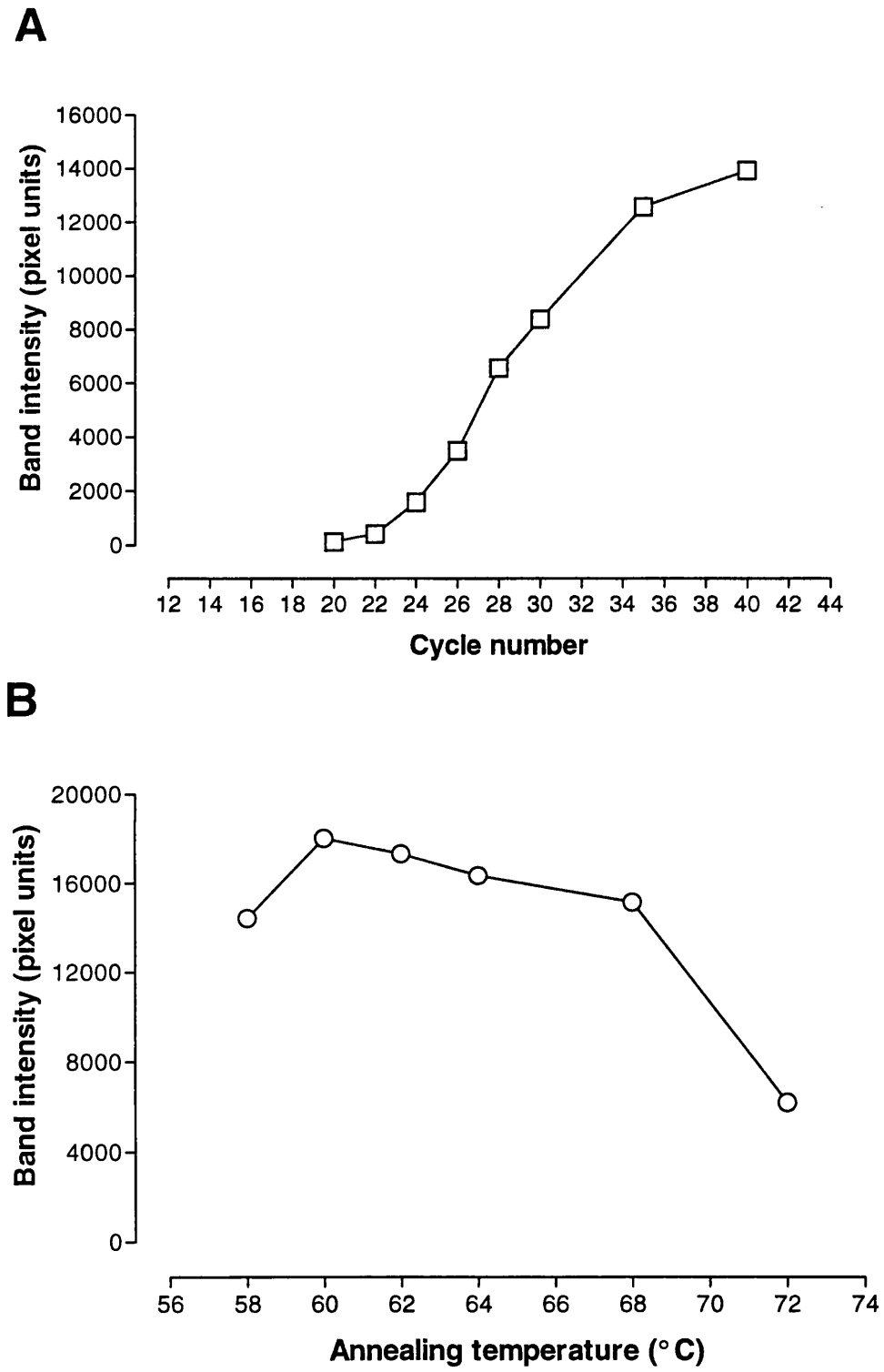
Cycle number
B
Band intensity (pixel units)
1000
800
600
400
200
0
54 56 58 60 62 64 66 68 70 72 74
Annealing temperature (°C)

