

Characterization of the interaction between monocytes and synovial fibroblasts in rheumatoid arthritis

by

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Abstract

A continuous presence of monocytes/macrophages in association with intimal synovial fibroblasts is observed in rheumatoid arthritis. Mononuclear cells infiltration is conspicuous in the earliest examples of synovitis and this observation points to a possible role for the two cell types in the onset of chronic inflammation. The chronic inflammation is characterized through the high content of inflammatory mediators such as IL-1 β , IL-6 or TNF- α and the release of metalloproteinases and toxic oxygen metabolites. These latter products are thought to be instrumental in the ongoing erosion of cartilage and bone. The intimal synovial fibroblasts, with their constitutive VCAM-1 expression, may have a role in retention and subsequent priming or activation of monocytes in the synovial membrane. We developed a cell co-culture system, comprising of isolated synovial fibroblasts, monocytic U937 cells, hyaluronan (a component abundantly present in the synovial joint space) and human plasma rather than fetal bovine serum. Under these conditions, U937 cells adhere tightly to synovial fibroblasts (mediated by β 1 and β 2 integrins) and differentiate towards mature monocytes. We next analysed release of inflammatory mediators and toxic oxygen metabolites. The co-culture expresses high amounts of IL-6 but no detectable amounts of IL-1 β or TNF- α . As a positive control we used phorbol ester treated U937 cells, which do release significant amounts of these mediators. Co-cultured cells did not release toxic oxygen metabolites, but did induce the synthesis of metalloproteinases in the synovial fibroblasts. In order to evaluate if the synovial fibroblasts distinguished themselves from other fibroblasts, with regards to the above mentioned parameters, we performed similar experiments with primary cultures of dermal fibroblasts. There was a quantitative difference, less monocytic cells bound to the dermal fibroblasts and a lower level of maturation was obtained, but there was no qualitative difference between the two co-cultures.

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Abbreviations

adh	adherent cells
anti-RANA	anti-rheumatoid arthritis nuclear antigen
bp	base pairs
BSA	Bovine Serum Albumin
CaCl ₂	Calcium chloride
CD	Cluster of differentiation
CD11a/CD18 (LFA-1)	Integrin- α L
CD11b/CD18 (Mac-1)	Integrin- α M
CD11c/CD18 (p150,95)	Integrin- α X
CD14	Cluster of differentiation antigen 14
CD18	Integrin β 2
CD31 (PECAM)	Platelet / Endothelial cell adhesion molecule-1
CD44	Cluster of differentiation antigen 14
c.m.	conditioned medium
CR3	Complement receptor 3
D	Dermal fibroblasts
DNA	Deoxy
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetracetic acid
ELISA	Enzyme linked immunosorbant assay
E-selectin (ELAM-1)	Endothelial selectin
FACS	Fluorescent Activated Cell Sorter
FBS	Foetal Bovine Serum
FcR1	Fc Receptor 1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GIT	Guanidinium Isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HA	Hyaluronan
HABP	Hyaluronan binding protein
HLA DR	Human Leukocyte Antigen -DR
HEPES	4-(-2-Hydroxyethyl)-1-piperazine ethanesulfonic acid
HPDS	Human Plasma Derived Serum (also referred to as plasma)
ICAM-1	Intercellular Adhesion Molecule -1
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFN- γ	Interferon - γ
IL-1 β	Interleukin - 1 β
IL-2	Interleukin - 2

IL-4	Interleukin - 4
IL-6	Interleukin - 6
IL-8	Interleukin - 8
INCAM-110	Inducible Cell Adhesion Molecule-110
LAD	Leukocyte Adhesion Deficiency
L-selectin	Leukocyte selectin
LFA-1	Leukocyte Function Associated antigen -1
LFA-3	Leukocyte Function Associated antigen -3
LPS	Lipopolysaccharide
MHC (II)	Major histocompatibility complex (Class II)
M-LuLv	Moloney Leukemia Virus
Mn ²⁺	Manganese
mRNA	messenger RNA
MSE	Monocyte Specific Esterase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBT	Nitro Blue Tetrazolium
NK	Natural Killer cells
non-adh	Non-adherent
PAF	Platelet Activating Factor
P-selectin	Platelet selectin
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDBu	Phorbol 12,13-dibutyrate
PE	Phycoerthrin
PEG	Polyethylene Glycol
PMA	Phorbol -12-myristate-13-acetate
P/S	Penicillin and streptomycin
RA	Rheumatoid Arthritis
RANA	Rheumatoid Arthritis Nuclear Antigen
RF	Rheumatoid Factor
RT	Reverse Transcriptase
S	Synovial fibroblasts
SCR	Short Consensus Repeats
SDS	Sodium Dodecyl sulphate
SEM	Standard Error of the Mean
TBS	Tris buffered saline
TCR	T Cell Receptor
TGF β1/2	Transforming Growth Factor β1/2
TIMP	Tissue Inhibiting Metalloproteinase
TNF-α	Tumour necrosis factor - α
TPA	12-o-tetradecanoyl-phorbol-13-acetate
U	U937 cells
VBS	Veronal Buffer Saline
VCAM-1	Vascular Adhesion Molecule -1
VCAM-6D	Vascular Adhesion Molecule -6 domain
VCAM-7D	Vascular Adhesion Molecule -7 domain
VLA-4	Very Late Adhesion Molecule -4
v/v	volume per volume
w/o Ca ²⁺ and Mg ²⁺	without calcium and magnesium
w/v	weight per volume

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Chapter One

Introduction

1.1. Types of Joints

There are three types of joint found in man: fibrous joints (synarthroses) which provide little movement; cartilaginous joints (symphyses) which are found in the spine are covered with hyaline cartilage and separated by fibrocartilage, providing limited movement but withstanding a large amount of pressure; synovial joints (diarthroses), which are the predominant type of joint found within the body and allows a wide range of movement (Sledge, C.B., 1989). The introduction will focus on the synovial joints.

1.2. Structure of Synovial Joints

Synovial joints consists of five types of tissue; bone, cartilage, ligament, synovium and synovial fluid. Their main characteristic is the presence of a cavity which is surrounded by specialised connective tissue, synovium. The synovium is attached to the bone at the junction with the articular cartilage and is reflected off this junction to line the whole capsule. The joint space is filled with a small volume of synovial fluid, which owes it high viscosity to hyaluronan, a glycosaminoglycan. Articular cartilage covers the bone ends. The joint is able to maintain its stability by ligament and muscle attachments to the bone (Edwards, J.C.W., 1994) (see Fig. 1.1. A synovial joint).

1.2.1. Synovial Fluid

The normal joint contains a small volume of synovial fluid which consists of a dialysate of blood plasma that passes through the sub-synovial capillary endothelium into the extracellular space where it joins with hyaluronan (HA). Hyaluronan is produced by the synovial lining cells (type B cells, see 1.2.4.) (Pitsillides *et al.*, 1993) and is a high molecular weight, highly anionic polysaccharide composed of repeating disaccharide units of β -1,4-glucuronate- β -1,3-N-acetylglucosamine. HA has unique physiochemical properties and it can interact with hyaluronan binding proteins (HABP's), HA binding proteoglycans and link proteins contribute to the structure of extracellular matrices via

interactions with HA (Toole, B.P., 1990). The cell surface HABP's are thought to act as HA receptors which mediate the effects of HA on cell behaviour such as cell adhesion and migration (Toole, B.P., 1990). It has also been shown that CD44 is another HA receptor (Culty *et al.*, 1990, Aruffo *et al.*, 1990). Hyaluronan provides the synovial fluid with a more viscous consistency, enhancing its lubricating qualities as well as acting as a molecular filter (Henderson and Edwards., 1987). The plasma is filtered through hyaluronan, excluding large solute molecules such as fibrinogen and IgM from passing into the synovial fluid. Both the concentration of HA (3.5 mg/ml) and its molecular weight are reduced in conditions of inflammatory synovitis, particularly in patients with rheumatoid arthritis, resulting in a decrease in fluid viscosity and an increase in the presence of large molecules (Hay, E.D., 1991). Most of the protein in normal synovial fluid is albumin (60-75%), with higher molecular weight proteins present in lower concentrations. However when the synovium becomes inflamed, the protein content in the synovial fluid increases and the quantity of high molecular weight proteins becomes greater than in normal synovial fluid.

1.2.2. Articular (hyaline) Cartilage

Of the tissues in the joint, articular cartilage is the more highly specialised matrix that forms a smooth, glistening surface over which lies the lubricating film of synovial fluid. It is avascular and contains small numbers of chondrocytes whose nutritional requirements are obtained from synovial fluid. Cartilage consists of types I, II and III collagen and giant molecular aggregates of proteoglycan (Edwards, J.C.W., 1993). The latter absorbs large amounts of water and swells forming a gelatinous matrix and it gets trapped between the collagen fibres that binds the cartilage to bone (Hazleman, B., 1995).

1.2.3. Synovium

The cells of normal synovium include fibroblasts, macrophages and a small number of mast cells and lymphocytes (Henderson and Edwards., 1987). The cell distribution of the synovium can vary from a scattering of flattened cells through to a dense band packed with cells several layers deep (Henderson and Edwards., 1987). However the sub-synovial layer is relatively acellular and contains vessels, some fibroblasts and connective tissue, consisting of collagen I, III, IV and VI, laminin and fibronectin.

Deeper layers of the synovium may also contain fat or may have the appearance of loose areolar or dense fibrous tissue (Reith *et al.*, 1970). In normal synovium, the synovial surfaces are smooth and non-adherent, providing frictionless movement between tissue, and the volume ratio of interstitial matrix to cells is very high. Synovium has three types of appearances. It may be 1) flat, 2) broken up into folds or villi, which do not disappear when stretched, or 3) it could be in the form of parallel surface pleats that can be stretched out (Henderson and Edwards., 1987). In all cases the function of the synovium is to provide a frictionless surface, produce synovial fluid and to provide nutrition for articular cartilage. The surface is permeable to crystalloids and proteins but has no macroscopic gaps in it and therefore prevents synovial fluid from being squeezed out of the cavity during movement (Edwards, J.C.W., 1993).

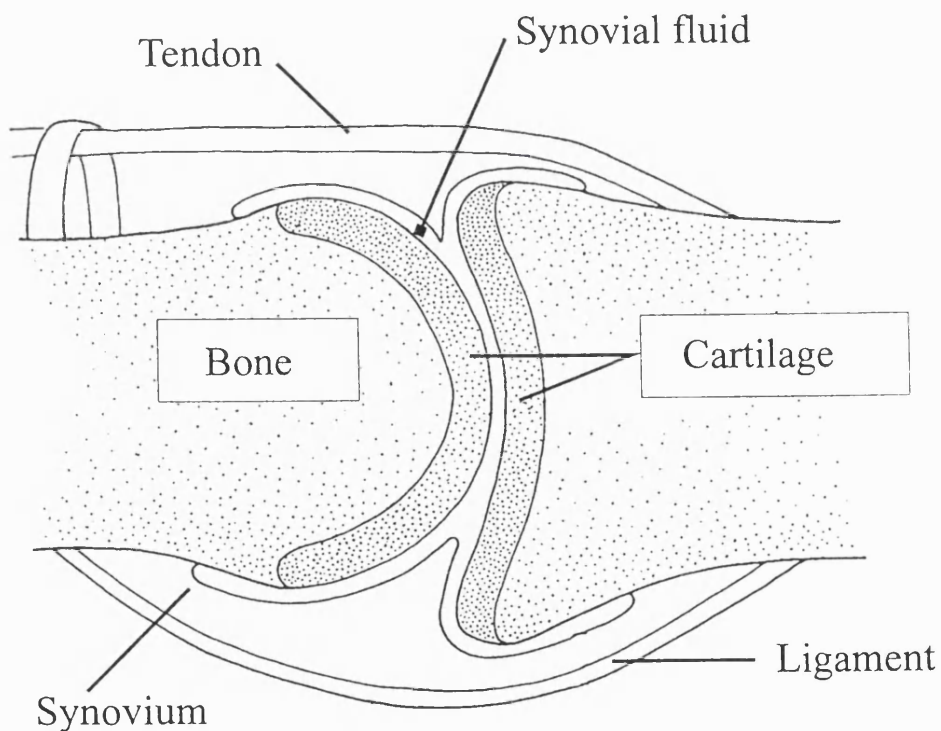


Figure 1.1. A synovial joint

1.2.4. Synovial Lining Cells

The surface of the synovium is covered with a thin layer of cells (1-3 cells thick), which is a mixture of Type A and Type B cells, constituting two-thirds of the lining cells. The Type A cells resemble macrophages and are derived from blood monocytes. Therefore they are capable of phagocytosis so they can remove debris presented at the fluid-cell interface (Edwards, J.C.W., 1982a; Kelley *et al.*, 1989). They contain prominent Golgi complexes and many large vacuoles, dense chromatin in the nuclei and thin cell processes that stretch into the adjacent extracellular matrix (Cutolo *et al.*, 1993). These aspects suggest a cell mainly equipped for phagocytosis and for some synthetic activity. Like monocytes, they also contain surface receptors for the Fc chains of immunoglobulins, for complement and HLA DR gene products, and they are able to present antigen. Macrophage-like synoviocytes originate from the bone marrow and are constantly replaced via the circulation. In normal synovium 20-30% of the lining cells have type A characteristics and are HLA-DR positive (Cutolo *et al.*, 1993). Type A synoviocytes are completely replaced every twenty weeks. The second cell type is a fibroblast-like cell (Type B cell or synoviocyte, in this thesis referred to as the fibroblasts-like synoviocytes) and contains large amounts of rough endoplasmic reticulum with few cell processes and vacuoles, but more developed nucleoli indicating protein synthesis (Cutolo *et al.*, 1993). Although mitotic figures are rarely seen in the normal synovial lining, fibroblast-like synoviocytes proliferate locally in the synovial tissue (Edwards, J.C.W., 1982a). The rate of turnover of type B cells is slow and can take an average of 361 days (Coulton *et al.*, 1980). Synovial lining cells synthesise and secrete fibronectin, of a higher molecular weight than normal or longer cultured rheumatoid synovial fibroblasts (Linck *et al.*, 1983, Lavietes *et al.*, 1985), which is thought to play an role in the attachment of intimal (superficial) synovial lining cells to the underlying matrix (Scott *et al.*, 1981). Collagen VI and III, which are found in the lining layer have also been suggested to help in the binding of the synovial cells together, in the absence of tight junctions or desmosomes (Okada *et al.*, 1990). Furthermore, rheumatoid synovial cells synthesise collagen I and III (Goldring *et al.*, 1987), prostaglandins (Dayer *et al.*, 1986), metalloproteinases (Okada *et al.*, 1989), and their inhibitors (Hembry *et al.*, 1995) and hyaluronan (Pitsillides *et al.*, 1993), in cell culture. In normal synovial joints it is been implicated that synovial fluid hyaluronan is synthesised from the synovial lining cells and is secreted directly in to the joint cavity,

and evidence shows hyaluronan in the Golgi complex and large secretory vacuoles in the type A cells (Roy *et al.*, 1967).

1.2.4.1 Intimal synovial fibroblasts have the unique characteristic of VCAM-1 expression.

One important marker for intimal synovial fibroblasts is the constitutive expression of the adhesion molecule of the immunoglobulin superfamily, vascular adhesion molecule-1 (VCAM-1) (Morales-Ducret *et al.*, 1992, Wilkinson *et al.*, 1992, Wilkinson *et al.*, 1993, Kriegsmann *et al.*, 1995). Such constitutive expression is so far only observed on myoblast cells (Iademarco *et al.*, 1993), follicular dendritic cells (Freedman *et al.*, 1990) and bone marrow stromal cells (Miyake *et al.*, 1991b). For the latter it was shown that this expression mediated binding of VLA-4 expressing lymphocyte precursors in culture (Miyake *et al.*, 1991a). The adhesion molecule vascular cell adhesion molecule-1 (VCAM-1) is a 90-110 kDa glycoprotein of the immunoglobulin supergene family (Osborn *et al.*, 1989, Rice *et al.*, 1989). VCAM-1 was originally designated as inducible cell adhesion molecule-110 (ICAM-110). This was because it was shown to be expressed on inflammatory cytokine stimulated endothelial cells (Rice *et al.*, 1989). For instance, VCAM-1 expression on endothelial cells can be transiently induced by inflammatory cytokines such as TNF- α or IL-1 β (Osborn *et al.*, 1989), with the expression persisting for around two days. In addition, these cytokines are also able to induce other cell adhesion molecules, such as ICAM-1 (Chin *et al.*, 1990). The ligand for VCAM-1 is the integrin $\alpha 4 \beta 1$ also known as Very Late Antigen-4 (VLA-4), an adhesion molecule discovered on T cells (Rice *et al.*, 1990; van Dinther-Janssen *et al.*, 1991) but is also expressed on monocytes (Carlos *et al.*, 1991) and eosinophils (Elices *et al.*, 1990). The transient expression of VCAM-1 has an important role in the recruitment of leukocytes at sites of inflammation (Osborn *et al.*, 1989, Carlos *et al.*, 1991, Bochner *et al.*, 1991, Luscinskas *et al.*, 1994). In addition, evidence has been presented that VCAM-1 is an important component in the proliferative response of CD4⁺ T cells after activation by antigen-presenting cells through the CD3-TCR complex (Damle *et al.*, 1991). Lymphoid germinal centres contain VCAM-1 expressing cells (follicular dendritic cells) and VCAM-1 has been shown to be involved in binding of B lymphocyte precursors in culture (Freedman *et al.*, 1990). The VCAM-1 molecule exists as two isoforms: one containing six Ig-like domains (VCAM-6D) and the other

containing seven Ig-like domains (VCAM-7D) (Hession *et al.*, 1991, Cybulsky *et al.*, 1991). These two isoforms arise from alternative splicing of mRNA from the VCAM-1 gene (Hession *et al.*, 1991, Cybulsky *et al.*, 1991). The two isoforms are identical in amino acid sequence except for the absence of domain 4 in VCAM-1 6D. Both the 6D and 7D forms are found in cytokine stimulated endothelial cells (Hession *et al.*, 1991), where the 7D form is predominant. The importance of adhesion molecules in regulation of the immune response will be further discussed in section 1.4.

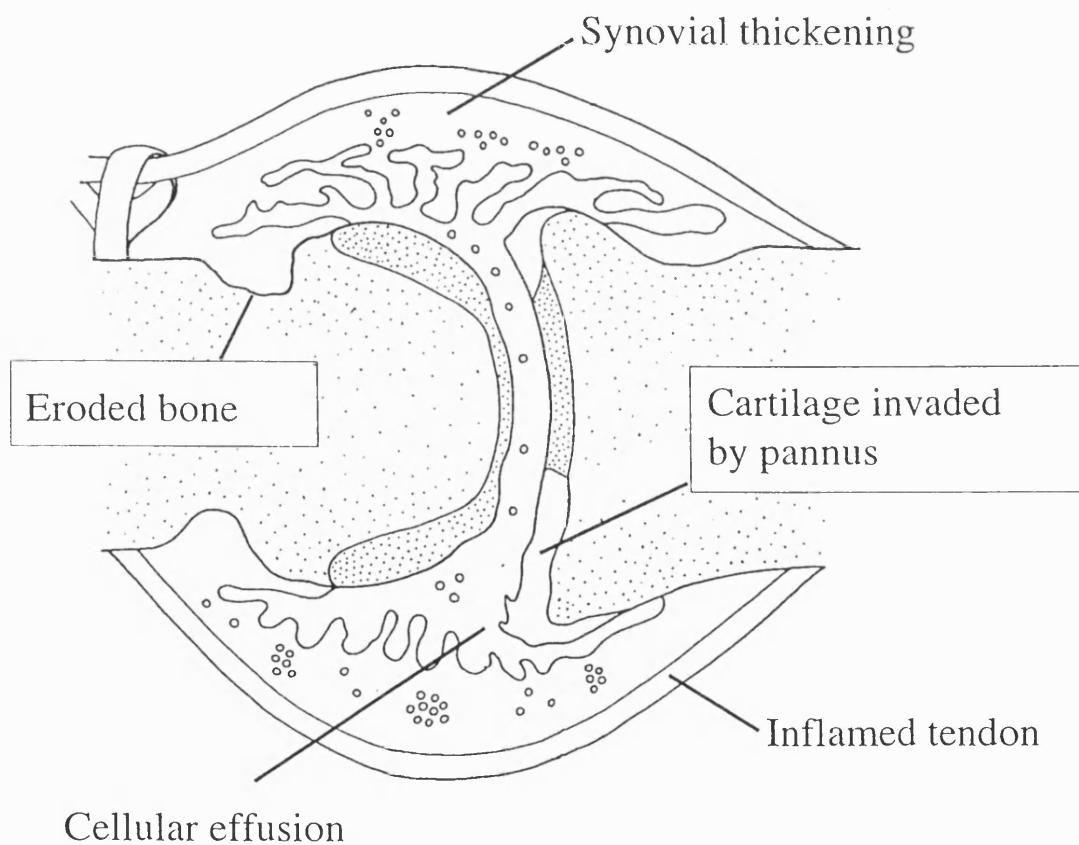


Figure 1.2. A rheumatoid arthritic synovial joint.

1.3. Rheumatoid Arthritis: An inflammatory disease of unknown etiology

Rheumatoid arthritis is the commonest chronic inflammatory synovial disease (Harris, E.D., 1989), especially females (3:1). The disease is systemic and a variety of tissues may be affected but synovium is the main target. Unfortunately the cause of this disease is still unknown, but it appears that a combination of environmental and genetic factors are relevant. Based on available data it is suggested that several environmental stimuli, such as bacterial, mycoplasmal or viral agents, may infect an individual and through some mechanism the inflammatory response is focused in joints (Harris, E.D., 1989, Maini *et al.*, 1993). The synovitis may then persist even in the absence of the offending agent because of autoimmunity and the cyclic automaticity that enables the disease to become self-perpetuating through cross-reactivity with normal proteoglycans (Harris, E.D.Jr., 1984). For example, the components of bacterial cell walls have been found to be similar to those of certain endogenous proteoglycans components (Kohaski *et al.*, 1976). There has also been an association of anti-rheumatoid arthritis nuclear antigen (anti-RANA) with rheumatoid arthritis and it has been suggested that antibodies against part of an internal repeat sequence in RANA may cross-react with auto-antibodies to structural proteins such as cytokeratin and proteoglycans (Baboonian *et al.*, 1989). More patients with RA have antibodies to RANA in their serum than non-rheumatoid patients, although anti-RANA is not specific for the disease (Baboonian *et al.*, 1989). It has also been postulated that RA occurs as a consequence of infection with mycobacteria (Maini *et al.*, 1993, Rook *et al.*, 1988, Cohen *et al.*, 1985) in particular, *M. tuberculosis* and *Proteus mirabilis* (Maini *et al.*, 1993), but there is no evidence to show their role in the etiology of RA. Two possible endogenous factors that are considered to be partly responsive for RA are collagen and rheumatoid factor. Ample data has accumulated showing collagen-induced arthritis in rats, which appears to be both a cell-mediated and an antibody mediated process (Harris, E.D.Jr., 1984, Stuart *et al.*, 1982, Trentham *et al.*, 1978). Type II collagen is more effective than other types in inducing the disease, and the immune response is generally greater against type II than against the other collagen types. Rheumatoid factor is another possible endogenous initiator of RA, as it is possible that small alterations in the conformation of IgG induced by non-specific inflammation could make it antigenic (Johnson *et al.*, 1974). There is also an association of increased morbidity and mortality in RA with the presence of rheumatoid factor. The deposition of immune complexes containing IgG and IgM are found in

vessel walls in the vasculitis of this disease. It is also apparent that IgG can self associate (Brown *et al.*, 1982, Randen *et al.*, 1993), forming complexes with other IgG molecules large enough to be phagocytosed, thus facilitating the generation of an inflammatory response within the joint space (Harris, E.D.Jr., 1984). In addition, IgE-Rheumatoid Factor has also been found in the serum of rheumatoid arthritic patients (Zuraw *et al.*, 1981, Herrmann *et al.*, 1991). This therefore provides a possibility that if IgE rheumatoid factor complexes with IgG, mast cell degranulation may occur contributing acute inflammation to the arthritic joint (Harris, E.D.Jr., 1984). It is unclear as to the role of the synovial mast cell in the inflammation of arthritis but there are several reasons implicating these cells in the inflammatory process (Kopicky-Burd *et al.*, 1988). The mast cells contain in its granules a variety of pro-inflammatory substances, and thus are equipped to intensify acute and chronic synovitis. It has been recently shown that there are differences between synovial mast cells from tissues of patients with rheumatoid arthritis and osteoarthritis, and it has been suggested that synovial mast cells may be activated in clinically active rheumatoid arthritis (Bridges *et al.*, 1991). Furthermore, Faaber *et al.*, (1989) found that hyaluronan increases the avidity of rheumatoid factor, resulting in the formation of IgG-IgM-RF complexes. In addition, approximately 37 times more IgM and 14 times more IgG was found in extracts from rheumatoid cartilage compared with normal cartilage extracts. These findings indicate that the immune complexes sequestered in the rheumatoid cartilage contain autoantibodies that are probably synthesised locally by cells infiltrating the inflamed synovium (Jasin H.E., 1985). These processes would therefore facilitate and perpetuate complex formation and hence intensifying the inflammatory response. The principle known immunogenetic association with RA is the HLA-Dw4 locus or the related HLA-DR4 allotype on lymphocytes. In addition, the HLA-Dw4 locus seems not to be associated with rheumatoid factor itself, because patients who have rheumatoid factor but do not have rheumatoid arthritis, do not have a higher frequency of HLA-Dw4 (Harris, E.D.Jr., 1984, Edwards, J.C.W., 1990). The susceptibility to developing RA is associated with the third hypervariable region of DR β chains (Nepom *et al.*, 1989). This susceptibility epitope is glutamine-leucine-arginine-alanine-alanine (QKRAA) which can be found in Dw4, Dw14 and DR β 1 chains. This epitope may also code for the severity of RA once it is established. HLA-DR β 1*0401/*0404 genotype also carries a substantially high risk for RA development specifically in its more severe forms. The

risks are particularly increased in young men. (MacGregor *et al.*, 1995). However although it is likely that every person who develops RA will share an important common epitope on the DR β chain, only a small percentage of these susceptible individuals will develop RA. Other possible factors suggested to have an effect are sex hormones which may accelerate or retard the onset (Cutolo *et al.*, 1993) and diet and stress have also been considered but their role in initiating the disease is debatable (Maini *et al.*, 1993).

In summary, although the inciting agent in RA is not known, the data implicates a number of potential antigens, resulting in a final common pathway and a number of different stimuli in the genetically predisposed host may generate a chronic sustained inflammation.

1.3.1. Synovial Structure in rheumatoid arthritis

In rheumatoid arthritis, the synovium loses its smooth, glistening pinkish-white appearance and instead becomes brown in colour, friable and engorged. The surface area is greatly increased due to the hypertrophied villi of the synovium, which is normally two cells thick but has increased in numbers of type A (macrophage-like) and type B (fibroblastic-like) cells, thus distending the joint capsule (Edwards, J.C.W., 1993). Fibrin may also be present on the synovial surface and within the substance of the tissue along with rice bodies. These are pearly white, smoothly-rounded elements, often found in large numbers and formed from shed fibrin and fragments of tissue debris. Microscopically, the tissue is densely cellular, due to the large influx of mainly T cells and macrophages, with a few follicular dendritic cells, plasma cells and B-lymphocyte lineage cells, which migrate from the vascular compartment rather than from local proliferation, as few cells are found to be mitotically active (Lalor *et al.*, 1987). The increase in cell number is believed to be due to a combination of cellular infiltration by chronic inflammatory cells and hyperplasia of resident fibroblasts-like cells. Studies using antibodies to several cell proliferation markers e.g. proliferation cell nucleus antibody (PCNA) have shown some *in situ* proliferation of fibroblast-like synoviocytes (Mohr *et al.*, 1975). However the enormous increase in cells which phenotypically and morphologically appear to be macrophages remains to be explained (Qu *et al.*, 1994). It has also been suggested by Burmester *et al.*, (1983), that there is a third major cell

population within inflamed synovial lining, which have been characterised according to the expression of surface antigens detected by various monoclonal antibodies (see Fig. 1.2. A rheumatoid arthritic synovial joint). Many of the cells in inflamed synovium are in an activated state and nearly all cell types have a higher expression of HLA class II than compared with normal joints. The deeper layers also have an increased cellularity of T-cells and plasma cells in particular, with macrophages found in the follicles and in between. Vascularity is also greatly increased and high endothelial venules are present (Freemont *et al.*, 1983), facilitating the diapedesis of lymphocytes and monocytes. Clearly, this mass of inflammatory synovial tissue cannot function as normal synovium and deterioration in joint function is seen early as painful restriction of the range of movement. Large volumes of synovial fluid are formed, but the fluid is low in viscosity, owing to the reduced concentration of hyaluronan (Balazs *et al.*, 1967) and increased content of tissue debris, effete cells and inflammatory mediators (Sledge, C.B., 1989).

In severe disease, the mass of inflammatory synovial tissue (known as “pannus” when it forms at the synovio-cartilage junction) encroaches on cartilage and bone, causing destruction of both in tissues. Histological and histochemical studies have revealed the macrophage-like cells appear to be closely involved in this destructive process which may result from the release of a variety of hydrolytic enzymes (Qu *et al.*, 1994).

Generally, two types of pannus: lining layer pannus and vascular pannus (Maini *et al.*, 1993) are usually found in patients with rheumatoid arthritis. The former is a continuation of the lining layer of hypercellular synovium and this so called cellular pannus is regarded as being derived from it. This pannus forms a distinct junction with underlying cartilage, which shows many characteristics of degradation, such as erosion of matrix and loss of water content and chondrocyte depletion. However a third type of pannus can also be found which is very similar in cell composition but it also has proliferating blood vessels and perivascular mononuclear cells present (Kobayashi and Ziff, 1975). Often this cellular pannus is seen infiltrating the cartilage. It is thought that this degradative effect is mediated by the secretions of enzymes, such as metalloproteinases, which are induced by pro-inflammatory cytokines that are released from the inflamed synovium. This degradative effect is amplified by the lack of synthesis of newly formed matrix. It is thought that cytokines may play a role in the

maintenance of balance in cytokine production between cartilage component synthesis and breakdown (Chu *et al.*, 1992) as well as in response to the state of inflammation or an immune reaction. TNF- α , IL-1, IL-6, GM-CSF and TGF- β were all detected in cells in the pannus, along the surface of the cartilage and at the site of cartilage erosion (Chu *et al.*, 1992, Alsalameh *et al.*, 1994). These cytokines were also found in normal chondrocytes, therefore the additional production of IL-1, TNF- α , IL-6 and GM-CSF may be involved in the immune and inflammatory responses and induce cartilage destruction (Akira *et al.*, 1990). The phenotype and distribution of the macrophage-like cells in the pannus are quite different from those reported from the 'synovium proper' and that the cells most associated with areas of cartilage degradation express the phenotype and characteristics of classical macrophages (Salisbury *et al.*, 1987). Furthermore, it has been suggested that specific cells of the macrophage family, osteoclasts, are involved in cartilage destruction (Bromley *et al.*, 1984).

Vascular pannus is usually found overlying cartilage with an indistinct multilayered zone of fibroblast-like cells. In contrast, the underlying cartilage of this pannus does not show any degradative activity but represents a fibrotic healing phase, as cells in this fibroblastic zone contain keratan sulphate, chondroitin sulphate and collagen type II. There is also very little pro-inflammatory cytokines such as TNF α and IL-1 β present, compared with the degradative cellular pannus. TGF- β is the only cytokine found in fibroblast-like cells and chondrocytes in the transitional fibroblastic zone, which induces cartilage synthesis, fibroblastic growth, fibrosis and inhibits collagenase, implying that this cytokine may be involved in initiating tissue repair and counteracting the effects of the little pro-inflammatory cytokines present (Chu *et al.*, 1992). The predominance of this cytokine with little pro-inflammatory cytokines would enable repair mechanisms to occur. In addition, although TGF- β is present in rheumatoid tissue, it is also found in normal synovial tissue, and it has been suggested that it may also play a role in the regulation of normal synovial tissue physiology (Lafyatis *et al.*, 1989).

Generally cytokines, which are predominantly macrophage products, are abundant in the RA joint at both the mRNA and protein levels, while on the contrary cytokines produced by T cells are detectable at the mRNA level, but are barely detectable at the protein level e.g. IL-2 (Firestein *et al.*, 1988) and IL-4 (Chen *et al.*, 1993). Studies using *in situ*

hybridization to quantify cytokine production at the level of gene expression have confirmed high concentrations in supernatants of synoviocyte cultures (Firestein *et al.*, 1990b). Macrophages are the major cell type which have been found to express IL-1 (Miossec *et al.*, 1986) and TNF- α (Saxne *et al.*, 1988) genes. These cytokines increase cultured synoviocyte proliferation, and TNF- α can stimulate GM-CSF synthesis (Xu *et al.*, 1989) in synovial fibroblasts, which in turn activate macrophages to synthesise and express MHC class II and increases the expression of membrane-bound IL-1. The GM-CSF may also exert an augmented antigen presentation function in RA (Fischer *et al.*, 1988).

1.3.2. Macrophages in RA synovium

Rheumatoid arthritis is characterised by marked mononuclear infiltration and microvascular proliferation. Available evidence supports the idea that bone marrow-derived cells come to the joint as circulating monocytes, traveling via the peripheral blood to synovial capillaries, where they egress from the circulation and migrate into the synovial lining (Edwards, J.C.W. *et al.*, 1982a; Hogg *et al.*, 1985; Lalor *et al.*, 1987; Revell, P.A., 1989; Cutolo *et al.*, 1993). These monocytes are activated and differentiate into more mature macrophage-like synoviocytes after they enter the rheumatoid joint (Ridley *et al.*, 1990) and a number of observations suggest that 80-100% of the synovial lining cells are HLA-DR positive, mature macrophage-like cells (type A) (Cutolo *et al.*, 1993, Hogg *et al.*, 1985) and are capable of antigen presentation. The synovial fluid and rheumatoid synovium are densely populated with cells, virtually all of which carry macrophage markers MHC class II antigens (Barkley *et al.*, 1989, Hogg *et al.*, 1985, Koch *et al.*, 1991b). The macrophages also have receptors for complement (C3bi), and immunoglobulins and stain with monoclonal antibodies to CD11a, CD11b, CD11c, CD14 and CD68 (Zvaifler, N.J., 1995). Studies have shown that the integrin profile of the lining cells in inflamed synovium reflects the increased number of monocyte / macrophages (Zvaifler, N.J., 1995). There is also a change in phenotype of the macrophage in inflamed tissue compared with normal, with weaker staining for CD14 and FcR1 in contrast to the intense staining with antibodies to MHC II (HLA class II molecules). This change in phenotype reflects the influence of the many soluble factors found in the inflammatory environment. For instance, normal blood monocytes exposed to IFN- γ and GM-CSF increase their expression of HLA-DR and DQ, while showing

less CD14 staining (Firestein *et al.*, 1987). Since IFN- γ is minimally present in the RA synovial environment, but GM-CSF is abundant, the latter is probably responsible for the observed changes (Alvaro-Gracia *et al.*, 1989). In the subintima, monocytes are found in two characteristic locations, exiting from small blood vessels or at the periphery of lymphoid collections. Characteristically, in these locations they show more CD14 staining, suggesting that they may be more newly arrived into the synovium (Edwards, J.C.W., 1994).

Monocyte / macrophage also show other signs of activation on the findings that cells secrete various proteolytic enzymes, eicosanoids, superoxide anion and numerous cytokines such as IL-1 and TNF- α (Arend and Dayer, 1990 and 1995). Infiltrating macrophages in RA joints may thus be essential to antigen presentation, persistence of inflammation, and tissue destruction (Akahoshi *et al.*, 1993). Thus resulting in the recruitment of circulating monocytes to both the immune and inflammatory components of rheumatoid arthritis. Zvaifler *et al.*, (1995) have suggested that macrophages have a very important role in the propagation of RA. Studies have shown that after therapy for the removal of lymphocytes by total lymphoid irradiation, the disease was diminished temporarily but not ameliorated. In contrast, it has been shown that macrophages constitute a large majority of the infiltrating cells and are highly activated, and therefore more evidence supports the hypothesis that macrophages have more of a major role in rheumatoid arthritis than T cells (Firestein and Zvaifler, 1990a).

1.3.3. Lymphocytes in RA synovium

The presence of lymphocytes within rheumatoid synovium indicates an immune process and lymphocyte populations have been intensely investigated (see review Harris 1989, Panayi *et al.*, 1992). T cell activation is one of the first major amplification steps in the pathogenesis of RA. It is thought that T lymphocytes together with the emigration of monocytes into the synovium create the first symptoms in rheumatoid arthritic patients. However this activated state is not specific for RA, although the lymphocyte profile for RA is prior activation of T cells with persistent MHC II expression, down-regulation of the T cell receptor and IL-2 receptor expression.

Within the synovium, large numbers of lymphocytes are seen to accumulate around blood vessels. Immunoelectron microscopic studies on the distribution of T cell subsets has shown that the majority of lymphocytes present have CD4 surface proteins, indicating that they are helper cells. Peripheral to these sites is a transitional zone where there is a large mixture of cells, including lymphocytes, undifferentiated blast cells, plasmablasts, plasma cells, and macrophages. Here it is thought that there are a mixture of CD4⁺ and CD8⁺ cells amidst the macrophages (Kurosaka and Ziff, 1983, Meijer *et al.*, 1982). However over all, T cells are predominant over B cells (van Boxel and Paget, 1975). The clonal expansion of the T cells will subsequently drive B lymphocyte proliferation and production of antibodies, including rheumatoid factor. Synovial lymphocytes also bear adhesion molecules of the VLA and LFA super-family of integrins, which may enable the inflammatory response to localize and persist within the synovium (Cush and Lipsky, 1988).

1.4. The role of cell adhesion molecules in inflammation

In the last decade, an enormous amount of experimental data has been gathered on the molecules and mediators that control the adhesion of different leukocyte subsets to the endothelium. This has resulted in the description of a broad collection of adhesion molecules. Based on their structural properties, these molecules can be divided into families. Since several excellent reviews on this subject have recently been published (Arnaout, M.A., 1989; Springer, T.A., 1990; Cronstein and Weissmann, 1993) we will confine ourselves here to a brief description. Table 1.1. represents an overview of the adhesion molecules discussed hereafter. A schematic drawing of representative members of each of the families of adhesion molecules is shown in Figure. 1.3.

