CHARACTERIZATION OF THE RESPONSES OF HUMAN SYNOVIAL FIBROBLASTS IN VITRO

By

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ABSTRACT

The synovial membrane (SM) is a specialized tissue which lines the joint capsule surrounding the interface between bone and cartilage. The intimal layer of the SM, a 1 to 2 cell thick layer of myeloid and mesenchymally-derived cells, has generally been characterized as the population of cells responsible for the secretion of synovial fluid (SF) and with maintaining structural stability within the capsule of diarthrodial joints.

In pathological states, the SM is transformed into an aggressive tissue inundated by inflammatory cells and their soluble mediators. The intimal layer of the SM and its resident cells, Type A and Type B synoviocytes, undergo an alteration in phenotype during disease, particularly chronic joint inflammation. These synoviocytes expand in number and activity and have been demonstrated to be involved in part with the arthritic lesions associated with inflammatory joint disease. The purpose of this thesis is to illustrate that the human synovial fibroblast (HSF or Type B synoviocyte) is a dynamic cell both responding to and influencing its local environment. More specifically, it is documented that HSF activity is significantly affected by a host of locally produced soluble mediators released during arthritic diseases. In addition, HSF are shown to respond to their environment by possessing the capability to alter the activity of other inflammatory cells which are present in the joint milieu. We also show that the pathological state of the originating SM is significant in determining the response and activity of HSF. That is, HSF-derived from normal SM respond to some stimuli differently than do HSF derived from SM originating from diseased joints.

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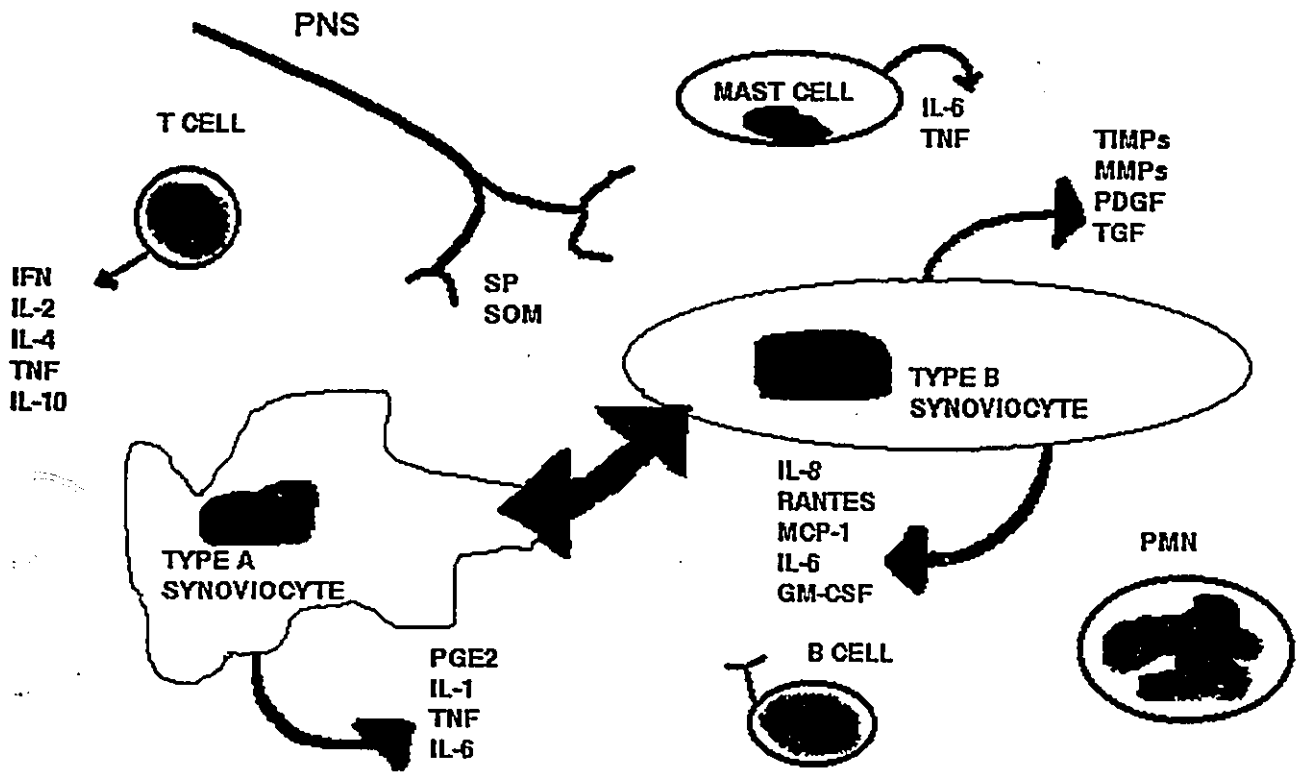

FIGURE 1:

The Cellular Interactions of Cells Within the Inflamed Synovial Membrane. **PGE**, prostaglandins E; **IL**, Interleukin; **MCP-1**, Monocyte Chemoattractant Peptide-1; **TNF**, Tumour Necrosis Factor, **MMP**, Matrix Metalloproteinase; **TIMP**, Tissue Inhibitor of Matrix Metalloproteinase; **GM-CSF**, Granulocyte Macrophage Colony-stimulating Factor; **PDGF**, Platelet Derived Growth Factor; **FGF**, Fibroblast Growth factor; **TGF**, Transforming Growth Factor; **SP**, Substance P; **SOM**, somatostatin; **PNS**, peripheral Nervous System; **PMN**, Polymorphonuclear Cell