Figure A.2

Figure A.3 The effect of cycle number and annealing temperature on the amplification of cDNA using GAPDH-specific oligonucleotide primers. Total RNA was isolated from dermal fibroblasts and reverse transcribed (see Methods and Materials). Hot start PCR was performed with first strand cDNA (equivalent to 100 ng of total RNA) and specific GAPDH primers (gap.67 and gap.406) (see Table 2.1). [α^{33}P]-dATP was added to the PCR reaction mix (see Methods and Materials) to allow direct quantification of the amount of PCR product. PCR was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer). The number of amplification cycles was varied with each cycle consisting of 30 sec at 94°C, 30 sec at an annealing temperature of 58°C, and 30 sec at 72°C (**A**). The annealing temperature of each cycle (30 sec at 94°C, 30 sec at an annealing temperature, and 30 sec at 72°C) was altered over a wide range using a cycle number in the linear phase of amplification (**B**). Following amplification, PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid and dried under vacuum. Gels were exposed to phosphorimager plates for 4-6 hours and scanned using a Fuji Bas 1000 phosphorimager. Band intensity (pixel units) represents the total pixel volumes generated by analysis of the area enclosing the bands using BAS 1000 software. Results shown are representative of three separate PCR experiments performed.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2000
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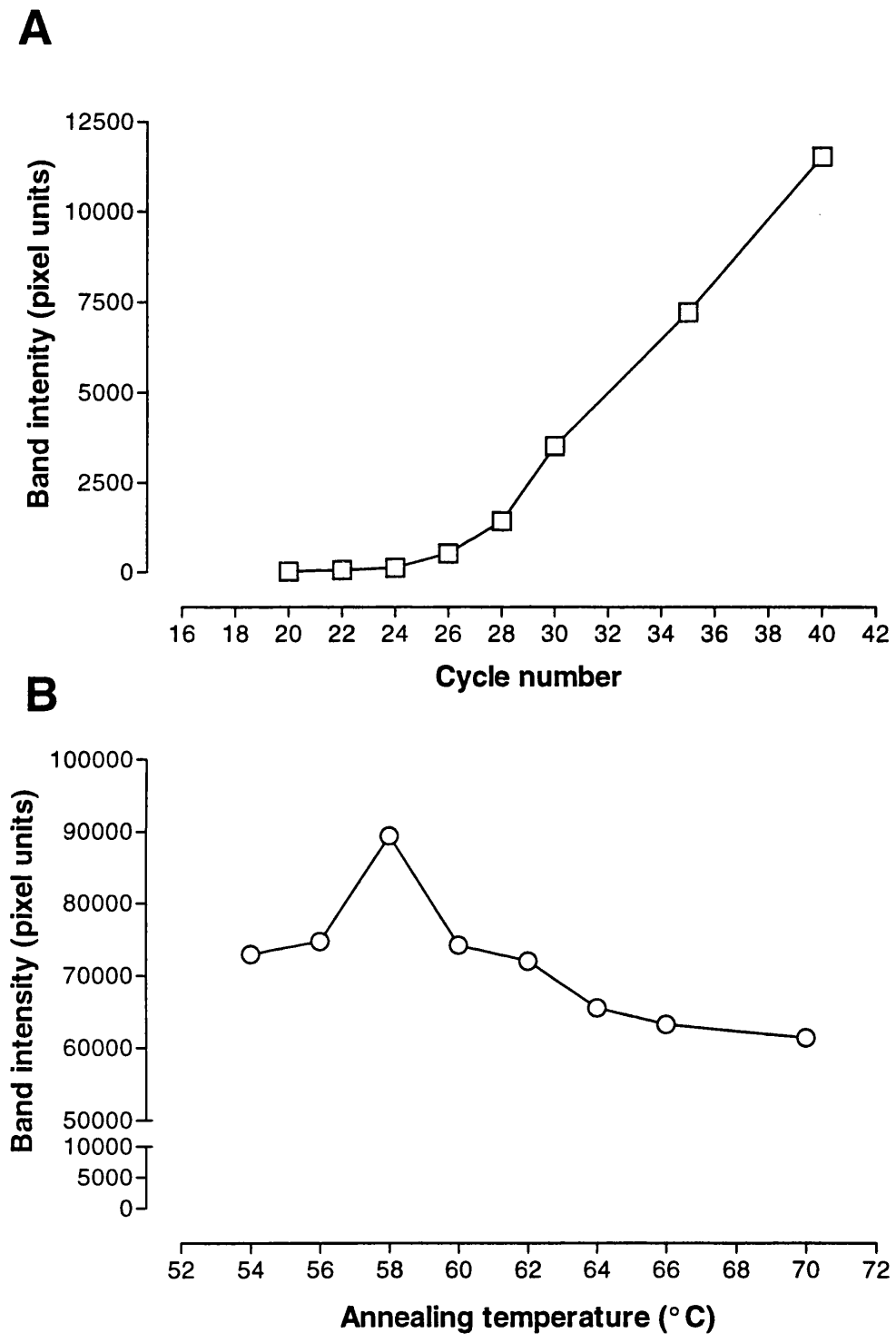
Cycle number
B
Band intensity (pixel units)
20000
16000
12000
8000
4000
0
56 58 60 62 64 66 68 70 72 74
Annealing temperature (° C)

Figure A.3

Figure A.4 The effect of cycle number and annealing temperature on the amplification of cDNA using MSE-1-specific oligonucleotide primers. Total RNA was isolated from differentiated U937 cells and reverse transcribed (see Methods and Materials). Hot start PCR was performed with first strand cDNA (equivalent to 100 ng of total RNA) and specific MSE-1 primers (mse.46 and mse.469) (see Table 2.1). [α^{33}P]-dATP was added to the PCR reaction mix (see Methods and Materials) to allow direct quantification of the amount of PCR product. PCR was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer). The number of amplification cycles was varied with each cycle consisting of 30 sec at 94°C, 30 sec at an annealing temperature of 58°C, and 30 sec at 72°C (**A**). The annealing temperature of each cycle (30 sec at 94°C, 30 sec at an annealing temperature, and 30 sec at 72°C) was altered over a wide range using a cycle number in the linear phase of amplification (**B**). Following amplification, PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid and dried under vacuum. Gels were exposed to phosphorimager plates for 4-24 hours and scanned using a Fuji Bas 1000 phosphorimager. Band intensity (pixel units) represents the total pixel volumes generated by analysis of the area enclosing the bands using BAS 1000 software. Results shown are representative of three separate PCR experiments performed.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Cycle number
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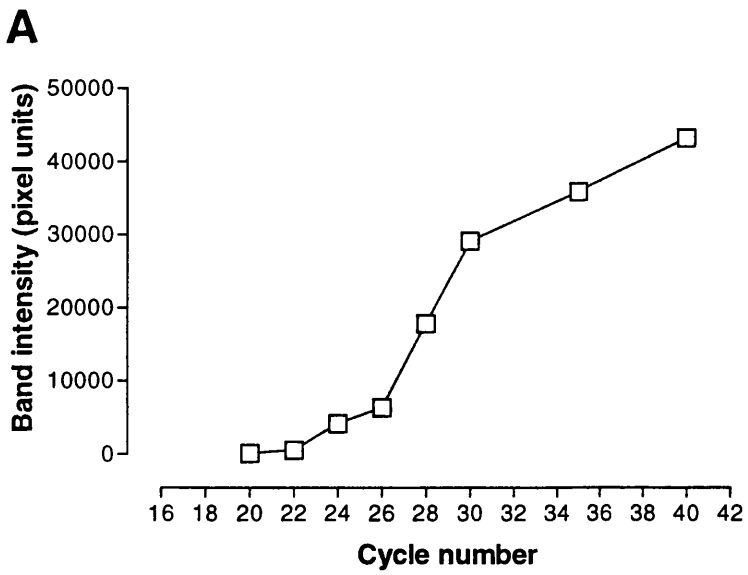
90000
80000
70000
60000
50000
10000
5000
0
Band intensity (pixel units)
52 54 56 58 60 62 64 66 68 70 72
Annealing temperature (° C)