Table 1.1. Families of adhesion molecules and their counterstructures.

Family	Adhesion molecule (CD nomenclature)	Chain composition	Alternative name(s)	Counterstructure
Ig superfamily	CD2		LFA-2	LFA-3
	LFA-3 (CD58)			CD2
	ICAM-1 (CD54)			LFA-1; Mac-1
	ICAM-2 (CD102)			LFA-1

	VCAM-1 (CD106)		INCAM-110	VLA-4
	CD4			MHC class II
	CD8			MHC class I
	NCAM (CD56)			NCAM, <i>hs</i>
	PECAM-1 (CD31)			?
Integrin family	VLA-1 (CD49a/CD29)	$\alpha 1/\beta 1$		lm, cl
	VLA-2 (CD49b/CD29)	$\alpha 2/\beta 1$	gpIa/IIa; ECMR II	lm, cl
	VLA-3 (CD49c/CD29)	$\alpha 3/\beta 1$	ECMRI	fb, lm, cl
	VLA-4 (CD49d/CD29)	$\alpha 4/\beta 1$	LPAM-1	fb, VCAM-1
	VLA-5 (CD49e/CD29)	$\alpha 5/\beta 1$	FNR; gpIc/IIa; ECMR VI	fb
	VLA-6 (CD49f/CD29)	$\alpha 6/\beta 1$	gpIc/IIa	lm
	CD51/CD29	$\alpha V/\beta 1$		fb
	LFA-1 (CD11a/CD18)	$\alpha L/\beta 2$		ICAM-1, -2, -3
	Mac-1 (CD11b/CD18)	$\alpha M/\beta 2$	CR3	ICAM-1, C3bi, fx, fg
	p150,95 (CD11c/CD18)	$\alpha X/\beta 2$	CR4	C3d
	CD41/CD61	$\alpha IIb/\beta 3$	gpIIb/IIIa	fb, fg, vWF
	CD51/CD61	$\alpha V/\beta 3$	VNR	vt, fg, vWF
	CD49f/CD-	$\alpha 6/\beta 4$?
	CD51/CD-	$\alpha V/\beta 5$		vt, fb
	CD49d/CD-	$\alpha 4/\beta p$	LPAM-2	?
Selectin	L-selectin (CD62L)		LAM-1; PLN-HR	carbohydrate moiety
	P-selectin (CD62P)		GMP-140; PADGEM	carbohydrate moiety
	E-selectin (CD62E)		ELAM-1	sialyl-Lewis ^x antigen
Unclassified	CD44		Hermes; ECMR II; Pgp-1	hyaluronic acid

Abbreviations: *cl* : collagen; *fb* : fibronectin; *fg*: fibrinogen; *fx* : factor X; *hs* : heparan sulphate; *lm* : laminin; *vt* : vitronectin; *vWF* : von Willebrand factor.

Leukocyte migration, activation and effector function depends on cell adhesion molecules. Adhesion molecules are glycoproteins expressed on the cell surface which mediate the contact between two cells or between the cell and the extracellular matrix. They regulate intercellular interactions, serve as contact molecules during the arrangement of complex tissues and mediate the interaction between the composition of the intercellular network. In addition, they mediate the interaction between static and migrating cells; e.g. lymphocyte homing (Ager, A., 1994) and localizing of neutrophils (Lo *et al.*, 1991) eosinophils or lymphocytes in inflamed tissue. More recently, it has been realised that some of the adhesion molecules also serve as signaling molecules (Pardi *et al.*, 1992). There are several main classes of adhesion molecules that are based on common characteristics. In this thesis we concentrate on the adhesion molecules responsible for the cell-cell interactions which might be crucial for the development of inflammation observed in rheumatoid arthritic patients. The three major groups of receptors are the selectins, integrins and the immunoglobulin superfamily. An additional molecule, CD44, lymphocyte homing receptor also contributes to the migration of leukocytes (Underhill, C., 1992).

1.4.1. Selectins

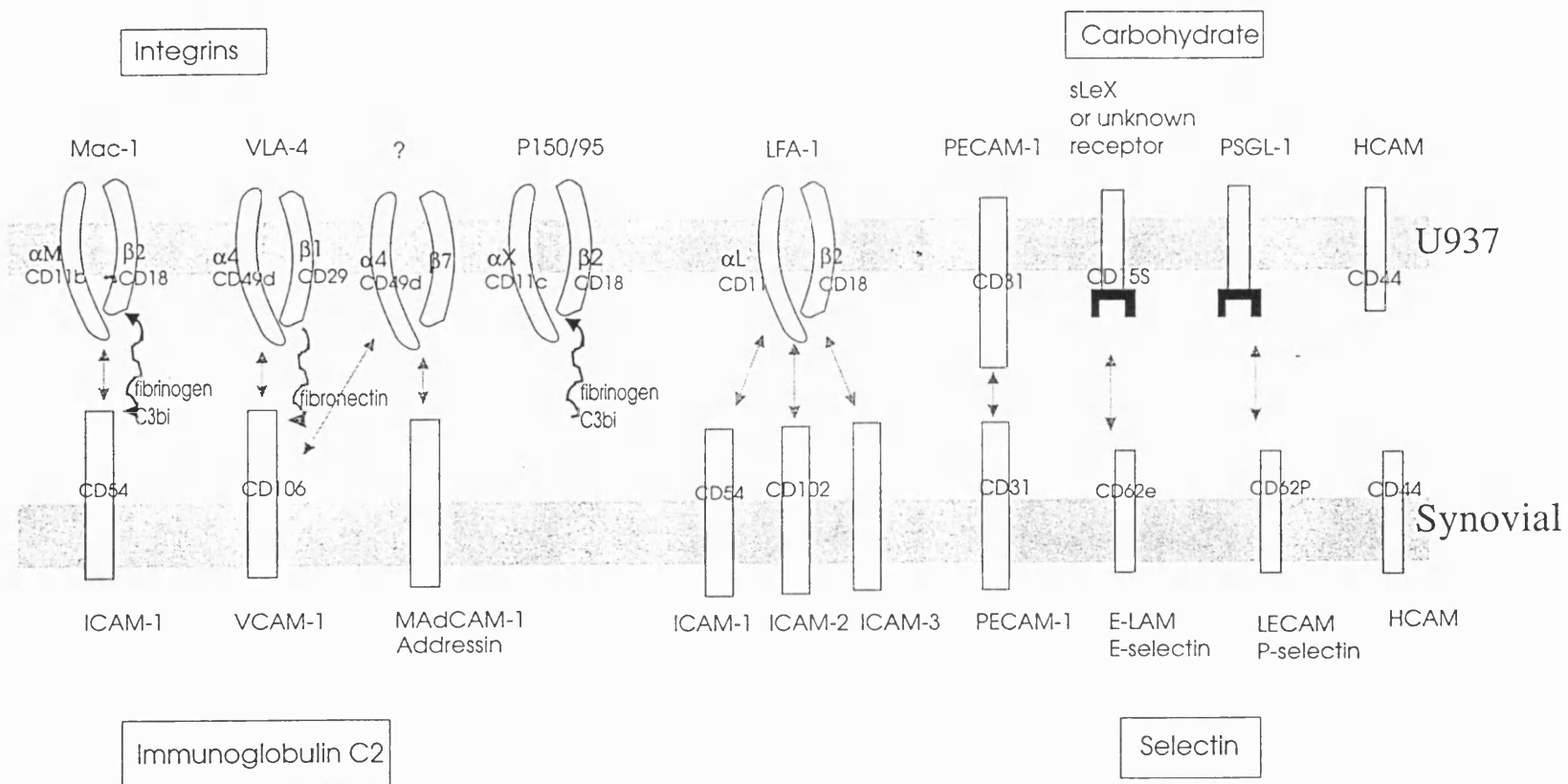
The selectin family is comprised of three proteins; i.e. E- (endothelial), P- (platelet), and L- (leukocyte) selectin. E- (CD62E) and P- (CD62P) are expressed on activated endothelial cells, while L-selectin (CD62L) is constitutively expressed on leukocytes (Hogg and Landis, 1993). Structural features that are common to the selectins are the presence of an amino-terminal (calcium dependent) carbohydrate-binding (lectin) domain, followed by an epidermal growth factor-like domain, and then a variable number of short consensus repeats (SCR), of sequences similar to those appearing in complement-regulatory proteins. This is then followed by a transmembrane domain and a short cytoplasmic carboxyl tail. The lectin part is responsible for binding of the selectin to its counter-receptor e.g. sialyl lewis X and other fucose-containing carbohydrate moieties. It has also been shown that the lectin domain is directly involved in cell binding as antibodies directed against this domain inhibit adhesion (Gibson *et al.*, 1995). The E-selectin molecule (ELAM-1) has only been shown to be expressed on activated endothelial cells and this expression can be induced *in vitro* by IL-1 and TNF, but not IFN- γ . Expression of this molecule mediates adhesion of granulocytes (Lund-

Johansen *et al.*, 1992), T cells (Shimizu *et al.*, 1991) and monocytes (Hakkert *et al.*, 1991) to endothelial cells, demonstrating the initial rolling effect. P-selectin is stored in Weibel-Palade bodies of endothelial cells and upon cell activation they are rapidly transported to the plasma membrane (Smith, C.W., 1993, Subramaniam *et al.*, 1993). Both P- and E- selectin upregulation are only transient and induced by cytokines and consequently they are thought to mediate acute inflammatory responses. In addition, selectins are thought to influence neutrophil function, for example, cross-linking of L-selectin can induce a rapid increase in calcium influx and superoxide anion production by neutrophils (Waddell *et al.*, 1994).

1.4.2. Integrins

The integrin family have an important role in the cell-cell and cell-matrix interactions and most integrins are expressed on a wide variety of cells, with most cells expressing several integrins (Hynes, R.O., 1992). Molecules belonging to this family all consist of heterodimers composed of an α subunit non-covalently associated with a β -chain subunit. The extracellular domain of the α subunit has three or four divalent cation binding sites that require calcium or magnesium for function. These cation binding sites play a crucial role in ligand binding (Springer, T.A., 1990). The integrins are divided further into eight β subfamilies. One of their crucial properties is their ability to adhere to molecules showing the amino acid sequence (Arg-Gly-Asp) RGD sequence (Pucillo *et al.*, 1993).

Figure 1.3.
A schematic drawing of representative members of each of the families of adhesion molecules.



The $\beta 2$ integrins are more commonly named leukocyte integrins since they are uniquely expressed on the different types of leukocytes. The common β -chain ($\beta 2$) can bind to three different α chains resulting in three subfamily members; i.e. lymphocyte function associated antigen-1 (LFA-1; CD11a/CD18), complement receptor 3 (Mac-1; CD11b/CD18) and p150,95 (CD11c/CD18) (Sanchez-Madrid *et al.*, 1983). Peripheral blood lymphocytes express primarily LFA-1, whereas basophils, mast cells (Hamawy *et al.*, 1994), neutrophils, eosinophils, monocytes and NK cells express all three $\beta 2$ integrins. Intracellular pools of Mac-1 and p150,95 are present in neutrophils and monocytes, whereas there is a much smaller storage pool for LFA-1 (Miller *et al.*, 1987). During activation of leukocytes, the $\beta 2$ integrins not only show an increased expression on the cell surface but also experience a conformational change and increased receptor affinity (Lollo *et al.*, 1994). In addition, Mac-1 and LFA-1 may also function as signaling molecules (Hogg and Landis, 1993). Activated LFA-1 has been shown to augment T cell responses (Van-Seventer *et al.*, 1990; van Seventer *et al.*, 1991; Shimizu *et al.*, 1991) and crosslinking of Mac-1 is reported to trigger oxidative burst in neutrophils (Crocket-Torabi *et al.*, 1995; Zhou and Brown, 1994) as well as degranulation of neutrophils and eosinophils (Schleffenbaum *et al.*, 1989; Horie and Kita, 1994). In addition, eosinophils have been shown to adhere to VCAM-1 on vascular endothelium, which resulted in the activation of superoxide anion generation (Nagata *et al.*, 1995).

Of the $\beta 1$ integrins, VLA-4 (very late antigen-4) is known for its function as a cellular matrix receptor i.e. for fibronectin, and as a cellular receptor for another adhesion molecule, VCAM-1 (vascular adhesion molecule-1) (Makarem *et al.*, 1994; Wayner *et al.*, 1989). However the binding sites for the two ligands are believed to be different (Elices *et al.*, 1990; Matsumoto and Hemler, 1993). VLA-4 can be found on lymphocytes, monocytes and eosinophils but not neutrophils. Furthermore, it has been shown that VLA-4 expressed on T cells can also support tethering and rolling on VCAM-1, a similar finding to the role of selectins (Alon *et al.*, 1995).

1.4.3. Immunoglobulin-gene superfamily

This superfamily consists of cell surface proteins that are involved in antigen recognition, complement binding or cellular adhesion. For endothelial-leukocyte

interactions, the most important members are intercellular adhesion molecule (ICAM-1(CD54), -2 (CD102) and VCAM-1 (CD106). VCAM-1 exists in two forms one with six Ig-like domains and one with seven (Hession *et al.*, 1991), however only the latter has a functional role in leukocyte adhesion. ICAM-1 and VCAM-1 expression can be induced by various cytokines, for example ICAM-1 is upregulated by interferon- γ , IL-1 β , TNF- α and LPS on endothelial cells. Whereas VCAM-1 can be selectively induced by IL-4 (Luscinskas *et al.* 1994) or IL-13 (Sironi *et al.*, 1994), but also by IL-1 β , TNF- α and LPS (Carlos *et al.*, 1990). All members of the ICAM family recognize the β 2-integrin and ICAM-1 additionally recognizes Mac-1. Recently it has been described that ICAM-1 can also bind to CD43 (Tiisala *et al.*, 1994). Moreover, Rothlein *et al.*, (1994) showed that crosslinking of ICAM-1 on CD14-positive mononuclear cells induces an oxidative burst suggesting that ICAM-1 is able to transmit a signal.

1.4.4. Adhesion cascade in inflammation

Under resting conditions leukocytes hardly bind to endothelium. However during inflammation, mediators such as histamine, leukotrienes, prostaglandins and cytokines are released by mast cells (Hamawy *et al.*, 1994), macrophages and T cells at the site of infection. Leukocytes are attracted by chemotaxis through these agents, thereby initiating emigration from the blood stream to extravascular sites of inflammation. The adhesion process is a sequence of events that initially prolongs leukocyte contact with the vessel wall, where it may respond to various chemoattractants eliciting transmigration and eventually an immune response (reviewed by Springer, T.A., 1994). The process begins with leukocyte tethering and rolling along endothelial cells and this first step of migration is mediated by selectins; L- and E- selectin. Leukocytes become activated by signaling through the selectins and cell membrane bound mediators such as PAF and IL-8, cause cell activation by chemoattractant-receptor interactions, thereby influencing β 2 integrin function (see mini-review Butcher, E.C., 1991). Activation of leukocyte integrins then triggers an increase in avidity caused by a conformational change in the integrin heterodimer, resulting in a greater affinity for their ligands. This is then followed by integrin-immunoglobulin interactions, which causes arrest and firm adhesion to the vessel wall (Springer, T.A., 1994). Finally, transmigration through the endothelial vessel wall is induced by both chemoattractant and integrin interactions with the leukocytes. Once the leukocytes cross the endothelium they are detached and begin

to migrate up a chemotactic concentration gradient and or by haptotaxis, when cells migrate to the region of highest adhesiveness.

Interestingly, expression of adhesion molecules is regulated at multiple levels. Firstly, different cytokines selectively induce specific adhesion molecules, such as IL-4 acts synergistically with IL-1 β to promote lymphocyte adhesion to microvascular endothelium by inducing VCAM-1 expression (Masinovsky *et al.*, 1990). The selectivity is also dependent on the cell type, for example IFN- γ is much more potent at inducing ICAM-1 on epithelial cells than on endothelial cells. Moreover, combinations of the different cytokines may produce additive, synergistic or antagonistic effects. These studies demonstrate that there are numerous pathways mediating cell adhesion and migration and that the dominance / hierarchy of these pathways varies dramatically with the activation state of the interacting cells and the differentiation state of the infiltrating cell. The diverse levels of regulation thus create unique sequence of events for certain inflammatory situations.

1.5. Cell adhesion molecules in rheumatoid arthritis

Immunohistochemical evidence has shown that a vast array of cell adhesion molecules are present in an inflamed synovial joint. So far, E- (Koch *et al.*, 1991a), P-, L- selectin, have all been found on the vascular endothelium in synovial tissue, however no significant difference in expression between disease groups was observed (Johnson *et al.*, 1993). However Grober *et al.*, (1991) found that P-selectin played a role in monocyte binding to frozen RA synovial tissues. Leukocyte adhesion molecules CR3 (CD11b) and p150,95 (CD11c) have also been evaluated immunohistochemically and a significant proportion of synoviocytes in normal, osteoarthritic and rheumatic patients express the leukocyte common antigen and CR3, but few express the p150,95 molecule (Johnson *et al.*, 1993). Expression of this molecule seemed to be more prominent when vast numbers of macrophages and mononuclear phagocytes infiltrated the joint and accumulated at the joint surface (Allen *et al.*, 1989). This pattern in expression seems to suggest that leukocyte adhesion molecules may be important in the diapedesis of mononuclear phagocytes into and through inflamed synovium. Modulation of cell surface molecules on lymphocytes and on endothelial cells of synovial capillaries may determine lymphocyte access to synovial tissue. One specific molecule that contributes

to lymphocyte-endothelial cell adhesion is lymphocyte function-associated antigen 1 (LFA-1). The LFA-1 molecule, together with Mac-1 and p150,95, form a family of related cell adhesion molecules that are important in leukocyte-cell interactions (Allen *et al.*, 1989). LFA-1/ICAM-1 and CD2/LFA-3 interactions have been shown to occur between T-lymphocytes and synovial cells. LFA-3 and ICAM-1 are widely distributed among macrophage-like synovial cells, synovial fibroblasts, macrophages, and endothelial cells (Hale *et al.*, 1989), and this evidence lends support for the hypothesis that the two adhesion pathways could be involved in the migration of lymphocytes to synovium and could participate in the maintenance of T and B cell responses in synovium (Hale *et al.*, 1989).

Other evidence has also accumulated showing that and VCAM-1/VLA-4 interaction can occur since high levels of VCAM-1 are expressed on the synovial lining cells (Wilkinson *et al.*, 1993, Marlor *et al.*, 1992) and synovial-like macrophages (Koch *et al.*, 1991, Cutolo *et al.*, 1993, Wilkinson *et al.*, 1993). Therefore this provides another possible pathway in which T lymphocytes and monocytes are able to migrate into an inflamed joint. Furthermore, the high expression of VCAM-1 by RA lining layer activated macrophages and therefore possible VCAM-1/VLA-4 interaction could also contribute to synovial tissue hyperplasia and the resulting cellularity of synovial fluid (van Dinther-Janssen *et al.*, 1994 and Veale *et al.*, 1993).

In addition, CD31 (PECAM), CD44 (hyaluronan receptor) and $\beta 3$ (vitronectin receptor) are up-regulated on RA macrophages and lining cells, CD44 on RA fibroblasts and $\beta 3$ -integrin on RA blood vessels (Johnson *et al.*, 1993; Haynes *et al.*, 1991). CD31 may be of some significance to the causal effect of infiltration of leukocytes because it is expressed on endothelial cells, platelets, granulocytes, monocytes and some lymphocytes, but more interestingly, CD31 is expressed on a greater percentage of synovial lining cells and sub-synovial macrophages in RA than in OA patients. It has also been noted that a CD31 related molecule has been shown to function in the chemotaxis of monocytes and neutrophils (Ohto *et al.*, 1985). Thus the increased expression of CD31 in RA synovium may be the result of chemotaxis of CD31⁺ cells into the diseased joint.

It has also been postulated that CD44 may have a role in the pathogenesis of RA, from its functions as a homing receptor (Underhill, C., 1992) and mediates transendothelial migration (Streeter *et al.*, 1988). Thus CD44 may function in a similar manner directing monocyte trafficking in to the RA joint. CD44 also enhances monocyte-lymphocyte interactions (Haynes *et al.*, 1989), directly stimulates monocyte interleukin 1 production (Haynes *et al.* 1989), which may be responsible for the presence and role of IL-1 in mediating joint inflammation and hence augmentation of persistent infiltrating inflammatory cells.

1.6. Aim of research project

The aim of the research project is to study the role of the synovial fibroblast in the propagation of inflammation in the synovial joint. We will develop a co-culture system comprising synovial fibroblasts obtained from patients with rheumatoid arthritis and the monocytic cell line U937. The U937 cells have been chosen because they resemble monocyte progenitor cells and because they are clonal cells, they will show less variation in response compared to peripheral blood monocytes. We will study the co-culture in the presence of human plasma derived serum rather than foetal bovine serum and we will assess the influence of the presence of hyaluronan. In the co-culture system we will study: the adhesion molecules involved in cell-cell interaction; U937 differentiation; the release of inflammatory cytokines and the role therein of the rheumatoid factor; the synthesis of metalloproteinases; and the activation of the NADPH oxidase. We will compare the capacity of synovial fibroblasts to affect any of the above mentioned parameters with that of a co-culture with primary dermal fibroblasts, cells that lack expression of VCAM-1.

Chapter Two

VCAM-1 expression by synovial fibroblasts in culture: a dual mode of regulation

2.1. Abstract

Intimal synovial fibroblasts *in situ* constitutively express VCAM-1. The VCAM-1 expression may have an important role in retention of mononuclear leukocytes in the synovial joint and may contribute to an unresolved inflammation as seen in rheumatoid arthritis. In synovial fibroblasts cultures, VCAM-1 expression can no longer be discerned, except transiently, after addition of inflammatory cytokines, such as IL-1 β or TNF- α . We have performed a time-course analysis of VCAM-1 expression using flow cytometry. Synovial fibroblasts express VCAM-1 at high levels in the first week but thereafter expression declines and is absent after four to six weeks. The VCAM-1 expression is independent of the presence of monocytes or IL-1 β and TNF- α and can be upregulated by culturing the cells on a collagen I matrix. We conclude that VCAM-1 expression is under dual control; a constitutive control which is gradually lost as soon as the cells are removed from the synovial membrane and a transient control, by inflammatory cytokines, which only becomes apparent when VCAM-1 expression has declined sufficiently.

2.2. Introduction

The adhesion molecule vascular cell adhesion molecule-1 (VCAM-1) is a 90-110 kDa glycoprotein of the immunoglobulin supergene family (Osborn *et al.* 1989, Rice *et al.* 1989). VCAM-1 was originally designated as inducible cell adhesion molecule-110 (INCAM-110). This was because it was shown to be expressed on inflammatory cytokine stimulated endothelial cells (Rice *et al.*, 1989). For instance, VCAM-1 expression on endothelial cells can be transiently induced by inflammatory cytokines such as TNF- α or IL-1 (Osborn *et al.*, 1989). Expression persists for around two days. The ligand for VCAM-1 is the integrin α 4 β 1 also known as Very Late Antigen-4 (VLA-4), an adhesion molecule discovered in T-cells but which is also expressed on

monocytes (Carlos *et al.*, 1991) and eosinophils (Elices *et al.*, 1990). The transient expression of VCAM-1 has an important role in the recruitment of leukocytes at a site of inflammation (Osborn *et al.*, 1989; Carlos *et al.*, 1991; Bochner *et al.*, 1991; Luscinckas *et al.*, 1994). In addition, evidence has been presented that VCAM-1 is an important component in the proliferative response of CD4⁺ T cells after activation by antigen-presenting cells through the CD3-TCR complex (Damle *et al.*, 1991). Lymphoid germinal centres contain VCAM-1 expressing cells (follicular dendritic cells) and VCAM-1 has been shown to be involved in binding of B lymphocyte precursors in culture (Freedman *et al.*, 1990). The VCAM-1 molecule exists as two isoforms: one containing six Ig-like domains (VCAM-6D) and the other containing seven Ig-like domains (VCAM-7D) (Hession *et al.*, 1991). These two isoforms arise from alternative splicing of mRNA from the VCAM-1 gene (Hession *et al.*, 1991; Cybulsky *et al.*, 1991). The two isoforms are identical in amino acid sequence except for the absence of domain 4 in VCAM-1 6D. Both the 6D and 7D forms are found in cytokine stimulated endothelial cells (Hession *et al.*, 1991), where the 7D form is predominant.

In contrast to those cells which show inducible expression of VCAM-1, a number of cell types show constitutive expression. For instance myoblasts cells (Iademarco *et al.*, 1993), follicular dendritic cells (Freedman *et al.*, 1990) or murine bone marrow stromal cells. For the latter it was shown that this expression mediated binding of VLA-4 expressing lymphocyte precursors in culture (Miyake *et al.*, 1991). The fibroblast-like intimal cells of the synovial membrane are also characterised by constitutive expression of VCAM-1 (Morales-Ducret *et al.*, 1992; Wilkinson *et al.*, 1992 and 1993; Kriegsman *et al.*, 1995). In the case of rheumatoid arthritis, an increased number of VCAM-1 expressing synovial fibroblasts is observed and the location of the cells is no longer restricted to the intima (Wilkinson *et al.*, 1993). A continuous presence of monocytes/macrophages in association with these fibroblasts is also observed (Wilkinson *et al.*, 1993; Kriegsman *et al.*, 1995). Mononuclear cell infiltration is conspicuous in the earliest examples of synovitis and this observation points to a possible role for the two cell types in the onset of chronic inflammation (Zvaifler *et al.*, 1994; see also Thomas and Lipsky, 1993). The synovial fibroblast and its VCAM-1 expression may have a role in retention of monocytes in the synovial membrane. In culture, however, VCAM-1 expression on synovial fibroblasts appears to be low or

absent (Morales-Ducret *et al.*, 1992; Blue *et al.*, 1993; Marlor *et al.*, 1992; Bombara *et al.*, 1993). Although inflammatory cytokines can up-regulate VCAM-1 expression, it seems unlikely that cytokine stimulation can explain the constitutive expression seen *in vivo* for two reasons: synovial fibroblasts in non-inflamed joints also express VCAM-1 and the up-regulation by cytokines seen *in vitro* is only transient (Morales-Ducret *et al.*, 1992; Marlor *et al.*, 1992; Bombara *et al.*, 1993). Regulation of the VCAM-1 promoter has not been studied in synovial fibroblasts and it therefore remains unknown what extra- or intra-cellular signal drives the VCAM-1 expression. In this manuscript we study the detailed time course of VCAM-1 expression in synovial fibroblasts in culture. We compare VCAM-1 expression in the presence of foetal bovine serum with that in the presence of human plasma derived (platelet free) serum at concentrations comparable to that expected in human connective tissue. We analyse the role of the extracellular matrix, in particular fibronectin, collagen, laminin and hyaluronan, in VCAM-1 expression and we show that collagen I has a significant up-regulatory effect on VCAM-1 expression. Importantly, we provide evidence that VCAM-1 expression in synovial fibroblasts occurs in the absence of relevant levels of pro-inflammatory cytokines.

2.3. Methods and Materials

2.3.1. Materials

DMEM, RPMI 1640, L-glutamine, Streptomycin sulphate, benzyl penicillinate and foetal bovine serum were obtained from Gibco BRL Life Technologies, UK.

2.3.2. Antibodies

Phycoerythrin -conjugated anti-mouse IgG antibodies were obtained from Sigma. Anti-CD14 was obtained from DAKO. Anti-ICAM-1, Anti-VCAM-1 and anti-VLA-4 were obtained from Becton Dickinson.

2.3.3. Human plasma derived serum preparation (HPDS)

Human plasma was obtained from male AB donors. Fresh citrated blood was spun at 10,000 rpm for 20 min at 4°C, and the supernatant (plasma) was microscopically tested

for the presence of platelets. If platelets were detected, the plasma was spun again for 20 min at 4°C. To the plasma, 4 mm CaCl₂ was added and the plasma was left at 4°C for overnight. The fibrin clot was spun at 10,000 rpm for 20 min at 4°C and the plasma was then heat inactivated at 54°C for 40 min. The plasma was cleared one more time of insoluble components by centrifugation at 10,000 rpm for 20 min at 4°C. The plasma is aliquoted and kept at -20°C until use. This product is referred to as plasma derived serum (HPDS) (Raines and Ross, 1985).

2.3.4. Synovial fibroblast isolation and cell culture

Rheumatoid synovium tissue was obtained after hip or knee joint replacement surgery and collected from University College London Hospital. The tissues described herein are all derived from rheumatoid arthritic joints. Tissue was processed within two hours after removal from the patient. The inner layer of the synovial membrane was removed with a scalpel and the tissue was washed extensively in PBS (w/o Ca²⁺ and Mg²⁺). The tissue was then chopped into small pieces (1mm³) washed again in PBS and left to digest in 2mg/ml collagenase in sterile DMEM supplemented with 1% penicillin-streptomycin in a shaking incubator at 37°C. The tissue was sheared using a sterile syringe, filtered using a fine sterile gauze and then washed and resuspended in DMEM containing 10%FBS and 1% penicillin-streptomycin. The cells were counted, (the viability of the cells were tested using 0.4% trypan blue solution and seeded into a 75cm³ flask. The cells were allowed to adhere overnight. The following day the cells were washed with PBS and then passaged with trypsin and the cells were seeded into 6 well tissue culture plates (Nunc), uncoated or coated with collagen, fibronectin and laminin (Becton Dickinson-Collaborative Biomedical Products). The cells were cultured with either 10% FBS or 10% HPDS. In the case of hyaluronan (1 mg/ml), 100mg of human umbilical cord hyaluronan (Sigma) was dissolved in 100ml DMEM supplemented with either 10% FBS or 10% HPDS. The cell culture was continued for various time periods. Medium was replaced every five days, but cells were not passaged during this period. For most of the cases, the synovial fibroblasts proliferate during the first two weeks and then divided only slowly.

2.3.5. Flow cytometry

After the appropriate time period in culture, the fibroblasts were washed in PBS (without Ca^{2+} or Mg^{2+}) and removed from the dish by a 20 min incubation in ice-cold PBS supplemented with 4 mM EDTA and the cells were scrapped off. All further cell manipulations took place on ice. The cell suspension, approximately 0.2×10^6 cells per dish (19.6cm^2) was incubated in 1 ml of 10% human AB serum for 20 minutes, washed in PBS containing 1% (w/v) BSA and then separated into various aliquots for separate incubations with monoclonal antibodies against: VCAM-1 (Becton Dickinson) ($10\mu\text{g/ml}$) ICAM-1 ($10\mu\text{g/ml}$) or CD14 (monocyte marker, DAKO) ($20\mu\text{g/ml}$) in a 96 well plate. The plate was then left on ice for 40 minutes, washed twice in PBS /1% BSA followed by a second incubation with the second anti-mouse IgG-antibody conjugated with Phycoerythrin (PE) (Sigma) ($5\mu\text{g/ml}$). PE was used because the synovial fibroblasts have a higher autofluoresence in the green region. The incubation time of each antibody was 40 minutes, and cells were washed three times in PBS containing 1% (w/v) BSA. Cell surface marker levels were measured using a FACscan flow cytometer (Becton Dickinson). Expression of the two antigens is depicted as mean channel fluorescence of Phycoerythrin per 2000 cells analysed.

2.3.6. RNA isolation

RNA was isolated by the method of Chomczynski and Sacchi (1987). A brief description follows, (all procedures were performed on ice); Cells were washed twice with PBS (w/o). The cells were solubilized in 2ml of GIT (25g guanidinium isothiocyanate, 29.3ml sterile DEPC water, 1.76ml sodium citrate 0.75M (pH 7.0) and 2.64ml 10% sarcosyl solution. This solution was then dissolved at 65°C and 0.36ml of 2-mercaptoethanol solution was added) and the solution was sheared through a needled syringe. At this stage the suspension could be frozen at -73°C . Then 200 μl of 2M sodium acetate was added, inverted, and an equal volume of water saturated phenol was added and mixed thoroughly. 400 μl of chloroform-isoamylalcohol (49:1) was added, vortexed and then left on ice for 20 minutes. The suspension was centrifuged at 13000rpm for 30 minutes at 4°C . The suspension separates into 2 phases : an aqueous upper layer containing the RNA, and an organic lower phase. At the boundary between the two phases there is a white interface containing genomic DNA. The upper aqueous phase was carefully removed and added to a fresh tube. 2 μl of glycogen ($20\mu\text{g}/\mu\text{l}$) was

added, mixed thoroughly. This was followed by the addition of 2ml of propan-2-ol, mixed and then left at -20°C for 1 hour. The samples were further centrifuged at 13000rpm for 30 minutes at 4°C and then the supernatant was removed by inversion. 400µl of GIT was added to the pellet and mixed thoroughly, then 400µl of propan-2-ol was added and mixed and left at -20°C for a further 1 hour. The samples were centrifuged at 13000rpm for 30 minutes at 4°C and washed with 75% ethanol. The samples were then air dried and resuspended in 20µl of DEPC water.

2.3.7. RT-PCR of VCAM-1 and GAPDH

All PCR experiments were carried out in collaboration with Dr. Peter McIntyre (Sandoz Institute for Medical Research). Total RNA (3 µg) was reverse transcribed to cDNA using a Ready to Go T-Primed First-Strand kit (Pharmacia Biotech). This kit utilises the Moloney leukemia virus (M-LuLv) reverse transcriptase and an oligo (dT)₁₈ primer to generate first strand cDNA. The reaction mix provided is a room temperature stable preparation containing dATP, dCTP, dGTP, dTTP, murine reverse transcriptase, RNAGuard, RNase/DNase-free BSA and not I-d(T)₁₈ primer (5'-d [AAC TGG AAG AAG AAT TCG CGG CCG CAG GAA T₁₈] - 3'). 3µg of the RNA was made up to a volume of 33µg using DEPC water. The samples were then heated to 65°C for 5 minutes, and then transferred to a 37°C bath and incubated for a further 5 minutes. The RNA solutions were transferred to their respective First-Strand Reaction Mix tubes and incubated for 5 minutes at 37°C. The tubes were vortexed to mix the RNA solution with the reaction mix and incubated for 1 hour at 37°C. The completed first strand reaction was heated to 95°C for 5 minutes in order to inactivate the reverse transcriptase. The reaction mixture is now ready to use immediately for the PCR reaction. (See Appendix I for the criteria that were used that determined the PCR reaction.) Specific primers for VCAM-1 (Hession *et al.*, 1991) were used for the PCR reaction. Oligomer P259 (anti-sense), which hybridises to a region 3' to the domain 4 insertion site, 5' GGA ACC TTG CAG CTT ACA GTG 3' and oligomer VC16 (sense) which hybridises to a region 5' of the domain 4 site, 5' CAA GTC TAC ATA TCA CCC AAG 3'. The oligomer P259 was shortened from primer 370-9 used by Hession *et al.*, 1991, so that both primers used in the amplification had a similar melting temperature and G C content. The amplified PCR product for VCAM-6D was 355-bp and VCAM-7D was 631-bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also monitored in order to quantitate the VCAM-1 RNA

levels in synovial fibroblasts. The GAPDH primers were purchased from Strategene. The GAPDH primers used produced a PCR product of 356 bp. Amplification was performed by adding 28.7 µl of sterile DEPC water, 8 µl of 1.25 mM each dNTP mix (Perkin-Elmer), 0.25 µl of ³³P-dATP (Amersham), 2.5 µl of 4 mM primer VC16, 2.5 µl of 4 mM primer P259, 5 µl of 10 x PCR buffer (Perkin-Elmer) and 0.25 µl of Amplitaq DNA polymerase (Perkin-Elmer) at 5 U/ml to 3 µl of each completed first strand reaction. The reaction tubes were heated to 94°C for 5 min and incubated in a GeneAmp PCR 96000 (Perkin-Elmer) thermal cycler using 28 cycles of 94°C (30 sec), 62°C (30 sec) and 72°C (60 sec). The GAPDH PCR cycle was as follows ; 28 cycles of 94°C (30 sec), 62°C (30 sec) and 72°C (60 sec). Fragments were finished off by incubating for 10 min at 72°C. Linearity of the amplification of the PCR products was determined by doing a cDNA dilution curve, annealing temperature curve and PCR cycle curve. The following dilutions were used for determining the correct concentration of cDNA; 1/100, 1/50, 1/30, 1/20, 1/10, 2, and 1 and the dilution that showed the best specificity was the 1/20 as this dilution showed the best discrete band, whilst the other dilutions showed more smearing of bands. The appropriate number of cycles was assessed by testing a cycle range from 15, 20, 22, 24, 26, 28, 30, 32, 35 and 40 cycles and the best number was 28. Analysis of the PCR reaction was determined by electrophoresing the PCR products through a 4% polyacrylamide gel. The gel was fixed for 15 min in acetic acid-methanol and dried under vacuum at 80°C. The gels were put against a phosphor screen for 2-4 hrs and primer-specific bands were then analysed using a BAS1000 BioImager (Fuji).

2.3.8. Southern blot analysis of D6 and D7

For Southern blot analysis, identical PCR reactions were performed with the exclusion of ³³P-dATP. 15 µl of each PCR product was run on a 1% agarose gel and blotted onto Hybond N⁺ nylon membrane (Amersham). For the six-domain form, an anti-sense primer was used whose centrepont falls at the junction of domain 3 and 4 (oligo 3-4), 5' CTA GGG AAT GCT TGA ACA AT (T_m 53°C) (the oligo 3-4 probe has a low specificity for the 7 domain VCAM-1 and hybridises to bands at 355 and 631 bp produced by amplification). For the seven-domain, an anti-sense unique to the sequence of domain 4 (oligo 3b) was used, 5' CAA TCC GGG GTC CAG GGG AGA TCT CAA CAG (T_m 70°C) (see Hession *et al.*, 1991). The probes were end-labelled with a T4 polynucleotide kinase (New England BioLab) in the presence of 1.5 µl of ³²P-γ-ATP

(Amersham). Hybridization with the blot was performed at the calculated primer T_m (See Appendix I for the formula for calculating the T_m) - 10 °C overnight in 6 x SSPE (Saline Sodium Phosphate EDTA buffer) (20x stock solution: 175.3g NaCl, 27.6g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 7.4g EDTA at pH 7.4 up to 1 Litre), 5 x Denhardt's reagent (50x stock solution; 5g Ficoll (Type 400), 5g polyvinylpyrrolidone, 5g BSA (Fraction V and 500ml H_2O), 0.5% (w/v) SDS, 100 $\mu\text{g/ml}$ salmon sperm DNA and 50% (w/v) formamide (Sambrook, Fritsch & Maniatis). (The calculated T_m in 1M salt for oligo 3b and oligo 3-4 are 70°C and 53°C respectively.) The membranes were washed with 6 x SSPE and 0.5% SDS briefly at room temperature. The blots were washed at the calculated oligo T_m -5 °C for 10 minutes. This high stringency wash was performed in order to remove any oligo non-specifically bound to the membrane. The blots were analysed by placing against a phosphorscreen and scanned using a BAS1000 BioImager (Fuji).