ABBREVIATIONS

CNS = Central Nervous System

DMARDs = Disease Modifying Anti-Rheumatic Drugs

ECM = Extracellular Matrix

GM-CSF = Granulocyte Macrophage Colony - Stimulating Factor

HLA = Human Leukocyte Antigens

HSF = Human Synovial Fibroblast

IL-1 = Interleukin-1

IL-1R = Interleukin-1 Receptor

IL-1RA = Interleukin-1 Receptor Antagonist

IL-6 = Interleukin - 6

IL-8 = Interleukin - 8

LPS = Lipopolysaccharide

MCP-1 = Macrophage Chemoattractant Peptide

MIPs = Macrophage Inflammatory Proteins

MMP = Matrix Metalloproteinases

MMP-1 = Interstitial Collagenase

MMP-2 = Gelatinase

MMP-3 = Stromelysin

MMP-9 = 92kDa Gelatinase

OA = Osteoarthritis

PG = Prostaglandin

PGI2 = Prostacyclin

PGE2 = Prostaglandin E2

RA = Rheumatoid Arthritis

RF = Rheumatoid Factor

SF = Synovial Fluid

SM = Synovial Membrane

SP = Substance P

TGF = Transforming Growth Factor

Th1 = T helper 1

TIMP = Tissue Inhibitor of Matrix Metalloproteinases

TNF = Tumor Necrosis Factor

Chapter 1

INTRODUCTION

1.1 ARTHROLOGY

Skeletal joints vary significantly in form and structure and are specialized tissues which vary in function. Joints which are characterized by immobility are commonly known as Fibrous joints (O'Rahilly, 1986). These include bones within the skull which are connected by several fibrous layers making up the calvarial sutures. These articulations do not move and are usually spared from any arthritic involvement. Cartilaginous joints are joints of limited mobility where skeletal elements are united by cartilage (either fibrous or hyaline). Vertebral joints as well as the pubic symphysis are members of this family. Degeneration of joint space due to the aging process and excessive movement are the main factors initiating pathologies within these joints (Altman et al, 1986).

Articulating diarthrodial joints, also known as synovial joints, have been studied in detail. These possess a cavity and are specialized to permit free movement. Synovial joints are classified according to their axes of movement and the shape of their bony articulating surfaces. The Condylar joint, an example of which is the knee, consists of two bony surfaces interfacing through articular cartilage. The articular surface of the bones are lined with a layer of cartilage. The architecture of the synovial joint is based within a capsule. The inside of the capsule is lined by a unique, highly vascularized tissue known as the synovial membrane (Ghadially, 1969). An important contribution of the SM to the joint is to provide synovial fluid, the lubricating, viscous fluid composed of non-sulfated mucopolysaccharide, most notably, hyaluronic acid (Swann et al., 1974). In addition to hyaluronic acid, synovial fluid can be considered as a dialysate of plasma as albumin and gamma globulins are present.

In addition, mononuclear cells are also present as normal constituents of the fluid (Swann et al. 1985).

1.2 SYNOVIAL MEMBRANE

The morphology and cellularity of the SM contributes significantly to the uniqueness of this tissue. In the normal joint, the SM consists of an undulating villous-like surface covered by a one or 2-deep cellular layer (Ghadially, 1969, Henderson et al, 1985). These cells are known as synoviocytes (Type A and Type B) and they will be discussed in detail in the following section. Underlying this thin surface of cells is a loose arrangement of connective tissue which is permeated by a variety of mononuclear cells, sensory nerves, interdigidating dendritic cells, lymphatics and a network of vascularization (Barrett et al, 1975, Ghadially, 1983) . The connective tissue compartment underlying the SM consists mainly of type II collagen fibers, with proteoglycans (chondroitin sulfate) and glycosaminoglycans. The consistency and rigidity of the underlying matrix is maintained by its continued remodeling. Proteolytic enzymes and their specific inhibitors, which are derived in part from the SM, as well as a host of soluble mediators (cytokines and growth factors), aid in the regulation of this remodeling process (Mauviel et al. 1993).

1.2.1. Type A Synoviocytes

Most commonly regarded as macrophage-like synoviocytes in the normal state, this cell population possesses phagocytic characteristics and are believed to be derived from bone marrow precursors which inundate the joint during early embryological development (Allard et al., 1990). They have been shown to have the classical monocyte macrophage cell surface markers in situ (Henderson et al, 1985, Allard et al., 1990, Burmester et al., 1983) and their proliferative rate in the normal joint is quite low. Broker and colleagues has confirmed the presence of a variety of Fc type receptors on these cells suggesting they are functionally important in immune complex activation (Broker et al., 1990). Allard et al demonstrated that these macrophage-like synoviocytes also express class II MHC antigens further implicating these cells as potential mediators of immune and inflammatory reactions which are ever present during joint disease (Allard et al., 1990). Evidence that these macrophage-like cells are derived from the bone marrow has come from published works showing staining with marrow specific precursor monoclonal antibodies. In addition, the absence of CD11c markers further suggested that this population of cells was macrophage-derived (Allard et al., 1987 and 1990). The intimal layer of the SM has been shown to contribute to the making of the extracellular matrix (ECM) by the production and release of proteoglycans, glycosaminoglycans and collagens type 1 and III (Hamerman et al, 1985, Myers et al., 1983) . However, in the normal synovium, Type A synoviocytes do not appear to contribute significantly to the production of any of these molecules (Henderson et al., 1985).

The in vitro activity of normal Type A HSF has been difficult to assess since a sufficient number and purity of these cells is very difficult to attain for in vitro examination. The majority of studies examining the role and activity of monocyte/macrophage cells in vitro from arthritic patients have utilized the monocyte population obtained from the periphery. In vitro studies of inflamed synovium have made the examination of the role of Type A HSF easier to interpret and assess.