Figure A.4

Figure A.5 The effect of cycle number and annealing temperature on the amplification of cDNA using VCAM-1-specific oligonucleotide primers. Total RNA was isolated from freshly dispersed RA synovium and reverse transcribed (see Methods and Materials). Hot start PCR was performed with first strand cDNA (equivalent to 100 ng of total RNA) and specific VCAM-1 primers (vcam.751 and vcam.1361) (see Table 2.1). [α^{33}P]-dATP was added to the PCR reaction mix (see Methods and Materials) to allow direct quantification of the amount of PCR product. PCR was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer). Amplification of the 631 bp (**A**) and 355 bp (**B**) products was tested over a wide range of cycle numbers. Each cycle consisted of 30 sec at 94°C, 30 sec at an annealing temperature of 58°C, and 30 sec at 72°C. The annealing temperature of each cycle (30 sec at 94°C, 30 sec at an annealing temperature, and 30 sec at 72°C) was altered over a wide range using a cycle number in the linear phase of amplification (**C**). Following amplification, PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid and dried under vacuum. Gels were exposed to phosphorimager plates for 4-6 hours and scanned using a Fuji Bas 1000 phosphorimager. Band intensity (pixel units) represents the total pixel volumes generated by analysis of the area enclosing the bands using BAS 1000 software. Results shown are representative of three separate PCR experiments performed.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Band intensity (pixel units)
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30000
20000
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0
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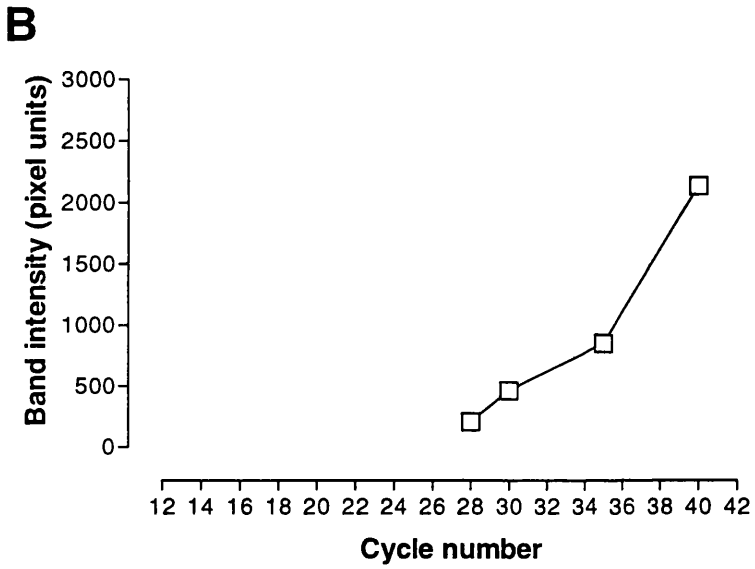
Cycle number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Band intensity (pixel units)
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1500
1000
500
0
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Cycle number

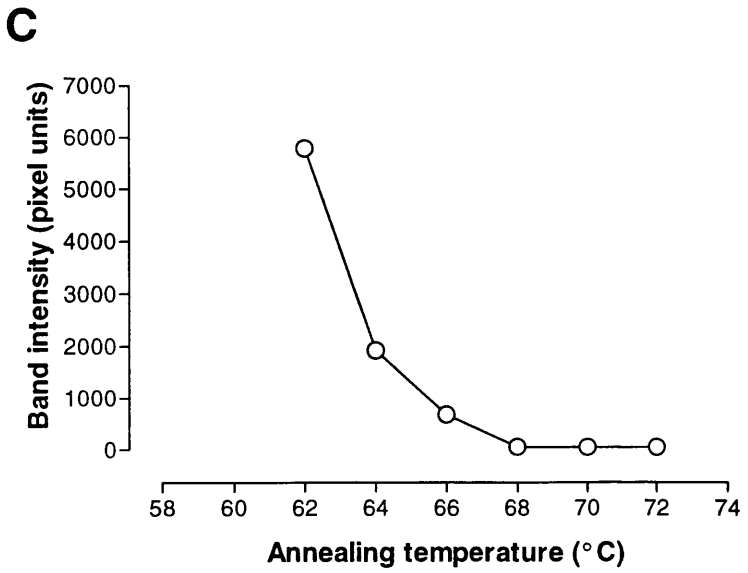

C
Band intensity (pixel units)
7000
6000
5000
4000
3000
2000
1000
0
58 60 62 64 66 68 70 72 74
Annealing temperature (°C)

Figure A.5

Figure A.6 The effect of cycle number on the amplification of cDNA using CD14, CD44, GAPDH, MSE-1, and VCAM-1-specific oligonucleotide primers. Total RNA was isolated from freshly dispersed RA synovium and reverse transcribed (see Methods and Materials). Hot start PCR was performed with first strand cDNA (equivalent to 100 ng of total RNA) and the respective oligonucleotide primer pair (see Table 2.1). [α^{33}P]-dATP was added to the PCR reaction mix (see Methods and Materials) to allow direct quantification of the amount of PCR product. PCR was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer). The number of amplification cycles was varied between 20 and 40 cycles. Each cycle consisted of 30 sec at 94°C, 30 sec at an annealing temperature of 58°C, and 30 sec at 72°C. Water only controls (Con), where cDNA was omitted from the PCR reaction, were performed for 35 cycles. Following amplification, PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid and dried under vacuum. Gels were exposed to phosphorimager plates for 4-24 hours and scanned using a Fuji Bas 1000 phosphorimager. Results shown are representative of three separate PCR experiments performed.