2.3.9. Cytokine measurement

IL-1 β and TNF- α were measured using cytokine assay kits provided by R&D Systems.

2.3.10. Statistical analysis

Statistical analyses were performed using analysis of variances (ANOVA). P values less than 0.05 were considered significant. If there were significant findings using ANOVA, a series of student paired t tests were conducted to identify differences among groups.

2.4 Results

2.4.1. Characterization of rheumatoid synovial fibroblasts

Fig. 2.1 shows the morphology of cultured rheumatoid synovial fibroblasts grown in 10% FBS and 10% HPDS. There is no difference between the morphology of the rheumatoid synovial fibroblasts when grown under the two culture conditions. These fibroblasts-like cells do not show any difference from other fibroblast cell types and they can grow to confluency. In addition, rheumatoid synovial fibroblasts are able to produce hyaluronan (Pitsillides *et al.*, 1993) due to the presence of the enzyme, uridine diphosphoglucose dehydrogenase (UDPGD), which is the rate limiting enzyme for the synthesis of hyaluronan. One of the cell surface markers that has been shown by immunohistochemistry studies to be constitutively expressed on the rheumatoid synovial fibroblasts is VCAM-1 (Wilkinson *et al.*, 1993). Therefore, we decided to use VCAM-1 as our marker for the presence of rheumatoid synovial fibroblasts (see Figure. 2.2). In addition we also measured ICAM-1, but only low levels of ICAM-1 were detectable (See Figure 2.2.)

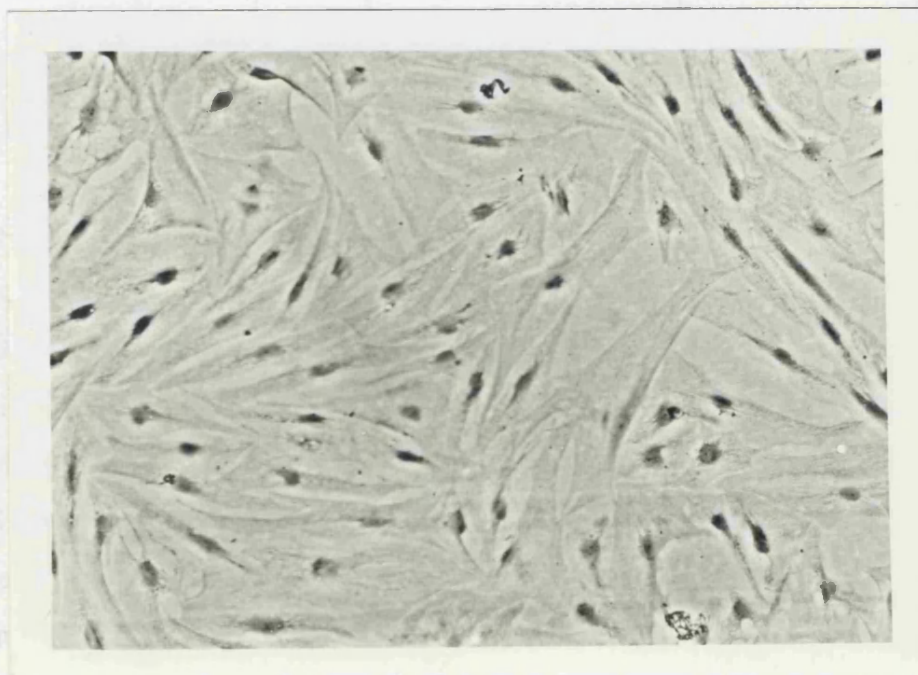


Figure 2.1. Rheumatoid synovial fibroblasts were cultured in 10% HPDS for 1 week. The morphology of the cells did not differ from cells grown in 10% FBS. The above figure is a typical result from 5 experiments.

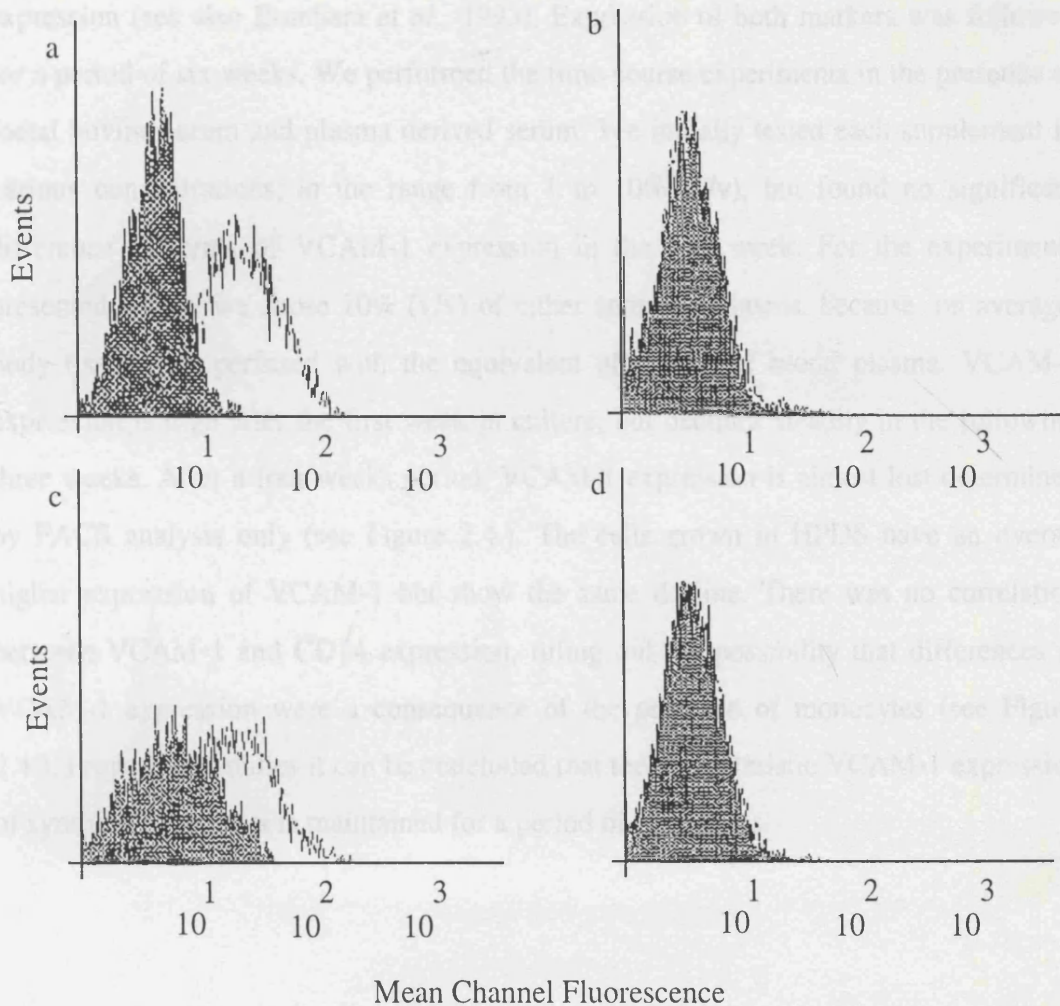


Figure 2.2.

Flow cytometric analysis of a) VCAM-1 b) IgG1 subtype (control) and c) ICAM-1 and d) IgG2b subtype (control) on rheumatoid synovial fibroblasts. The results shown are of synovial fibroblasts cultured for 1 week in 10%HPDS medium. The cells were detached from the dish as described in Methods and Materials. The shaded area is denoted as the FACS control, i.e. cells incubated with the 2nd antibody only, and the unshaded area denotes cells incubated with the 1st and 2nd antibodies. Statistical analysis ; paired student t-test. N=4

2.4.2. VCAM-1 expression is stable for two weeks in culture

Primary synovial fibroblasts cell cultures were analysed with VCAM-1 antibodies (see Figure 2.1.) and antibodies against the monocyte-macrophage marker CD14 (see Figure 2.3.). We wished to assess monocyte contamination because of the possible release of

inflammatory cytokines, from these cells, which could have an effect on VCAM-1 expression (see also Bombara *et al.*, 1993). Expression of both markers was followed for a period of six weeks. We performed the time-course experiments in the presence of foetal bovine serum and plasma derived serum. We initially tested each supplement in various concentrations, in the range from 1 to 10% (v/v), but found no significant difference in terms of VCAM-1 expression in the first week. For the experiments presented below, we chose 10% (v/v) of either serum or plasma, because, on average, body tissues are perfused with the equivalent of 5-10% of blood plasma. VCAM-1 expression is high after the first week in culture, but declines steadily in the following three weeks. After a four weeks period, VCAM-1 expression is almost lost determined by FACS analysis only (see Figure 2.4.). The cells grown in HPDS have an overall higher expression of VCAM-1 but show the same decline. There was no correlation between VCAM-1 and CD14 expression, ruling out the possibility that differences in VCAM-1 expression were a consequence of the presence of monocytes (see Figure 2.4.). From these studies it can be concluded that the characteristic VCAM-1 expression of synovial fibroblasts is maintained for a period of two weeks.

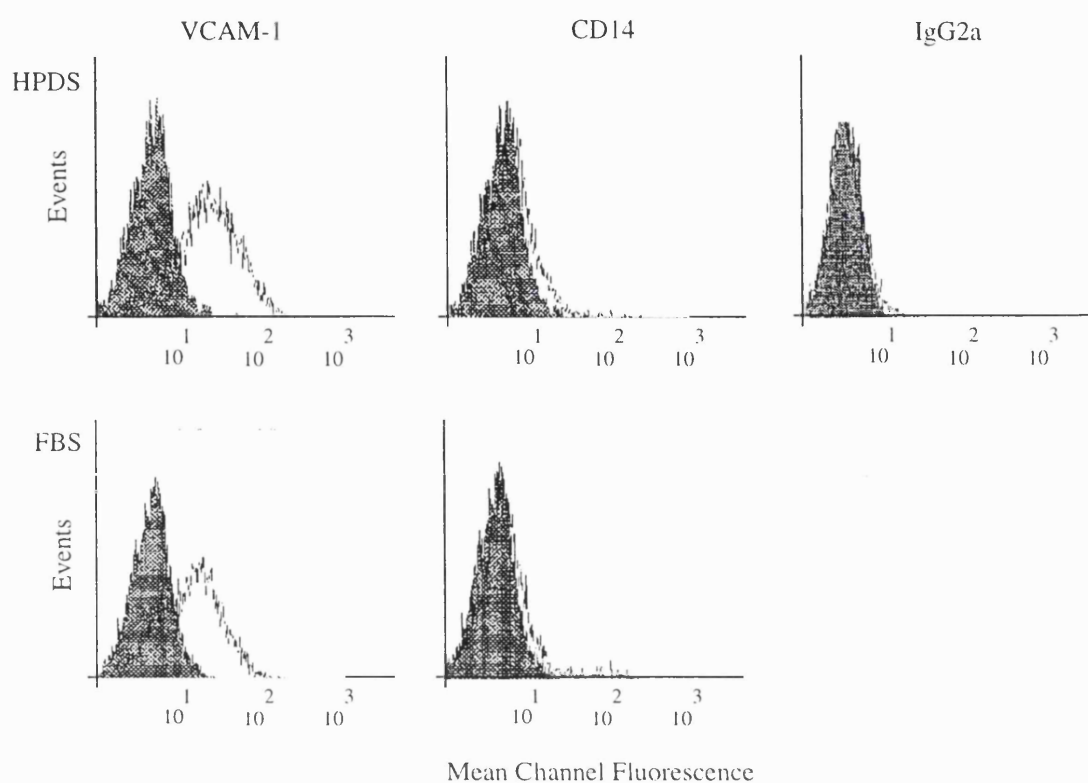


Figure 2.3.

Flow cytometric analysis of a) VCAM-1, CD14 and IgG2a sub-type (Control) cultured in 10% HPDS for 1 week and VCAM-1, CD14 cultured in 10% FBS for 1 week, determined by FACS analysis. The results shown are of cell adhesion molecules expressed on synovial fibroblasts. Anti-CD14 antibodies were used as a marker for the presence of monocytes in the culture. The cells were detached from the dish as described in Methods and Materials. The shaded area is denoted as the FACS control, i.e. cells incubated with the 2nd antibody only, and the unshaded area denotes cells incubated with the 1st and 2nd antibodies. N=5

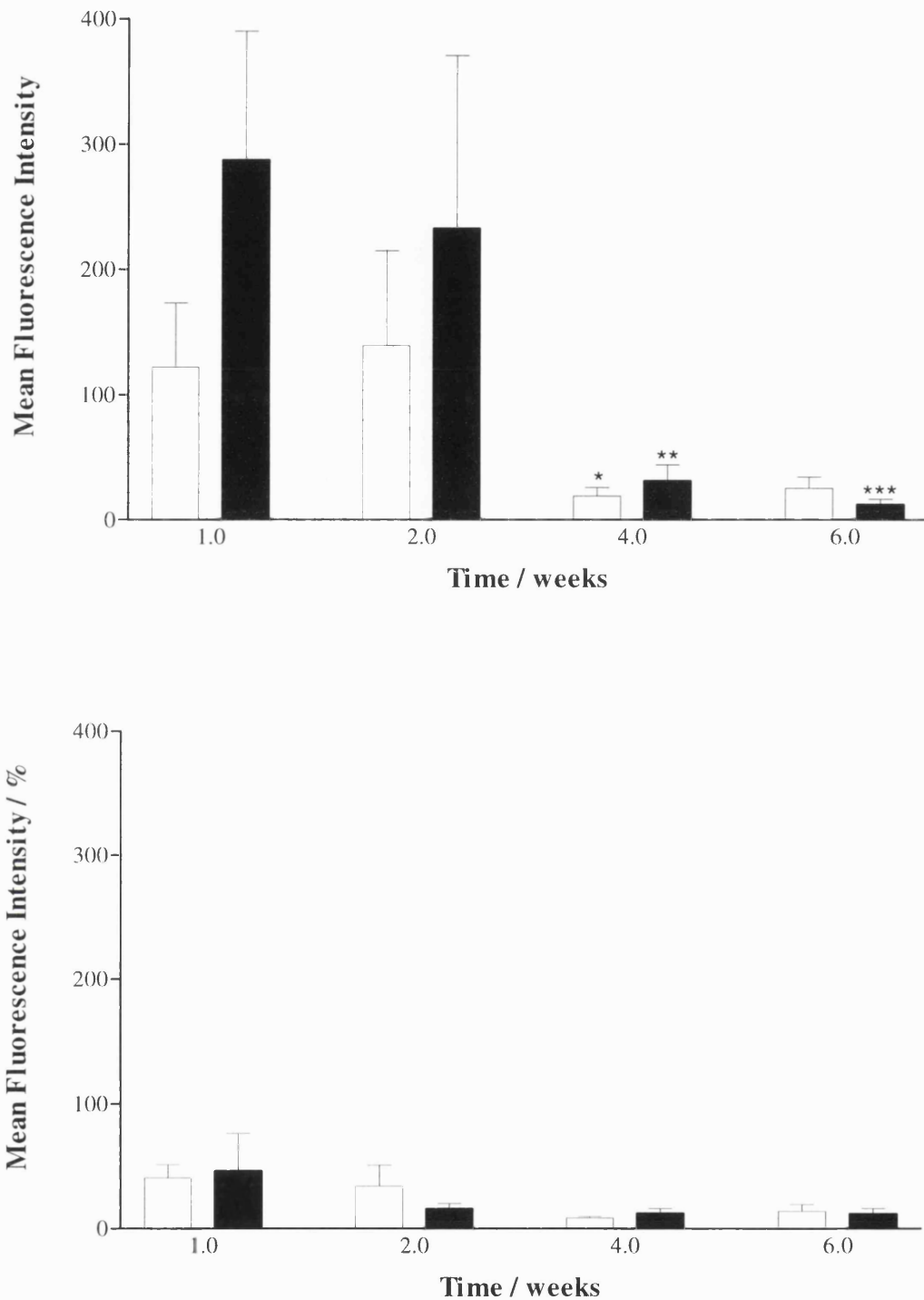


Figure 2.4.

Time course of a) VCAM-1 and b) CD14 expression on rheumatoid synovial fibroblasts in culture supplemented with 10% HPDS (full bars) and 10% FBS (empty bars). Cells were treated as described in Methods and Materials. N=4 Statistical analysis : Student paired t-test. * P = 0.05, ** P = 0.01, *** P = 0.001 (compared with the results in week 1).

2.4.3. Synovial fibroblasts in culture express both the 6 and 7 domain of VCAM-1

In order to get insight into which splice variants of VCAM-1 are expressed, we performed a semi-quantitative RT-PCR and analysed the amplified products in a Southern blot with a 6 or 7 domain-specific oligonucleotide probe. Messenger expression has been normalized by analysis of expression of GAPDH. The amplification conditions for both the VCAM-1 and GAPDH primers were optimised using cDNA from a day 0 patient sample. The annealing temperature was increased and the highest temperature (62°C) which gave the specific product was used (See Figure 2.5). Serial dilutions of the cDNA were amplified to avoid product saturation due to the primer concentration becoming limiting. The cycle number was also varied in order to determine the linear phase of the amplification (See Figure 2.6.). Controls were used in each amplification, to check that PCR and primer solutions had not become contaminated. We compared messenger expression in plasma derived serum and foetal bovine serum of the same day, with VCAM-1 cell surface expression analysed by flow cytometry (except for day 0, where we could not adequately measure VCAM-1 expression with the flow cytometer). At day 0, synovial cells express both the 6 and 7 domain and even after 2 weeks under culture conditions VCAM-1 expression can still be detected (See Figure 2.7). Synovial fibroblasts in culture, without the addition of stimuli to induce VCAM-1 expression, express messenger for both the 6 and 7 domain. Messenger expression for the 7D form was much higher than compared with the 6D form (see Fig. 2.7.). These results therefore support our FACS analysis data showing that VCAM-1 is still constitutively expressed after 2 weeks. From these results we decided to use rheumatoid synovial fibroblasts between 0 and 2 weeks old.

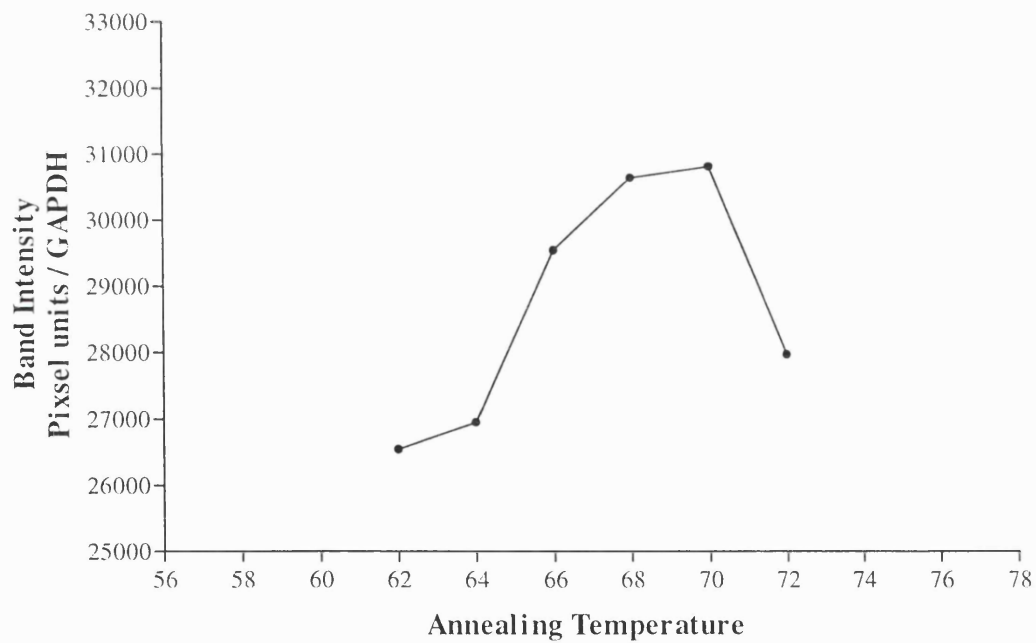
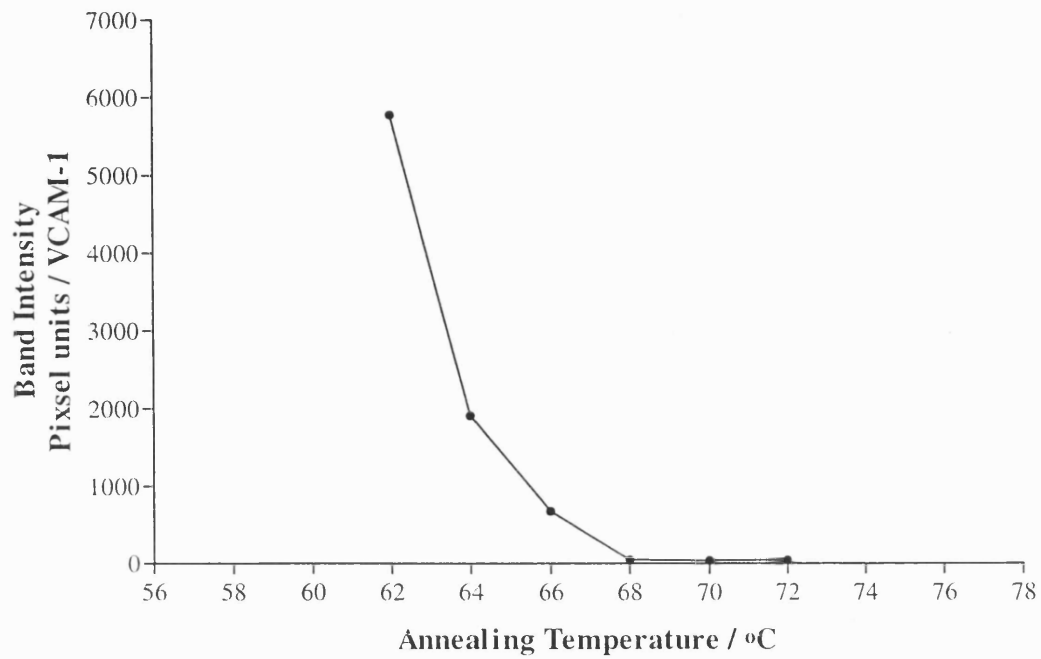


Figure 2.5. Annealing temperature curves for a) VCAM-1 b) GAPDH in the PCR reaction. Statistical analysis : Student paired t-test. N= 3 patient samples. This is a representation from one experiment.

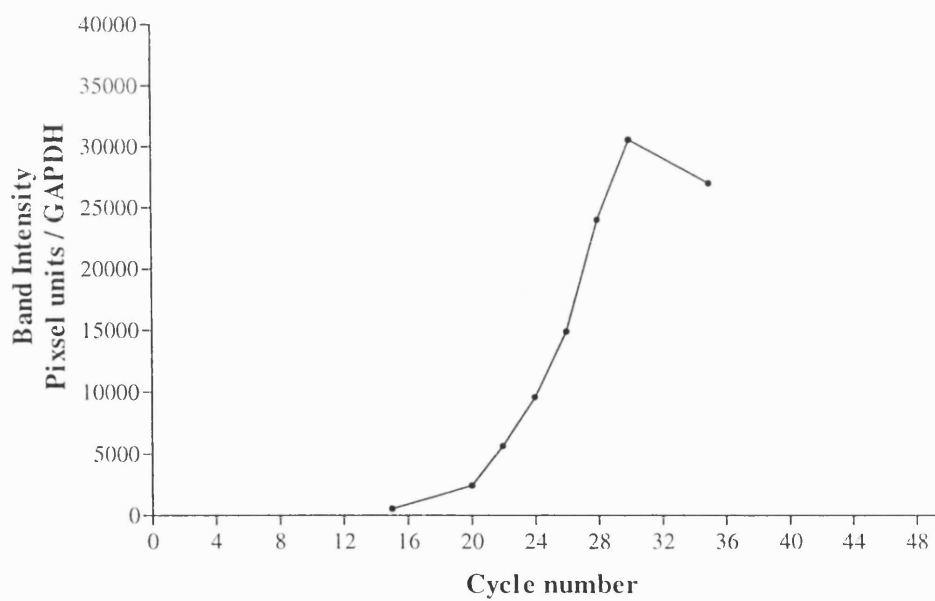
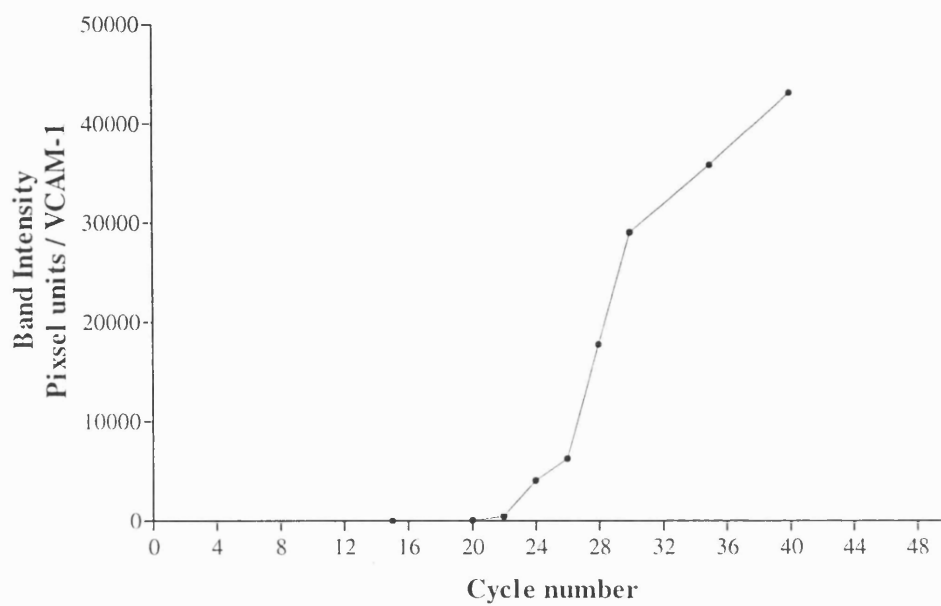


Figure 2.6. PCR cycle curve for a) VCAM-1 b) GAPDH in the PCR reaction. Statistical analysis : Student paired t-test. N=3 patient samples. This is a representation from one experiment.

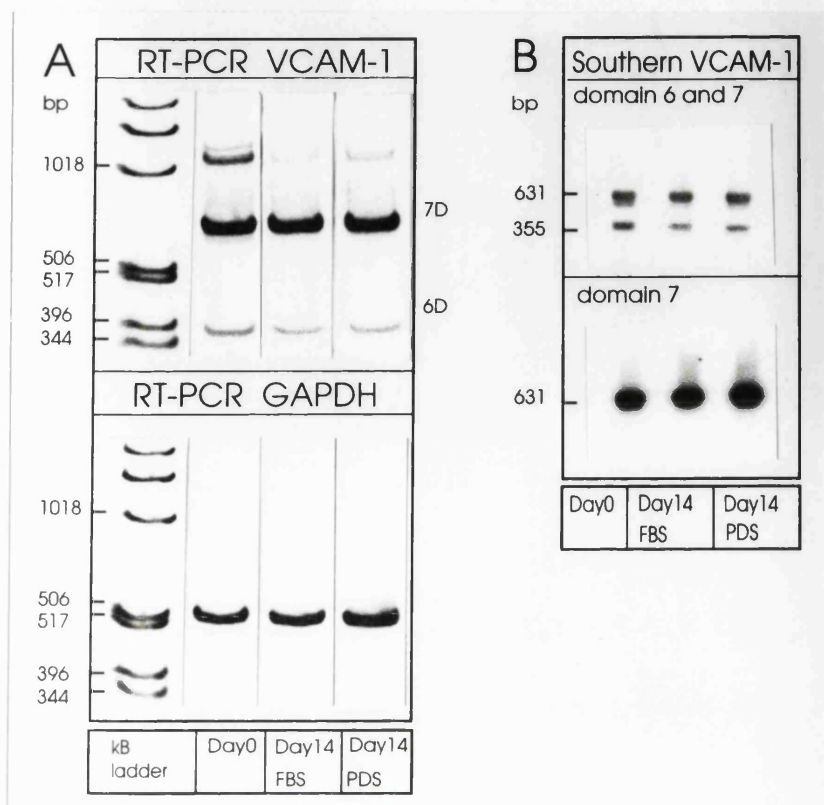


Figure 2.7.

a) RT-PCR analysis of VCAM-1 and GAPDH expression in rheumatoid synovial cells after zero or 14 days in culture with either 10% FBS or 10% HPDS. Cells were washed in PBS and lysed in GIT. The isolated total RNA was reverse transcribed to cDNA. The completed first strand cDNA was amplified by PCR, in the presence of a trace of ^{33}P -dATP, using specific primers for VCAM-1 (oligomer P259 anti-sense, oligomer VC16 sense). To normalise the VCAM-1 data, a RT-PCR was performed in parallel with primers specific for GAPDH. The PCR products for VCAM-1 6 domain = 355 bp and VCAM-1 7 domain = 631 bp.

b) Southern blot analysis of VCAM-1 6- or 7-domain expression in rheumatoid synovial cells after zero or 14 days of culture. The RT-PCR products as described above, were used for the southern blot analysis but it was blotted in the absence of ^{33}P -dATP onto nylon membrane. For the six-domain form, the same anti-sense primer was used whose centerpoint falls at the junction of domain 3 and 4 (oligo 3-4). For the seven-domain, an anti-sense unique to the sequence of domain 4 (oligo 3b) was used. The probes were ^{32}P - γ -ATP end-labelled with a T7 kinase. The above results are a representation of a typical experiment. $N=3$.

2.4.4. Collagen I up-regulates VCAM-1 expression

Because the synovial fibroblasts are embedded in an extracellular matrix containing collagen I, fibronectin and laminin, and because they are situated adjacent to the synovial space that contains high levels of hyaluronan, we tested if these extracellular matrix factors have an effect on constitutive VCAM-1 expression. Again we compared cells grown in medium supplemented with human plasma derived serum or with foetal bovine serum. Although cells grown on collagen or fibronectin express on average a higher level of VCAM-1 than cells grown on non-coated dishes, in the presence of HPDS, this difference appeared not to be statistically significant. However, when cultured in FBS, collagen significantly increased VCAM-1 expression ($p < 0.05$, $n=5$). The addition of the soluble matrix factor hyaluronan had no significant effect on VCAM-1 expression (Figure 2.8.). Furthermore, over the six weeks culture in 10% HPDS or 10% FBS VCAM-1 expression was lost (See Figure 2.8.)

2.4.5. Expression of VCAM-1 is independent of the presence of inflammatory cytokines IL-1 or TNF- α

It has been suggested that VCAM-1 expression is a consequence of the presence of inflammatory cytokines in the inflamed joint. We set out a series of experiments to evaluate cytokines expression in synovial tissue in order to determine whether these cytokines were responsible. We could not detect IL-1 β nor TNF α in the culture medium at day 6 or at day 14, suggesting that the presence of VCAM-1 is not a consequence of release of any of these mediators (see Table 2.1.).

	Day 6 HPDS	Day 14 HPDS	Day 14 FBS
TNF- α (pg/ml)	1.4	0.0	0.0
IL-1 β (pg/ml)	2.1	1.0	1.5
	n=2	n=4	n=4

Table 2.1.

Measurement of the TNF- α and IL-1 β levels in cultures of rheumatoid synovial fibroblasts. Cytokine levels were measured using an cytokine ELISA kit according to the manufacturers protocol (R&D Systems)(N=4).

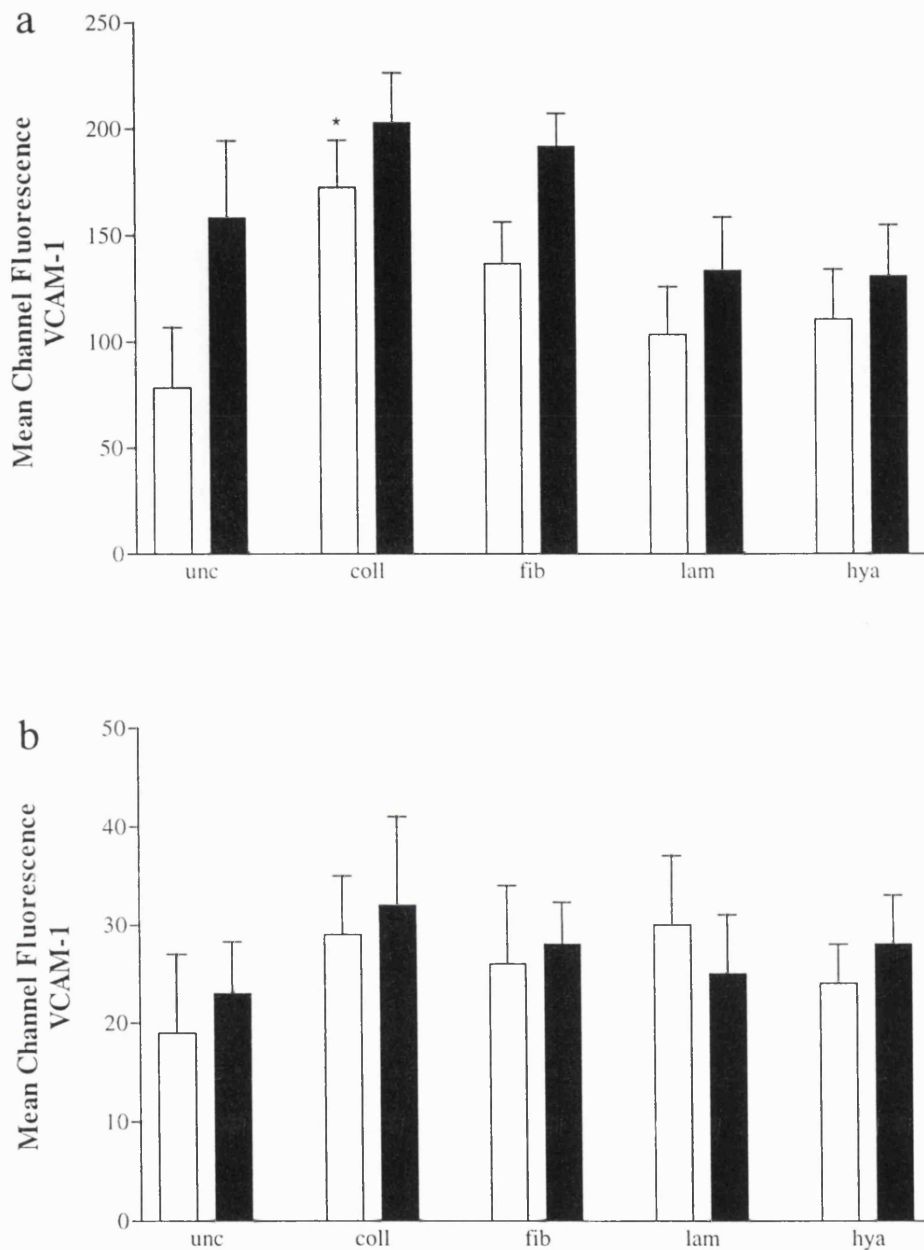


Figure 2.8. VCAM-1 expression of rheumatoid synovial cells cultured in the presence of various extracellular matrix components with 10% HPDS (full bars) or 10% FBS (empty bars). The synovial fibroblasts were cultured in dishes; unc, uncoated; precoated with coll, collagen I; fib, fibronectin; or lam, laminin. Hya, hyaluronan was added to the culture medium at 1mg/ml. Cells were harvested after a) 2 weeks and b) 6 weeks (see Methods and Materials) through an incubation in PBS with 4 mM of EDTA at 4°C. The suspension was incubated with VCAM-1 antibodies, washed with PBS/1%BSA, followed by an incubation with anti-mouse IgG conjugated with PE. N=4, Statistical analysis : student paired t-test, * indicates a significant difference with a *P* value of 0.05.

2.5. Discussion

Synovial fibroblasts in culture maintain their VCAM-1 expression for a period of two weeks, thereafter expression appears to decline, as no expression could be discerned at 4 or 6 weeks of culture. These data confirm earlier reports where VCAM-1 expression was measured using flow cytometry and it was shown that after 4 passages, which coincides with four to five weeks of cell-culture in our hands, synovial cells had no or low levels of VCAM-1 (Morales-Ducet *et al.*, 1992; Bombara *et al.*, 1993). This loss of expression could relate to a change in extracellular matrix environment. However, the presence of the extracellular matrix molecule collagen I only partly rescues the decline, as a significantly higher expression was seen after two weeks in culture in the presence of FBS but not after six weeks (see Fig. 2.8). A number of studies have indicated that VCAM-1 expression can be restored through addition of inflammatory cytokines (Morales-Ducet *et al.*, 1992; Marlor *et al.*, 1992) or through addition of T lymphocytes or monocytes (Bombara *et al.*, 1993; Blue *et al.*, 1993). We feel that this elevation cannot explain constitutive VCAM-1 expression as seen *in situ*. Firstly, we observed high levels of VCAM-1 expression, with very little detectable IL-1 β or TNF- α in the supernatant of the synovial cell culture at 1 week (see Table 2.2). Secondly, in the case of addition of inflammatory cytokines, VCAM-1 expression is only transiently up-regulated and thirdly, in non-inflamed tissue, synovial fibroblasts still express VCAM-1 (Wilkinson *et al.*, 1993). In addition, further studies by Dr. Kramer showed no VCAM-1 expression (detected by FACS analysis) was induced by 1 week old rheumatoid synovial fibroblast conditioned medium when used as culture medium for “VCAM-1 run-down” 5 week old rheumatoid synovial fibroblasts. This indicates that the low levels of TNF- α and IL-1 β detected in the early rheumatoid synovial fibroblast cultures can not induce any cell surface expression of VCAM-1.

The constitutively expressed VCAM-1 in culture, in our hands, is predominantly of the 7D form, very little 6D could be detected, in either plasma or serum. Cells isolated directly from the synovial membrane (day 0) also predominantly express the 7D form. RT-PCR analysis has been performed on TNF- α -induced VCAM-1 expression, and it was shown that the predominant splice variant is also the 7D form (Marlor *et al.*, 1992).