1.2.3 Type B Synoviocytes

The other major cell lining the SM is believed to be a mesenchymally derived fibroblast-like cell (Linblad et al., 1987). Within the normal human SM, variability as to the number of Type B synoviocytes exists. This population can make up anywhere between 20% to 80% of the total cells in the intimal layer, however in a variety of other species including mice and rats, the percentage of Type B synoviocytes is significantly less (Ghadially, 1983). Much like the Type A cell, limited mitotic events are seen within this cell population within the normal joint (Ghadially, 1978 and 1983). Embryological studies in mouse joint development have documented that the Type A and Type B synoviocytes do infiltrate the joint lining on or about the same day (Linck et al., 1978). This is suggestive of a close histogenetic relationship between the two populations of synoviocytes, however, the exact derivation of the Type B cell is still uncertain. Within the normal joint, the absence of macrophage-like markers and the lack of other macrophage-like morphological characteristics also suggest that the Type B cell is not derived from the Type A population once within the SM (Allard et al. 1990). An active Golgi and abundant endoplasmic

reticulum are consistent features of this and other cells that are active in protein synthesis and secretory functions (Ghadially, 1983). As such, Type B cells do contribute to the ECM by producing, tenascin (Engel, 1989), hyaluronate (Myers et al, 1983), collagen type I fibrils (Linck et al., 1983), fibronectin (Linck et al., 1983) other proteins and complex polysaccharides. Immunohistochemical studies show that the normal Type B HSF cell stains well with antibodies against Human Leukocyte Antigens (HLA) II antigens (Burmester et al., 1983). The removal of fibrin by the action of plasminogen activator (Hamilton et al, 1981) and active pinocytosis (which Type A and B cells demonstrate) are two other important "housekeeping" functions of this population (Ghadially, 1983).

1.3 DEGENERATIVE JOINT DISEASE

1.3.1 Rheumatoid Arthritis

The main clinical manifestation of Rheumatoid arthritis (RA) is bilateral joint pain, swelling and dysfunction. While mainly a disease of the joints, Rheumatoid arthritis is considered a systemic disease since a number of organs and tissues are significantly affected (Harris, 1990). Continued research notwithstanding, the etiology of RA is still not clear. Despite a variety of inbred, genetically susceptible animal conditions and various rodent/ lapine arthritic models which are dependent on the introduction of various microorganisms and adjuvents, repeated investigations into the role of various viruses and other foreign antigens in eliciting RA have so far proved unsuccessful. Though early diagnosis is difficult, there are hallmark clinical features which accompany a differential diagnosis of RA. Clinical manifestations of symmetrical morning stiffness, musculoskeletal pain and peripheral joint

swelling is supportive of further radiological and laboratory investigation (Harris, 1990). The most common radiological feature is joint space narrowing (Martel, 1981). Blood analysis usually demonstrates an increased erythrocyte sedimentation rate, an eosinophilia and the presence of autoantibodies against specific antigenic determinants in the Fc portion of the IgG molecule known as Rheumatoid factor (RF) (Harris, 1990). Synovial fluid biopsy usually reveals a clouded opaque sample with an increased marked pan-leukocytosis due to inflammation and positive rheumatoid factor (Schumacher, 1981). Synovial biopsy of an established rheumatoid joint reveals an edematous synovium protruding into the joint space. The gross appearance of the lesion demonstrates typical villous inundation of the cartilage/bone interface. Closer examination illustrates a significant tissue neovascularization with a inflammatory cell infiltration of the sub-synovial space (Haraoui et al., 1991). In addition, collections of lymphocytes/monocytes into nodular aggregates surrounding new small vessels is also evident. This new tissue is often referred to as a pannus based on its granulation-like characteristics. This "transformed-like" tissue, composed of activated Type A and B synoviocytes, a variety of inflammatory/immune cells and actively proliferating vascular tissue, is thought, by its continued release of pro-inflammatory mediator and destructive enzymes, to be responsible for the progressive deterioration of the rheumatoid joint (Hamilton, 1983, Lafyatis et al., 1989).

1.3.2. Osteoarthritis

The degenerative condition of osteoarthritis is a progressive worsening of articular cartilage due in part to a variety of mechanical factors as well as the aging process (Linblad

et al., 1987). Initial examination of the OA patient initially reveals limited inflammation however, severe pain, impaired function and progressive joint deformity are diagnostic features of the disease (Altman et al., 1986). The clinical changes are not usually symmetrical which aids in differentiating osteoarthritis from other rheumatic (bilateral) syndromes. Some radiological changes depicting new bony formation and cartilage degeneration are sometimes seen in the susceptible patient though they often tend to appear late in the disease (Altman, 1995).

There is still a great deal of debate as to the inflammatory status within the joints of patients suffering from OA. Linblad et al (1987) examined a small group of arthroscopically diagnosed OA patients and showed that synovial inflammation varied quite significantly however the degree and intensity was always far less then that described for patients with RA. Revel and colleagues performed a detailed histological examination of the SM in patients with OA (Revell et al., 1988). They demonstrated that an inflammatory infiltrate, usually confined to the SM coupled with diffuse fibrosis was seen in the majority of patients studied. Using immunohistochemical techniques, they also confirmed the presence of lymphoid follicles which contained activated T and B cells and macrophages staining for HLA-DR antibodies. A more recent study by Haraoui et al further supported the hypothesis that the OA synovium was indeed an inflamed tissue, however, when compared to synovial membrane derived from RA tissue, the synovial lining thickening, inflammatory infiltrate and neovascularization is somewhat less in OA-derived tissue (Haraoui et al., 1991). Irrespective of inflammatory infiltrate, isolated cells from the SM as well as this tissue in

situ demonstrates an enhanced expression of proteases, various growth factors and pro-inflammatory cytokines suggesting that the OA joint is an activated tissue in OA. While the Osteoarthritis lesion is notably a degeneration of the articular cartilage and secondary synovitis and its inflammatory characteristics are observed, a granulation-like pannus as seen in RA is not a characteristic of the OA joint.