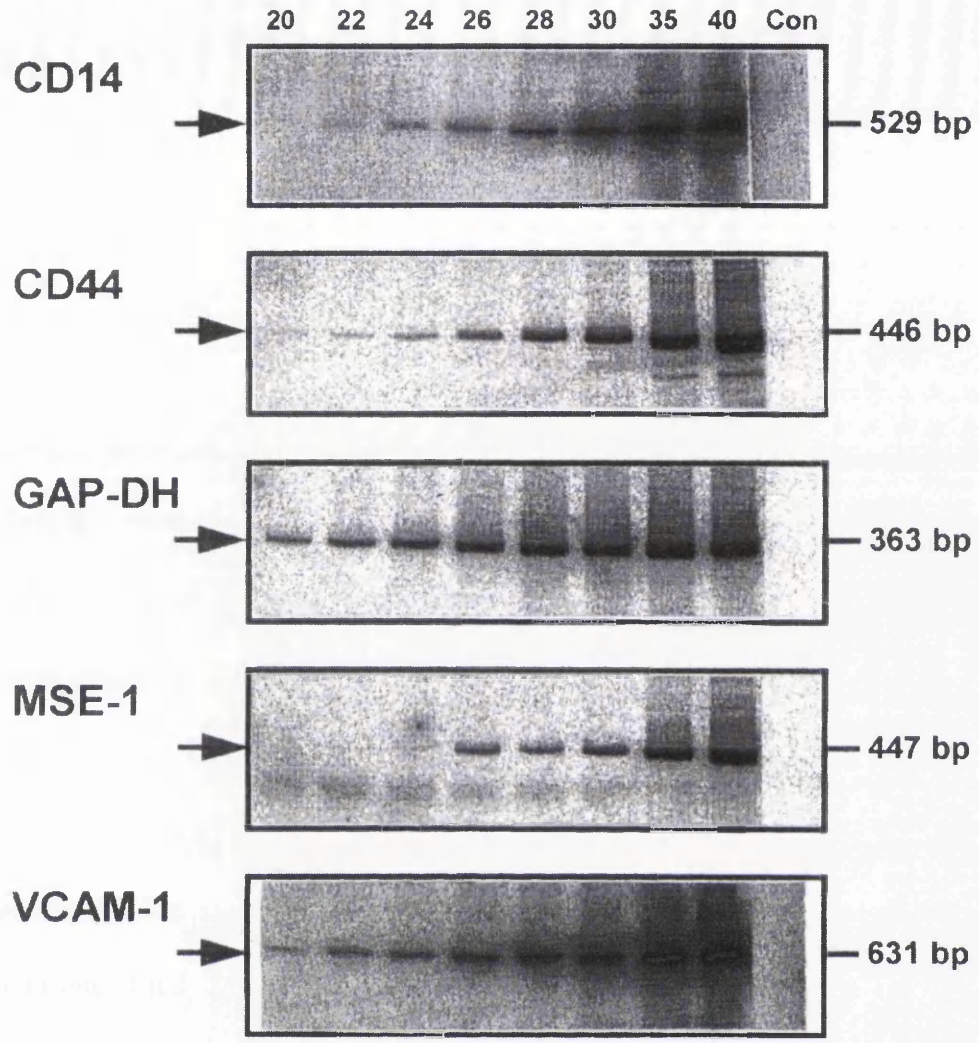

20
22
24
26
28
30
35
40
Con
CD14
529 bp
CD44
446 bp
GAP-DH
363 bp
MSE-1
447 bp
VCAM-1
631 bp

Figure A.6

Figure A.7 The effect of annealing temperature on cDNA amplification using CD14, CD44, GAPDH, MSE-1, and VCAM-1-specific oligonucleotide primers. Total RNA was isolated from freshly dispersed RA synovium and reverse transcribed (see Methods and Materials). Hot start PCR was performed with first strand cDNA (equivalent to 100 ng of total RNA) and the respective oligonucleotide primer pair (see Table 2.1). [α^{33}P]-dATP was added to the PCR reaction mix (see Methods and Materials) to allow direct quantification of the amount of PCR product. PCR was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer). The annealing temperature of each cycle (30 sec at 94°C, 30 sec at various annealing temperatures, and 30 sec at 72°C) was altered over a wide range using a cycle number in the linear phase of amplification. Water only controls (Con), where cDNA was omitted from the PCR reaction, were performed for 30 cycles using an annealing temperature of 58°C. Following amplification, PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid and dried under vacuum. Gels were exposed to phosphorimager plates for 4-24 hours and scanned using a Fuji Bas 1000 phosphorimager. Results shown are representative of three separate PCR experiments performed.