Our findings combined with those published on TNF- α -induced VCAM-1 expression, suggest that in synovial fibroblasts, the VCAM-1 promoter is under two forms of control. One form that regulates transient expression and one that is responsible for long term expression. The VCAM-1 promoter has been isolated from human lung fibroblasts and its regulation has been studied in endothelial and skeletal muscle cells (Iademarco *et al.*, 1992 and 1993). In endothelial cells, octamer binding sites at positions -1,641 and -288 base pairs, act as silencers and prevent VCAM-1 expression in unstimulated cells. TNF- α overcomes the negative effects of these octamers and activates the promoter through two adjacent NF- κ B binding sites, at positions -77 and -63 base pairs. It was shown that the two NF- κ B sites, which differ slightly in their sequence, form distinct complexes in gel retardation assays, suggesting that they interact with different NF- κ B-site binding proteins. The distribution of these proteins could control activity of the NF- κ B sites (Iademarco *et al.*, 1992) and explain why for example endothelial and synovial cells respond to TNF- α , but dermal fibroblasts or Jurkat cells do not (Marlor *et al.*, 1992). In myoblasts, which have a constitutive expression of VCAM-1, a position-specific enhancer located between bp -21 and -5 overrides the effect of other promoter elements (Iademarco *et al.*, 1993). It was shown that a nuclear protein binds the position-specific enhancer in myoblasts but not in endothelial cells, which indicates that the pattern of expression of this protein could control “constitutive” enhancer activity. That the long and transient expression are not regulated by the same enhancer element is also emphasised with the finding that TNF- α does not up-regulate VCAM-1 expression in muscle. It is of interest to find out whether or not synoviocytes employ a similar mode of gene regulation. However, because synovial fibroblasts in culture constitute a mixed population of cells, of both intimal and subintimal origin, a further selection, for instance on their level of VCAM-1 expression, may be necessary before enhancer element studies can be performed.

Although we may understand the TNF- α -induced expression of VCAM-1 in some detail, the question remains, however, what extracellular signal is responsible for the constitutive expression of VCAM-1 in the synoviocytes. Embryological studies may provide some clues. Site specific VCAM-1 expression is not seen on synovial intimal cells until well after joint cavity formation (Edwards *et al.*, 1994a). This indicates that VCAM-1 expression indeed may be dependent on the micro-environment of the internal

surface of the joint. In conclusion, the VCAM-1 promoter in synovial fibroblasts seems to be under dual control, a transient and constitutive. In culture the cells lose constitutive expression and collagen I only partly rescues this loss. It has been suggested that the accumulation of inflammatory cells in rheumatoid synovium may be driven by VCAM-1-dependent interactions between synovial fibroblasts and monocytes (Zvaifler *et al.*, 1994). The observation that VCAM-1 expression declines in culture indicates to us that, if these cells were to be used in co-culture experiments with mononuclear leukocyte populations (as described in Bombara *et al.*, 1993; Blue *et al.*, 1993; Dechanet *et al.*, 1995), they should be used within two weeks after removal from the synovial membrane, in order to have the unique characteristic of constitutive VCAM-1 expression still present .

Chapter Three

Human plasma inhibits VLA-4 binding to VCAM-1

3.1. Abstract

Human monocyte adherence to vascular endothelium is initiated with an LFA-1-mediated binding followed by a contribution of Mac-1 and VLA-4. Compared to foetal bovine serum, binding to endothelium was much reduced when human plasma, devoid of complement and fibrinogen, was present during the binding assay. It was left unresolved whether this reduction in binding was a consequence of changes in the monocyte or the endothelium. We studied the contribution of human plasma derived serum to the binding of U937 cells to ICAM-1, VCAM-1 and fibronectin. We cultured cells in either foetal bovine serum or human plasma derived serum and measured their binding capacity in either foetal bovine or human plasma derived serum. Cells cultured in human plasma derived serum up-regulate their VLA-4 and Mac-1 expression and show a two to three fold increase in ICAM-1, VCAM-1 or fibronectin binding. The upregulation is not a consequence of an altered sensitivity to PDBu, because the EC₅₀ data show no significant change between cells grown in human plasma derived serum or foetal bovine serum. However, the actual presence of human plasma derived serum during the binding assay, selectively inhibits the VLA-4/VCAM-1 interaction. This inhibition may explain the reduced binding of monocytes to endothelium in the presence of plasma devoid of fibrinogen. We conclude that plasma contains a factor that inhibits binding and that the local release of platelet products (as in clotting of blood) may instantaneously up-regulate the VLA-4/VCAM-1 interaction.

3.2. Introduction

Human monocytes adhere with their $\beta 1$ and $\beta 2$ integrins to the counter receptors ICAM-1 and VCAM-1 on the vascular endothelial cells, (for a review see Springer, T.A., 1994). It has been shown that the initial binding is predominantly a LFA-1-mediated event, followed by a more sustained binding involving both Mac-1 and VLA-4 (Beekhuizen *et al.*, 1990; Diamond *et al.*, 1990; Hakkert *et al.*, 1990; Meerschaert *et al.*, 1994). The relative importance of the $\beta 2$ integrins in the initial binding process may

be dependent on the source of endothelial cells, as it was shown that compared to Mac-1, LFA-1 has a greater contribution in binding to venous endothelial cells but an equal contribution in binding to arterial endothelial cells (Beekhuizen *et al.*, 1990). The physiological role of the $\beta 2$ integrins is clearly illustrated in the disease called leukocyte adhesion deficiency (LAD), where leukocytes lack expression of the common $\beta 2$ -chain (CD18) resulting in a reduced presence of neutrophils and monocytes at the site of inflammation or infection (Arnaout *et al.*, 1990). In *in vitro* binding studies it was shown that heat inactivated human plasma (also indicated as plasma derived serum or platelet free serum) greatly reduced monocyte adherence to vascular endothelium (Beekhuizen *et al.*, 1990, Doherty *et al.*, 1987). In these experiments the positive contribution of fibrinogen to the binding of monocytes (Languino *et al.*, 1993) was eliminated because of the use of heat inactivated human plasma, which is devoid of fibrinogen. It was left unresolved whether or not this effect was due to changes in the binding capacity of the monocyte or the endothelial cell. It was also left unresolved if the presence of serum during culture of the endothelial cells may have influenced the binding studies. The above mentioned findings indicated to us that, apart from fibrinogen, other plasma components may contribute to the capacity of integrins to bind to the counter receptors VCAM-1 or ICAM-1. To address whether serum or plasma have distinct effects on integrin binding, we have cultured monocytic U937 cells in foetal bovine serum or in human plasma derived serum and tested their binding to recombinant ICAM-1, VCAM-1 or fibronectin in the presence of plasma or serum. We have deliberately chosen the human monocytic U937 cell line because, unlike blood monocytes, the culture history of these cells can be standardized and in addition these cells were shown to be useful for binding studies to vascular adhesion molecules (DiCorleto *et al.*, 1985, Masumoto and Hemler, 1993). U937 cells are the neoplastic derivative of committed progenitors of monocytes (Harris and Ralph, 1985). Compared to mature monocytes, these cells lack high expression of a number of monocyte markers but which can be induced by a variety of differentiating agents (Bohbot *et al.*, 1993, Testa *et al.*, 1993). In this study we report the characterization of U937 cells grown in foetal bovine serum or human plasma derived serum. We have studied cell proliferation, expression of CD14, expression of members of the NADPH oxidase enzyme complex and cell surface expression of $\beta 1$ and $\beta 2$ integrins. We report that U937 cells grow at a similar proliferation rate in human plasma derived serum as they do in foetal bovine

serum. However, cells grown in human plasma derived serum up regulate their Mac-1 and VLA-4 expression but down regulate expression of LFA-1 and no longer express the NADPH oxidase component p40^{phox}. Up regulation of Mac-1 and VLA-4 expression is reflected in an enhanced binding of PDBu-activated U937 cells to VCAM-1 and ICAM-1 as tested in human plasma derived serum. However, when adherence is compared in the presence of foetal bovine serum or human plasma derived serum, it was shown that plasma greatly reduces VCAM-1 and fibronectin binding, without significantly affecting binding to ICAM-1. We conclude that the reduction in VLA-4 mediated binding could explain the reduction of binding of monocytes to endothelium as seen in the presence of human plasma derived serum. These findings would imply that during an inflammatory response and in the presence of chemokines, VLA-4/VCAM-1 binding could be immediately increased, without the delay in up regulation of VCAM-1 expression.

3.3. Materials and methods

3.3.1. Materials

DMEM, RPMI 1640, L-glutamine, Streptomycin sulphate, benzyl penicillinate and foetal bovine serum were obtained from Gibco BRL Life Technologies, UK. Nitrocellulose, Hybond-C super, and I¹²⁵-Protein A and ECL were obtained from Amersham. Fibronectin, a 120 kDa-chymotryptic fragment was obtained from Calbiochem Ltd.

3.3.2. Antibodies

Anti-human IgG (Fc specific), anti mouse IgG conjugated with horseradish peroxidase and PE-conjugated anti-mouse IgG antibodies were obtained from Sigma. Anti-CD14 was obtained from DAKO. Anti-ICAM-1, anti-LFA-1 β (CD18), anti-LFA-1 α , anti-Mac-1 (CD11b), anti-p150/95 (CD11c) and anti-VLA-4 (CD49) were obtained from Becton Dickinson. Anti-p40^{phox}, anti-p47^{phox} and p67^{phox} were a gift from Prof. Tony Segal and Dr. Frans Wientjes, Department of Medicine, UCL, London.

3.3.3. Human plasma derived serum preparation (HPDS)

This protocol is based on that described by Raines and Ross (1985). Human plasma has been obtained from male AB donors. Fresh citrated blood was spun at 10,000 rpm for 20 minutes at 4°C, and the supernatant (plasma) was microscopically tested for the presence of platelets. If platelets were detected, the plasma was spun again for 20 minutes at 4°C. To the plasma, 4 mM CaCl₂ was added and the plasma was left at 4°C for overnight. The fibrin clot was spun at 10,000 rpm for 20 minutes at 4°C and the plasma was then heat inactivated at 54°C for 40 min. The plasma was cleared one more time of insoluble components by centrifugation at 10,000 rpm for 20 minutes at 4°C. The plasma is aliquoted and kept at -20°C until use. This product devoid of fibrinogen or fibrin is referred to as human plasma derived serum (HPDS).

3.3.4. Cell culture

U937 cells were maintained in RPMI/10% FBS (GibcoBRL). For the comparison experiments, an aliquot of U937 cells was either cultured in DMEM/10% FBS or DMEM/10% HPDS. The cells were followed for expression of CD14, β 1 and β 2 integrins and NADPH oxidase components for a period of 60 days during which they were passaged (1/8) every other 3rd or 4th day. Density varied from 0.125 to 1.0×10^6 cells/ml during the culture. Cell samples were taken for measurement when cells were at a density in between 0.4 to 1.0×10^6 cells/ml.

3.3.5. Flow cytometry

U937 cells were centrifuged, resuspended in human AB serum for 20 minutes at 4°C, washed in ice cold PBS/1%(w/v) BSA and subsequently incubated for 40 minutes at 4°C with the following antibodies: anti-CD14 (20 μ g/ml), anti-LFA-1 β (CD18) (10 μ g/ml), anti-LFA-1 α (10 μ g/ml), anti-Mac-1 (CD11b) (10 μ g/ml), anti-p150/95 (CD11c) (10 μ g/ml), and anti-VLA-4 (5 μ g/ml) (also see Chapter 2 Methods and Materials for more detail). The cells were washed again in PBS/1% BSA and incubated with anti-mouse IgG conjugated with PE for another 40 minutes at 4°C. After 4 washes in PBS/1% BSA the cells were fixed with paraformaldehyde (1% w/v final concentration) and stored in the dark at 4°C until analysed on a Becton Dickinson flow cytometer. Data are presented as mean channel fluorescence in the presence of both antibodies minus mean channel fluorescence of second antibody only.

3.3.6. Binding assay

Recombinant VCAM-1 was obtained from Dr. A. Shock, Celltech, Slough, UK and is a chimera of domain 1-4 VCAM-1 linked to IgG Fc. A dilution curve of the recombinant VCAM-1 was done (See Figure 3.3.). Recombinant ICAM-1 was a gift from Dr. N. Hogg, ICRF, London and is a chimera of the 5 extracellular Ig domains linked to IgG Fc. The ICAM-1 was purified through a protein A column and presented to us with a concentration of 1mg/ml. A dilution curve was done to determine the final concentration to use in the assay (See Figure 3.3). The final concentration of VCAM-1 and ICAM-1 used in the assay was 100ng/ml. In addition, a concentration curve was also done for the goat anti-human IgG antibody (See Figure 3.3.) Maxisorb (Nunc) 96 well plates were coated overnight at 4°C with 20µg/ml of goat anti-human IgG (Fc specific) dissolved in 0.1M NaHCO₃ (pH~8.4). The plates were blocked by incubation with 1% BSA in PBS for 60 minutes at room temperature. VCAM-1 or ICAM-1 was added at a concentration of 100 ng/ml and left at room temperature for 60 min. Fibronectin was solubilized in PBS and coated overnight at a concentration of 100 µg/ml and blocked with 1% BSA in PBS for 60 minutes at room temperature. The plates were washed in PBS and U937 cells were added at 0.2x10⁶/200 µl after the addition of 30 nM of PDBu or an equivalent volume of DMSO as a control. In case of a PDBu response, various concentrations were added as indicated in figure legends. Non-specific binding was determined by binding to IgG/BSA-coated plates (or BSA-coated in the case of fibronectin). The cells were left at 37°C for 60 minutes then washed in PBS three times, fixed in ethanol, 5 minutes room temperature, and stained with Rose Bengal 0.25% (w/v) in PBS for 10 minutes. The plates were washed with PBS and the dye was dissolved in 100 µl of 50% ethanol/PBS. Dye intensity was read at 540 nm in a Titertek scanner. The data are presented as binding to VCAM-1 or ICAM-1 minus aspecific binding. To assess the contribution of the VLA-4 and Mac-1 in the binding to VCAM-1 or ICAM-1, U937 cells were pre-incubated with 10 µg/ml of blocking antibodies against VLA-4 or Mac-1 for 20 minutes at 4°C.

3.3.7. Western blot analysis

U937 cells were cultured in 10% HPDS or FBS, washed in PBS and solubilized in Laemmli sample buffer at 10⁶ cells/100 µl. The lysate was heated at 100°C for 5 minutes and run on a 10% acrylamide gel. Protein was blotted onto nitrocellulose and the blot

was blocked with 5% skimmed milk in Tris Buffered Saline 25mM (TBS) ; (8g NaCl, 0.2g KCl, 3g Tris base at pH 8.0). First antibody e.g. p21^{phox}, p40^{phox}, p47^{phox}, and p67^{phox} was incubated for overnight at 4°C. The blots were then washed three times with TBS and then antibody binding was detected with a 2 hr incubation with ¹²⁵I-protein A (Amersham). The blots were washed 3 more times with TBS, followed by exposure to a phosphor screen and analysis with a Fuji BioImager.

3.3.8. Statistical Analysis

Paired student's t-test (two tailed) were used to analyse results and the significance level was set at a *P* value of * 0.05; ** 0.01; *** 0.001.

3.4. Results

3.4.1. U937 cells cultured in HPDS or FBS have similar characteristics, except for the expression of p40^{phox}, which is absent in cells cultured in HPDS

To compare growth rates, U937 cells were seeded at 0.1x10⁶ cells per ml and cell numbers were determined after three days of culture. U937 cells grown in 10% HPDS showed a similar division rate as cells grown in 10% FBS, with an average population doubling time of 26 hrs. The cells remain round and non-adherent and do not express the monocyte differentiation marker CD14 (See Figure 4.7i.) and very little MSE (see Figure 4.9.) when cultured in 10% HPDS. We analysed expression of NADPH-oxidase components and found that the cells grown in FBS or HPDS, lack expression of p47^{phox} (see Figure 4.4.), p67^{phox} and p40^{phox} (see Figure 4.5.). The results in Table 3.1 indicate that U937 cells without stimulation by PDBu produce little p47^{phox}, p67^{phox} and p40^{phox}, but when PDBu stimulated their expression, is increased dramatically. In addition, the cells cultured in HPDS differ from the cells grown in FBS with respect to the expression of p40^{phox} (Figure 3.1.). We could only detect a minimal expression of p40^{phox} in U937 cell cultured in 10% HPDS, whereas cells cultured in FBS expressed a higher amount of p40^{phox}. As an internal control, similar samples were tested for the presence of p21^{phox}, the α-subunit of cytochrome b₅₅₈, which is constitutively expressed in the U937 cells (Cassatella *et al.*, 1989). Cells grown in HPDS express equal amounts of p21^{phox} compared to cells grown in FBS (Figure 3.1.).

	Background (Band Intensity / pixel units)	U937 Control /10% HPDS (Band Intensity / pixel units)	U937 + PDBu (100nM) (Band Intensity / pixel units)
p47 ^{phox}	26	38	792
p67 ^{phox}	11	15	620
p40 ^{phox}	43	51	537

Table. 3.1.

The table shows the pixel units of the radioactive band intensity results of protein expression of p47^{phox}, p67^{phox}, p40^{phox}, after Western blot analysis. These are results from a typical experiment. N=4

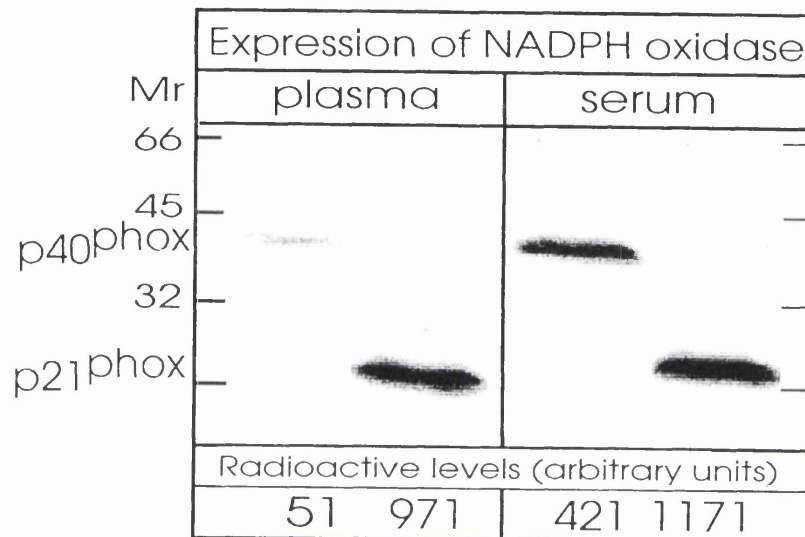


Figure 3.1.

p21^{phox} and p40^{phox} expression in U937 cells after culture in the presence of 10% FBS or 10% HPDS. Cells passaged in RPMI/10% FBS and transferred 10% HPDS and cultured for three days. The cells were washed and solubilized in Laemmli sample buffer. Protein was separated on a 10% polyacrylamide gel and p21^{phox} or p40^{phox} expression was evaluated in a Western blotting protocol with the inclusion of I¹²⁵-protein A, followed by quantitation of radioactivity using a Fuji Bas1000 Bio-Imager. "Plasma" represents human plasma derived serum (HPDS); "Serum" represents foetal bovine serum. This is a representation of a typical experiment. N=4

3.4.2. Cells cultured in HPDS have an upregulated Mac-1 and VLA-4 but down regulated LFA-1 expression

Having established that U937 can be cultured in HPDS, we next studied expression of adhesion molecules of the integrin family. These molecules are known to be involved in monocyte binding to vascular endothelium. Compared to cells grown in FBS, U937 cells grown in HPDS express a significantly higher level of Mac-1 (CD11b) and VLA-4, but a decrease was observed in the expression of LFA-1 (CD11a), although the latter one only had a low significance ($p < 0.1$) due to a high standard deviation (Figure 3.2.). We could not detect expression of p150/95 (CD11c).

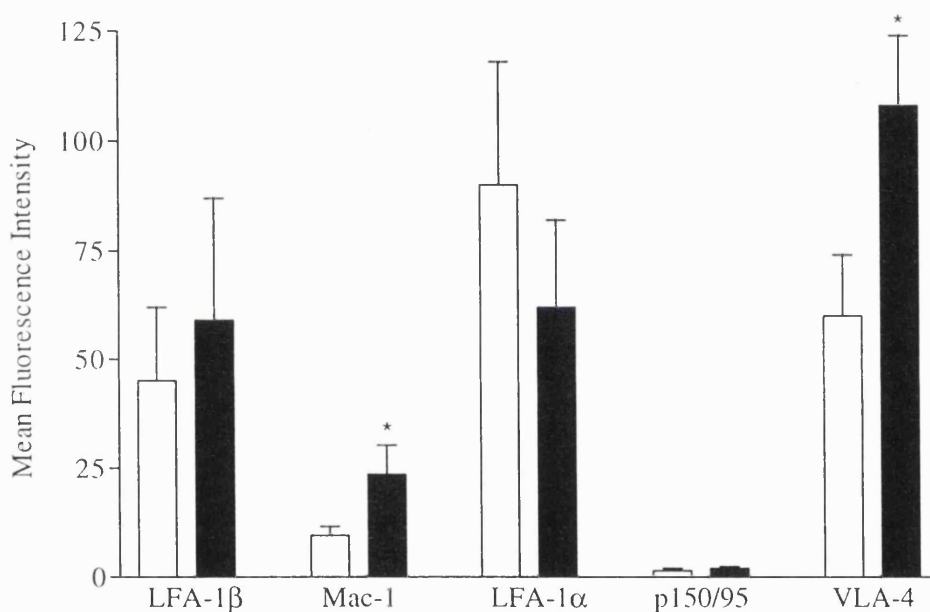


Figure 3.2.

Integrin expression on U937 cells cultured in 10% FBS (empty bars) and 10% HPDS (full bars) for 1 week. Cells were taken from a stock culture passaged in RPMI/10% FBS, and transferred to RPMI/10% HPDS. Samples were taken and assayed by FACS analysis with anti-LFA-1β (CD18), anti-Mac-1 (CD11b), anti-LFA-1α (CD11a) and anti-p150/95 (CD11c) or anti-VLA-4 (α4β1) antibodies, followed by an incubation with anti-mouse IgG conjugated with PE. Antibody binding was analysed in a Becton Dickinson FACScan.* : $P < 0.05$ indicates the significant difference between the two culture mediums of the expression of the various cell adhesion molecules analysed using the paired student t-test. N=5

Expression of the common $\beta 2$ chain (CD18) does not change, indicating that the regulation mainly takes place at the level of cell surface expression of the α -chains. We followed a time-course of integrin expression (Mac-1 and VLA-4) after changing from FBS to HPDS and found that a new expression level is obtained after three days (Table 3.2.). We also tested a range of plasma concentrations for an incubation period of three days, from 5% to 80% (v/v) and found that around 10 to 20% of HPDS a maximum change was obtained (see Table 3.2.). In none of these cases could we detect CD14 expression. In the next series of experiments we will focus on the difference between HPDS and FBS, indicated in the figures as “plasma” and “serum”.

	FBS day 3	HPDS day 1	HPDS day 2	HPDS day 3
Mac-1	8.58 \pm 3.5	9.2 \pm 2.0	12.3 \pm 3.1	23.5 \pm 6.8 *
VLA-4	62.7 \pm 10.7	58.4 \pm 12.5	121.4 \pm 23.6	122.4 \pm 25.1 *

Table 3.2. Time-course of altered integrin expression on U937 cells cultured in 10% HPDS. The data are expressed as mean channel fluorescence of an average of eight experiments. The upregulation of both Mac-1 and VLA-4 after 3 days culture in 10% HPDS is statistically significant with a $*P < 0.05$. Statistical analysis by student paired t-test. FBS, foetal bovine serum; HPDS, human plasma derived serum.

	CD14	Mac-1	VLA-4
FBS 10% (v/v)	0.92	8.5	57.9
HPDS 5% (v/v)	0.84	9.7	136.6
10%	0	21.3	125.8
20%	0	22.6	129.7
40%	0	21.4	93.8
80%	0	23.8	121.0

Table 3.3. Integrin expression at various concentrations of human plasma derived serum. The data are expressed as mean channel fluorescence of an average of two experiments. FBS, foetal bovine serum; HPDS, human plasma derived serum.

3.4.3. HPDS inhibits VLA-4 binding to VCAM-1 and fibronectin

The adhesion molecules VCAM-1 and ICAM-1, expressed on endothelial cells, are the counter receptors of the leukocyte integrins. We therefore looked at binding of U937 cells to recombinant protein under FBS and HPDS conditions. The assay was characterised by doing dilution curves for the recombinant VCAM-1, ICAM-1 and goat anti-human IgG antibody (See Figure 3.3.) used to bind the recombinant VCAM-1 or ICAM-1. Although the U937 cells express the integrins VLA-4, LFA-1 and Mac-1, they are not able to bind to either VCAM-1 or ICAM-1 without the addition of a stimulus. We used the phorbol ester PDBu at a concentration of 30 nM, which is a near optimal concentration for stimulation of binding activity. U937 cells cultured in 10%HPDS bind three fold better to either VCAM-1 ($P<0.001$) or ICAM-1 ($P<0.001$) coated dishes, compared to cells grown in 10%FBS, when assayed in FBS (Figure 3.4.). However, there is a significant decrease ($P<0.001$) in VCAM-1 binding when the actual assay is tested in the presence of HPDS (rather than FBS) whereas binding to ICAM-1 remains unaltered. The inclusion of antibodies against Mac-1 blocked binding to ICAM by 20% when cells were cultured under FBS conditions, but this was not found to be significant due to the high standard deviation values (Figure 3.5ai and Figure 3.5bi). However, when cells were grown in HPDS, binding could be reduced by 50% ($P<0.001$) when assayed in HPDS or FBS (Figure 3.5ai. and 3.5bi.). In addition, as seen above, there was no difference in ICAM-1 binding when tested in the presence of HPDS or FBS, whereas there was a significant difference in VCAM-1 binding ($P<0.001$). We could completely block adherence to VCAM-1 by inclusion of antibodies against VLA-4 under both HPDS and FBS culture conditions and in the presence HPDS (Figure 3.5aii.) or FBS (Figure 3.5bii.) during the binding assay. These findings illustrate that in the case of VCAM-1 binding, VLA-4 is the predominant molecule involved in binding, but in the case of binding to ICAM-1, the Mac-1 contribution is culture-condition dependent.

Because the CS1 region of fibronectin is another ligand of VLA-4 (Wayner *et al.* 1989) and the binding seems to occur at a similar binding site on the VLA-4 molecule (Clements *et al.*, 1994; Makarem *et al.*, 1994; May *et al.*, 1993) we assayed binding of U937 cells to fibronectin in a similar fashion as we tested binding to VCAM-1 or ICAM. When U937 cells are cultured in HPDS, a three fold increase in binding to fibronectin again can be observed ($P<0.001$) compared to cells grown in FBS. However,

but this binding is again significantly reduced by the presence of HPDS ($P<0.01$ for cells cultured in HPDS and $P<0.001$ for cells grown in FBS) during the binding assay compared to the presence of FBS (Figure 3.6.).

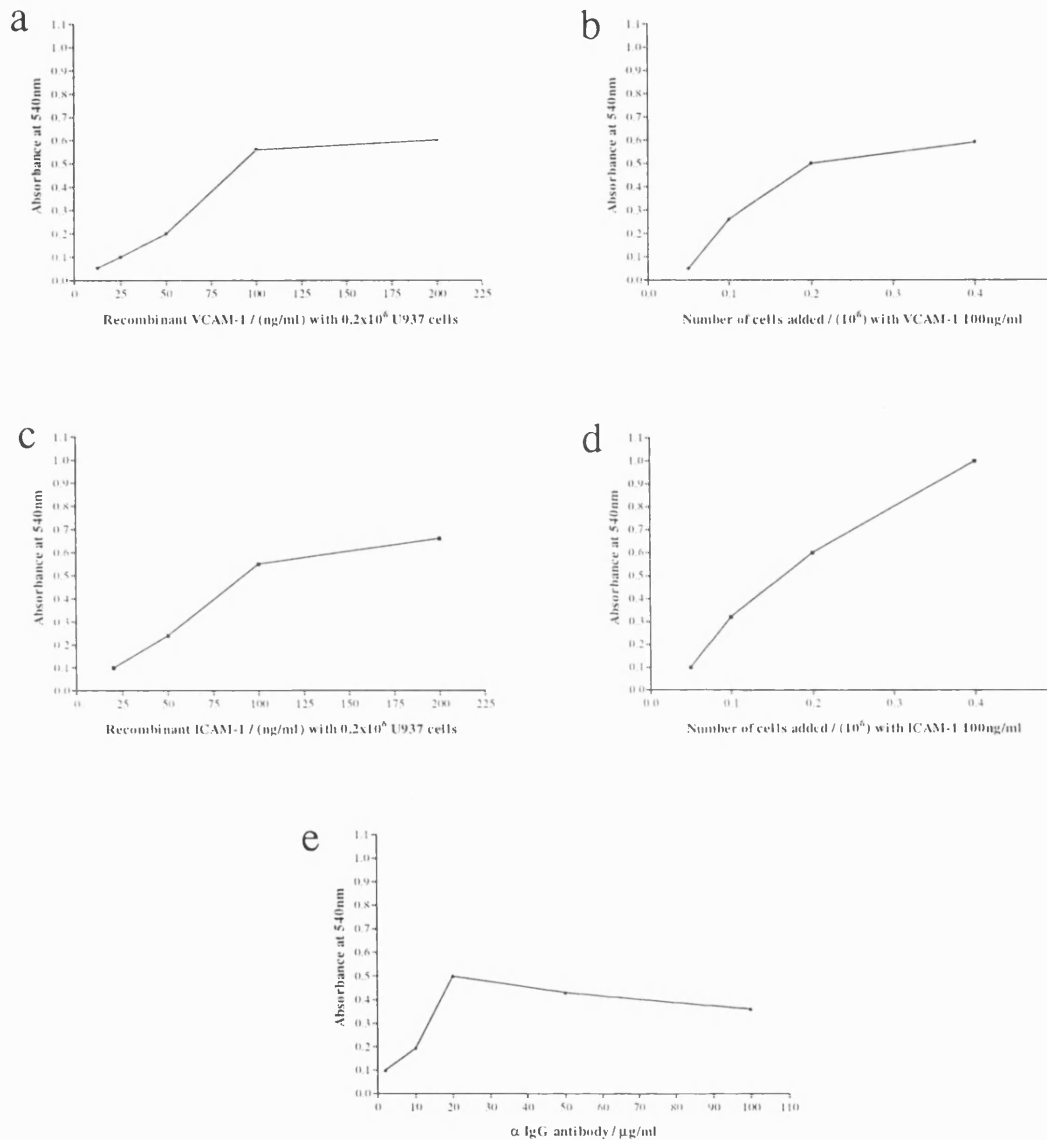


Figure 3.3.

Dilution curves for recombinant VCAM-1, ICAM-1 and IgG antibody used in the binding assay. a) and c) shows the dose curves for VCAM-1 and ICAM-1 respectively, determining the final concentration used in the assay. b) and d) shows the cell number curve for VCAM-1 and ICAM-1 respectively determining the ideal number of cells to add to 100ng/ml of recombinant protein. e) shows the dose curve for goat anti-human IgG antibody determining the concentration for coating the plates. N=2

Figure 3.4.

Comparison of cell binding to ICAM-1 and VCAM-1 of U937 cells cultured in either HPDS or FBS. Cells were passaged and cultured in 10% FBS or 10% HPDS for a minimum of three days. Each batch was separated into two and after the cells were spun and washed once in PBS, each pellet was resuspended in either HPDS or FBS. Maxisorb 96 well plates were coated overnight at 4 °C with 20mg/ml of goat anti-human IgG (Fc specific). VCAM-1 or ICAM-1 was added at a concentration of 100ng/ml and left at room temperature for 60 minutes. The plates were washed in PBS and the U937 cells were added at 0.2×10^6 /ml shortly after the addition of 30nM of PDBu. The plates were left at 37 °C for 60 minutes then washed in PBS three times, fixed in ethanol, stained with Rose Bengal 0.25% (w/v) and after washing, the dye intensity was read at 540nm in a titertek scan. PBS; phosphate buffered saline, PDBu ; phorbol 12,13-dibutyrate, “plasma” represents human plasma derived serum, “serum” represents foetal bovine serum. Statistical analysis ; paired student t-test, ** indicates a significant difference of $P < 0.01$. N=4.

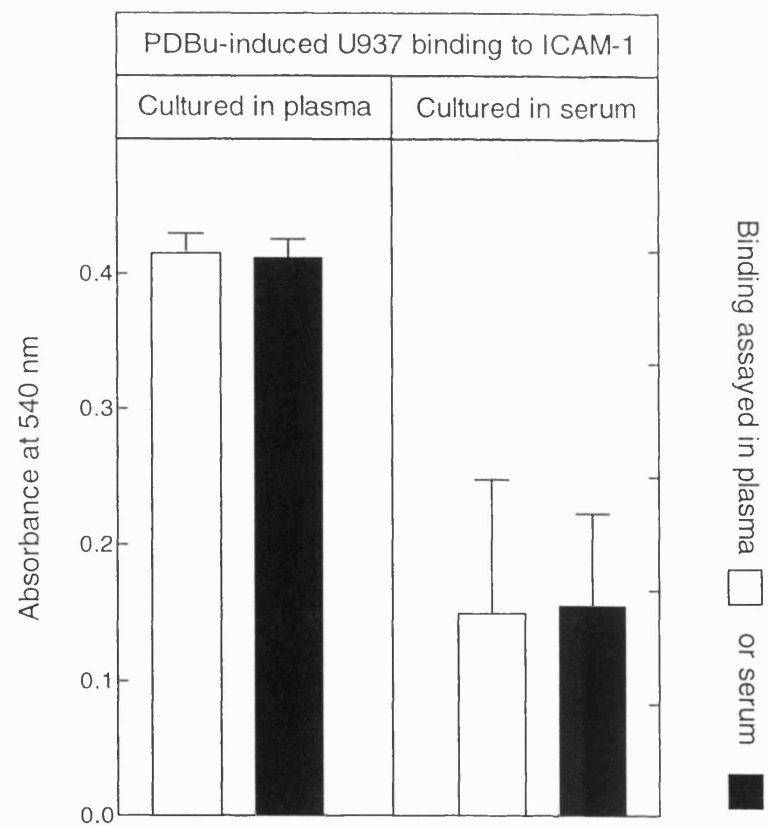
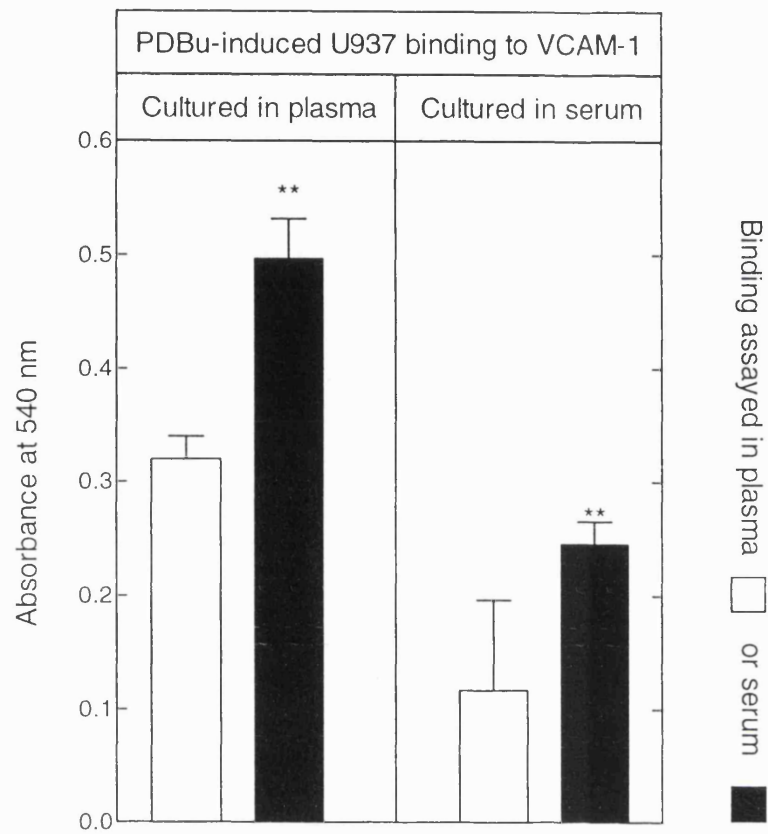
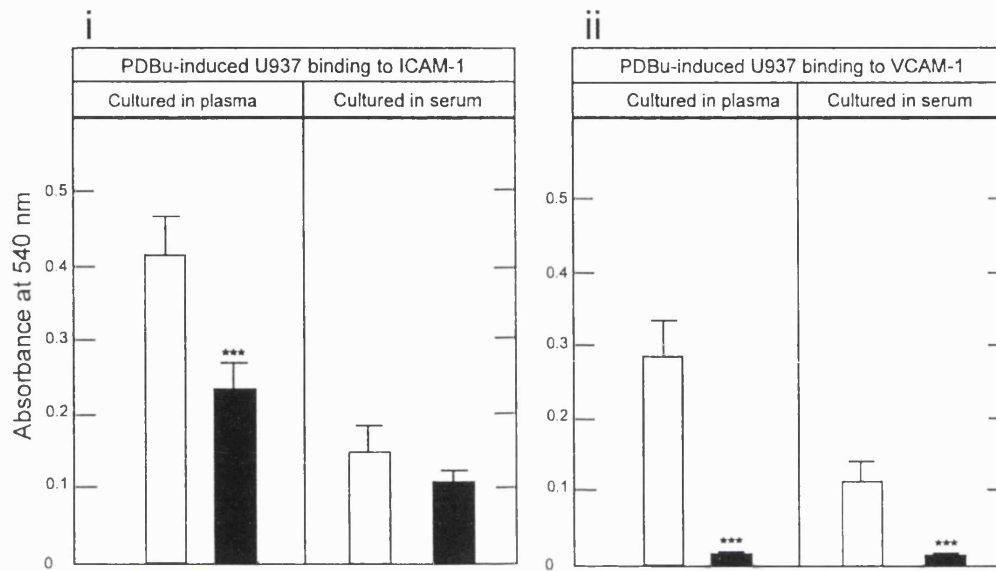


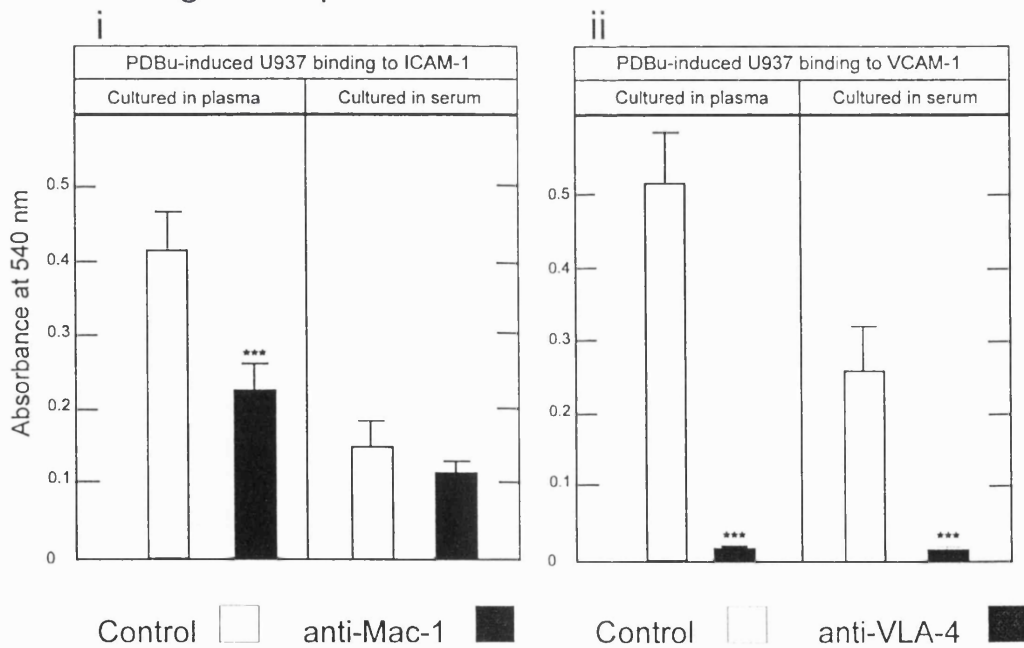
Figure 3.5.