1.4 THE INFLAMED SYNOVIAL MEMBRANE AND ITS CONSTITUENTS

Pathological conditions of the joint pre-dispose the SM to significant morphological and cellular changes. The intimal layer of synoviocytes, usually 1-2 cells thick, becomes 8-10 cells thick or more in the RA joint (Harris, 1990). The increase in cell number within the SM is due mostly to an influx of peripheral monocyte/macrophage like cells which ultimately contribute to the type A synoviocyte pool (Henderson et al., 1985). Broker et al (1990) has suggested that mature peripheral monocytes make up the majority of the expanded intimal layer based on immunohistochemical analysis. Coupled with the increased cellularity of the intima there exists a major increase in tissue vascularization and a marked influx of lymphocytes into the SM and neutrophils into synovial fluid with greater numbers seen in RA than in OA (Ghadially, 1983). In RA, the aggressive "tumour like" pannus escapes the boundaries of the cartilage/bone interface and invades each of these tissues with enhanced growth kinetics driven by cytokines, growth factors, and matrix degrading enzymes (Harris, 1990, Richards et al, 1993).

1.4.1. Type A Synoviocytes in the Chronically Inflamed Joint

Of the cells within the pannus, some 40% are monocyte derived and most are considered to be macrophage-like type A synoviocytes (Broker et al. 1990). Therefore, the postulation that these have an important role in the arthritic lesion is reasonable. They exist in an activated state expressing increased MHC class II antigens above normal (Burmester et al., 1983, Alvaro-Gracia et al., 1991). Histological examination of the SM has demonstrated that activated macrophages lie in close proximity to T cells within the nodular regions of the RA synovium (Iguchi et al., 1986, Haraoui et al. 1991). Coupled to this data, Broker et al determined that SM Type A HSF populations express three different populations of Fc receptor molecules in both RA and OA; i) CD64 (FcγRI) the cytokine inducible-monocyte/macrophage high affinity IgG receptor molecule; ii) CD32 (FCγRII) which is also present on macrophage and monocytes and iii) CD16 (FCγRIII) which has not been observed on monocytes but is a marker of mature macrophage populations (Broker et al. 1990). Three important observations can be drawn from this morphological data within the SM. Firstly, there is an abundance of mature macrophage residing in the intimal layer of the SM. Secondly, the association of these Type A HSF populations with T cell compartments is supportive of an immune-related (ie. antigen presenting) phenomena. Finally, the presence of FcR receptors is suggestive that the macrophage populations within the SM could provide a fertile source for immune complex damage within the inflamed joint.

Irrespective of the morphological importance of Type A HSF populations within the RA or OA SM, the production of soluble messengers (cytokines, proteolytic enzymes and

autocoids) by these cells is considered to be their most important contribution to the local environment (Arend et al., 1989). The production and release of a variety of cytokines deems the macrophage population of the SM an important player within the normal physiology of the joint and during the pathologies associated with various arthrides. In addition the Type A synoviocyte is a major source or neutral and serine proteases critical in cartilage/joint remodeling/destruction (Keyszer et al., 1995).

1.4.2. Mast Cells

In the normal SM, mast cells are seen deep within the ECM of synovial tissue (Ghadially, 1983). The presence of degranulated mast cells has not been confirmed. Not until recently has the increased presence of mast cells been documented in inflammatory arthritis (Crisp et al. 1994) and a distinct mastocytosis has been seen within the SM of patients with RA. Malone et al suggested that the presence and number of mast cells within the SM of RA patients was closely correlated to their inflammatory indices and their treatment regime since the use of steroid therapy was seen to significantly reduce the number of mast cells within the SM (Malone et al., 1987). Mastocytosis has also been documented in non-rheumatoid arthritis (OA) as well (Wasserman, 1984).

The potential contribution of mast cell products to the pathogenesis of arthritis has only recently been realized. Documentation of these cells as a major source of bioactive amines, proteases and more recently cytokines in vitro, suggests that these cells could contribute to the inflammatory processes within the SM (Leal-Berumen et al 1995). While the quantitative contribution of cytokines by mast cells is still under scrutiny, their presence

and potential role within the arthritic lesion can not be ignored.

1.4.3. Lymphocytes

The presence of various populations of lymphocytes is a characteristic commonly found within chronically inflamed tissue. Their ability to precipitate damage via immune mediated mechanisms is well understood. In RA, systemic presentation of plasma cell derived rheumatoid factor (RF) and the local accumulation of lymphocytic aggregates within the SM are suggestive that lymphocytes contribute to the disease process (Harris, 1990). In fact, passive transfer of lymphocytes from arthritic animals to irradiated naive animals is sufficient to initiate disease and therefore suggests that T cells are vital in disease progression in such models (Sack et al., 1994, Mima et al., 1995). The pathological lesion within the RA synovium is typified by CD4+ T cells which aggregate around burgeoning blood vessels (Harris, 1990). These are often surrounded by RF secreting plasma cells. More blast-like T and B cells are scattered diffusely throughout the SM. Since a specific common antigen that stimulates immune cells within RA patients has yet to be identified, the exact immunological role of T and B lymphocytes in RA and other inflammatory arthrides (including OA) is still unclear.

With the number of lymphocytes both within the SF and SM significantly increased in RA and other degenerative joint diseases, but their functional significance still unclear, attempts to further understand the role of T cells within the inflamed SM have compelled researchers to analyze the characteristics of various T cell receptors within RA. In vitro analysis of T cell receptor repertoires within antigen-induced rodent models of chronic

arthritis have demonstrated that restricted lineages of T cell receptors are used (Chiocchia et al., 1991, Kakimoto et al., 1988). This was suggestive that a small number of T cell clones may mediate the lesion. In addition, in many other classically defined human autoimmune diseases, activated T cell populations possess an increased presence of activation markers coupled with enhanced production of T cell-specific cytokines (Sumida et al, 1992). While these phenomenon were found in other chronic diseases and animal models of autoimmune origin, examination of T cell receptor usage and T cell-derived cytokine activity in human RA has yet to yield conclusive results (Yamamoto et al., 1992). Within the RA lesion, major T cell derived cytokines are not produced or found at significant levels. IL-2, IL-4 and IFN-γ are all absent from the joint milieu and synovial tissue T cells do not express either GM-CSF or IL-10 (Firestein et al., 1990, Cush et al., 1995, Cicuttini et al., 1995). This strongly suggests a lack of T cell activation. Thus, while the predominate T cell population within the SM of RA patients are CD4+ T cells, their primary role in the inflammatory process in human disease is not proven and suggests that T cell independent mechanisms may be responsible for the perpetuation of damage seen in joint disease (Firestein et al 1990). Although the activity of local T cells in inflamed joints are diminished in established RA, their increased presence and morphological association likely reflect an important role possibly in the early initiation and pathology of chronic joint disease.