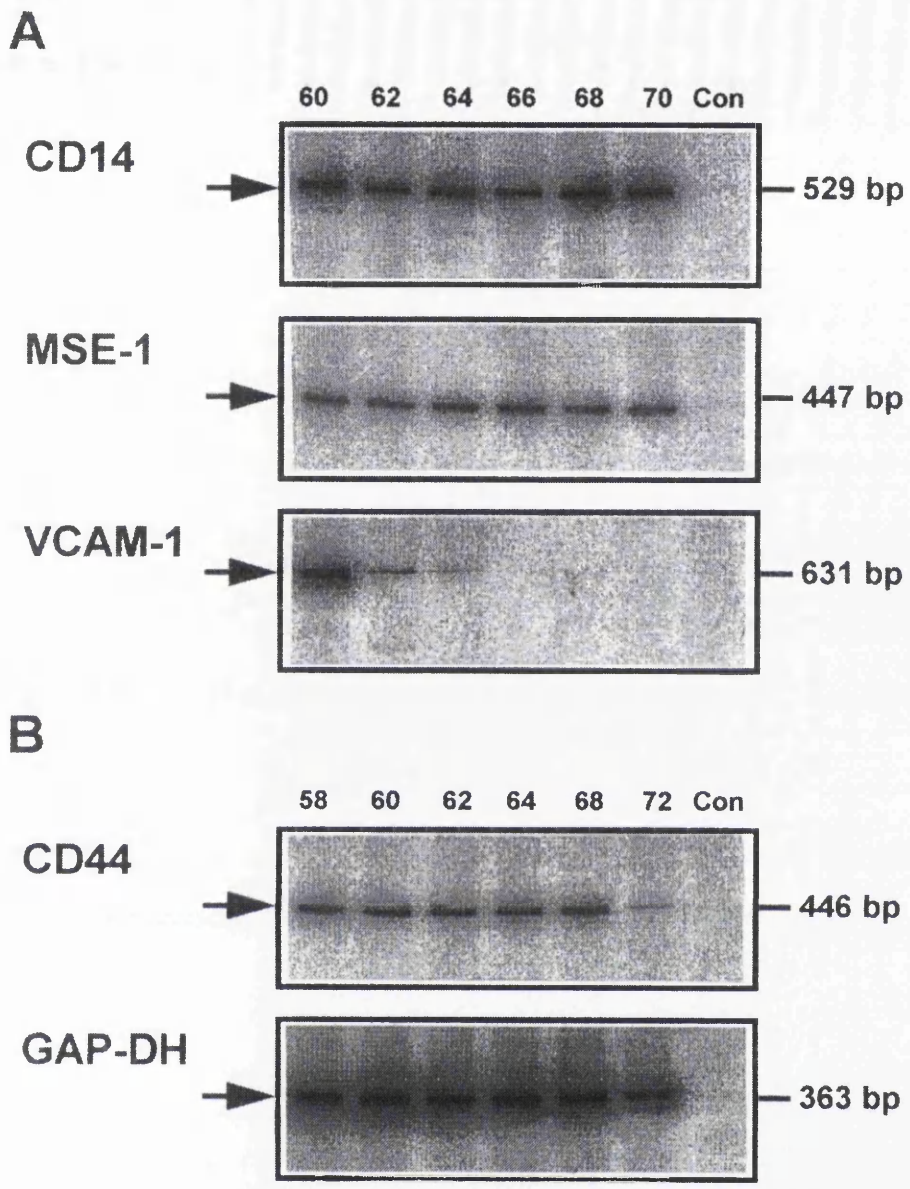

A
60 62 64 66 68 70 Con
CD14
529 bp
MSE-1
447 bp
VCAM-1
631 bp
B
58 60 62 64 68 72 Con
CD44
446 bp
GAP-DH
363 bp

Figure A.7

Figure A.8 The effect of cDNA dilution upon amplification. Total RNA was isolated from freshly dispersed RA synovium and reverse transcribed (see Methods and Materials). Hot start PCR was performed with various amounts of first strand cDNA and oligonucleotide primer pairs (see Table 2.1). [α^{33}P]-dATP was added to the PCR reaction mix (see Methods and Materials) to allow direct quantification of the amount of PCR product. PCR was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer). Each cycle of amplification consisted of 30 sec at 94°C, 30 sec at 58°C, and 30 sec at 72°C. Following amplification, PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid and dried under vacuum. Gels were exposed to phosphorimager plates for 4-24 hours and scanned using a Fuji Bas 1000 phosphorimager. Band intensity (pixel units) represents the total pixel volumes generated by analysis of the area enclosing the bands using BAS 1000 software. Results shown are representative of three separate PCR experiments performed.

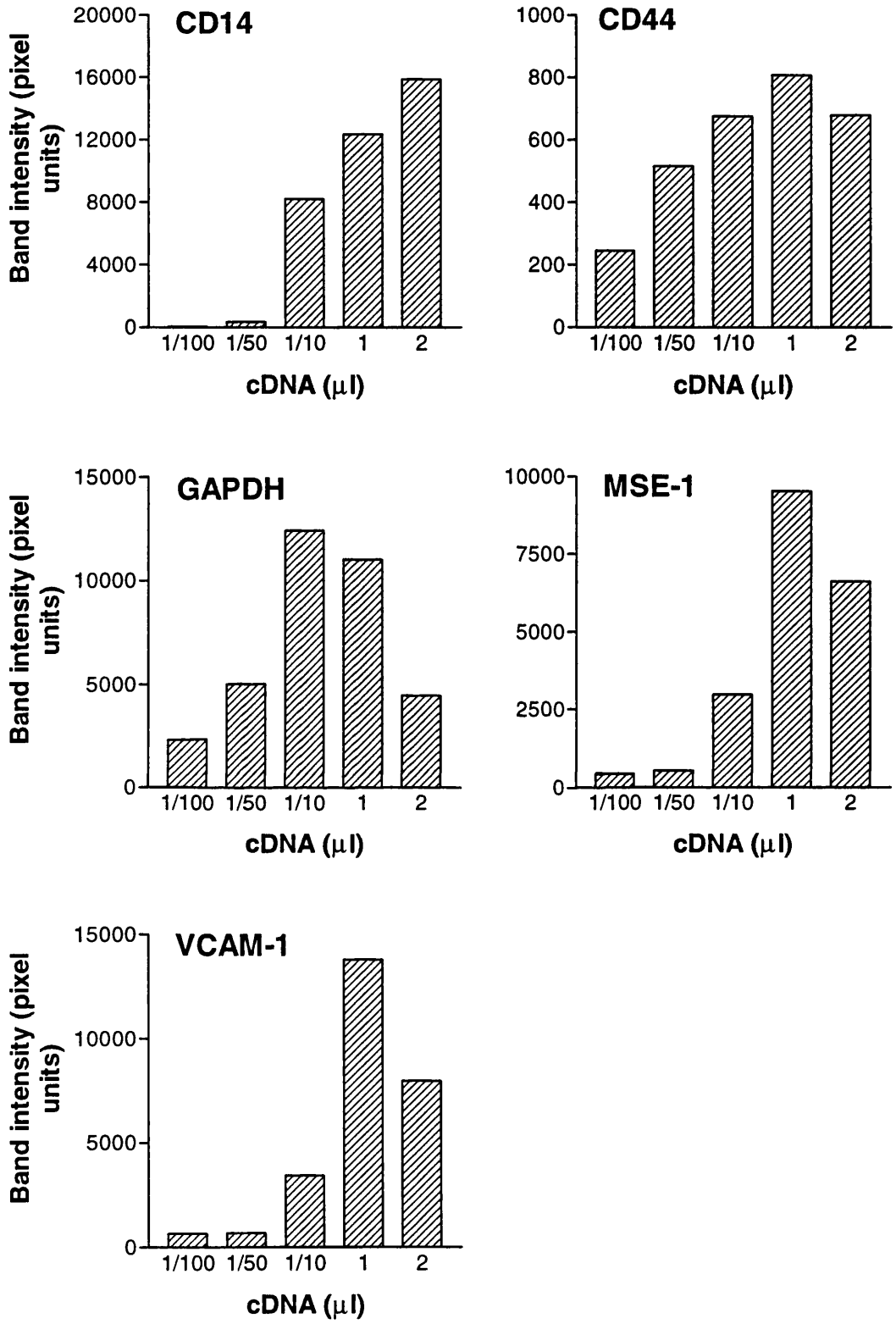

CD14
Band intensity (pixel units)
20000
16000
12000
8000
4000
0
1/100 1/50 1/10 1 2
cDNA (µl)
CD44
1000
800
600
400
200
0
1/100 1/50 1/10 1 2
cDNA (µl)
GAPDH
15000
10000
5000
0
1/100 1/50 1/10 1 2
cDNA (µl)
MSE-1
10000
7500
5000
2500
0
1/100 1/50 1/10 1 2
cDNA (µl)
VCAM-1
15000
10000
5000
0
1/100 1/50 1/10 1 2
cDNA (µl)