Inhibition of U937 adherence to ICAM-1 or VCAM-1 by inclusion of antibodies against Mac-1 or VLA-4 respectively. Cells were prepared exactly as described in Figure 3.4, except that after they had been resuspended in either HPDS (Figure 3.5ai and ii; i.e. assayed in HPDS), or FBS (Figure 3.5bi and ii; i.e. assayed in FBS), they were put at 4°C and 10µg/ml of blocking anti-Mac-1 or anti-VLA-4 antibodies were added 20 minutes prior to the onset of the adhesion assay. Cells were added at $0.2 \times 10^6 / 200 \mu\text{l}$ shortly after the addition of 30 nM of PDBu. The plates were left at 37°C for 60 minutes then washed in PBS three times, fixed in ethanol, stained with Rose Bengal 0.25% (w/v) and after washing, the dye intensity was read at 540 nm in a titertek scan. PBS, phosphate buffered saline; PDBu, phorbol 12,13-dibutyrate; “plasma” represents human plasma derived serum (HPDS); “serum” represents foetal bovine serum (FBS). Statistical analysis by student paired t-test *** indicates a significant difference of $P < 0.001$. N=4

A Binding in the presence of plasma



B Binding in the presence of serum



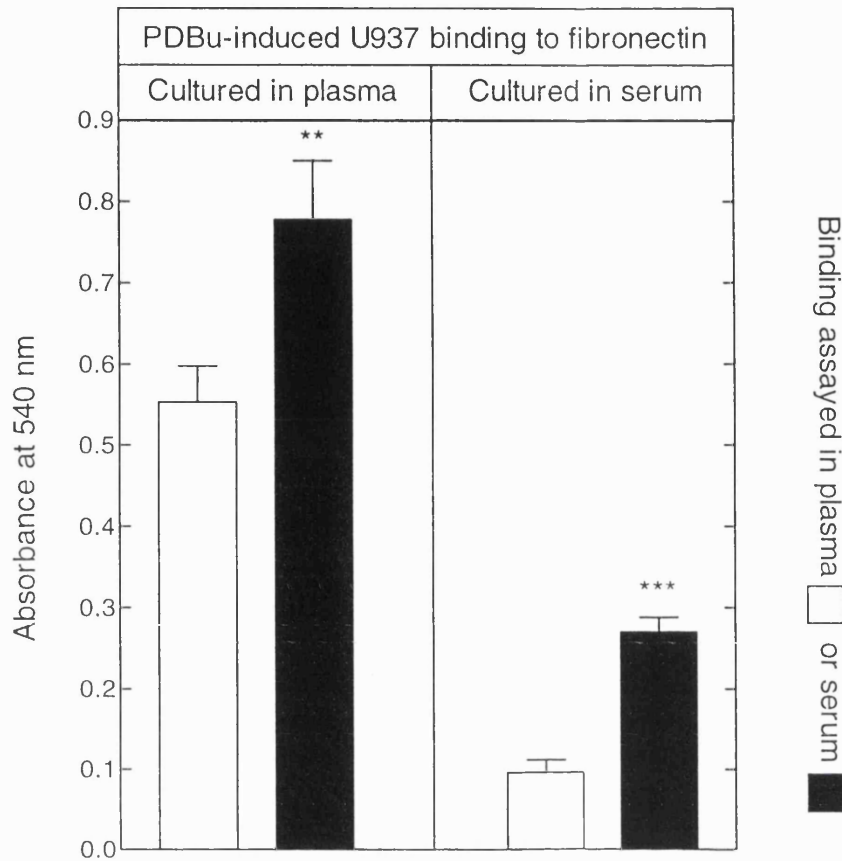


Figure 3.6.

Comparison of binding to fibronectin of U937 cells cultured in either 10%HPDS or 10%FBS. Cells were passaged and cultured in either HPDS or FBS for a minimum of three days. Each batch was separated into two and after the cells were spun and washed once in PBS, each pellet was resuspended in either HPDS or FBS. Maxisorb 96 well plates were coated overnight at 4°C with 100 µg/ml of fibronectin 120 kDa chymotrypsin proteolytic fragment followed by a 60 minutes blocking with 1% BSA in PBS. The plates were washed in PBS and U937 cells were added at $0.2 \times 10^6/200 \mu\text{l}$ shortly after the addition of 30nM of PDBu. The plates were left at 37°C for 60 minutes then washed in PBS three times, fixed in ethanol, stained with Rose Bengal 0.25% (w/v) and after washing, the dye intensity was read at 540 nm in a titertek scan. PBS, phosphate buffered saline; PDBu, phorbol 12,13-dibutyrate; “Plasma” represents human plasma derived serum (HPDS); “serum” represents foetal bovine serum (FBS). Statistical analysis by student paired t-test ** indicates a significant difference of $P < 0.01$ and *** $P < 0.001$. N=4

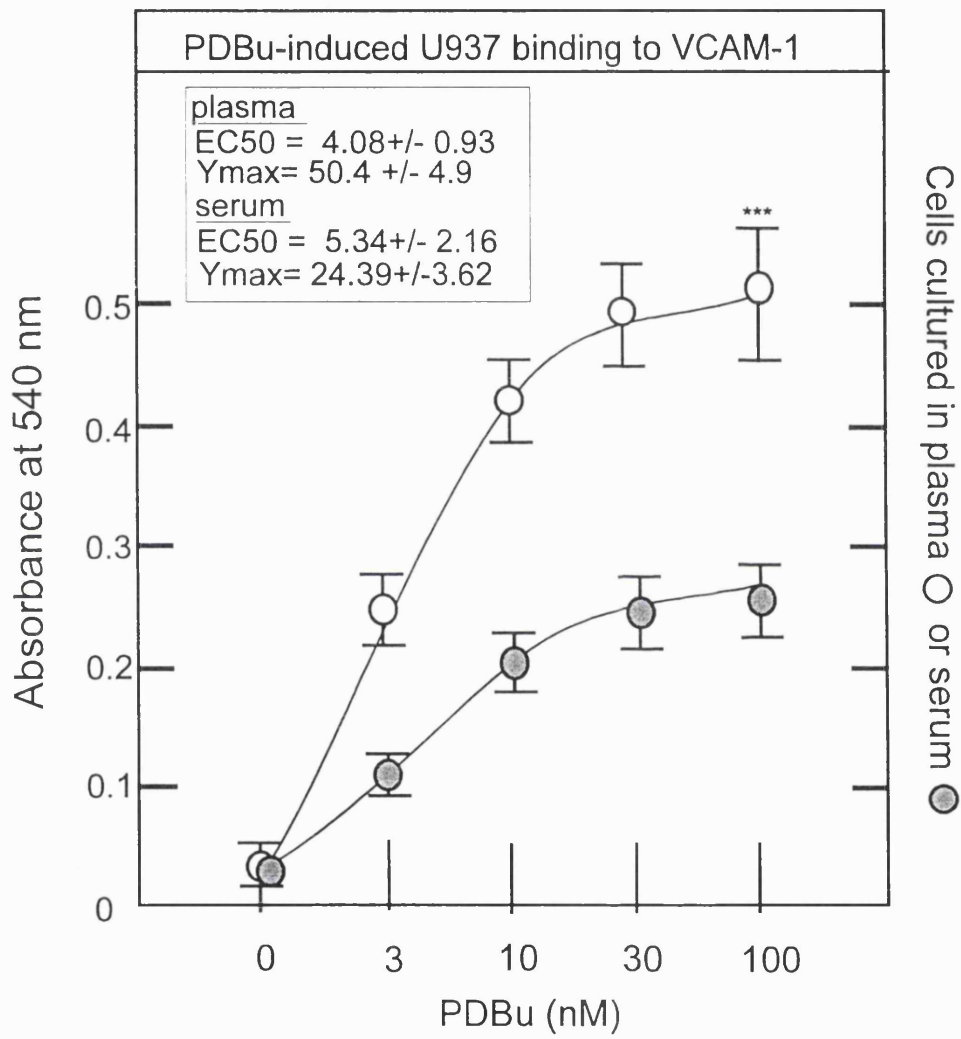
3.4.4. Increased binding to ICAM-1 or VCAM-1 is not a consequence of an altered activation state of the HPDS-cultured U937 cells

The increase in binding of cells cultured in HPDS could be a consequence of a partly activation of the U937 cell by a HPDS-induced component involved in activation of the integrins. To find out if the increased binding was a consequence of an altered cell activation pattern, we performed a PDBu dose response curve for the binding to VCAM-1 or ICAM-1 measured in the presence of FBS. We reasoned that if the cells were in some way activated, lower doses of PDBu would be needed to reach maximal binding. Cells cultured in HPDS require the same concentration of PDBu for maximal stimulation compared to cells grown in FBS when binding was measured in the presence of FBS (Figure 3.7.). The curves have been analysed in a curve fitting programme using the Hill equation (cv-fit programme from Prof. David Colquhoun, Department of Pharmacology, University College London, UK). There was no significant difference between the EC50 values, but there was a significant difference in the maximal binding (Ymax); $P < 0.001$ between HPDS and FBS for VCAM-1 and $P < 0.01$ for ICAM-1 binding. We take these data to mean that cells cultured in HPDS are not in some way activated but nevertheless have a higher capacity to bind to VCAM-1 or ICAM-1 (Figure 3.7.). In addition we tested activation of binding by Mn^{2+} , a mode of activation which is believed to be independent of inside-out signaling events. Stimulation of binding by Mn^{2+} showed the same difference between HPDS- and FBS-cultured cells as seen with PDBu (Figure 3.8.). These findings, together with the observation that there is absolutely no binding in the absence of PDBu, exclude the possibility that U937 cells grown in HPDS are somehow activated and therefore bind better to VCAM-1 or ICAM-1.

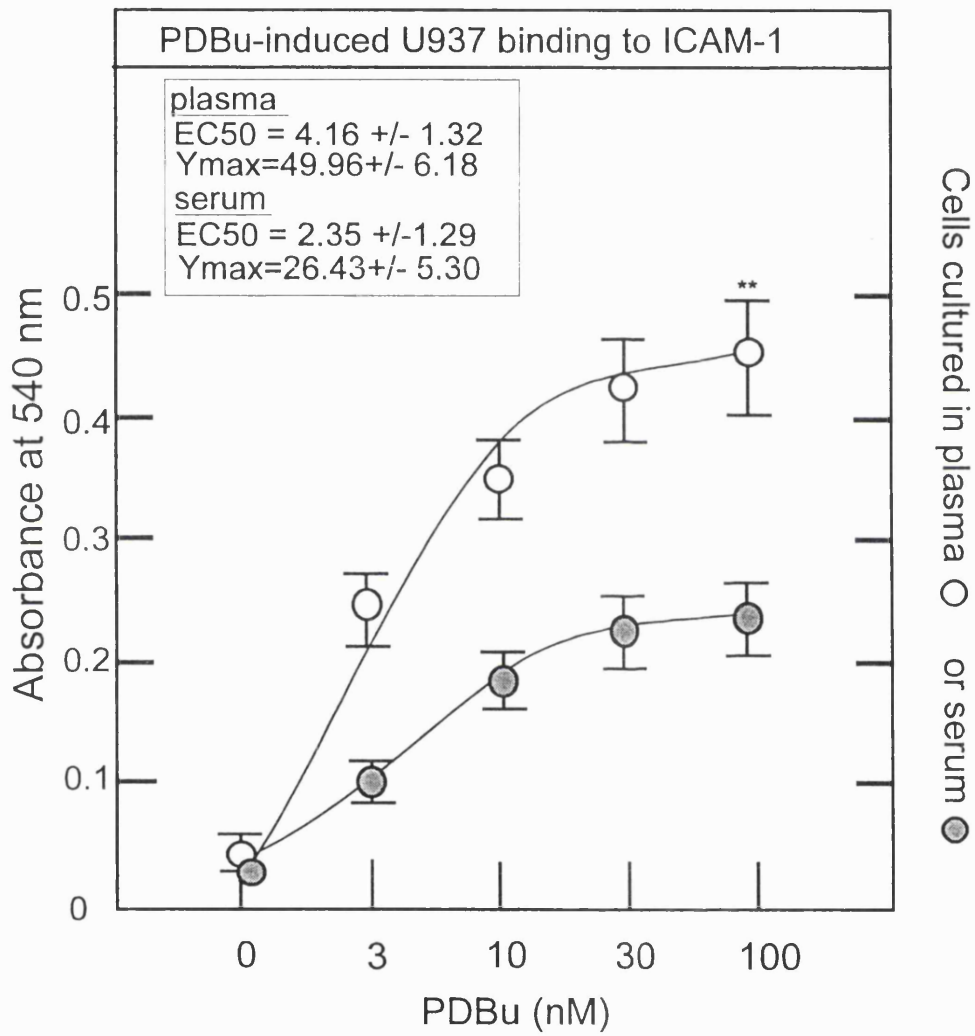
Figure 3.7.

Dose response of PDBu-induced binding to a) VCAM-1 or b) ICAM-1 of U937 cells cultured in either HPDS or FBS. Cells were passaged and cultured in either HPDS or FBS for a minimum of three days. Each batch was spun and washed once in PBS, and both pellets were resuspended in FBS. Maxisorb 96 well plates were coated overnight at 4°C with 20µg/ml of goat anti-human IgG (Fc specific). VCAM-1 or ICAM-1 was added at a concentration of 100 ng/ml and left at room temperature for 60 min. The plates were washed in PBS and U937 cells were added at $0.2 \times 10^6/200 \mu\text{l}$ shortly after the addition of PDBu at concentrations as indicated. The plates were left at 37°C for 60 minutes then washed in PBS three times, fixed in ethanol, stained with Rose Bengal 0.25% (w/v) and after washing, the dye intensity was read at 540 nm in a titertek scan. The data were analyzed using the Hill equation. Ymax depicts maximal binding and EC50 depicts that concentration of PDBu that gives half maximal binding. PBS, phosphate buffered saline; PDBu, phorbol 12,13-dibutyrate; “Plasma” represents human plasma derived serum (HPDS); “serum” represents foetal bovine serum (FBS). Statistical analysis ; paired student t-test ** indicates the significant difference of $P < 0.01$; *** $P < .001$. N=3

a)



b)



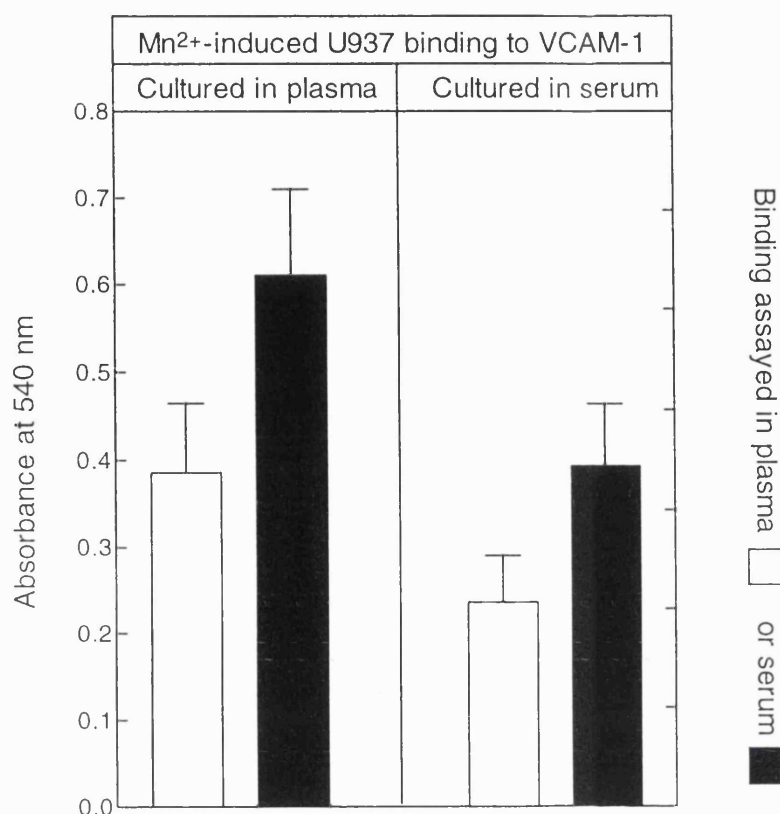


Figure 3.8.

Comparison of Mn^{2+} -induced binding to VCAM-1 of U937 cells cultured in either HPDS or FBS. Cells were passaged and cultured in either HPDS or FBS for a minimum of three days. Each batch was separated into two and after the cells were spun and washed once in PBS, each pellet was resuspended in HPDS or FBS. Maxisorb 96 well plates were coated overnight at 4°C with $20\mu\text{g/ml}$ of goat anti-human IgG (Fc specific). VCAM-1 was added at a concentration of 100 ng/ml and left at room temperature for 60 min. The plates were washed in PBS and U937 cells were added at $0.2 \times 10^6/200\text{ }\mu\text{l}$ shortly after the addition of 1 mM of Mn^{2+} . The plates were left at 37°C for 60 minutes then washed in PBS three times, fixed in ethanol, stained with Rose Bengal 0.25% (w/v) and after washing, the dye intensity was read at 540 nm in a titertek scan. PBS, phosphate buffered saline; "Plasma" represents human plasma derived serum (HPDS); "serum" represents foetal bovine serum (FBS). Statistical analysis ; paired student t-test
 * indicates a significant difference of $P < 0.05$ analysed by student paired t-test. $N=4$.

3.4.5. The presence of HPDS during binding inhibits inside-out signaling and maximum binding of the U937 cells to VCAM-1

We next compared PDBu requirements for cells that were cultured in HPDS and where binding was studied in the presence of HPDS or FBS. VCAM-1 binding in the presence of HPDS is significantly reduced by 53% ($P < 0.05$), and now a clear shift in the EC50 can be discerned (Figure 3.9.). VCAM-1 binding measured in the presence of HPDS requires higher concentrations of PDBu, the EC50 value shifts from 2.54 to 15.6 nM. However, the Hill equation also estimates a difference in maximum binding (Y_{max}). We tested Mn^{2+} -induced VCAM-1 binding and found that under HPDS assay conditions in the presence of 1 mM Mn^{2+} , a 40% inhibition was observed. These findings present evidence that the inhibition of VCAM-1 binding in the presence of HPDS is twofold; 1) a reduction in sensitivity to the stimulus PDBu and 2) a reduction in maximal binding.

3.4.6. Comparison between human serum and human plasma derived serum

We next compared binding to VCAM-1 in the presence of human serum or human plasma derived serum to find out whether the same differences apply. The possibility existed that foetal bovine serum had a stimulatory action which is not present in human serum. We therefore prepared human serum and tested binding. Binding in the presence of human serum does not differ from that in the presence of foetal bovine serum (See Figure 3.10.) but again a great inhibition is seen in the presence of human plasma derived serum. Human serum contains factors that eliminate the inhibitory action of plasma.

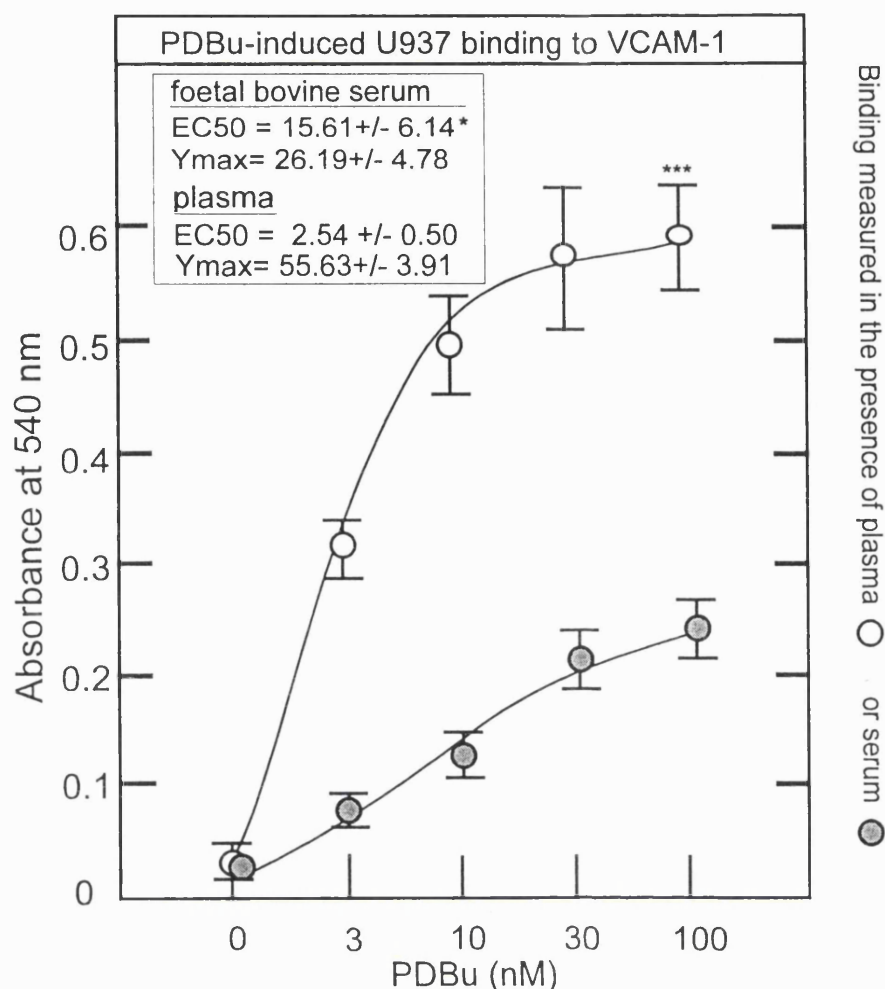


Figure 3.9. Dose response of PDBu-induced binding to VCAM-1 of U937 cells in the presence of HPDS or FBS. Cells were passaged and cultured in HPDS for a minimum of three days. Each batch was spun and washed once in PBS, and the pellet was resuspended in HPDS or FBS. Maxisorb 96 well plates were coated overnight at 4°C with 20 µg/ml of goat anti-human IgG (Fc specific). VCAM-1 or ICAM-1 was added at a concentration of 100 ng/ml and left at room temperature for 60 min. The plates were washed in PBS and U937 cells were added at $0.2 \times 10^6/200 \mu\text{l}$ shortly after the addition of PDBu at concentrations as indicated. The plates were left at 37°C for 60 minutes then washed in PBS three times, fixed in ethanol, stained with Rose Bengal 0.25% (w/v) and after washing, the dye intensity was read at 540 nm in a titertek scan. The data were analyzed using the Hill equation. Ymax depicts maximal binding and EC50 depicts that concentration of PDBu that gives half maximal binding. PBS, phosphate buffered saline; PDBu, phorbol 12,13-dibutyrate; “Plasma” represents human plasma derived serum (HPDS); “serum” represents fetal bovine serum (FBS). * indicates a significant difference of $P < 0.05$ and *** $P < 0.001$, analysed by paired student t-test. N=4.

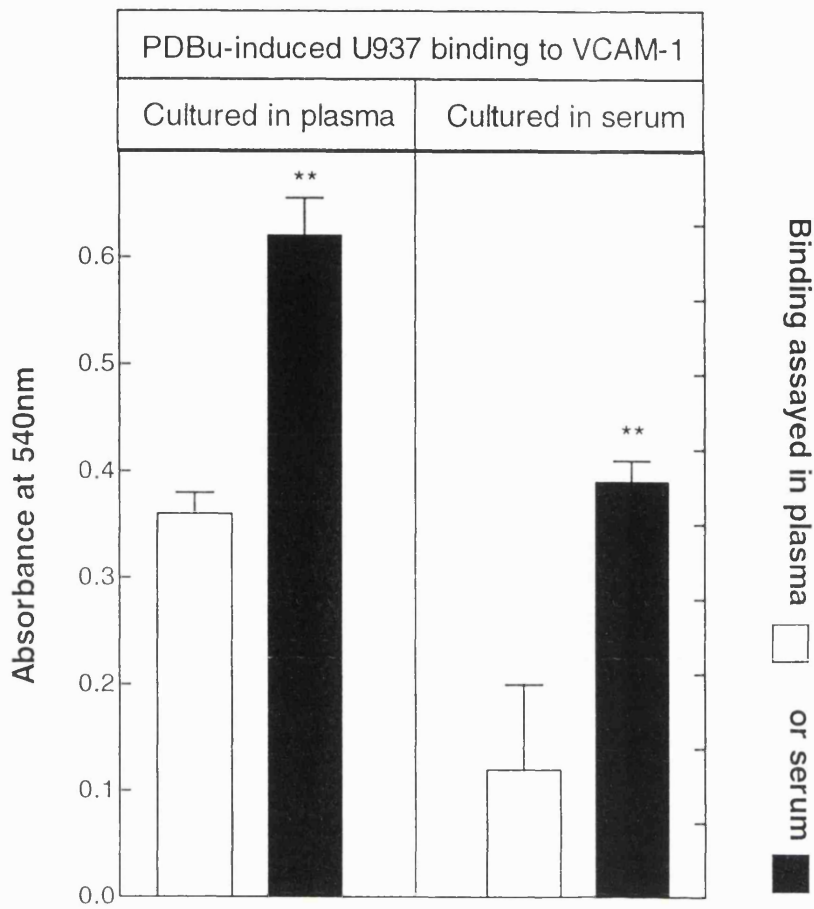


Figure 3.10. Cell binding of U937 to recombinant VCAM-1. U937 cells were cultured in either 10% HPDS (plasma) or 10% Human Serum (serum). Statistical analysis ; paired student t-test ** indicates the significant difference of $P < 0.01$, $N=3$

3.5. Discussion

We have established that U937 cells can be grown in 10% human HPDS for a period of up to two months, with a doubling rate which is similar to cells grown in 10% FBS, a medium-supplement used under standard culture conditions. With respect to expression of differentiation markers (CD14 and NADPH oxidase components), the cells cultured in HPDS display a similar expression pattern as cells grown in FBS except for a markedly lower expression of the NADPH oxidase component, p40^{phox}. Which serum component(s) have a role in regulation of expression of the p40^{phox}, and whether or not this is important in regulation of the respiratory burst activity of neutrophils or monocytes, remains to be studied. Although p40^{phox} is found associated with the NADPH oxidase complex after stimulation of peripheral blood neutrophils or monocytes (Wientjes *et al.*, 1993), it so far has not been elucidated what role it has in the NADPH oxidase complex. The p40^{phox} protein is not necessary for, nor has it any effect on, the NADPH oxidase activity in an *in vitro* reconstitution assay, (Dr. F. Wientjes and Prof. A.W. Segal, unpublished observation).

When U937 cells are cultured in the presence of HPDS, they show a marked increase in binding to ICAM-1, VCAM-1 or fibronectin, when compared to cells grown in FBS. This difference is manifest independent of whether or not adherence is measured in FBS or in HPDS. The experiments with Mn²⁺ and PDBu clearly indicate that the U937 cells cultured in HPDS are not somehow activated, an activation that possibly could synergize with the PDBu-induced inside-out activation of the integrins. Because if they were, there should be a lower requirement for PDBu to induce adhesiveness. The EC50 values for cells cultured in either HPDS or FBS does not differ significantly. In addition, in the presence of 1 mM Mn²⁺, a way of maximum activation of integrins where inside-out signaling is not required, we in principle should have observed a difference in binding. The increased binding to VCAM-1 or fibronectin could in part be explained by an increase in VLA-4 expression but the level of expression is not a measure for the binding capacity *per se*. It has been shown that different cells have a wide range of constitutive activities of VLA-4. There is no clear biochemical understanding of the cellular processes by which $\beta 1$ integrins are produced with different constitutive activities (Masumoto and Hemler, 1993). Thus the possibility exists that culture

conditions have an effect on the activation state of the integrins themselves, independent of inside-out signaling. The different activation states would then result in a higher degree of binding when additional stimuli are added such as PDBu or Mn^{2+} . Recently, Diamond and Springer have shown that the percentage of active Mac-1, as detected by an “activation-specific antibody”, is only in the range of 10% of the total number of Mac-1 molecules expressed on the cell surface (Diamond and Springer, 1993). Therefore, in theory, the possibility exists that U937 cells cultured in HPDS have a higher percentage of integrins involved in binding to VCAM-1, which may in turn explain the increased binding. We are not in the position to test this hypothesis. It would be of interest to find out whether or not the two culture conditions greatly affect the percentage of integrin molecules engaged in binding. The observation that ICAM-1 binding is increased could be explained by the increased contribution of Mac-1 in the binding process, from 20 to 50% as indicated by blocking of Mac-1 binding by antibodies. As shown, the total CD18 expression is not changed, suggesting that the total number of $\beta 2$ integrins available for binding has not changed. This could be taken to mean that the presence of LFA-1 alone may not give as high a binding as the combination of LFA-1 and Mac-1. It has been suggested that the early contact between monocytes and endothelial cells is primarily a LFA-1-mediated event. Subsequently, with time, an increased contribution of Mac-1 is seen (Hakkert *et al.*, 1990; Meerschaert *et al.*, 1994). Together they may form a more stable bond which, measured over a one hour incubation period, could result in a higher percentage of cells bound to the ICAM-1. Alternatively, of course, it could be that the cells grown in plasma have a higher percentage of $\beta 2$ integrins actively participating in the binding to ICAM-1, in analogy with the findings of Diamond and Springer (1993).

When U937 cells adherence is measured in the presence of HPDS, a marked reduction in binding of VLA-4 to VCAM-1 and fibronectin is observed. This inhibitory action is independent of whether the cells were cultured in HPDS or FBS. Interestingly, HPDS had no effect on ICAM-1 binding. The inhibitory action of HPDS is unlikely a consequence of a possible difference in the free Ca^{2+} concentration because Ca^{2+} had no effect on binding of U937 cells to VCAM-1 (unpublished observation and Masumoto and Hemler, 1993). The inhibitory action of HPDS on VCAM-1 binding is at the level of inhibition of an inside-out signaling event, because in the presence of HPDS, the

EC50 value for PDBu shifts greatly to a higher concentration. However, HPDS also has an effect on maximum binding, because inhibition was still observed in the presence of high concentrations of PDBu and Mn^{2+} .

In conclusion HPDS has two effects on U937 cells. One, when present during cell culture, it greatly up-regulates the capacity of these cells to bind to ICAM-1, VCAM-1 or fibronectin. Two, when present during the binding assay itself, it inhibits binding to VCAM-1 and fibronectin but leaves binding to ICAM-1 unchanged. Human serum gave similar data, with respect to VCAM-1 binding, as foetal bovine serum. An important functional implication of this latter finding is that local high levels of inflammatory mediators released from platelets during for instance an infection or wounding, may eliminate the inhibitory action of plasma, thereby inducing an immediate upregulation of VLA-4/VCAM-1-mediated binding of monocytes to endothelium. Together with the presence of chemokines, this immediate upregulation would allow increased adherence to - and subsequent migration through - the endothelium, without a necessary upregulation of VCAM-1 expression. At present we have no indication about the identity of the plasma factor(s) that has this specific inhibitory effect on VCAM-1 binding.

Chapter Four

Development of a co-culture system comprising synovial fibroblasts and U937 cells

4.1. Summary

Based on the knowledge of integrin expression already obtained from previous studies on interactions between endothelial cells and monocytes we wanted to see if there was any difference in interaction with rheumatoid synovial fibroblasts. Therefore after characterising both the rheumatoid synovial fibroblasts and U937 cells when cultured in human plasma derived serum, we were able to subsequently develop a co-culture system. This allowed us to investigate the cell-cell interactions between the synovial fibroblasts and U937 cells, by performing binding assays, to see which integrins were involved. In addition, we were able to analyse the differentiation state of the U937 cells to see if the culture conditions caused maturation towards monocytes. We concluded that the U937 cells did differentiate towards monocytes after three days in co-culture with rheumatoid synovial fibroblasts and that differentiation was mediated by soluble factors released in to the culture medium, rather than by adherence to the synovial fibroblasts.

4.2. Introduction

The rheumatoid chronic inflammatory response has features of a cellular immune response and is characterised by the infiltration and accumulation of monocytes and lymphocytes. In addition, a large increase in the number of proliferating fibroblasts is observed, amongst which, the VCAM-1 expressing intimal synovial fibroblast. There is evidence that the monocytes in the rheumatoid synovium contribute significantly to the pathology of rheumatoid arthritis (van Lent *et al.*, 1995), by providing accessory functions for T lymphocytes, and as a source of pro-inflammatory mediators (for instance, TNF- α , IL-1 β) (Cutolo *et al.*, 1993) that indirectly affect the release of degradative enzymes, such as collagenase and stromelysin, by other resident cell types,

e.g. chondrocytes, (Schnyder *et al.*, 1987) or extracellular matrix, (Shapiro *et al.*, 1993). Histological staining of inflamed synovial tissue reveals that the monocytes are in close proximity to the VCAM-1 expression synovial fibroblasts (Edwards *et al.*, 1982c.). In fact, both the monocytes and synovial fibroblasts express adhesion molecules that may act as each others counter receptors. For instance, VLA-4 and VCAM-1, but also LFA-1 and ICAM-1. The role of VCAM-1 and ICAM-1 in inflammation has been studied extensively with regards to monocyte adhesion to vascular endothelium. Upon the presence of LPS, TNF- α or IL-1 β , endothelial cells up-regulate expression of either VCAM-1 alone or both ICAM-1 and VCAM-1. The integrins on the circulating monocytes are inactive, but the release of chemokines by the endothelial cells are thought to activate both VLA-4 and LFA-1 resulting in binding (Springer, T.A., 1994). ICAM-1 and VCAM-1 also play an important part in the subsequent passage of the monocytes through the vascular endothelial layer (extravasation). Apart from a role in acute inflammation, VCAM-1 also has a role in muscle development and in maturation of T-cells in the lymph node. (Osborn *et al.*, 1989). Thus we envisaged that upon entering the synovial membrane, monocytes could attach to the intimal synovial fibroblasts. However, exactly how monocytes interact with the synovial fibroblasts and what activates them to release inflammatory mediators and proteases, remains elusive.

We have set up a synovial fibroblasts culture system (Croft *et al.*, manuscript submitted) where we preserve VCAM-1 and ICAM-1 expression. We have used U937 cells as a source of monocytes and studied binding and differentiation in the presence of the synovial fibroblasts. The experiments are performed in the presence of human plasma derived serum rather than foetal bovine serum to reduce the contribution of a number of known/unknown inflammatory mediators released during the blood clotting process. Because intimal synovial fibroblasts are bathed in hyaluronan, we have included this glycosaminoglycan in the co-culture studies. Hyaluronan can bind to CD44, a molecule abundantly expressed on both monocytes and synovial fibroblasts. In this study we report that only after a delay of 12 hrs, U937 cells adhere to the synovial fibroblasts. Co-culture results in an arrest of proliferation and a complete differentiation of the U937 cells towards monocytes. Cell-cell interaction was found not to be inhibited by a combination of anti-CD18 and anti-VLA-4 antibodies.

4.3. Methods and Materials

4.3.1. Materials

DMEM, RPMI 1640, L-glutamine, Streptomycin sulphate, benzyl penicillinate and foetal bovine serum were obtained from Gibco BRL Life Technologies, UK. Fibronectin was purchased from Calbiochem, UK.

4.3.2. Antibodies

Antibodies against VLA-4, CD18, ICAM-1, ELAM-1, P-selectin or CD44H used for FACS analysis were purchased from Becton Dickinson, UK. MHC II was obtained from DAKO and anti-fibronectin was obtained from Boehringer Mannheim.

4.3.3. Human plasma derived serum preparation (HPDS)

Human plasma was obtained from male AB donors. Fresh citrated blood was spun at 10,000 rpm for 20 min at 4°C, and the supernatant (plasma) was microscopically tested for the presence of platelets. If platelets were detected, the plasma was spun again for 20 min at 4°C. To the plasma, CaCl₂ was added (4 mM) and the plasma was left at 4°C for overnight. The fibrin clot was spun at 10,000 rpm for 20 min at 4°C and the plasma was then heat inactivated at 54°C for 40 min. The plasma was cleared one more time of insoluble components by centrifugation at 10,000 rpm for 20 min at 4°C. The plasma is aliquoted and kept at -20°C until use. This product is referred to as human plasma derived serum (HPDS) (Raines and Ross, 1985).