1.4.4. Nerves

While inflammatory and mesenchymal cells and their soluble mediators predominate

within the joint microenvironment, peripheral nerves and their products are present within the inflamed synovium and thus their role as players in the scheme of inflammatory joint disease has been considered (Levine et al., 1985). Earlier in this century, a variety of case studies documented that hemipalegics who later developed RA were spared of any pathological changes to the denervated side (Thompson et al., 1962). This data, coupled with later reports which demonstrated that peripheral neurologic maladies such as poliomyelitis, lessened the arthritic lesion in affected limbs, suggested that the nervous system may potentially be involved in mediating this chronic inflammatory disease (Glick, 1967). More recently, a closer examination of the joint infrastructure demonstrated the presence of sensory nerve fibers and their neurotransmitters in many forms of joint disease including RA and OA (Periera da Silva et al., 1990, Kidd et al., 1989, Walsh et al., 1992).

Immunohistochemistry studies have confirmed that nerves, particularly sensory type C fibers, are present within the inflamed synovium. Gronblad et al and da Silva et al have both confirmed that there appears to be a decrease in the number of nerve fibers existing in the rheumatoid synovium compared to normal synovium (Periera da Silva et al., 1990, Gronblad et al, 1988). OA synovium has been documented to have a slight decrease as compared to normal synovium. More recent work has shown that the innervation existing within the synovium of inflamed tissue is sequestered to the endothelial cells of the new vasculature and not within the intimal layer of the SM (Mapp et al., 1994). Others, however, have documented that nerve fibers do exist within the intimal layer of the SM derived from OA and RA tissue (Konttinen et al., 1989, Walsh et al., 1992).

While debate still exists as to the presence of nerve fibers in the synovium, products of these type C sensory fibers (ie. neuropeptides) are present and found in increased amounts in the synovium and synovial fluid of patients with RA or OA (Marshall et al., 1990, Gronblad et al., 1988). A variety of investigations have been dedicated to showing that many of these low molecular weight mediators can influence the dynamics of the synovial membrane and its cellular constituents in RA and OA (Levine et al., 1984, Lotz et al., 1987, Marabini et al., 1991).

1.4.3. Type B Synoviocytes-Synovial Fibroblasts

Enzymatic digestion of synovial biopsies results in the dispersion of the cells in the SM. Upon tissue culture, the adherent population consists of Type A (macrophage-like) and Type B (fibroblast-like) synoviocytes, however, by 72-96 hours, the Type B cells predominate due to their ability to survive and proliferate. This population is commonly referred to as the synovial fibroblast and in vivo, is thought to be a major contributor to pathological lesions associated with inflammatory joint disease (Zvaifler et al., 1994). For instance, in RA these cells contribute significantly to the breakdown of cartilage by their release of matrix degrading enzymes (Okada et al., 1989). As well, HSF have been postulated to influence the activity of a variety of local cells by their release and production of soluble mediators including various cytokine growth factors and autocoids (Richards et al., 1993). The newly described phlogistic characteristic of this cell population (once thought to be solely involved in joint stability and collagen deposition) have been reinforced with the finding that HSF are a significant source of cytokines in the joint milieu (Arend et al., 1990,

Farahat et al., 1993).

In RA, HSF manufacture a variety of pro-inflammatory modulators including cytokines, proteolytic enzymes, MHC class II and adhesion molecules as well as arachidonic acid liberating enzymes (Cyclooxygenase 1 and 2) (Farahat et al., 1993, Alvaro-Gracia et al., 1991, Crofford et al., 1994, Wilkinson, et al., 1993). In vitro, HSF-derived from RA tissue, have an increased capacity to proliferate in response to various stimuli (Butler et al., 1988, Butler et al., 1989). This coupled with their ability to grow under anchorage independent conditions and the increased capacity to secrete growth factors, cytokines and matrix-degrading enzymes are suggestive that this cellular population may exist in a "transformed state" (Hamilton, 1983, Lafyatis et al. 1989, Case et al., 1989). While it can not be denied that Type A synoviocytes, with its production and release of various monokines (IL-1, TNFα) and other mediators, has an important role as an effector population within the SM and pannus (see Figure 1), the Type B synoviocyte (HSF) population, which responds to and interacts with the Type A synoviocyte and contributes to the local cytokine pool within the joint space, is also an important cell contributing to the chronic inflammatory lesion. In fact, studies examining the SM in early onset RA have demonstrated that one of the earliest morphological changes associated with the joint infrastructure is synovial lining hyperplasia (Zvaifler et al., 1994, Soden et al., 1989).

1.5. INFLAMMATORY MEDIATORS WITHIN THE SYNOVIUM

Molecular biological techniques have advanced enough to assess the properties of the

synovial membrane and its constituents in situ. However, the majority of recent work aimed at understanding the biochemical processes occurring in the inflamed synovium have been performed on enzymatically dispersed synovium originating from synovectomy or joint replacement surgery in OA or RA patients. As mentioned above, cytokines, proteolytic enzymes and prostaglandins released by and effecting the HSF population in the joint are now considered to be integral players during pathological processes driving damage within the degenerative joint. The following sections will briefly review important synovium-derived modulators.