Figure A.8

Figure A.9 Representative gels showing the effect of cDNA dilution upon amplification. Total RNA was isolated from freshly dispersed RA synovium and reverse transcribed (see Methods and Materials). Hot start PCR was performed with various amounts of first strand cDNA and oligonucleotide primer pairs (see Table 2.1). [α^{33}P]-dATP was added to the PCR reaction mix (see Methods and Materials) to allow direct quantification of the amount of PCR product. PCR was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer). Each cycle of amplification consisted of 30 sec at 94°C, 30 sec at 58°C, and 30 sec at 72°C. PCR was carried out on a water only control (Lane 1), cDNA diluted 1/100 (Lane 2), cDNA diluted 1/50 (Lane 3), cDNA diluted 1/10 (Lane 4), 1 µl of cDNA (Lane 5), and 2 µl of cDNA (Lane 6). Following amplification, PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid and dried under vacuum. Gels were exposed to phosphorimager plates for 4-24 hours and scanned using a Fuji Bas 1000 phosphorimager. Results shown are representative of three separate PCR experiments performed.

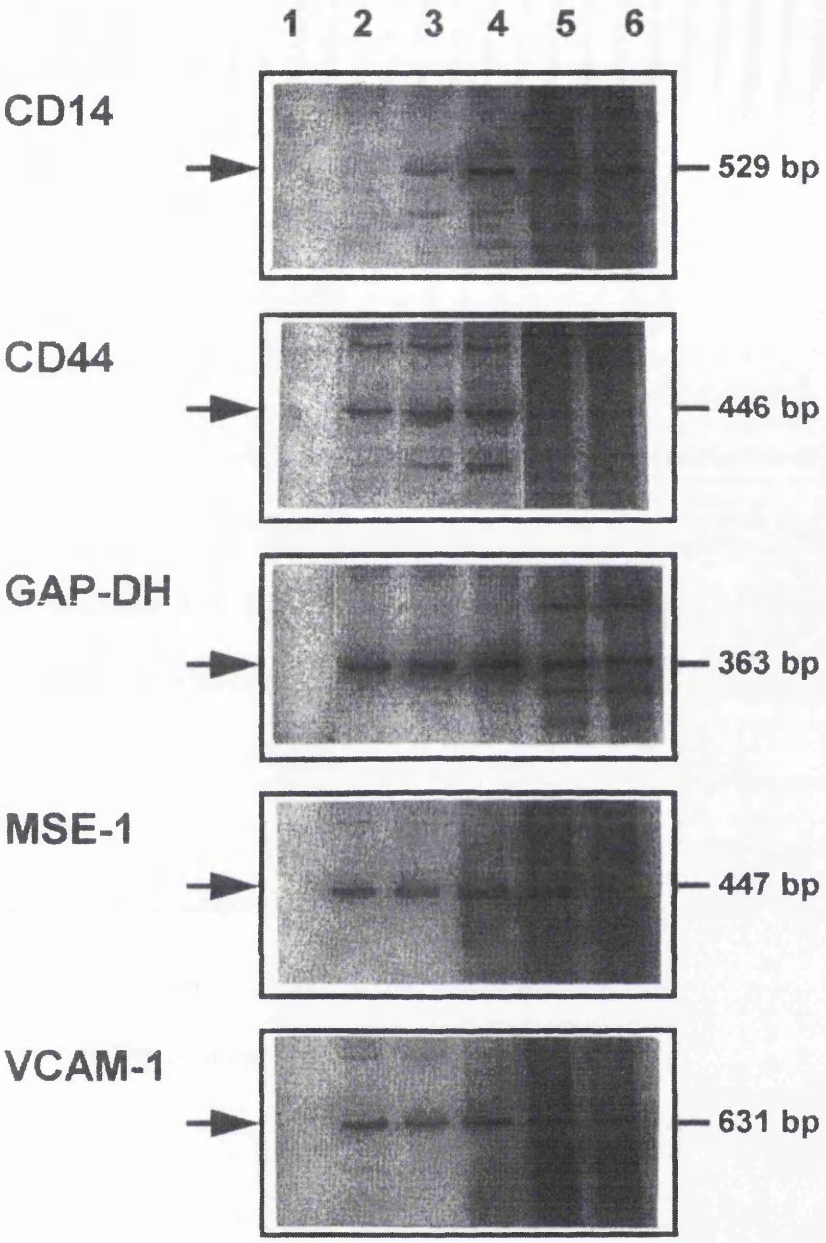
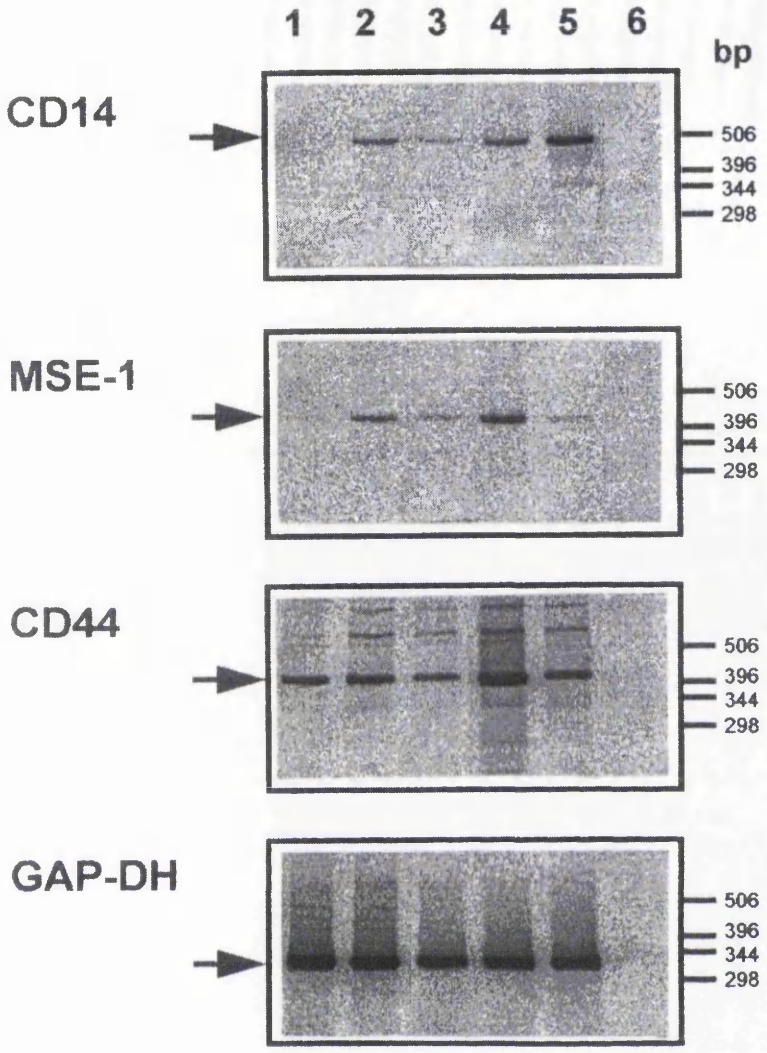