4.3.4. Synovial fibroblast isolation and cell culture

Rheumatoid synovium tissue was obtained after hip or knee joint replacement surgery and collected from University College London or the Whittington Hospital (London). The tissues described in this paper are all derived from rheumatoid arthritic joints. Tissue was processed within two hours after removal from the patient. The inner layer of the synovial membrane was removed with a scalpel and the tissue was washed extensively in PBS (w/o Ca²⁺ and Mg²⁺). The tissue was then chopped into small pieces (1mm³) washed again in PBS and left to digest in 2mg/ml collagenase in sterile DMEM supplemented with 1% penicillin-streptomycin in a shaking incubator at 37°C. The tissue was sheared using a sterile syringe, filtered using a fine sterile gauze and then

washed and resuspended in DMEM containing 10%FBS and 1% penicillin-streptomycin. The cells were counted and seeded into 150 cm² flasks (Nunc), and left overnight for the fibroblasts to adhere. The cells were then washed three times with PBS and fresh DMEM was added, supplemented with either 10% FBS or 10% heat inactivated human plasma (HPDS). In the case of hyaluronan, 1 mg/ml of human umbilical cord hyaluronan (Sigma) was dissolved in DMEM supplemented with either 10% FBS or 10% HPDS. The cell culture was continued for another week after which the cells were passaged into 6 well or 24 well plates. For co-culture studies, cells were used within three weeks.

4.3.5. Fibroblast-U937 cell co-culture

U937 cells were added per at 0.2×10^6 cells/well of cultured synovial fibroblasts in 6 well plates. The co-cultures were then left for a further three days. In addition, medium previously collected from the synovial fibroblasts was used as conditioned medium to culture U937 cells alone, in order to determine the contribution of soluble mediators in the process of U937 differentiation. Culture conditions were: 1) HPDS 10%, 2) HPDS (10%) plus hyaluronan (1mg/ml) or 3) FBS (10%). After the appropriate time of culture, U937 cells were removed from the synovial fibroblasts by incubation in PBS supplemented with 4 mM EDTA at 4°C for 15 min. Under a light shear flow (pipette PBS over cell surface) U937 cells detached, whereas synovial fibroblasts remain adherent.

4.3.6. Adhesion Binding Experiments

0.2×10^6 U937 cells were added to the rheumatoid synovial fibroblasts and cultured for 3 days in the appropriate medium. To determine the percentage of adherent U937 cells in co-culture, we counted the number of adherent and non-adherent U937 cells independently. Trypan blue solution was used to determine whether the U937 cells viability.

4.3.7. Radiolabelled Binding Experiments

Synovial fibroblasts were seeded at 0.01×10^6 per well of a 24 well plate with DMEM supplemented with 10% HPDS. Because the U937 cells only appeared to bind after an initial co-culture of twelve hours, we pre-incubated the 5×10^6 U937 cells in 10 ml of

synovial fibroblast-conditioned medium containing HPDS (10%) and hyaluronan (1mg/ml), for a similar time period before assessing binding. After the 12 hr incubation in synovial fibroblast conditioned medium with or without hyaluronan (i.e. 10% HPDS control), U937 cells were washed with PBS (without Ca^{2+} or Mg^{2+}), and incubated for a further 2 hours in pre-warmed DMEM + P/S with radioactive sodium cromate (^{51}Cr) (50 μCi to 10×10^6 cells), at 37°C in a 5% CO_2 incubator. The cells were washed in DMEM + P/S and left for 30 minutes in fresh medium in which they were cultured. The U937 cells were then washed and resuspended in RPMI + 1% BSA + P/S and for the inhibition experiments the cells were incubated with the appropriate antibody for cell adhesion molecules found on U937 cells: anti-CD18 (40 $\mu\text{g/ml}$), anti-VLA-4 (20 $\mu\text{g/ml}$), anti-fibronectin (40 $\mu\text{g/ml}$) or anti-IgG (40 $\mu\text{g/ml}$) for 30-45 minutes at 4°C. The synovial fibroblasts were washed and replenished with RPMI + 1% BSA + P/S. For the inhibition experiments on the synovial fibroblasts the appropriate amount of antibody was added; (Morales-Ducret *et al.*, 1992, DiCorleto *et al.*, 1985, Beekhuizen *et al.*, 1990, Meerschaert and Furie, 1995); anti-ICAM-1 (40 $\mu\text{g/ml}$), anti-ELAM-1 (40 $\mu\text{g/ml}$), anti-P-selectin (40 $\mu\text{g/ml}$), anti-CD44v5 (40 $\mu\text{g/ml}$), anti-CD44v7-8 (40 $\mu\text{g/ml}$) and anti-fibronectin (40 $\mu\text{g/ml}$) were added to the synovial fibroblasts and left for 45 minutes at 37°C in 5% CO_2 . The fibroblasts were then washed with RPMI + 1% BSA + P/S and 0.1×10^6 U937 cells were added per well. The co-culture was left for 1 hour at 37°C and then the non-adherent U937 cells were carefully removed and washed twice with PBS/1% BSA and the adherent cells were lysed with 0.5% SDS. Both non-adherent and adherent radiolabelled cells were counted using a gamma counter.

4.3.8. Flow cytometry

U937 cells were spun down, washed with PBS, resuspended in human AB serum for 20 min at 4°C, washed in PBS/1%(w/v) BSA and subsequently incubated for 40 min at 4°C with the following antibodies: anti-CD14 (20 $\mu\text{g/ml}$), anti-Mac-1 (CD11b) (10 $\mu\text{g/ml}$) and anti-VLA-4 (5 $\mu\text{g/ml}$). The cells were washed again in PBS/1% BSA and incubated with anti-mouse IgG conjugated with PE (5 $\mu\text{g/ml}$) for another 40 min at 4°C. After 4 washes in PBS/1% BSA the cells were fixed with paraformaldehyde (1%w/v final concentration) and stored in the dark at 4°C until analysed on a FACScan (Becton Dickinson flow cytometer). (see Chapter 2 Methods and Materials for more details.)

4.3.9. Fluorescein - Hyaluronan Binding Experiment

These experiments were done in collaboration with Dr. Claire Isaac from Imperial College London. Fluorescein-conjugated hyaluronan (Fl-HA) was prepared as described by De Belden and Wilk, (1975) but obtained as a gift from Dr. Jane Lesley, using rooster comb hyaluronan. U937 cells were incubated for 24 hours in rheumatoid synovial fibroblast and hyaluronan conditioned medium. The cells (approximately 10^6) were washed twice in PBS and 1% BSA followed by a one hour treatment at 37°C with hyaluronidase (2mg/ml) to remove any residual hyaluronan. The cells were washed twice again with ice cold fluorescence buffer (PBS containing 1% (w/v) BSA, 0.01% sodium azide and 10% FBS) and then incubated with Fl-HA for 30 minutes at 4°C at a concentration of 2µg/ml. The cells were then washed, fixed with 3% paraformaldehyde and viewed under a fluorescent microscope. As a positive control, the cell line BW5147 was used. The BW5147 cell line originates from a mouse T cell lymphoma and they constitutively express the CD44 receptor.

4.3.10. Western blot analysis

U937 cells were cultured in HPDS or FBS, washed in PBS and solubilized in Laemmli sample buffer at 10^6 cells/100 µl. The lysate was heated at 100°C for 5 min and run on a 10% acrylamide gel. Protein was blotted onto nitrocellulose and the blot was blocked with 5% skimmed milk in TBS. The blots were incubated overnight with the first antibody p47^{phox}, p67^{phox}, and p40^{phox} at 4°C. Antibody binding was detected after a 2 hr incubation with ¹²⁵I-protein A, followed by exposure to a phosphor screen and analysis with a Fuji Bioimager.

4.3.11. Nitro Blue Tetrazolium Test

U937 cells were added at 0.2×10^6 cells/well of cultured synovial fibroblasts in 6 well plates. The co-cultures were then left for a further three days. A fresh solution of NBT was made with DMSO:saline (1:1) with a final concentration of 20mg/ml. On day 3 of the co-culture, any non-adherent U937 cells were removed by careful washing with PBS and 10µl of the NBT solution was added to the medium for 20 minutes in the presence / absence of 100nM PDBu. The staining was quenched through the removal of medium and the addition of ice cold PBS.

4.3.12. RT-PCR of monocyte specific esterase

RNA was isolated by the method of Chomczynski and Sacchi (1987) and also see Chapter 2 Methods and Materials. The isolated total RNA (3 µg) was reverse transcribed to cDNA using a Ready to Go T-Primed First-Strand kit (Pharmacia Biotech). This kit utilises the Moloney leukemia virus (M-LuLv) reverse transcriptase and an oligo (dT) primer to generate first strand cDNA. The completed first strand cDNA was amplified by polymerase chain reaction (PCR) using specific primers for MSE. The sense primer is 5' AAT GCC ACC TCG TAC CCT CCT ATG, and the anti-sense primer is 3' GAC ACT TTC TCC TCC CGC TGA CTC. The MSE primers were designed by using a computer selection package called Primer Select (DNA Star). The same PCR primer selection criteria (See Appendix I) was used in order to chose the primers. The GAPDH primers were purchased from Strategene. By measuring GAPDH levels we can quantitate MSE RNA levels in synovial fibroblasts. The PCR amplification was again determined by doing dilution curves similar to those performed in Chapter 2, Methods and Materials (See Figure 4.6); by adding 28.7 µl of sterile DEPC water, 8 µl of 1.25 mM each dNTP mix (Perkin-Elmer), 0.25 µl of ³³P-dATP (Amersham), 2.5 µl of 4 mM primer, 5 µl of 10 x PCR buffer (Perkin-Elmer) and 0.25 µl of Amplitaq DNA polymerase (Perkin-Elmer) at 5 U/ml to 3 µl of each completed first strand reaction. The reaction tubes were heated to 94°C for 5 min and incubated in a GeneAmp PCR 96000 (Perkin-Elmer) thermal cycler using 28 cycles of 94°C (30 sec), 62°C (30 sec) an 72°C (60 sec). Fragments were finished off by incubating for 10 min at 72°C. 5 µl of each PCR reaction was electrophoresed through a 4% polyacrylamide gel. The gel was fixed for 15 min in acetic acid-methanol and dried under vacuum at 80°C. The gels were put against a phosphor screen for 2-4 hrs and primer-specific bands were then analysed using a BAS1000 BioImager (Fuji).

4.3.13. Immunohistochemistry

All the immunohistochemistry was done in collaboration with Drs. Theresa Coquerelle and Peter Angel at the Institute of Genetics, Karlsruhe. The co-culture system was grown on plastic chamber slides for three days, afterwards the cells were fixed with ice cold methanol for 4 minutes, dried with ice cold acetone and then allowed to air dry. They were stored at -73°C wrapped in foil, ready for immunohistochemical staining

performed by Dr. Coquerelle. The slides were incubated with 10% normal goat serum (DAKO) /PBS for 15 minutes, followed by the primary antibody (anti-collagenase I or anti-TIMP in 1% BSA/PBS (DAKO)) for 60 minutes at room temperature, washed three times with PBS over 5 minutes. It was then blocked in 0.3% H₂O₂/methanol for 15 minutes and then washed three times with PBS over 5 minutes. A dilution of 1:400 of the secondary biotinylated antibody (1% BSA/PBS) (DAKO) was added for 30 minutes and washed as before in PBS, followed by the streptavidin-biotin complex solution (DAKO) for 30 minutes. The slides were washed again three times with PBS and treated with AEC to develop the colour in the cells for 10 minutes. AEC solution : 10mg of 3,3-amino-9-ethyl carbazole in 1ml N,N-dimethylformamide with 20ml acetate buffer 50mM pH4.9, filtered and then 6.5µl of H₂O₂ was added. It was then briefly washed in bidest, counterstained with hematoxylin solution for 4 minutes and washed several times with tap water for 10 minutes, finally drying the sections and mounting the coverslips.

4.3.14. Statistical analysis

Statistical analyses were performed using analysis of variances (ANOVA). *P* values less than 0.05 were considered significant. If there were significant findings using ANOVA, a series of paired t-tests were conducted to identify differences among groups. * $P < 0.005$; ** $P < 0.01$; *** $P < 0.001$.

4.4. Results

4.4.1. Addition of hyaluronan increases adhesion of U937 cells to synovial fibroblasts

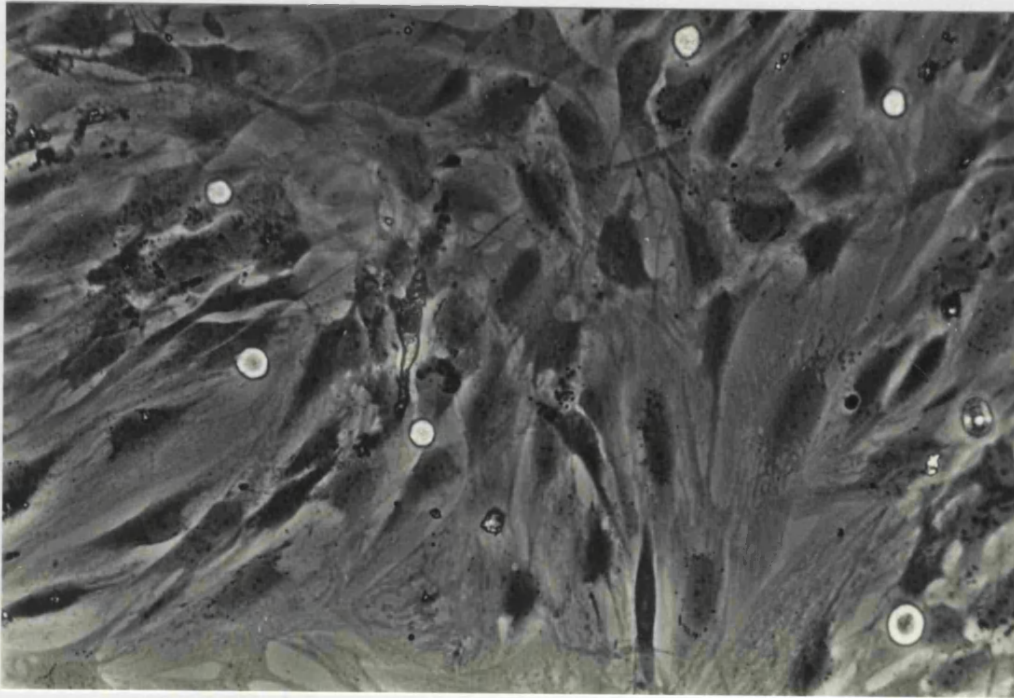
When U937 cells are combined with synovial fibroblasts, in the presence of HPDS or FBS for 3 days little binding occurred i.e. 33% (See Table 4.1). In an attempt to be as complete as possible with reconstruction conditions in the synovial joint, we decided to add hyaluronan to the medium. The addition of hyaluronan to either HPDS or FBS medium enhanced binding two fold from 33% to 60% (See Table 4.1). The percentage adherence was calculated by counting the non-adherent and adherent cells separately, using a light microscope and calculating the percentage of adherent cells. In addition, it was noticed that only an overnight co-culture incubation, i.e. 12 hours, was necessary to obtain a similar percentage of U937 cells binding when grown in the presence of HA (See Figure 4.1.), instead of the 3 days co-culture. Figure 4.1 illustrates that 12 hours is sufficient time for U937 cells to adhere to the rheumatoid synovial fibroblasts when grown in 10% HPDS and hyaluronan (1mg/ml).

Culture conditions	% Adherent U937 ±SEM
U / HPDS	2 ±0.5
U / HPDS+HA	5.2 ±2.4
U + PDBu / HPDS	91.9 ±1.3
S+U / HPDS	33.3 ±5.1 ***
S+U / HPDS+HA	60.8 ±6.9 ***
S+U / FBS	40.6 ±5.2
U / HPDS (c.m.)	0.7 ±0.4 **
U / HPDS+HA (c.m.)	16.5 ±8.4

Table 4.1. % of U937 cells that are adherent in culture with or without rheumatoid synovial fibroblasts for 3 days. Data are presented as mean ±SEM Statistical analysis : student paired t-test * $P<0.05$; ** $P<0.01$; *** $P<0.001$; N = 4; S ; Rheumatoid synovial fibroblasts, U ; U937 cells, PDBu ; Phorbol 12,13-dibutyrate.

A possible reason for the U937 cells binding to the synovial fibroblasts was thought to be through the interaction between the CD44 receptors found on both synovial fibroblasts and U937 cells, which were detected by FACS analysis (See Figure 4.2.). Fluorescent-hyaluronan binding experiments were done to investigate whether U937 cells incubated with rheumatoid synovial fibroblast conditioned medium and hyaluronan (1mg/ml) could bind fluorescent-hyaluronan via the CD44 receptor. The U937 cells were not found to bind to fluorescent-hyaluronan despite CD44 receptors being present. The cell line BW5147 was also used as a positive control, which showed positive hyaluronan binding. However because no binding occurred between the U937 cells and fluorescent-hyaluronan, binding experiments were not done on the rheumatoid synovial fibroblasts. The exact role of hyaluronan in this process remains unclear. It seems to be the combination of rheumatoid synovial fibroblast conditioned medium and hyaluronan that produces an increase in binding.

a)



b)

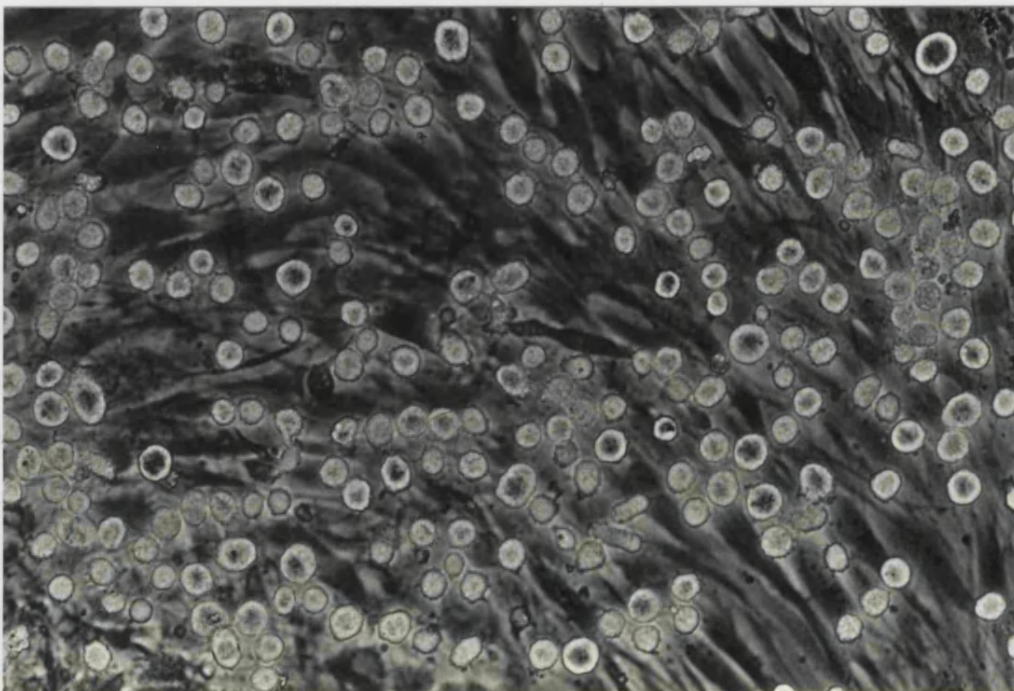


Figure 4.1.

U937 cells grown in co-culture with rheumatoid synovial fibroblasts for 12 hours in a) 10% HPDS, b) 10% HPDS + hyaluronan (1mg/ml). Results show that cultures grown in 10% HPDS do not induce U937 cell binding to the rheumatoid synovial fibroblasts after 3 days co-culture, whilst there is 60% increase in binding when hyaluronan is also added into the medium. This figure represents results of a typical experiment. N=5.

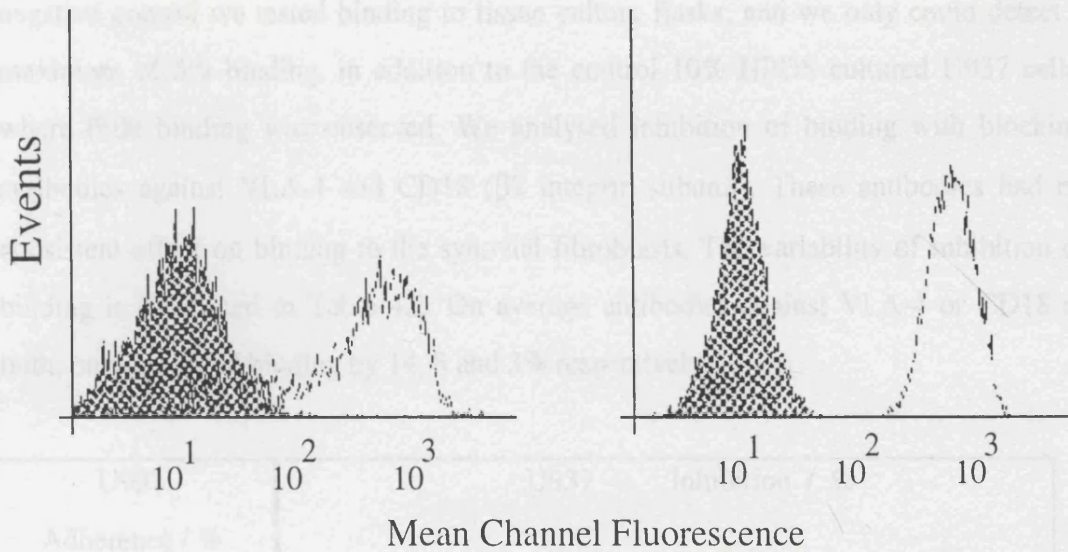


Figure 4.2. Expression of CD44 on a) rheumatoid synovial fibroblasts and b) U937 cells detected by FACS analysis. The rheumatoid synovial fibroblasts were cultured in 10% HPDS for 1 week and the U937 cells were cultured in 10% HPDS for 3 days. This figure represents results of a typical experiment. Statistical analysis ; Paired student t-test. N=5.

4.4.2. Initial binding does not occur through VLA-4 or $\beta 2$ integrins

As CD44 did not seem to have a role in U937 cell binding we wanted to investigate further if other cell adhesion molecules were instrumental in the adhesion to synovial fibroblasts. In order to determine the percentage of adherent U937 cells more accurately we performed radiolabelled binding (^{51}Cr) studies. To investigate which adhesion molecules may be involved, we studied radiolabelled binding in the absence and presence of blocking monoclonal antibodies. Synovial fibroblasts constitutively express VCAM-1 and low levels of ICAM-1 (see Chapter 2, Figure 2.2.) and both molecules are involved in monocyte binding to endothelium. Therefore we used anti-VLA-4 and CD18 blocking antibodies. U937 cells are cultured for 24 hr culture in synovial fibroblast-conditioned medium with hyaluronan (1mg/ml) as above, however only 1 hour was allowed for binding as this was showed maximum adherence (See Figure 4.3. for characterisation of the radiolabelled binding assay). 41% of the U937 cells was

found to bind to the synovial fibroblasts after one hour incubation (see Table 4.2.). As a negative control we tested binding to tissue culture flasks, and we only could detect a maximum of 5% binding, in addition to the control 10% HPDS cultured U937 cells, where little binding was observed. We analysed inhibition of binding with blocking antibodies against VLA-4 and CD18 ($\beta 2$ integrin subunit). These antibodies had no consistent effect on binding to the synovial fibroblasts. The variability of inhibition of binding is illustrated in Table 4.2. On average antibodies against VLA-4 or CD18 or both, only inhibited binding by 14, 8 and 3% respectively.

U937 Adherence / %		U937 Inhibition / %			
		α CD18	α VLA-4	α CD18 + α VLA-4	α IgG2b
	75.06 \pm 1.81	17.42 \pm 3.16	10.31 \pm 1.08	10.47 \pm 3.72	-0.78 \pm 1.53
	35.83 \pm 2.64	34.64 \pm 4.62	-1.03 \pm 2.64		-8.00 \pm 6.38
	55.13 \pm 6.33	-9.22 \pm 1.81	-9.94 \pm 4.11		
	16.45 \pm 4.53	16.27 \pm 5.72	29.36 \pm 1.06		
	50.36 \pm 1.09	26.5 \pm 0.89	31.44 \pm 3.69		3.85 \pm 2.13
	14.56 \pm 0.86	-3.66 \pm 10.06	-14.15 \pm 2.00	-4.28 \pm 7.79	
Mean	41.23	13.66	7.67	3.1	-1.64
SEM	\pm 9.62	\pm 6.95	\pm 7.97	\pm 7.38	\pm 3.45

Table. 4.2.

Inhibition of binding with blocking antibodies against VLA-4 and CD18 ($\beta 2$ integrin subunit) on U937 cells. Data are presented as mean \pm SEM of experiments done in triplicate. No statistical analysis was done as there was such a wide range in SEM. N=6

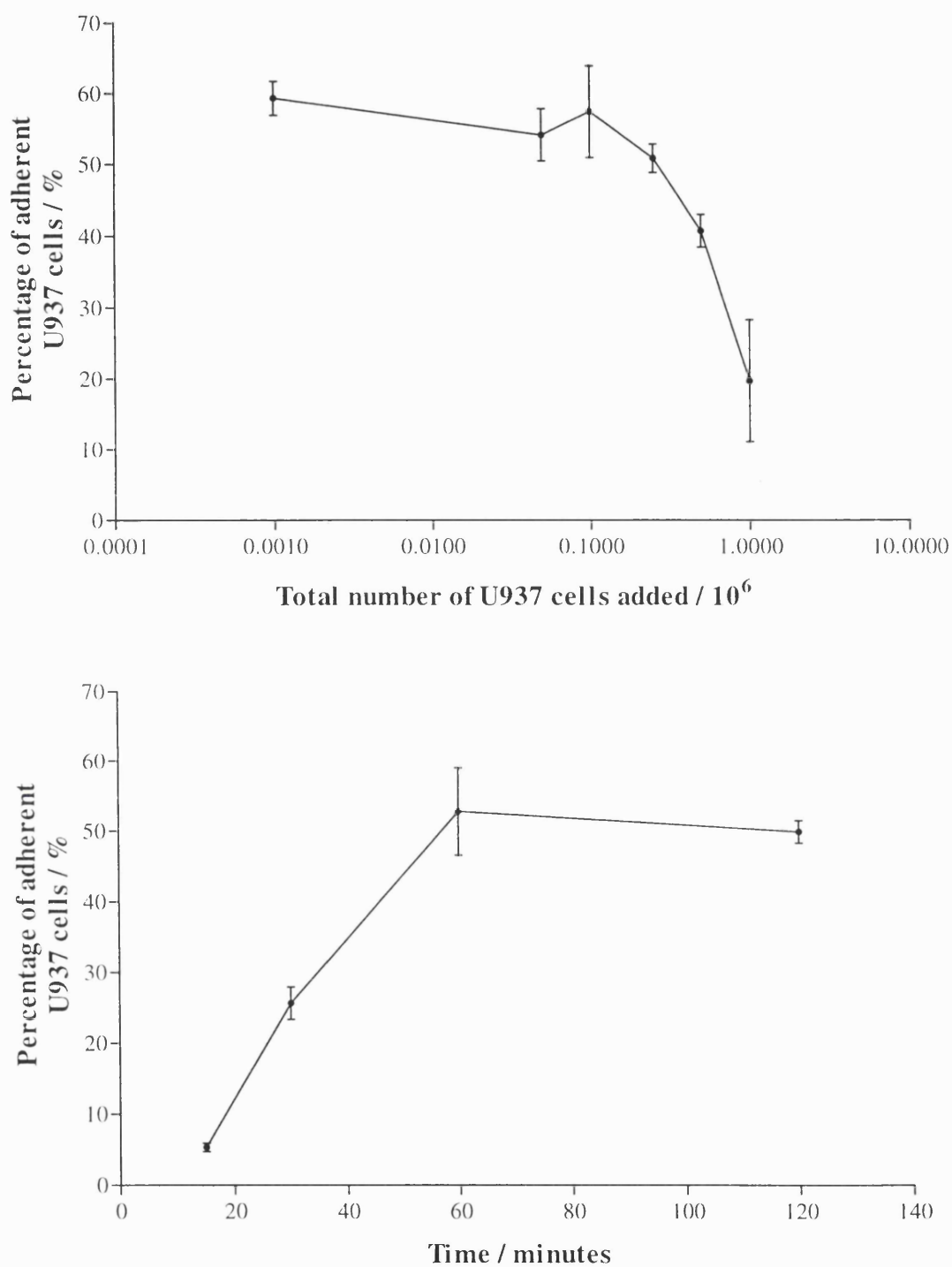


Figure 4.3. Characterisation curves for radiolabelled binding experiments. a) Dose curve of the total number of U937 cells (after the 24 hour incubation in conditioned medium) added after 1 hour incubation, b) Time curve of 0.1×10^6 U937 cells (after the 24 hour incubation in conditioned medium) added to rheumatoid synovial fibroblasts cultured in 10% HPDS. N=2

We also tested binding of the U937 cells after a 24 hr culture in conditioned medium with hyaluronan (1mg/ml) to ICAM-1 or VCAM-1. Inhibition of U937 cells binding with blocking antibodies against VCAM-1 or ICAM-1 on the rheumatoid synovial fibroblasts was not observed. These results are consistent with the above results, indicating that the binding between the U937 cells and rheumatoid synovial fibroblasts is not due to either VCAM-1/VLA-4 or ICAM-1/CD18. However, these results are rather surprising as they do not support the data shown in Chapter 3 where U937 cells show positive binding to recombinant VCAM-1 or ICAM-1 and the binding can be inhibited by using anti-VLA-4 and anti-CD18 antibodies. Therefore these data suggest that although VCAM-1 and ICAM-1 are expressed on the rheumatoid synovial fibroblasts, they are not involved in the initial contact between the synovial fibroblasts and the U937 cells.

In addition, we could not detect E or P selectin on the surface of synovial fibroblasts. To study if fibronectin may be involved in binding, we saturated fibronectin binding sites on both synovial fibroblasts and U937 cells through the presence of soluble fibronectin in the radioactive binding assay. Again there was no inhibition in U937 cells binding to the synovial fibroblasts (See Table 4.3.)

U937 Adherence / %		U937 with α - fibronectin	Syn Fib with α - fibronectin	Syn Fib+ U937 with α - fibronectin	IgG1
	55.13 \pm 6.3	0.44 \pm 1.0	2.91 \pm 0.9	5.41 \pm 1.1	-13.19 \pm 1.5
	16.45 \pm 4.5	5.56 \pm 4.3	-7.76 \pm 1.5	-15.0 \pm 1.2	-0.78 \pm 4.56
	50.36 \pm 1.1	-8.21 \pm 3.8	-1.36 \pm 0.1	-8.0 \pm 5.6	
	73.18 \pm 0.7	3.00 \pm 1.6	4.9 \pm 1.5		
Mean	48.78	0.198	-0.33	-5.87	-6.98
SEM	\pm 11.84	\pm 2.9	\pm 2.8	\pm 5.9	\pm 6.2

Table 4.3.

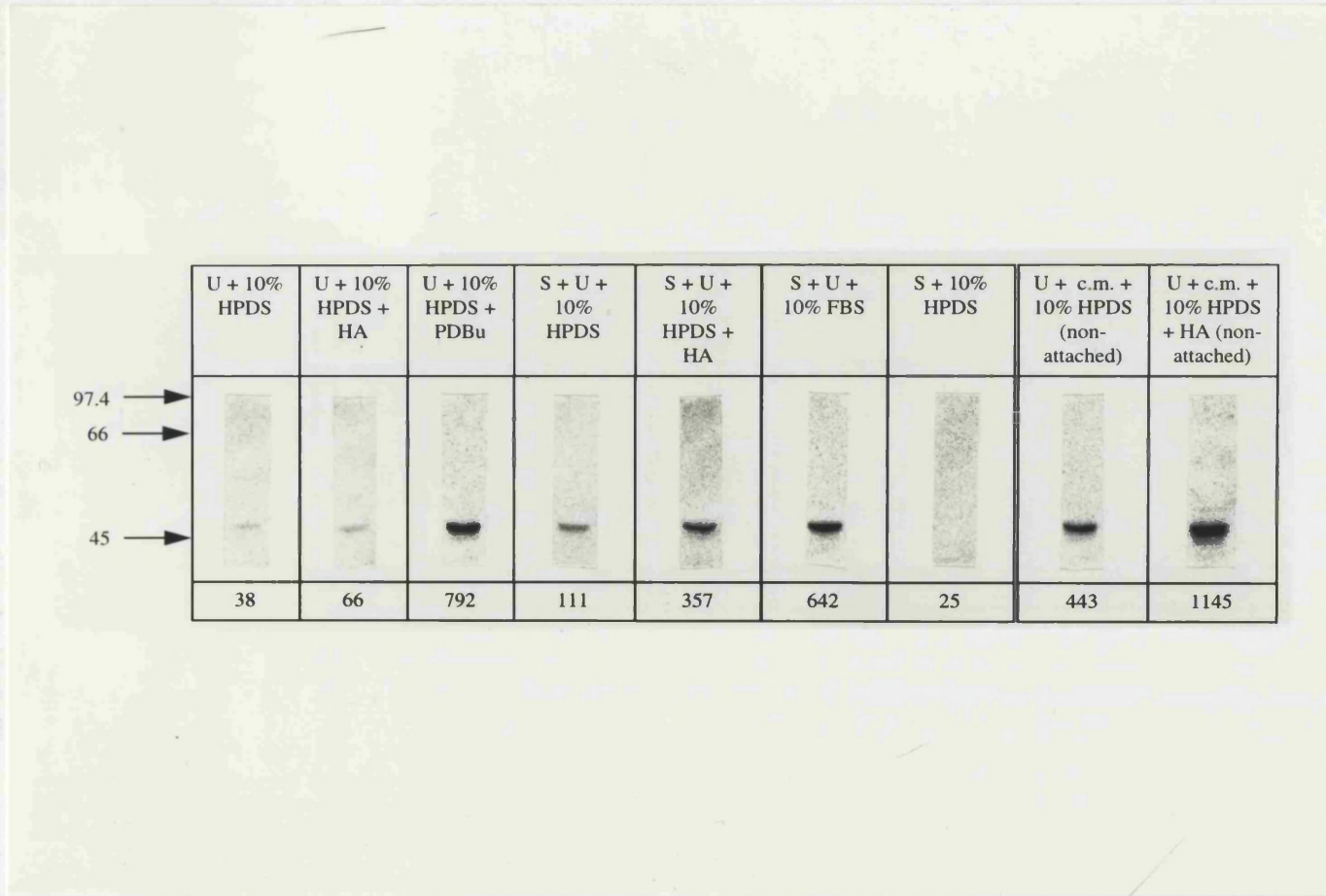
Inhibition of binding with blocking antibodies against fibronectin on U937 cells, rheumatoid synovial fibroblasts and U937 and synovial fibroblasts in co-culture. Data are presented as mean \pm SEM of experiments done in triplicate. N=4.

4.4.3. Adherent U937 cells fully differentiate towards monocytes

We next tested if the adherent cells would mature to monocytes. For this purpose expression of components of the NADPH : oxidoreductase, expression of CD11b (Mac-1), CD14, MHC II and expression of monocyte specific esterase (MSE) was assessed. Expression of p47^{phox} is greatly enhanced after three days of co-culture with 10% HPDS (see Figure 4.4.). This induction is accompanied by expression of p67^{phox}, p40^{phox} (See Figure 4.5.) as shown by the capacity to generate a respiratory burst determined by the reduction of NBT after stimulation with phorbol ester (see Figure 4.6.). The cells also highly express CD14, CD11b (Mac-1), VLA-4 and MHC II, as determined by flow cytometry (see Table 4.4. and Figure 4.7i. and 4.7ii.). Lastly, the cells express mRNA for MSE as determined by RT-PCR (see Figure 4.9.). These data indicate that adherent cells have fully matured and in fact resemble monocytes.

Figure 4.4.

Expression of p47^{phox} after three days of co-culture determined by western blot analysis. Samples were solubilized in Laemmli sample buffer at 10⁶ cells/100 µl and run on a 10% polyacrylamide gel. Protein was blotted onto nitrocellulose and the blot was blocked with 5% skimmed milk in TBS. The first antibody was incubated for overnight at 4°C and antibody binding was detected with a 2 hr incubation with ¹²⁵I-protein A, followed by exposure to a phosphor screen and analysis with a Fuji Bioimager. N=4. This is a representation of results obtained.



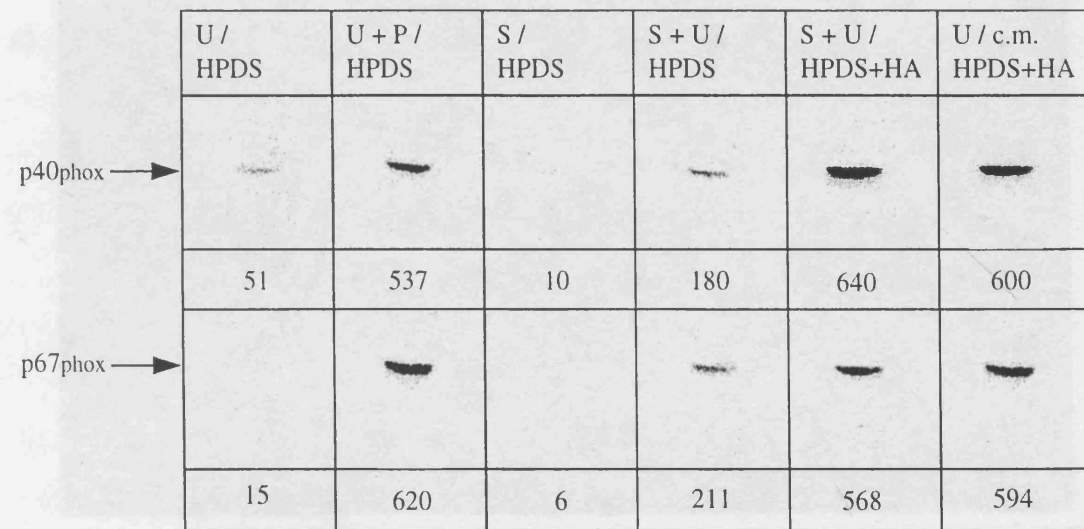


Figure 4.5.