1.5.1. Interleukin 1

A major intercellular mediator driving synoviocyte activation and cartilage degradation in chronic inflammatory diseases of the joint is Interleukin-1 (IL-1). IL-1 is a pleiotrophic cytokine mainly synthesized by activated monocytes and tissue macrophages (Dinarello, 1993). This 17 Kda polypeptide is active in two forms, α and β. Each form is a product of a 31 kDa precursor molecule which is cleaved by a specific converting enzyme to produce the biologically active isoforms (Dinarello, 1989, Arend et al., 1990, Arend, 1991).

Both forms of the 17 kDa IL-1 bind to the same membrane-specific receptor proteins which exists on a variety of cell types. The IL-1 receptor (IL-1R) , of which two forms exist, belongs to the larger family of immunoglobulin gene superfamily of receptors (Dower et al., 1987, Slack et al., 1993). A soluble form of the IL-1 receptor has been isolated in a number of biological fluids including the SF suggesting that a role for this molecule in inflammatory

regulating events warrants further investigation (Krzesicki et al, 1993, Deleuran et al., 1992).

IL-1 has clearly been shown to induce inflammatory responses in vitro and in vivo. To assess the pro-inflammatory characteristics of any cytokine causally linked to a chronic inflammatory/autoimmune disease, Hollander has established 6 criteria which must be met to identify the mediator as candidate initiating tissue destruction (Hollander, 1991). Recent data confirms that IL-1 possesses at least 5 of these criteria. The first two criteria assess whether the biological mediator is present in and around diseased tissue and is increased in this tissue when signs of damage are on-going. In fact, increased levels of IL-1 have been documented in the arthritic lesion (both RA and OA) and they have been linked to a cellular source with the inflamed joint. Miossec et al (1986) has established that IL-1-like activity was found in RA and OA synovial fluids in 1986 . More recently, it has been documented that IL-1-specific RNA was localized to the mononuclear cells of the inflamed SM by in situ hybridization in specimens originating from both RA and OA tissues (Koch et al., 1992).

In vitro studies have determined that IL-1 can significantly contribute to the pathophysiological destruction of the joint by directly and indirectly inducing tissue damage. This characteristic of IL-1 helps fulfill the third criteria which states that the active mediator should alter normal tissue in a manner which is indicative of diseased tissue. In vitro, IL-1 significantly enhances the production and release of matrix degrading enzymes responsible for cartilage and bone alterations typically seen in the arthritic joint (Gowen et al., 1983, Pettipher et al., 1986, Arend et al., 1993) Analysis of the catabolic effects of IL-1 in vivo involve administration of the mediator in various animal models. Repeated intra-articular

injection of IL-1 caused a transient inflammatory reaction in the joint of rabbits which was exacerbated by additional doses (Chandrasekhar et al., 1990). Serum and joint measures of inflammatory indices including PGE, SP, C-reactive protein, and protease activity as well as locally-derived changes reflected by enhanced bone resorption, proteoglycan synthesis and MMP release further implicate IL-1 as a major player in joint destruction. Van de Loo and colleagues have provided additional evidence implicating IL-1 as both an initiating and exacerbating factor in murine models of arthitis. Injections of IL-1 into antigen-induced arthritic mice caused a significant flare up of a variety of inflammatory indices (van de Loo et al., 1992). As well, IL-1 injections induced arthritic-like lesions in naïve mice (van de Loo et al., 1995).

A further criteria solidifying the involvement of IL-1 in the arthritic lesion has come from the recent studies examining the effect of specific IL-1 antagonists on the in vitro effects of IL-1 and on the clinical progression of antigen induced arthritis in animals and of rheumatoid/osteoarthritis. The naturally occurring soluble inhibitor of IL-1, IL-1 Receptor Antagonist (IL-1RA), has been shown to possess potent inhibitory effects on IL-1 activity in vitro by inhibiting the IL-1-induced production of various inflammatory mediators including PGE, GM-CSF, MMP production, and various fibrogenic growth factors including Platelet-Derived Growth Factor from macrophage and fibroblast-derived cultures (Seckinger, 1990, Arend et al, 1995). The effect of IL-1RA in vivo also shows that this product of activated monocytes and macrophages posesses IL-1-inhibiting activities. For instance, in a variety of animal models of arthritis, administration of IL-1RA resulted in decreased joint

swelling and significantly inhibited IL-1-induced tissue damage (Matsukawa et al., 1993). A limited number of clinical trials assessing the efficacy of IL-1RA as a disease modifying anti-inflammatory agent are under way. Early results suggest that some inflammatory indices are decreased by local injection of IL-1RA and that endogenous IL-1 levels both peripherally and locally are moderately reduced (Lebsack et al., 1993).

1.5.2. Tumor Necrosis Factor

While IL-1 is well defined as a pro-inflammatory cytokine driving the arthritic lesion, TNF's role as a potential pro-inflammatory signal in arthritis is based on its in vitro abilities to elicit the release of IL-1 and GM-CSF from synovial derived macrophage populations during RA (Brennan et al., 1989) as well as many activities which overlap with IL-1, such as chondrocyte activation, MMP regulation and PGE production by fibroblasts (Arend et al., 1995). Based on the Hollander criteria, TNF does cause joint inflammation in animal models of arthritis and can augment human cartilage degradation in in vitro studies; however, the presence of a secondary stimuli (either IL-1 or others) often appears necessary (Henderson et al., 1989). Interestingly, over expression of TNF in a murine transgenic model results in excessive joint destruction and administration of antibodies to TNF limit the joint destruction (Keffer et al., 1991). In similar studies to that reported for IL-1, intraarticular injection of TNF into collagen-induced arthritic mice or rats results in an accelerated disease course (Brahn et al., 1992, Cooper et al., 1992).