1 2 3 4 5 6
CD14
529 bp
CD44
446 bp
GAP-DH
363 bp
MSE-1
447 bp
VCAM-1
631 bp

Figure A.9

Figure A.10 Expression of CD14, CD44, MSE-1, and GAPDH mRNA transcripts by U937 cells. Total RNA was isolated from U937 cells treated with medium only (FBS), or medium supplemented with either HA (1 mg/ml), conditioned medium (CM), IFN-γ (200 units/ml), or RA-D_3 (10^{-7}M) for 4 days prior to RNA isolation. Total RNA was isolated and reverse transcribed (see Methods and Materials). First strand cDNA was amplified by hot start PCR using oligonucleotide primers specific for CD14 (14.533 and 14.1038), MSE-1 (mse.46 and mse.469), CD44 (44.513 and 44.934), and GAPDH (gap.67 and gap.406) (see Table 2.1). [α^{33}P]-dATP was added to the PCR reaction mix (see Methods and Materials) to allow direct quantification of the amount of PCR product. PCR was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer) using optimized amplification conditions (see Table 3.1). Following amplification, PCR-products were electrophoresed on a 6% polyacrylamide gel, fixed in methanol-acetic acid and dried under vacuum. Gels were exposed to phosphorimager plates for 4-24 hours and scanned using a Fuji Bas 1000 phosphorimager. Results shown are representative of three separate PCR experiments performed.



Lane 1= FBS only

Lane 2= FBS + CM

Lane 3= FBS + HA (1 mg/ml)

Lane 4= FBS + IFN-γ (200 Units/ml)

Lane 5= FBS + RA-D_3 (10^{-7}M)

Lane 6= no cDNA

Figure A.10

Appendix III

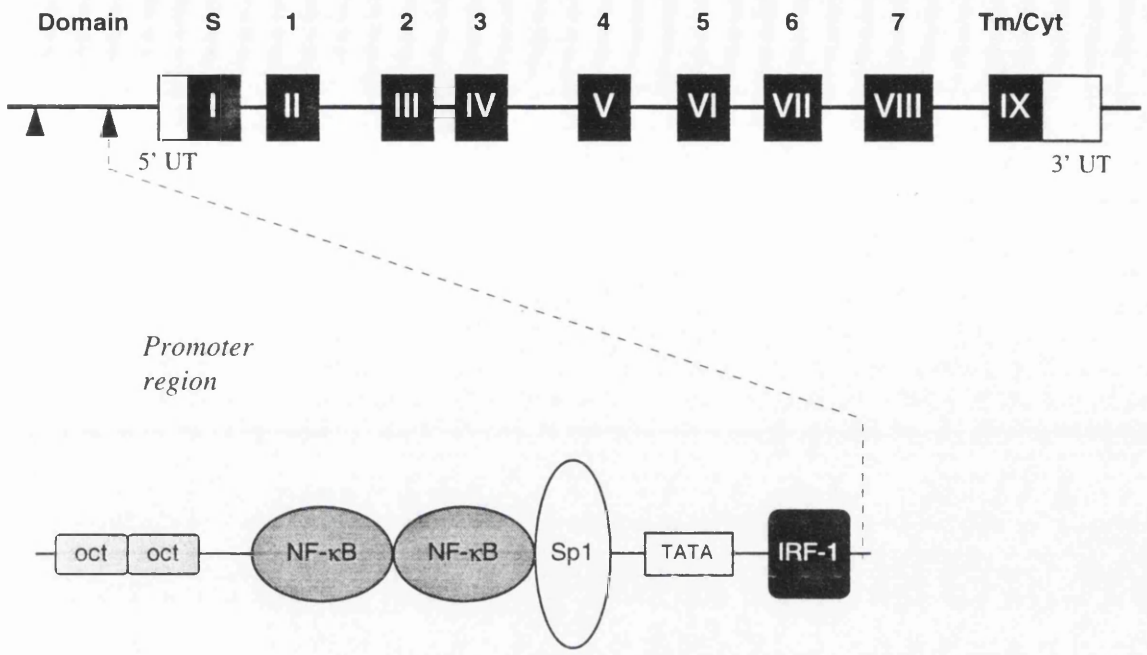


Figure A.11 Schematic diagram of the VCAM-1 gene showing the promoter region. Exons are denoted by black boxes with the interconnecting lines representing the intron sequence. Exon 1 which encodes the signal peptide (S). Arabic numerals represent the immunoglobulin-like domains. The transmembrane (Tm) and cytoplasmic (cyt) domains are encoded by exon IX. Open boxes represent 5′ and 3′ untranslated regions. The expanded region of the 5′-flanking sequence shows the regulatory elements present in the human VCAM-1 gene promoter (Adapted from Iademarco et al., 1992, 1993; Collins *et al.*, 1995).