Expression of p67^{phox} and p40^{phox} after three days of co-culture determined by western blot analysis. Samples were solubilized in Laemmli sample buffer at 10^6 cells/100 μ l and run on a 10% polyacrylamide gel. Protein was blotted onto nitrocellulose and the blot was blocked with 5% skimmed milk in TBS. The first antibody was incubated for overnight at 4°C and antibody binding was detected with a 2 hr incubation with 125 I-protein A, followed by exposure to a phosphor screen and analysis with a Fuji Bioimager. N=3. This is a representation of results obtained. These results show that p67^{phox} and p40^{phox} expression are increased in co-culture with rheumatoid synovial fibroblasts.

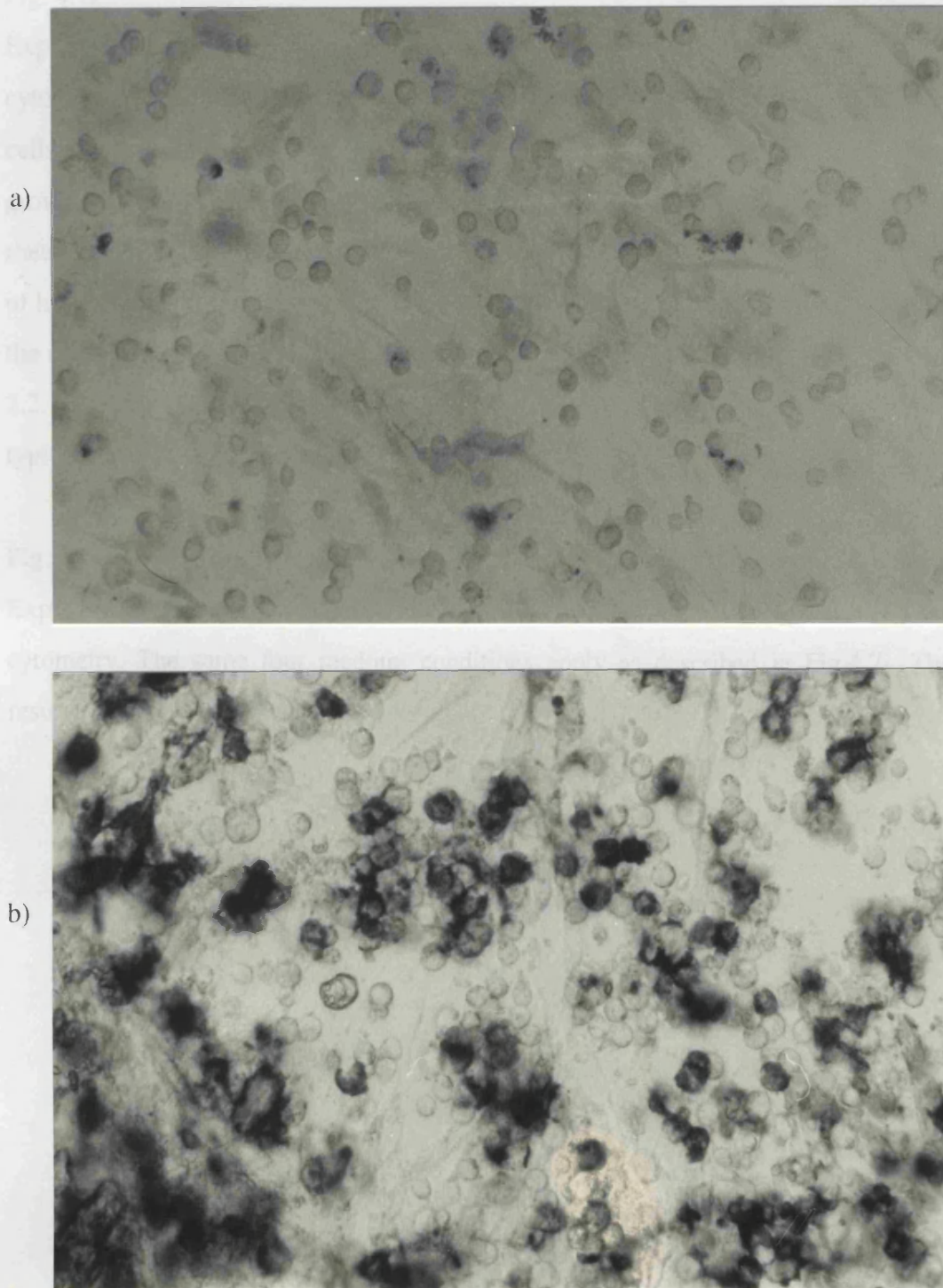


Figure 4.6.

U937 cells grown in co-culture with primary cultured rheumatoid synovial fibroblasts in a) 10% HPDS medium b) 10% HPDS with hyaluronan (1mg/ml) for 3 days. U937 cells are capable of generating a respiratory burst determined by the reduction of NBT after stimulation with a phorbol ester, PDBu (see Methods and Materials). N=5 This is a representation of results obtained from one experiment.

Fig. 4.7i.

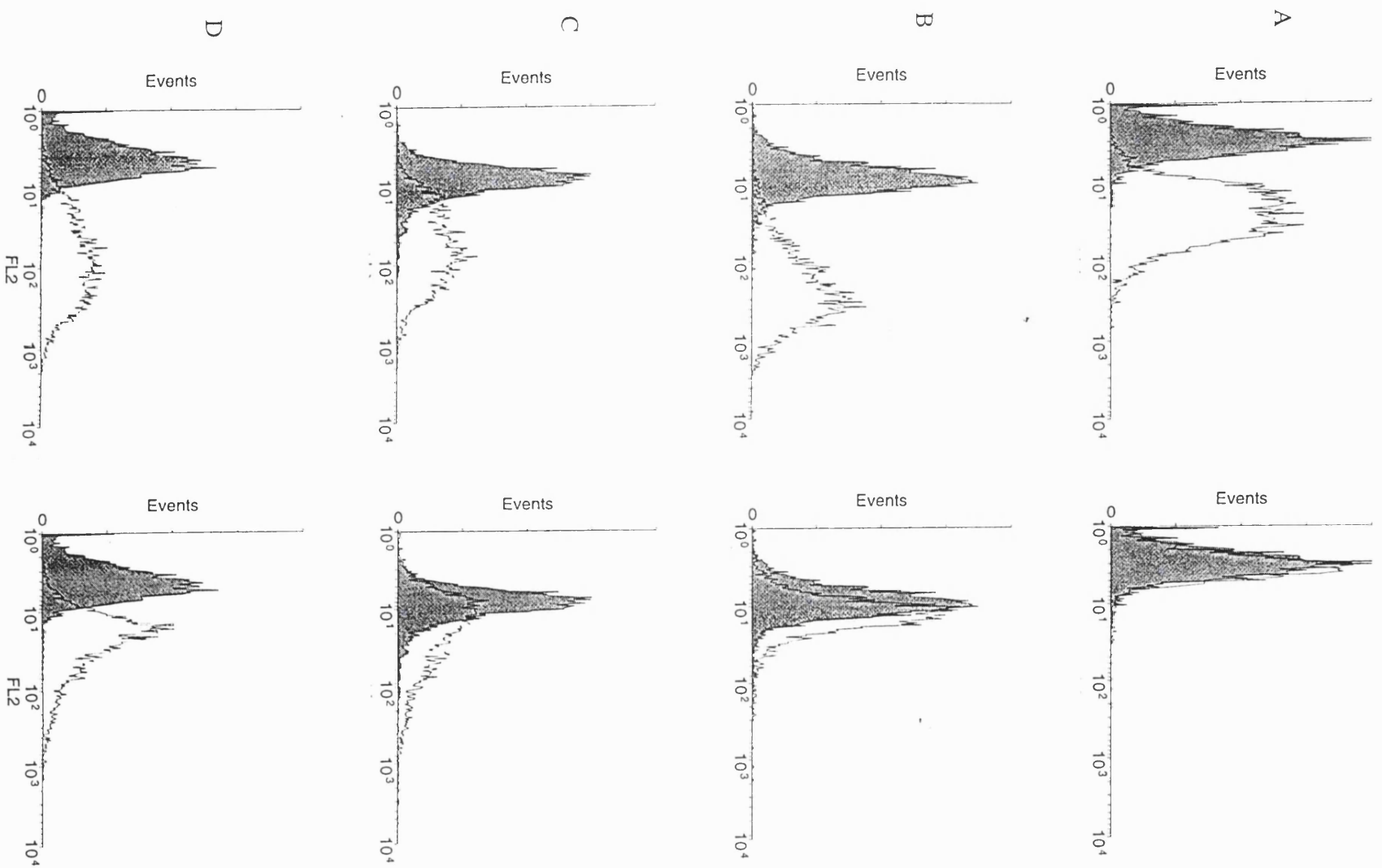
Expression of CD11b and CD14 on differentiated U937 cells as determined by flow cytometry. Cells were cultured in four different medium conditions for 3 days: (A) U937 cells grown in 10% HPDS medium; (B) U937 cells differentiated with 100nM PDBu grown in 10% HPDS medium; (C) U937 cells grown in co-culture with primary cultured rheumatoid synovial fibroblasts in 10% HPDS medium; (D) as in (C) with the addition of hyaluronan (1mg/ml). The shaded area denotes the control for the FACS analysis and the unshaded area indicates the positive expression of the antibodies studied. See Figure 2.2. and Figure 2.3. for IgG sub-type controls. These results are a representation of a typical experiment. N=4

Fig. 4.7ii.

Expression of VLA-4 and MHC II on differentiated U937 cells as determined by flow cytometry. The same four medium conditions apply as described in Fig.4.7i. These results are a representation of a typical experiment. N=4.

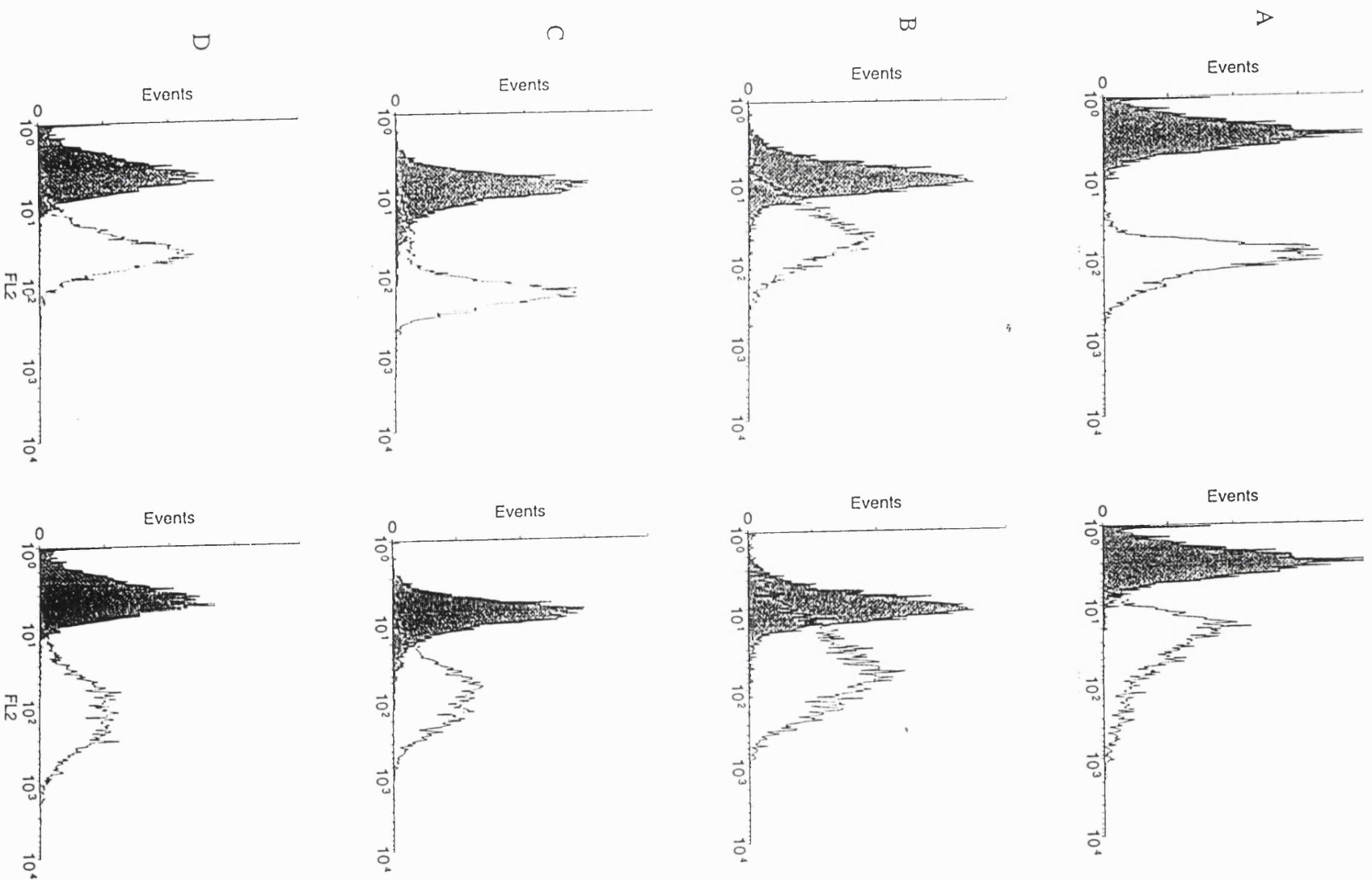
CD11b

CD14



VLA-4

MHC II



	CD11b	CD14	VLA-4	MHC II
U/HPDS	19.97 ±1.0	1.66 ±0.7	127.05 ±73.4	94.36 ±51.7
U/HPDS +HA	23.41 ±3.0	1.49 ±0.3	176.05 ±45.1	58.94 ±25.4
U/FBS	8.69 ±3.9	0.26 ±0.2	98.62 ±9.2	62.28 ±29.3
U+PDBu (100nM)/ HPDS	124.96 ±21.6	13.41 ±2.2	98.56 ±29.5	274.42 ±161.7
S+U(adh)/ HPDS	59.05 ±13.7 ***	54.86 ±20.4 **	133.04 ±21.3	105.38 ±16.2
S+U(adh)/ HPDS+HA	82.49 ±14.3 ***	127.28 ±34.8 ***	49.6 ±23.0 *	166.09 ±44.8 *
S+U(adh)/ FBS	53.36 ±12.0 ***	83.75 ±32.2 **	140.5 ±25.3	87.82 ±6.8
U/c.m. HPDS+HA	73.57 ±20.6*	57.63 ±17.7**	41.19 ±28.9*	147.07 ±38.4*

Table 4.4.

Monocyte differentiation marker expression determined by flow cytometry. Data are presented as Mean ±SEM (standard error of the mean). Statistical analysis ; paired student *t*-test, **P*<0.05; ***P*<0.01; ****P*<0.001; U, U937 cells; HPDS, Human plasma derived serum; HA, Hyaluronan; FBS, Foetal bovine serum; PDBu, Phorbol 12,13-dibutyrate; S, synovial fibroblast; adh, adherent cells; N = 4

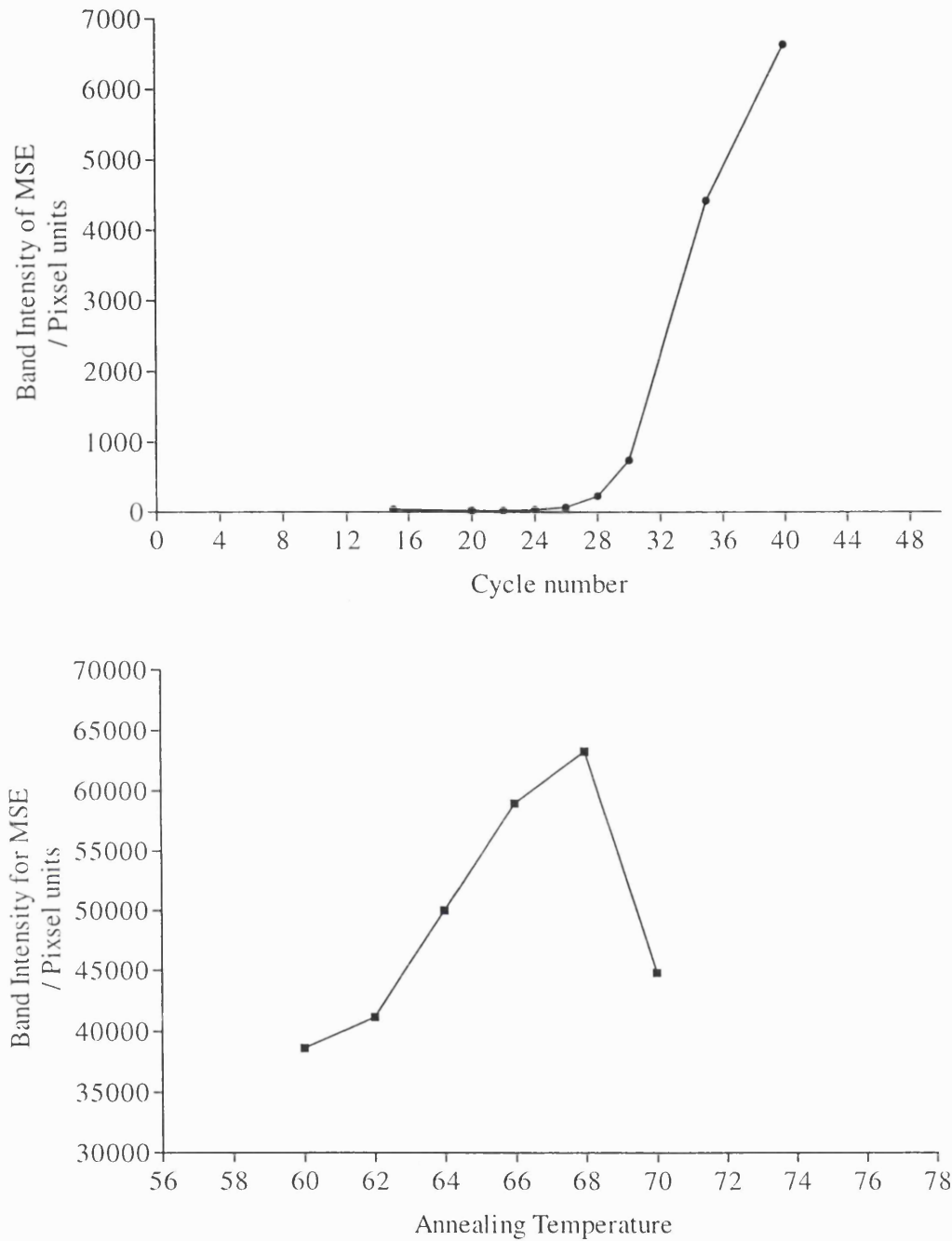


Figure 4.8. Amplification curve of the PCR reaction for MSE expression in U937 cells after being cultured in conditioned medium for 3 days. mRNA expression of MSE was determined by RT-PCR (see Methods and Materials). The U937 cells were washed, RNA was isolated and the PCR reaction was performed. The PCR product of MSE is 446 bp and GAPDH is 356 bp. The figure above represents results from a typical experiment. N=4.

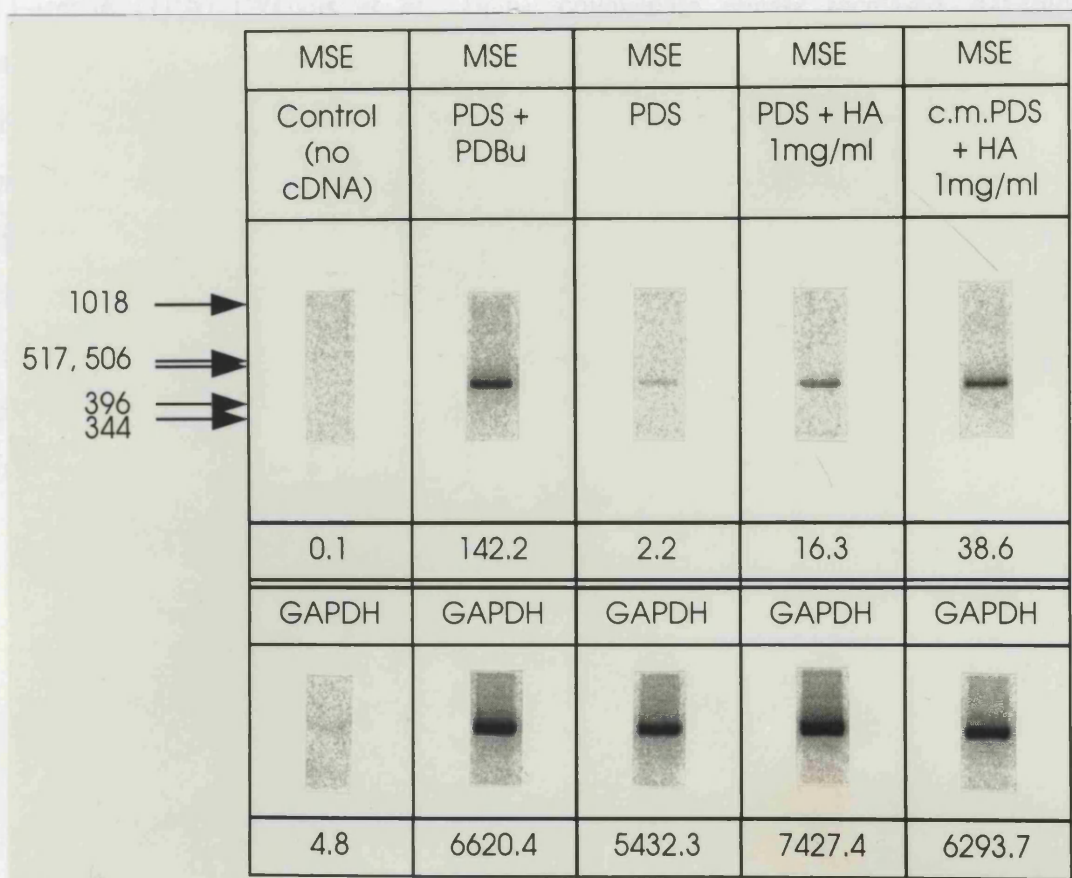


Figure 4.9.

mRNA expression of MSE determined by RT-PCR (See Methods and Materials) from U937 cells cultured under various conditions after 3 days co-culture. The PCR products of MSE 446 bp and GAPDH is 356 bp. Lane 1; no cDNA control for PCR, lane 2 ; U937 + PDBu cultured in HPDS, lane 3 ; rheumatoid synovial fibroblasts + U937 cultured in HPDS, lane 4 ; rheumatoid synovial fibroblasts + U937 cells cultured in HPDS + HA, lane 5 ; U937 cells cultured in rheumatoid synovial fibroblast conditioned medium + HA (1 mg/ml). The figure above represents results from a typical experiment. N=4.

4.4.4. Differentiated U937 cells also express collagenase

U937 cells secreted barely detectable quantities of enzyme, 10-12 ng/10⁶ cells per 24 hrs, under basal conditions. Upon incubation with 10 nM 12-o-tetradecanoyl-phorbol-13-acetate (TPA) (Welgus *et al.*, 1986), collagenase release increased 200-fold, comparable to the amount secreted by phorbol-stimulated human fibroblasts. Using immunohistochemistry with collagenase I-specific antibodies we assessed the induction of this metalloproteinase in the synovial fibroblasts co-culture. In culture, synovial fibroblast from rheumatoid arthritic patients express collagenase I when grown in 10% HPDS (interstitial or tissue collagenase) (Tetlow *et al.*, 1995; McCachren *et al.*, 1989) (see Figure 4.10a). After three days co-culture, the adherent U937 cells also express collagenase type I as seen by a perinuclear staining with the collagenase type-1 antibody (see Figure 4.10b.).

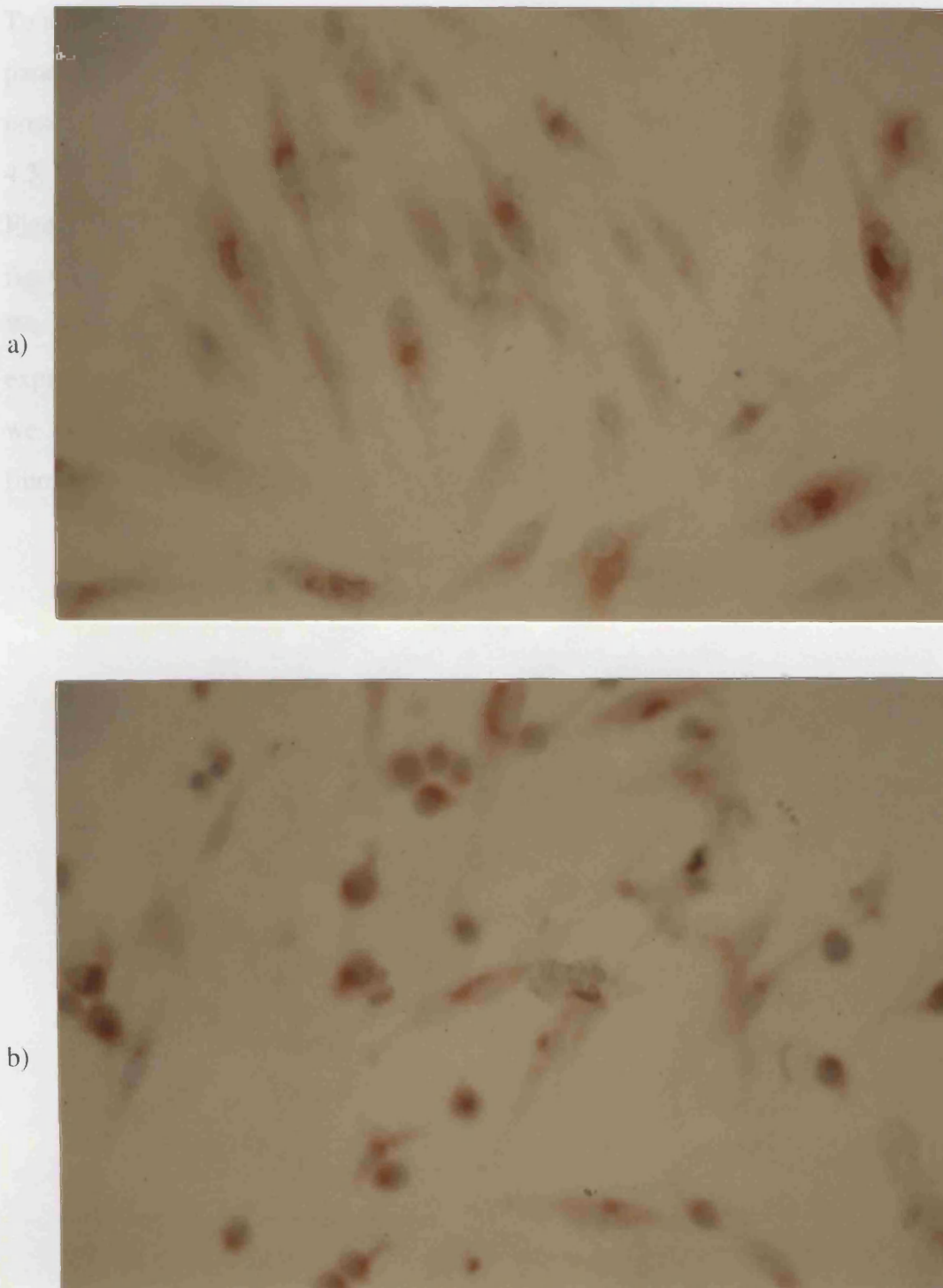


Figure 4.10.

a) Collagenase I expression on synovial fibroblasts grown in 10% HPDS, b) Collagenase I expression on adherent U937 cells grown in co-culture with the synovial fibroblasts in 10% HPDS for 3 days. The cells were then washed with PBS and fixed and frozen at -73°C (See Methods and Materials). The figure above represents results from a typical experiment. $N=5$.

4.4.5. Differentiation is mediated by soluble factors

To assess if differentiation was dependent on adherence, we tested the above mentioned parameters with U937 cells cultured in synovial fibroblast conditioned medium, in the presence of HPDS and hyaluronan. See Figures 4.2. for p47^{phox} (last column), Figure 4.3. for p67^{phox} and p40^{phox} (last column), Figure 4.7. for MSE expression (last column), Figure 4.11. for NBT reduction test and Table 4.4 for FACS analysis (last row) ; these figures and tables show results of U937 cells cultured in conditioned medium for 3 days. We could not detect any qualitative nor quantitative difference in the induction of expression of monocyte markers, between conditioned medium and adherent cells. Thus we conclude that differentiation is not dependent on adherence to the synovial fibroblasts *per se*.

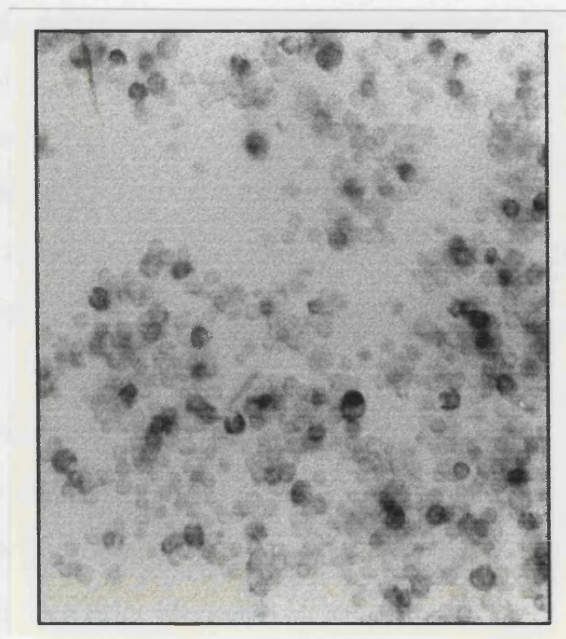


Figure 4.11. U937 cells are able to produce respiratory bursts after being cultured in rheumatoid synovial fibroblasts conditioned medium for 3 days. This is shown by the reduction of NBT which produces a purple precipitate coating the U937 cell surface. The figure above represents results from a typical experiment. N=4.

4.5. Discussion

We have demonstrated that incubation of the monocytic cell line U937 with synovial fibroblasts, in the presence of plasma derived serum and hyaluronan, results in U937 cell adherence which lasts for at least 6 days. During the first 3 days, the U937 cells stop proliferation and are committed to a programme of full differentiation, with high expression of CD11b (Mac-1), CD14 and a complete development of the NADPH : oxidoreductase. The induction of differentiation is independent of binding as it could also be obtained through incubation of the U937 cells in synovial fibroblasts conditioned medium. The contribution of hyaluronan is not understood. We initially believed that hyaluronan could bind to the CD44 receptor on both U937 cells and the synovial fibroblast, but we could not detect any binding to the U937 cells. In addition, binding of U937 cells to the synovial fibroblasts only occurs after an overnight culture, indicating that hyaluronan must have an indirect effect. A possibility is that hyaluronan acts through changing the release of cytokines from the synovial cells or through changing the action of the cytokines. Hyaluronan has been shown to be able to bind to cytokines and as such may modify their mode of action (Tanaka *et al.* 1993, Ruoslahti and Yamaguchi, 1991). We however felt that hyaluronan should be present in the co-culture, as it is an important component of synovial joint and, *in situ*, intimal synovial fibroblasts are in direct contact (Pitsillidis *et al.*, 1994).

The observation that neither antibodies against the $\beta 2$ (CD18) nor antibodies against the $\alpha 4$ integrin (VLA-4) reduce binding in a consistent matter is surprising. For each binding experiment we have assessed the presence of VCAM-1 or ICAM-1 on the synovial fibroblasts and the cells used for binding studies always expressed both adhesion molecules. Yet, only in certain cases did we see inhibition. The variation in inhibition could be due to variability in the source of the synovial fibroblasts. We also assessed the capacity of U937 cells (after culture in conditioned medium) to bind to VCAM-1 or ICAM-1 expressed on the rheumatoid synovial fibroblasts. We could not detect any binding. This finding suggests that the conditioned medium itself does not activate these integrins, but also makes it unlikely that VLA-4 or LFA-1 are involved in the initial binding to the synovial fibroblasts. In this respect, the findings differ from those found in binding studies with monocytes and U937 cells to endothelium

(Meerschaert and Furie, 1994) as well as to our own results in Chapter 3, which showed positive binding by the U937 cells to recombinant VCAM-1 and ICAM-1. This showed that U937 cells are capable of binding but only after stimulation by PDBu. Therefore a possible reason for the lacking in binding may be because the U937 cells still require further differentiation for further activation of the adhesion molecules. The potential problems with these binding experiments is that a combination of adhesion molecules may be involved, but due to financial restrictions, it was not possible to study all the combinations of adhesion molecules that may be involved in the role of U937 cell binding to rheumatoid synovial fibroblasts. We also analysed the expression of selectins by FACS analysis, but did not find them present on the rheumatoid synovial fibroblasts and therefore we conclude that they are involved in the initial contact.

A more accurate method of detecting binding would be to use antibodies that bind to the activated adhesion molecule rather than 'normal' antibodies, as the latter can bind to both active and non-active adhesion molecules. This would show the true percentage of adhesion molecules that were in fact active. In addition, the nature of the antibodies i.e. their affinity for the adhesion molecule, should also be taken into account, as this may or may not explain the inconsistent results obtained above. Therefore it remains to be studied which adhesion molecules are instrumental in the binding of the two cells.

The aim of setting up this co-culture system was to develop an assay system where we could study the role of synovial fibroblasts in the orchestration or perpetuation of the inflammatory state of the joint in rheumatoid arthritis. We decided to use U937 cells, rather than circulating monocytes, to limit the variables in the experiment to the type of synovial fibroblast, i.e. derived from rheumatoid arthritic patients or osteoarthritic patients. We have established a co-culture where we have reconstituted part of the characteristics of the inflamed joint, synovial fibroblasts intermingled with monocytes. This co-culture can now be used to study the release of pro-inflammatory cytokines, like IL-6, IL-1 β , IFN- γ or TNF- α and the role therein of for instance the rheumatoid factor. The co-culture could also be used for studying the production and release of proteases and toxic oxygen metabolites.

Chapter Five

Rheumatoid synovial fibroblasts are a potent source of IL-6, but not IL-1 or TNF- α

5.1. Summary

The co-culture system enabled us to further investigate whether the interactions between monocytes and synovial fibroblasts had any effect on cytokine release. To assess the capacity of synovial fibroblasts to release cytokines, we compared them with primary cultures of dermal fibroblasts. Our results indicate that rheumatoid synovial fibroblasts and dermal fibroblasts release IL-6. IL-1 β or TNF- α could not be detected in these cultures. Addition of hyaluronan dramatically augmented this production, an increase that could be mimicked by engagement of the CD44 receptor with a stimulating antibody. IL-6 release could be further increased with the addition of U937 cells, again without any measurable release of IL-1 β or TNF- α . The addition of immune complexes did not alter the pattern of cytokine release. Studies with phorbol esters indicate that the differentiated U937 cells are able to release both IL-1 β and TNF- α , but for some reason are not stimulated to do so in the co-culture with rheumatoid synovial fibroblasts. Synovial fibroblasts, particularly in the presence of hyaluronan or anti-CD44 antibodies released ten fold higher levels of IL-6 than dermal fibroblasts. This finding indicates that IL-6 release is a key capacity of synovial fibroblasts, but also that IL-6 release may not necessarily be a consequence of the presence of high concentrations of inflammatory cytokines.

5.2. Introduction.

Knowledge about cytokine regulation in inflammatory events like in rheumatoid arthritis has given valuable information about the processes that may underly joint destruction. Cytokine concentrations in synovial fluid exudates have been estimated by ELISAs or at the levels of mRNA. Cytokines that are thought to be important in the pathogenesis of rheumatoid arthritis, are IL-1 β , TNF- α and IL-6 (reviewed by Feldmann *et al.*, 1990). Both IL-1 β and TNF- α have been found to induce cartilage degradation, stimulate

synthesis of metalloproteinase by chondrocytes (Schnyder *et al.*, 1987, Shinmei *et al.*, 1989) and synovial cells (Dayer *et al.*, 1985, Dayer *et al.*, 1986). The major metalloproteinases released by macrophages are collagenase, gelatinase and stromelysin. These proteases can degrade several types of collagen (I, II, III, IV, V and X), insoluble elastin, proteoglycan, laminin and fibronectin. IL-1 and TNF- α were also shown to inhibit proteoglycan synthesis by chondrocytes (Saklatvala *et al.*, 1986). Recent studies have shown that IL-6 is necessary for IL-1 to inhibit proteoglycan synthesis, indicating that this cytokine may act as a co-factor in the process of cartilage degradation (Nietfeld *et al.*, 1990). IL-6 is implicated in B-cell maturation towards plasma cells and subsequent release of immunoglobulins (Burdin *et al.*, 1995), and thus may contribute to the formation of circulating immune complexes also known as the rheumatoid factor. It is known that the B-lymphocyte lineage, which constitutes 10-15% of the population of mononuclear cells in rheumatoid arthritis, produce auto-antibodies and it is thought that these cells are a possible source for the production of immune complexes. Hyaluronan was suggested to facilitate the formation of immune complexes in the joint cavity, by increasing the avidity of auto-antibodies resulting in the formation of IgG-IgM-RF complexes and then in an inflammatory reaction in the joints (Faaber *et al.*, 1989). Immune complexes are thought to cause inflammation because they probably become large enough to be phagocytosed and to activate complement (Brown *et al.*, 1982). The phagocytotic process itself, generates many inflammatory mediators such as lysosomal enzymes, free radicals produced from oxygen metabolism, prostaglandins, leukotrienes, platelet-activating factor, and other substances capable of initiating an inflammatory response.

To assess the contribution of synovial fibroblasts and monocytes to the release of these cytokines, we have developed a co-culture system comprising one week old cultures of synovial or dermal fibroblasts and U937 cells. We chose to use dermal fibroblasts we had a good access to these cells and they showed no expression of VCAM-1, an adhesion molecule that is implicated in the immune response (Morales-Ducret *et al.*, 1992). The dermal fibroblasts would be a source to compare with the rheumatoid synovial fibroblasts to distinguish if certain functions found with synovial fibroblasts were unique. The U937 cells develop towards monocytes within three days of culture. In this study we compare the induction of cytokines under various culture conditions.

Synovial fibroblasts are a rich source of IL-6 release, a release that can be induced by the presence of hyaluronan, anti-CD44 antibodies and in co-culture with U937 cells. For both cells types, under any of the conditions we could detect no IL-1 β or TNF- α release. The addition of rheumatoid factor did not change this pattern. These findings indicate that IL-6 may be independent of inflammation and appears a key capacity of the synovial fibroblast.