In human joint disease, increased expression of TNF is localized to joint tissue,

synovial fluid and serum in both OA and RA (Tetta et al., 1990). In situ hybridization studies confirm that TNF is localized to Type A cells lining the intimal layer of the SM (Chu et al., 1991, Brennan et al., 1992). In terms of an endogenous inhibitor of TNF, (as per IL-1RA), the recently described soluble TNF receptor molecule has been found within the inflamed microenvironment of the joint and appears to be enhanced druing RA (Cope et al., 1992, Cope et al., 1994). The effectiveness of this molecule and that of anti-TNF antibodies as therapeutics in chronic joint diseases are now being investigated in early clinical trials.

1.5.3. Interleukin-6

IL- 6 is a 26 kDa pleiotrophic cytokine produced by cells of an assortment of lineages including T cells, fibroblasts, endothelial cells, activated monocytes/macrophages from a variety of tissues (lung, liver, blood, skin), mast cells and synoviocytes (Wong et al., 1988, Elias et al., 1990, Elias et al., 1990a, Leal-Berumen et al. 1985). Physiologically, IL-6 is intimately involved in hematopoeisis, acute inflammatory reactions, the healing process and chronic inflammation (Kishimoto, 1992).

IL-6 responsive cells possess a receptor complex that initiates signals that are common to a family of cytokines including Oncostatin M, Leukemia Inhibitory Factor, Ciliary Neurotrophic Factor and IL-11 (Kishimoto, 1992, Zhang et al., 1994). A signal transducing protein known as gp 130 dimerizes with the IL-6-specific α subunit and allows signal transduction to ensue. While the gp130 signal transducing protein is a ubiquitous component of this group of cytokine receptors, the specificity of the responses elicited by the IL-6 family of cytokines is in part determined by cell expression of the unique α subunits of

the complexes (Kishimoto, 1992).

Increased concentrations of IL-6 protein have been documented in the SF, plasma and bone marrow of patients suffering from a multitude of arthrides but in particular, RA (Hirano et al., 1988, Tanabe et al., 1994). Within the inflamed joint, the cellular sources of IL-6 are quite diverse. Immunohistochemical studies of the rheumatoid pannus has shown that IL-6 activity is detectable within the synovial intimal layer (Wood et al., 1992). In vitro studies of isolated HSF and synovial macrophages have shown that IL-6 protein and mRNA expression are detectable (Guerne et al., 1992). An increased level of IL-6 has been linked to an increased presence of enhanced disease activity (Dasgupta et al., 1992, Heumann et al., 1995). Similarly, greater expression of IL-6 protein is seen in the synovial fluid, serum and pannus of patients suffering from RA over OA (Swaak et al., 1988). With these studies in mind and the data demonstrating that IL-6 is a potent stimulator of B cell immunoglobulin production (Kishimoto, 1991), it is reasonable to suggest that IL-6 could contribute to the pathogenesis of arthritis.

While the increased presence of IL-6 protein and mRNA seen in various arthrides helps fulfill some of the criteria nominating this mediator as a pro-inflammatory signal, its ability to induce/exacerbate an arthritic lesion is at best skeptical. Wendling et al. (1993) attempted to use a monoclonal antibody against IL-6 to treat the inflammation and modify the diseases in 5 patients with RA. While short term clinical effectiveness was noted in 4 of 5 patients, it was coupled with a significant increase in serum/synovial fluid IL-6 indicating that the treatment in some manner, actually enhanced levels of bioactive IL-6. The

authors speculated that IL-6 may have some in vivo anti-inflammatory role during RA. As well, Mihara et al (1991) examined the effects of IL-6 on adjuvent induced arthritis in mice. Mini-osmotic pumps containing IL-6 were placed sub-cutaneously in the backs of mice who simultaneously received adjuvant (liquid paraffin containing *Mycobacterium tuberculosis).* IL-6-treated animals had a significantly decreased mean arthritic score (which accounted for paw swelling, joint deformity and edema) over control treated animals over a 40 day period. Therefore, in this animal model, exogenously administered IL-6 significantly suppresses adjuvent-induced arthritis. In mice, genetically altered to be deficient in IL-6, Fattori et al (1994) demonstrated that LPS-treated IL-6 deficient mice had 3 times as much serum TNF-α suggesting that endogeonous IL-6 may aid in controlling TNF production during inflammatory conditions. In other chronic inflammatory conditions such as Alzeimers disease, increased IL-6 expression is noted within the senile plaques scaring the central nervous system (CNS) (Bauer et al 1991). It was suggested that IL-6 could therefore mediate the neurodegenerative processes typical to such pathologies. Since IL-6 and other members of the IL-6 family of cytokines possess the ability to both directly and indirectly modulate neurite outgrowth (Hama et al., 1989), it could be that local and/or systemic increases in IL-6 seen in CNS-specific chronic inflammatory conditions may be a host-induced salvage mechanism initiated to repair damaged CNS circuitry subsequent to neuronal injury. Additional information within this area may be further suggestive that IL-6 may possess additional protective roles during chronic inflammatory conditions.

Taken together, the role of IL-6 can not clearly be deemed pro/ or anti-inflammatory

in any one system. Further investigations of IL-6 as a regulator of inflammatory responses and a modulator of homeostasis during chronic conditions may lead to a greater understanding of this area.

1.5.4. Granulocyte Macrophage Colony-Stimulating Factor

Another important family of inflammatory mediators are the colony-stimulating factors. In particular, the increased presence of GM-CSF has been linked to the pathological lesions of chronic inflammatory conditions including arthritis (Alvaro-Gracia et al., 1990, Firestein et al., 1990). GM-CSF is a heavily glycosylated 22 kDa glycoprotein which was initially characterized based on its ability to induce the growth and differentiation of bone marrow derived hematopoeitic cells and appears essential for the proper maturation of monocytes/granulocytes (Wong et al., 1985). Apart from regulating early events in committed hematopoeisis, GM-CSF has also been shown to regulate the activity of mature myeloid and lymphoid lineages (Vancheri et al., 1989, Xing et al., 1992). Recombinant GM-CSF can stimulate functional activities of neutrophils including proliferation, maturation, cytokine release, oxygen radical production and phagocytosis (Yuo et al. 1990, Nathan, 1989). Vancheri et al demonstrated that the presence of GM-CSF was important for the survival of human eosinophils in vitro (Vancheri et al., 1989). Subsequent studies determined that recombinant GM-CSF was important for the survival and viability of neutrophils and macrophages in other systems (Xing et al., 1992).