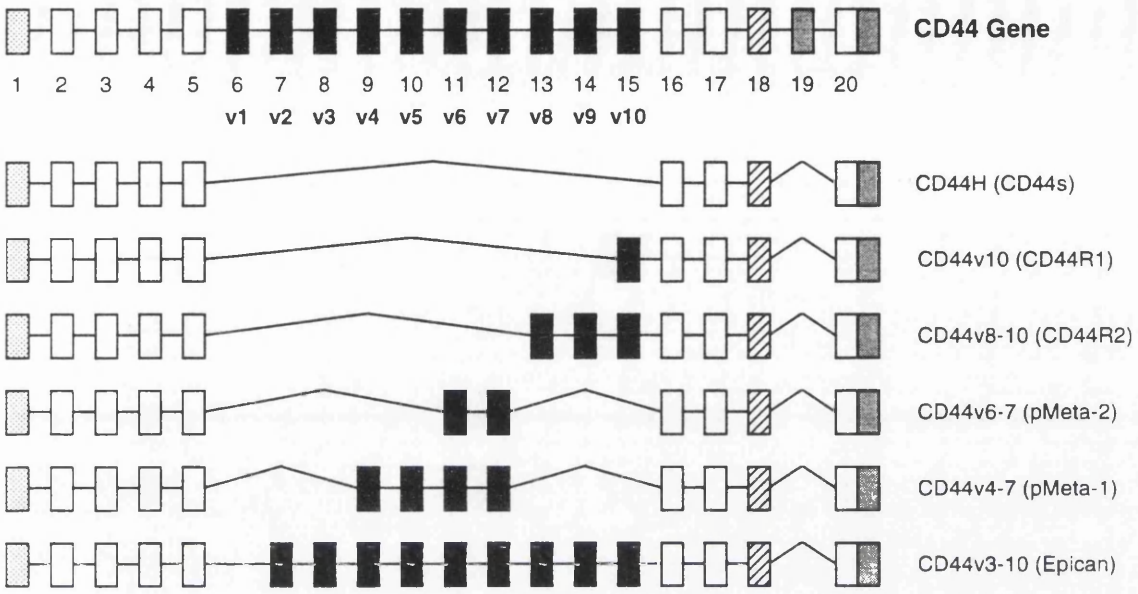


Figure A.12 Schematic diagram of the CD44 gene and some common CD44 isoforms showing variant exon utilization. Stippled boxes represent exon 1 which encodes the leader peptide; open boxes represent constitutive exons present in all isoforms of CD44; black boxes represent variant exons (v1-v10) which can be alternatively spliced at a site in the membrane proximal region; hatched boxes represent the exon encoding the transmembrane domain of all CD44 isoforms; grey boxes represent the 3′ untranslated region of the cytoplasmic exons. The common form of CD44, referred to as hematopoietic CD44 (CD44H) or standard CD44 (CD44s), has variant exons v1-v10 excised. Splice variant isoforms of CD44 show differential utilization of the variant exons v2-v10. In humans exon v1 contains a stop codon and does not appear to be expressed in any isoforms. (Modified from Screaton *et al.*, 1992 and 1993; Cooper and Dougherty, 1995).

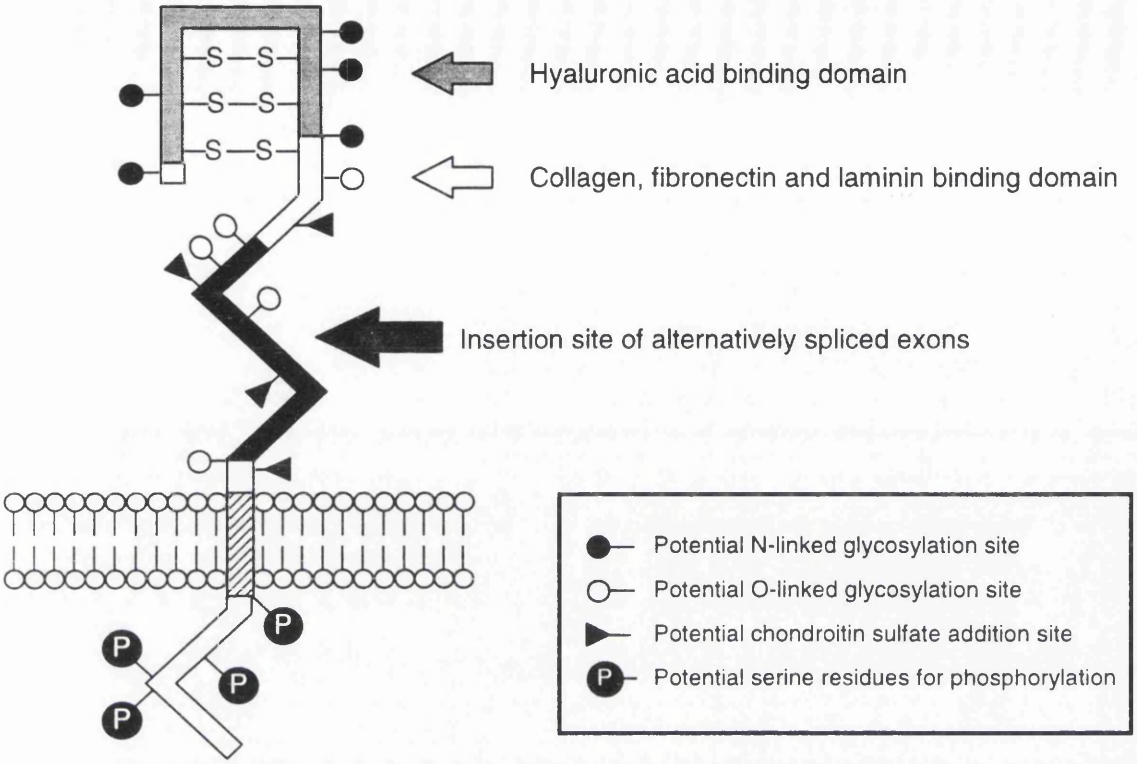


Figure A.13 Diagrammatic representation of the human CD44 protein. The solid grey region represents the hyaluronan binding region of the amino terminus of the extracellular domain. The solid black region represents the membrane proximal region where alternatively spliced exons can be inserted. The hatched region represents the transmembrane domain. Potential sites for chondroitin sulphate attachment, N-linked glycosylation, O-linked glycosylation, and phosphorylation are indicated. (Adapted from Lesley *et al.*, 1993; Sherman *et al.*, 1994).