5.3. Methods and Materials

5.3.1. Materials

DMEM, RPMI 1640, L-glutamine, Streptomycin sulphate, benzyl penicillinate and foetal bovine serum were obtained from Gibco BRL Life Technologies, UK.

5.3.2. Antibodies

Phycoerythrin conjugated anti-mouse IgG antibodies were obtained from Sigma. Anti-CD14 was obtained from Dako. Anti-Mac-1 (CD11b), anti-VCAM-1, anti-CD44H and anti-VLA-4 were obtained from Becton Dickinson. Anti-MHC II was obtained from DAKO.

5.3.3. Human plasma derived serum preparation (HPDS)

Human plasma was obtained from male AB donors. Fresh citrated blood was spun at 10,000 rpm for 20 minutes at 4°C, and the supernatant (plasma) was microscopically tested for the presence of platelets. If platelets were detected, the plasma was spun again for 20 minutes at 4°C. To the plasma, 4 mM CaCl₂ was added and the plasma was left at 4°C for overnight. The fibrin clot was spun at 10,000 rpm for 20 minutes at 4°C and the plasma was then heat inactivated at 54°C for 40 min. The plasma was cleared one more time of insoluble components by centrifugation at 10,000 rpm for 20 minutes at 4°C. The plasma is aliquoted and kept at -20°C until use. This product is referred to as plasma derived serum (HPDS) (Raines and Ross, 1985).

5.3.4. Synovial fibroblast isolation and cell culture

Rheumatoid synovium tissue was obtained after hip or knee joint replacement surgery and collected from University College London Hospital. The tissues described in this paper are all derived from rheumatoid arthritic joints. Tissue was processed within two hours after removal from the patient. The inner layer of the synovial membrane was removed with a scalpel and the tissue was washed extensively in PBS (w/o Ca^{2+} and Mg^{2+}). The tissue was then chopped into small pieces (1mm^3) washed again in PBS and left to digest in 2mg/ml collagenase in sterile DMEM supplemented with 1% penicillin-streptomycin in a shaking incubator at 37°C . The tissue was sheared using a sterile syringe, filtered using a fine sterile gauze and then washed and resuspended in DMEM containing 10% FBS and 1% penicillin-streptomycin. The cells were counted and seeded into 6 well tissue culture plates (Nunc), uncoated or coated with collagen, fibronectin and laminin (Becton Dickinson-Collaborative Biomedical Products), and left overnight for the fibroblasts to adhere. The cells were washed three times with PBS and fresh DMEM was added, supplemented with either 10% FBS or 10% heat inactivated human plasma (HPDS). In the case of hyaluronan, 1 mg/ml of human umbilical cord hyaluronan (Sigma) was dissolved in DMEM supplemented with either 10% FBS or 10% HPDS. The cell culture was continued for various time periods. Medium was replaced every five days, but cells were not passaged during this period. For most of the cases, the synovial fibroblasts proliferate during the first two weeks and then divided only slowly. The culture dishes never reached confluence during these experiments.

5.3.5. Dermal fibroblast cell culture

Dermal fibroblasts were obtained from fresh skin biopsies. A piece of skin was removed under sterile conditions and placed into a petridish. Fibroblasts grow out of the corium layer onto the dish and the skin is removed after three days. The cells were continued to be cultured in DMEM, supplemented with either 10% FBS or 10% HPDS. Before use in the experiments the cultures were screened CD14 expression which was absent in all cases. Only early passages between 2-4 weeks were used for the experiments. When hyaluronan was added this was dissolved in DMEM supplemented with either 10% FBS or 10% HPDS at a concentration of 1 mg/ml.

5.3.6. Polyethylene Glycol precipitation of Immune complexes

PEG 6000 polymer brings down aggregates, immune complexes, C1q, C3d, binding proteins. 300µl of positive rheumatoid factor sera was put into a 5 ml tube with 60µl of 12% (w/v) PEG 6000 in veronal buffered saline (VBS) (5 times stock VBS solution: sodium chloride 4.25g, sodium barbitone 0.1875g, barbitone 0.2875g dissolved in 100ml distilled water). The solution was mixed and left overnight at 4°C. It was then centrifuged at 2000rpm for 30 minutes at 4°C, put on ice, the precipitate was washed with 2% (w/v) PEG in VBS (2ml) and centrifuged again at 2000rpm for another 30 minutes at 4°C. The precipitate was resuspended in 150µl VBS, incubated for 1 hour at 37°C. The solution was then tested in an agglutination rheumatoid factor titre test and a positive result indicated that the solution contained high amounts of rheumatoid factor constituting approximately 80µg/ml of protein. A final concentration of 1.6µg/ml of protein was added to the cultures.

5.3.7. Fibroblast-U937 cell co-culture

The fibroblasts were seeded in to 6 well plates at 0.2×10^6 cells per well in DMEM supplemented with 10% FBS. Once the wells were half confluent the medium was changed or replenished with 10% FBS, 10% HPDS, 10% HPDS+HA. The medium was left for two days and then 0.2×10^6 U937 cells were added per well. The co-cultures were then left for a further three days. PEG precipitated immune complexes (rheumatoid factor (1.6µg/ml) was added on the third day of the co-culture and it was left for a further three days and then it was harvested for FACS analysis. In addition, medium was collected from the control fibroblasts, after the first two days incubation, and used as conditioned medium to culture U937 cells only in order to determine if any soluble mediators may be causing differentiation. Again these cells were left for three days.

Supernatants of all cultures were collected after the allocated time period. These supernatants were microfuged for 5 minutes at 1200rpm and then stored at -20°C, until later tested for IL-1β, IL-6 and TNF-α by ELISA kits.

5.3.8. Cytokine measurement

IL-1β, IL-6 and TNF-α were measured using cytokine assay kits provided by R&D Systems or Central Laboratory of the Netherlands Red Cross Blood Transfusion Service.

5.3.9. Statistical analysis

Statistical analysis was performed using paired students t-test (two tailed). P values less than 0.05 were considered significant. * $P < 0.005$; ** $P < 0.01$; *** $P < 0.001$.

5.4. Results

5.4.1. Characterisation of dermal fibroblasts

Dermal fibroblasts were characterised by the absence of VCAM-1 (Morales-Ducret *et al.*, 1992).

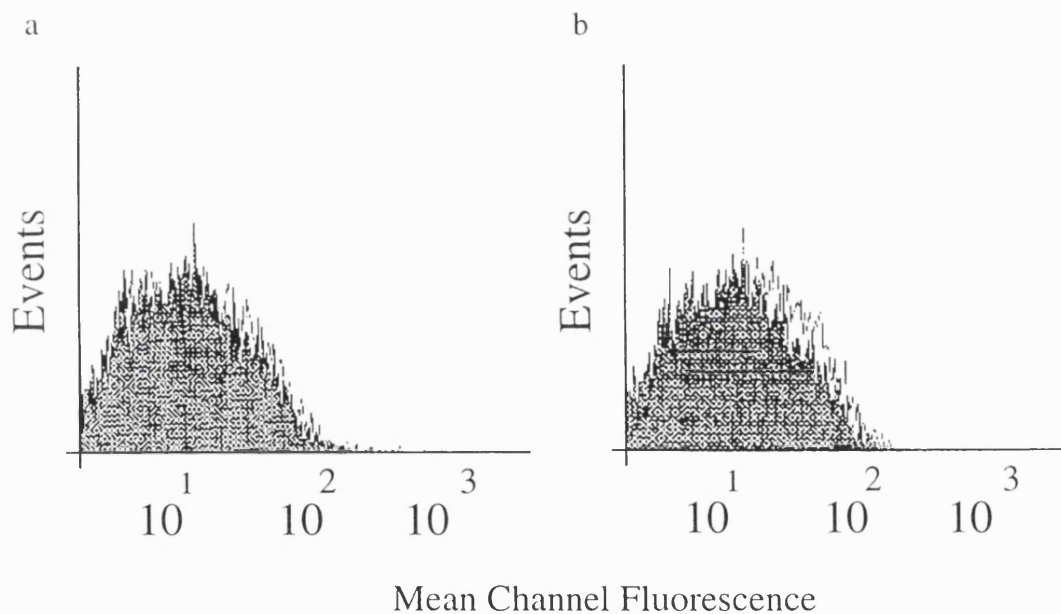


Figure 5.1.

Characterisation of dermal fibroblasts. Dermal fibroblasts cultured for 2 weeks do not express a) VCAM-1 and very little b) ICAM-1.

The dermal fibroblasts also secrete little IL-1 β , TNF- α i.e. they were undetectable with the ELISA assay and approximately 5 fold less IL-6 (See Table 5.1.) when cultured alone. In addition in co-culture the dermal fibroblasts are induced to secrete more IL-6 but again this is much lower than compared to the rheumatoid synovial fibroblasts.

a)

IL-6 (pg/ml)	FBS	HPDS	HPDS + Hyaluronan
U937	4 ±1	5 ±1	23 ±12
Dermal fibroblasts	625 ±210	165 ±86	2583 ±1093
U937 + dermal fibroblasts	1218 ±646	165 ±32	3650 ±507

b)

IL-6 (pg/ml)	FBS	HPDS	HPDS + Hyaluronan
U937	4 ±1	5 ±1	23 ±12
Intimal synovial fibroblasts	1,683 ±1,160	534 ±289	11,013 ±5,445
U937 + intimal synovial fibroblasts	2,773 ±140	1,625 ±658	40,075 ±8,103 *
U937 + syn fib + imm com	1,928 ±165	1,356 ±455	29,689 ±7,569

Table 5.1.

a) Dermal fibroblasts respond to the presence of U937 with high increase in IL-6 over a three day incubation period. b) Rheumatoid synovial fibroblasts release a high amount of IL-6 alone and in co-culture with U937 cells. Data is expressed as Mean ±SEM. N=3. Syn fib ; rheumatoid synovial fibroblasts, imm com ; immune complexes.

In addition, we investigated whether U937 cells were also differentiated to the same extent compared with U937 cells grown in co-culture with rheumatoid synovial fibroblasts. We found that U937 cells were differentiated which was detected by a slight increase in cell adhesion molecules but this was not seen to be as much as with the cell adhesion molecules expressed on rheumatoid synovial fibroblasts (See Table 5.2.). There was also not as much NBT precipitate produced on the cells surface compared with U937 cells co-cultured with synovial fibroblasts (See Figure 5.1.)

	CD11b	CD14	VLA-4	MHC II
U/HPDS	10.41 ±4.0	0.51±0.15	135.07±29.3	79.90±49.7
U/HPDS +HA	21.49 ±1.9	3.29±1.37	149.97±42.0	27.76±18.2
U/FBS	9.85±3.9	0.46±0.18	68.37±29.8	56.76±25.7
U+PDBu (100nM)/ HPDS	149.64±32.4	7.99±4.1	58.78±27.5	190.21±140.1
D+U(adh)/ HPDS	26.65±9.6	2.66±1.8	95.27±29.6	257.81±98.4
D+U(adh)/ HPDS+HA	37.14±14.7	12.42±3.9*	23.71±12.4**	238.26±146.5
D+U(adh)/ FBS	15.25±7.2	1.72±0.9	82.93±44.1	220.05±123.9
U/c.m. HPDS+HA	19.87±16.3	4.3±3.2	57.5±28.7	189.3±95.6

Table 5.2.

Monocyte differentiation marker expression detected by flow cytometry on U937 cells grown with or without dermal fibroblasts. Data are presented as Mean ±SEM (standard error of the mean). Statistical analysis ; paired student *t*-test, **P*<0.05; ***P*<0.01; ****P*<0.001; U, U937 cells; HPDS, Human plasma derived serum; HA, Hyaluronan; FBS, Foetal bovine serum; PDBu, Phorbol 12,13-dibutyrate; S, synovial fibroblast; adh, adherent cells; N = 5.

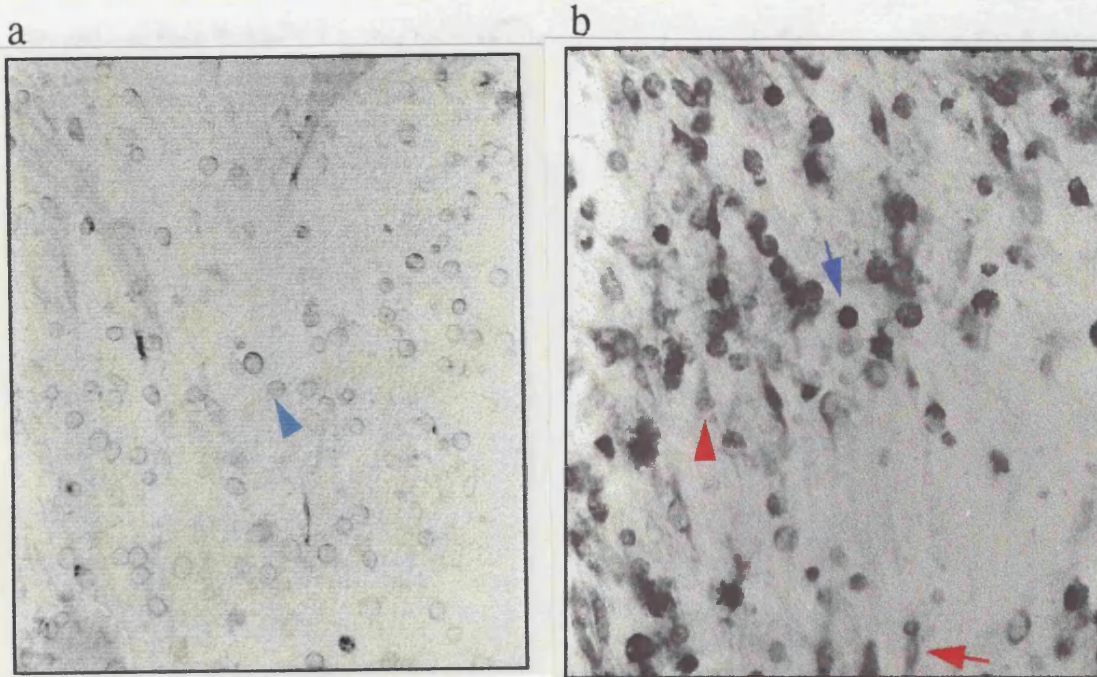


Figure 5.2. NBT reduction test of U937 cells grown in co-culture in a) 10% HPDS and b) 10% HPDS + HA with dermal fibroblasts for 3 days. The figure above represents results from a typical experiment. N=4. Red arrow points to a dermal fibroblast and blue to U937 cells.

5.4.2. Addition of U937 cells induces release of IL-6 but no release of IL-1 β or TNF- α

Cultured intimal synovial cells produced abundant amounts of IL-6 within a three day time period. In the presence of HPDS, the production was significantly lower. This finding indicates that serum has a contribution to the basal release of IL-6. The addition of U937 cells for a period of three days, in which these cells have fully differentiated towards monocytes, IL-6 production increases with 2 to 3 fold. Again in the presence of HPDS the release is lower (see Table 5.1b and 5.3.). Levels of IL-1 β or TNF- α were very low. We used PDBu as a positive control for the induction of expression of TNF- α by U937 cells, and under these conditions the cells produced 140 pg/ml in the same period of time. We next compared cytokine production with a primary culture of dermal fibroblasts. Dermal fibroblasts also have a basal release of IL-6 (See Table 5.1a) and no detectable amounts of IL-1 β or TNF- α . Addition of U937 cells, which in three days also fully develop towards monocytes, only increased IL-6 release in the presence of FBS, but had no effect on the release in the presence of HPDS. The levels of IL-6 released by dermal fibroblasts were 50% lower than the levels released by intimal synovial fibroblasts (see Table 5.1.). For both studies we have extended the co-culture for 6 days,

fibroblasts (see Table 5.1.). For both studies we have extended the co-culture for 6 days, and although an accumulative release of IL-6 was observed, we could not detect any TNF- α or IL-1 β . Over a six day period, the addition of U937 induced IL-6 release in synovial and dermal fibroblasts in both HPDS and FBS conditions.

IL-1 β (pg/ml)	FBS	HPDS	HPDS + Hyaluronan
U937	0	0	0
Intimal synovial fibroblasts	0	0	0
U937 + intimal synovial fibroblasts	0	0	8 \pm 1
U937 + syn fib + imm com	0	0	25 \pm 12.4

TNF- α (pg/ml)	FBS	HPDS	HPDS + Hyaluronan
U937	9 \pm 1	4 \pm 0.5	0
Intimal synovial fibroblasts	5 \pm 1	18 \pm 7	9 \pm 3
U937 + intimal synovial fibroblasts	0	27 \pm 6	0
U937 + syn fib + imm com	4.2 \pm 0.9	0	0

Table 5.3.

IL-1 β and TNF- α release from U937 cells, intimal rheumatoid synovial fibroblasts and both in co-culture (\pm immune complexes) for 3 days (or 6 days). Data is expressed as Mean \pm SEM. Statistical analysis ; paired student t-test. * indicates a significant difference with a *P* value of 0.05. The figure above represents results from a typical experiment. N=3. Syn fib ; rheumatoid synovial fibroblasts, imm com ; immune complexes.

5.4.3. Hyaluronan increases IL-6 but not TNF- α or IL-1 β which can be mimicked in part by stimulation of CD44 with anti-CD44 antibodies

Because hyaluronan had such a great effect on binding and differentiation of U937 cells to the synovial fibroblasts, we studied cytokine release in the co-culture in the presence of hyaluronan. In synovial fibroblasts, the addition of hyaluronan in the presence of HPDS, resulted in a massive increase in IL-6 release, without the slightest increase in

IL-1 β or TNF- α (Table 5.1b. last column). The addition of U937 cells, further increased IL-6 release almost 4 fold. A similar pattern was seen with dermal fibroblasts, albeit that the extent of release was much lower (only 25%) and the addition of U937 cells had less of an effect, only 1.25 fold (Table 5.1a.). The synovial fibroblasts express the hyaluronan receptor CD44, as detected by flow cytometry (see Figure 4.2.), and we added stimulatory anti-CD44 antibodies to both the synovial fibroblasts and the U937 cells, to study whether or not hyaluronan could contribute through this pathway (See Table 5.4. Anti-CD44 antibodies caused a 11 fold increase in IL-6 release which is 40% of the IL-6 release as seen with hyaluronan and thus, the effect of hyaluronan could in part be explained by activation of the CD44 receptor. The addition of a similar concentration of IgG had no effect on IL-6 release (Table 5.4.). We were unable to detect any IL-1 β or TNF- α under these conditions.

IL-6 (pg/ml)	HPDS
Intimal synovial fibroblasts	534 \pm 245
Intimal synovial fibroblasts + anti-CD44	6,553 \pm 460 ***
Intimal synovial fibroblasts + IgG	432 \pm 145

Table 5.4.

IL-6 induction can be mimicked by the addition of anti-CD44 antibodies, which was found to significantly different with a *P* value of 0.001 (***).The figure above represents results from a typical experiment. N=3.

5.4.4. Addition of rheumatoid factor does not alter pattern of cytokine release

Although TNF- α is abundantly present *in situ* in the rheumatoid joint, a co-culture in the presence of fully differentiated U937 cells is not enough to cause the release of this inflammatory cytokine. Because it was proposed that the presence of rheumatoid factor (immune complexes) may have a role in the pathogenesis of rheumatoid arthritis we studied cytokine release after the addition of rheumatoid factor to the co-culture. It was shown that U937 cells are able to bind aggregated IgG and IgM complexes with their respective Fc receptors (Mehta *et al.*, 1986) and that hyaluronan improved immune complex formation in the presence of rheumatoid factor (Faaber *et al.*, 1989). We allowed the U937 cells to fully differentiate and at day 3 of the co-culture rheumatoid

factor was added. We measured cytokine release after a further 3 days of co-culture. Although IL-6 release was prominent, the addition of rheumatoid factor did not induce IL-1 β nor TNF- α (See the bottom row in Table 5.1b. and Table 5.3.).

5.5. Discussion

Primary cultures of synovial or dermal fibroblasts release IL-6, but no appreciable amounts of TNF- α or IL-1 β could be detected. Under human plasma derived serum conditions, basal release was only 25% to 35% of that measured in the presence of foetal bovine serum. Addition of U937 cells, which in the co-culture with either synovial or dermal fibroblasts fully differentiate towards monocytes, resulted in an two to three fold increase in IL-6 release, but not of IL-1 β or TNF- α . When U937 cells were differentiated with PDBu, 140 pg/ml of TNF- α was released. The U937 cells also produced IL-6 at 128 pg/ml in three days, a finding which is in agreement with those published by Navarro *et al.*, (1989). The PDBu treated cells were adherent and showed a similar expression of differentiation markers as the U937 cells in the synovial or dermal co-culture. In a recent study where unstimulated elutriated normal blood monocytes were used in a co-culture with intimal synovial fibroblasts from patients with rheumatoid or osteoarthritis, a synergistic increase of the production of IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), and IL-8 was observed, when compared with their respective production in culture alone. In contrast, cytokines such as IL-10, IL-1 β , IL-1 α and TNF- α could not be detected (Chomarat *et al.*, 1995b). Chomarat *et al.*, (1995b) also added U937 cells but showed that it had a smaller effect on IL-6 release than blood monocytes over a two day incubation period. It was concluded that certain monocyte markers, in particular CD14, were essential for a cell contact-induced IL-6 release. Our observation that only after a delay of two days, differentiation markers like CD14 and Mac-1 become significantly upregulated on the U937 cells when co-cultured with synovial fibroblasts, explaining why we measure a three fold increase after a three days co-culture period. Thus, indeed, the expression of certain monocyte markers appears essential for the effect on IL-6 release. Synovial fibroblasts are more potent in inducing monocyte differentiation than dermal fibroblasts (see previous chapter) and that may explain why within a three days co-culture, U937 cells have a stronger IL-6 inducing capacity in a

co-culture with synovial fibroblasts, but this difference diminishes after six days of co-culture. Both our findings and the findings presented by Chomarat *et al.*, (1995b) indicate that the monocyte interaction with the synovial fibroblast in itself is not sufficient to explain the presence of IL-1 β or TNF- α in the inflamed synovial joint. We extended the study by adding rheumatoid factor, but again this failed to increase release of these two cytokines. Additional aspects of the synovial joint must be involved in the accumulation of TNF- α or IL-1 β . A defined *in vitro* cell culture system where cytokine release, as seen in the rheumatoid synovial joint, could be reconstituted would be of great value for assessment of new anti-inflammatory compounds specific for rheumatoid arthritis.

The observation that engagement of the CD44 molecule, with anti-CD44 antibodies, could increase IL-6 release by eleven fold reveals a novel stimulatory pathway for this cytokine. An even higher increase was observed with the addition of hyaluronan, and this finding would suggest that the presence of hyaluronan might dictate IL-6 release. The addition of hyaluronan had no significant effect on IL-6 production in differentiated U937 cells indicating that in the co-culture the synovial fibroblasts are the main source of IL-6. Intimal synovial fibroblasts are producing hyaluronan *in situ* (Pitsillides *et al.*, 1994), but this production is very low when cells are in culture, as estimated by the presence of the enzyme UDP-dehydrogenase, a key enzyme in hyaluronan synthesis (unpublished observation). In addition, intimal synovial fibroblasts are situated adjacent to the synovial fluid which contains approximately 1 mg/ml of hyaluronan and as such, a possible role of hyaluronan in regulation of IL-6 is relevant for the study of inflammation in the synovial joint. We have assayed IL-6 release with varying concentrations of hyaluronan and release is dose-dependent in the range from 0.03-1.0 mg/ml. The effect of hyaluronan may be through sequestering of inhibitory cytokines (see Yayon *et al.*, 1991, Tanaka *et al.*, 1993, Ruoslahti *et al.*, 1991, Ramsden *et al.*, 1992). However, because hyaluronan preparations have protein contamination and contain chondroitin sulphate components, the observation warrants further investigation. It has been shown that monocyte induced IL-6 release in synovial fibroblasts is largely dependent on the presence of IL-1, that is soluble IL-1 receptors (IL-1Ra) can reduce the monocyte-induced response (Chomarat *et al.*, 1995b). We were not in the position to test the role of IL-1 on IL-6 release, but given the observation that such high

concentrations of IL-6 could be obtained with no-detectable IL-1, indicate that low levels of IL-1 are sufficient to maintain a high production of IL-6. In that case, the presence of high amounts of IL-6 in the synovial joint in rheumatoid arthritis may not necessarily be a consequence of the inflammatory state of that joint, but a simple consequence of an increased number of IL-6 secreting intimal synovial fibroblasts. If IL-6 is instrumental in the propagation of the inflammatory response then the total number of intimal synovial fibroblasts may be a risk factor in this disease.

Chapter 6

General Discussion

Previous studies have clearly shown that monocytes are able to interact with endothelial fibroblasts and that this interaction result in diapedesis and subsequent infiltration of tissue by the monocytes (Grober *et al.*, 1993; Springer, T.A., 1994). How monocytes interact with rheumatoid intimal synovial fibroblasts within the joint and what the consequences are of this interaction, is less well studied. In this thesis I have described co-cultures between synovial or dermal fibroblasts together with U937 cells to study the consequences of this interaction with respect to monocyte differentiation, cytokine release, protease expression and respiratory burst activity. A number of aspects of these studies will be dealt with in the following paragraphs.

No qualitative difference between co-cultures in human plasma derived serum and foetal bovine serum

We have chosen to test human plasma derived serum for the reason that we believe that foetal bovine serum, which is a standard medium supplement for cell cultures, may contain a number of mediators that are released from platelets and leukocytes during the blood clotting, which are of a pro-inflammatory nature. These mediators therefore could obscure the contribution of the synovial fibroblasts, in their interaction with monocytes, to the release of inflammatory cytokines in the synovial joint. Although in theory this may be true, in our studies many similarities existed between cultures in FBS or HPDS. There were however a few significant differences. With synovial fibroblasts, the use of HPDS as a culture supplement significantly upregulated VCAM-1 expression and caused a threefold reduction in monocyte- or hyaluronan-induced IL-6 release. With U937 cells, HPDS increased expression of Mac-1 and VLA-4 and resulted in a three fold increase in PDBu-induced binding to ICAM-1 or VCAM-1. In the presence of HPDS, the expression of the NADPH oxidase component p40^{phox} was absent. However, in the co-culture, we could not detect a difference between the two media with respect to IL-1 β or TNF- α release, which was absent under both conditions. Nor could we see a

difference in collagenase expression, which was equally high under both conditions. Synovial fibroblasts have a different morphology in HPDS compared to FBS, in the presence of HPDS the cells keep their stellate morphology much longer. In long term cultures, the synovial fibroblasts become spindle shaped. We conclude that although differences between HPDS and FBS exist, where FBS potentiates a number of responses, they are not to such an extent that their presence or absence resulted in important different findings.

The loss of VCAM-1 as a marker for a changing phenotype?

In culture, synovial fibroblasts almost immediately started to loose mRNA expression of VCAM-1, followed by a loss of cell surface expression. In the presence of human plasma derived serum, the level of cell surface expression is higher but the decline of mRNA and cell surface expression cannot be prevented. It has been shown by others that VCAM-1 expression can be temporarily upregulated by TNF- α , IL-1 β or IFN- γ but because this upregulation only lasts for two days we feel it cannot explain the constitutive expression as observed *in situ*. The decline in VCAM-1 expression indicates that the fibroblasts change their phenotype in culture. This is not entirely surprising considering the fact that removal from the synovial membrane must resemble wounding of the tissue, to which the cells will respond. For instance, characteristics of skin wounding are an increase in fibroblast proliferation, a change in integrin expression (so the cells can move into the wound space) and abundant hyaluronan and collagen deposition. Not only that, the isolated cells may also have lost extracellular matrix contacts that could be relevant for gene expression. Assuming that cells in culture resemble cells from wounded tissue, prolonged cell culture may obscure the differences that could exist between fibroblasts from rheumatoid -, osteo- arthritic, control or dermal tissue. On the other hand, it may be an ideal testing situation, because if we assume that damage is part of the onset of an inflammatory response in general, then under culture conditions we may actually reveal the essential difference(s) between the cells. Differences that may explain the unresolved inflammation in the synovial joint. We conclude that primary cultures are a good source for the type of study we have presented, but it would be beneficial if more markers for synovial fibroblasts were tested in order to carefully define the state of the cells *in situ* and during the experimentation.

Can the co-culture system provide useful information about mechanisms underlying the propagation of inflammation in rheumatoid arthritis?

The studies described in this thesis are based on the presumption that somehow the resident synovial fibroblasts, in particular those that constitutively express VCAM-1, may have a role in the propagation of the inflammatory state of the synovial joint. We focused on the VCAM-1-positive synovial fibroblasts because studies have indicated that the intimal lining layer, where the VCAM-1-positive fibroblasts reside, may be the first station for monocytes during the process of infiltration into the synovial tissue. There they mature towards macrophages and then move into other layers of the synovium (Hogg *et al.*, 1985). The possible role of the VCAM-1 positive synovial fibroblast in propagation of inflammation may go some way in explaining why synovial joints in particular are prone to unresolved inflammations. The interaction between U937 cells and synovial fibroblasts, in the presence of hyaluronan, results in a perfect differentiation of the U937 cells towards monocytes. Maximum expression of differentiation markers, like CD14 or Mac-1, was obtained after three days of co-culture and the cells have developed a complete NADPH oxidase and express collagenase. Differentiation is not dependent on adherence and must therefore be induced by soluble factors. However, the co-culture of U937 cells and synovial fibroblasts from RA patients, even in the presence of rheumatoid immune complexes, did not induce release of TNF- α or IL-1 β (nor could we detect any non-phorbol ester-induced NADPH oxidase activity). Identical studies with peripheral blood monocytes gave similar results (Chomorat *et al.*, 1995b). These data suggest that the interaction between fibroblasts and monocytes per se does not explain the elevated levels of TNF- α or IL-1 β . Other factors present in the joint must be involved in the release of these cytokines. As such the study has been useful. The question remains, which factor(s)? The monocyte is the main source of IL-1 β and TNF- α and therefore the co-culture could be used as a screening system where fractions of synovial fluid devoid of these two cytokines, are tested for the induction of cytokine release. However, although the assay system is good enough to test fractions, it will be very difficult to obtain enough synovial fluid to subsequently identify a putative factor. Only a concerted effort between a large number of rheumatology departments could make such an event possible. Another approach could be the addition of lymphocytes to assess their role in TNF- α and IL-1 β release. It has been shown that U937 cells release IL-1 upon culture in conditioned medium from

cloned human lymphocytes or lectin stimulated peripheral blood lymphocytes (Amento *et al.*, 1995), and therefore a three-cell model may be needed to explain release of IL-1 β or TNF- α . Lastly, known cytokines could be added for the same purpose. The putative TNF- α and/or IL-1 β -inducing factor should have a higher position in the cytokine hierarchy and its function could therefore be less redundant. This is of great importance when assessing the suitability of a cytokine as a target for pharmacological intervention. If the release of inflammatory cytokines can be reconstituted *in vitro* then the culture can also be a useful test system for the development of novel anti-rheumatic compounds and initially replace the use of animal models.

The human dermal fibroblast as a control

Essential in the co-culture studies is a comparison with either fibroblasts from non-inflamed joints or with primary fibroblasts cultures from other human tissues. We have chosen dermal fibroblasts for this purpose because we had a good access to these cells and they showed no expression of VCAM-1, an adhesion molecule that is implicated in the immune response. The rationale behind the use “control fibroblasts” is that if certain functions were found with synovial fibroblasts, a comparison would inform whether this function is unique or not. It being unique is a prerequisite for it being important in our understanding of arthritis. Many types of fibroblasts may contribute somehow to an inflammatory responses but that knowledge only will give information on their possible role in inflammation in general. In most of the cases their contribution *in situ* is unlikely to result in an unresolved inflammatory state as seen in the synovial joint. In other words, the contribution to inflammation in itself is not explaining arthritis if other fibroblasts could do the same. In our study it became clear that whatever synovial fibroblasts could do, dermal fibroblasts could do as well, although to a lesser extent. The one difference that did shine through is the 10 fold higher release of IL-6 by the synovial fibroblasts.

IL-6 release is a key capacity of synovial fibroblasts

Addition of hyaluronan or anti-CD44 antibodies plus the presence of differentiated U937 cells, induces high release of IL-6 from synovial fibroblasts (40,000 pg/ml), a release that, on an equal cell number basis, is not matched by dermal fibroblasts (3,600 pg/ml). Clearly, synovial fibroblasts are a good source of IL-6 and the observation that both anti-CD44 and hyaluronan can potentiate its release indicate that IL-6 release may always be present in the synovial joint, or for that matter in any tissue where fibroblasts are present and surrounded by sufficient amounts of hyaluronan. Similar findings were obtained by Chomarat *et al.*, (1995) where it was shown that peripheral blood monocytes induce IL-6 release from synovial fibroblasts without measurable release of IL-1 β or TNF- α . It therefore can be questioned if IL-6 is a good marker of the inflammatory state of the joint, it is more likely a marker for synovial fibroblast cell numbers. IL-6 levels are higher in the synovial joint of rheumatoid arthritic patients (Guerne *et al.*, 1989; Houssiau *et al.*, 1988), but in culture, on an equal cell number basis, IL-6 release does not differ significantly between the different diagnostic groups, RA, OA and trauma (Rosenbaum *et al.*, 1992). This finding supports the idea that total cell number may be the key factor that determines the concentration of this cytokine. Assuming that IL-6 contributes to inflammation in some way (Dechanet *et al.*, 1995; Guerne *et al.*, 1989), hyperplasia of synovial fibroblasts as such could therefore be an important factor in the onset and/or propagation of the inflammatory state. Once the high numbers are there, little can be done to revert the process. Hyperplasia is regarded as one of the first disease markers in rheumatoid arthritis (Zvaifler *et al.*, 1994) and that illustrates the possible importance of mere cell number. If mere synovial fibroblast number is a risk factor in the development of rheumatoid arthritis then mopping up the action of cytokines like TNF- α or IL-1 β will never resolve the disease (Lorenz *et al.*, 1996). It will modify its progression but as soon as therapy ends, inflammation will pick up at an equal rate. It therefore remains doubtful whether or not these type of therapies will ever be effective or economical. Targeting the synovial fibroblasts through induction of apoptosis could be a more economic way to intervene with the inflammatory events in the synovial joint. In that respect, IL-4 could be an interesting interleukin because it has been demonstrated that this interleukin reduces IL-6 release and induces increased cell death of synovial fibroblasts (Dechanet *et al.*, 1993). Future experiments on induction of apoptosis and on the precise mechanism by which IL-6

release is regulated in synovial fibroblasts could be an important direction in future rheumatoid research. Synovial fibroblasts in rheumatoid and osteo-arthritis are believed to be in some kind of transformed state, for instance elevated oncogene levels (Kinne *et al.*, 1995; Qu *et al.*, 1994), and should therefore be sensitive to apoptosis-inducing factors. The apoptosis studies could line up with similar studies in cancer cells and the mutual effort may be productive (Mountz *et al.*, 1994).

Differential library of synovial - and dermal fibroblast-induced mRNA expression in attached U937 cells

In the presented experiments we have analyzed differentiation of U937 cells and subsequent release of cytokines that are thought to be involved in the ongoing inflammation in the synovial joint. A different approach could be to use the same co-culture technique, with dermal or synovial fibroblasts and analyse the differences in mRNA expression of the adherent U937 cells between the two co-cultures. Essential to this approach is that, at the time of sampling, the U937 cells have reached an equal level of differentiation, for instance an equal level of CD14 or NADPH-oxidase expression, otherwise differences in differentiation are scored. Another essential aspect is prior selection of synovial cell. The adherent synovial cells are a heterogeneous population of fibroblasts, dendritic cells and macrophages. The contribution of each cell type may be different. To focus on the VCAM-1-positive synovial fibroblasts, flow cytometry could be used to isolate these fibroblasts and amplify it in culture for a period of one week prior to the addition of the U937 cells. The dermal fibroblasts appear very homogeneous and require little further selection. After the co-culture period, the differentiated U937 cells are selectively removed from the fibroblasts with a short ice cold PBS/EDTA treatment. Using a Dyna beads panning method, CD14 positive cells could then be selected to obtain a CD14-enriched population. A similar approach could be performed with peripheral blood monocytes. The difference between the two mRNA patterns could indicate a difference between the inducing capacities of the two fibroblast types. This difference may reveal new genes that are possibly involved in the interaction of synovial fibroblasts with monocytes and as such may open new lines of research into rheumatoid arthritis.

Chapter Seven

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Appendix I

Primer selection criteria

A computer programme called DNA Star was used to design the primers used in the PCR reactions. These calculate the DNA duplex stability based on pairwise interacting nearest neighbour analysis.

Primer design has an important influence on the specificity and efficiency of amplification with respect to each amplimer, such as the base composition, length, chemical modifications and purity. The factors will influence the generation of amplimer secondary structures and primer-dimer formations. The concentration of primers added to a reaction will also influence the yield, and usually it is necessary to titrate the final concentrations in the range of 0.1-1.0 μM . Primers used in PCR are generally between 18 and 30 nucleotides in length, which allows a reasonably high annealing temperature to be used. Primers within this range are extremely specific provided the annealing temperature of the PCR reaction is a few degrees below the actual melting temperature. Primers should also if possible be made such that the GC content is in the range 40-60%. In addition, regions of unusual sequence, such as polypurines, polypyrimidines, repetitive motifs or significant secondary structure should be avoided. Primer pairs should also be designed so that there is no complementarity of the 3' ends either intra or inter individual primers or have a sequence of a group of GC bases. This precaution will reduce the incidence of primer-dimer formation.

Generally, optimum enzyme concentration lie in the range of 0.005-0.025 units μl^{-1} . Higher concentrations may cause an increase in no-specific product generation.

Calculation for T_m

$$T_m = 81.5 + 16.6 (\log_{10} [\text{Na}^+]) + 0.41 (\text{fraction G + C}) - 600 / N$$

N = chain length predicts reasonably well the T_m for oligonucleotides as long as 60-70 nucleotides and as short as 14 nucleotides (Sambrook, Fritsch & Maniatis).

DNA STAR PROGRAMME : Primer selectInitial conditions

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

Primer Characteristics

Primer length	Min ; 17 bp	Max ; 24 bp
3' Pentamer	8.5 -Kc/M	
Stability		
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 with 7 bp of 3'	
Accept dimers of	3 bp or less
Accept hairpins of	3 bp or less

Primers are then scored by ideal product length (approx 0.3 - 1.0 Kb), primer T_m match, product T_m difference and internal stability.