GM-CSF is the product of a number of cell types including activated macrophages

(from a variety of tissues), various populations of stromal cells including fibroblasts (Alvaro-Gracia et al., 1989), epithelial cells (Wong et al., 1985), and some populations of lymphocytes (Firestein et al., 1990). An increased presence of GM-CSF has been documented in the bronchioalveolar lavage fluid of patients with sarcoidosis and within the synovial fluid and synovial membrane of patients suffering from various forms of acute and chronic arthrides (Alvaro-Gracia et al., 1990, Hamilton et al., 1992, Itoh et al., 1993) . Of interest are the findings that the presence of increased levels of GM-CSF within the SF of RA joints is closely correlated to disease activity and intensity (Firestein et al., 1990, Leizer et al., 1990).

Localization of GM-CSF protein and mRNA expression within the inflamed joint has been recently documented using elaborate immunohistochemical and in situ hybridizations studies of both RA and OA SM (Firestein et al., 1990). Both techniques have demonstrated that the protein and mRNA appears to be sequestered to the cells of the intimal layer of the SM. As well, Firestein and colleagues have confirmed the finding that in fact, little if any GM-CSF is produced by any immune/inflammatory cell within the synovial fluid (Firestein et al., 1990a). These studies fostered the hypothesis by Zvaifler and Firestein that GM-CSF derived from the SM was one of the most important locally derived soluble mediators within the inflamed joint. These authors propose that GM-CSFs role in propagating neovascularization and enhancing MHC expression on local cells, coupled with GM-CSF's ability to perpetuate the survival of a variety of pro-inflammatory (eosinophils, neutrophils and lymphocytes), supports its role as a key molecule driving arthritis by perpetuating the

chronicity of the arthritic lesion.

The control of GM-CSF expression has been recently scrutinized. Its synthesis and release has been demonstrated in a number of cells therefore a great deal of literature exists which examines both the transcriptional and post-transcriptional regulation of the glycoprotein (Shaw et al., 1986, Bickel et al., 1990, Schreck et al., 1990, Wang et al., 1994, Fraser et al., 1994). As with many cytokines, GM-CSF is significantly stimulated by IL-1 both at the protein and mRNA level (Leizer et al., 1989). Further examination of the regulation of GM-CSF has demonstrated that specific DNA binding sites within the 5'-flanking region of the GM-CSF gene and their respective binding proteins are important in regulating GM-CSF transcription (Fraser et al., 1994, Rizzo et al., 1994).

In vitro production of GM-CSF in stromal cell populations, in particular, HSF, has been documented by Hamilton et al (1992). They showed that IL-1-induced GM-CSF protein release was enhanced by indomethacin treatment and further inhibited by the addition of PGE suggesting that arachidonic derivatives (both endogenous and exogenous) could effectively regulate the local inflammatory milieu during the arthritic lesion. Taken together, these data suggest that GM-CSF is present within the inflamed joint, is produced by resident and infiltrating inflammatory/stromal cells and can effectively modify its environment by mediating stromal, myeloid, and lymphoid cell activation and survival.

1.5.5. Chemokines

The recruitment of activated leukocytes to an area of chronic inflammation appears,

in part, to be controlled by locally produced mediators which are released in a dysregulated manner (Rot, 1991) . A family of low molecular weight chemotactic factors has been described and their presence and activity within the inflamed synovium suggests that they play a pivotal role in mediating cellular infiltration within the synovium and synovial fluid, ultimately leading to the destruction of the joint (Baggiolini et al., 1992, Miller et al., 1992). Based on structural analysis, these chemotactic cytokines, or chemokines are subdivided into two groups. The C-X-C chemokines have an intervening amino acid residue between the first two cysteines in a four-cysteine conserved motif inherent in all family members (Schall, 1991). Members within this family include Interleukin-8 (IL-8) and Macrophage Inflammatory Proteins (MIPs). A second set do not have an interrupting amino acid residue between the first cysteines (C-C chemokines). Members of this group include RANTES and Macrophage Chemoattractant Peptide (MCP-1). The major biological effects of both chemokine families appears to be mediated through specific membrane receptors on target cells (Oppenheim et al., 1991).

1.5.5.1. C-X-C Chemokines

The leukocyte chemotactic capabilities and the ability to alter neoangiogenesis are these peptides' major pro-inflammatory qualities. The C-X-C family and specifically IL-8 mediate the chemotaxis and adherence of neutrophils and some populations of T cells (CD45RO) but have not been shown to elicit an effect on the chemotaxis of monocytes (Baggiolini et al., 1989, Larsen et al., 1989, Lloyd et al., 1996). In addition, IL-8 has been

documented to stimulate angiogenesis and this activity has been linked to the perpetuation of tumor growth and expansion (Koch et al., 1992, Hu et al., 1993, Smith et al., 1994). This characteristic coupled with findings which demonstrate an increased expression of IL-8 mRNA and protein within the inflamed SM (Koch et al., 1991, Loetscher et al., 1994), suggest that IL-8 may contribute to the tumour-like phenotype of the pannus tissue .

Many cell types including fibroblasts (Strieter et al., 1989), hepatocytes (Thorton et al., 1990), monocytes/macrophages (Peveri et al., 1988, Koch et al., 1991), and endothelial cells (Strieter et al., 1989a) have been shown to synthesize and secrete IL-8 in vitro and in vivo. In many instances, the increased presence of IL-8 within inflamed tissues and in isolated explants of inflamed tissue biopsies is closely coupled with disease activity (Breenan et al., 1990). For instance, Mazzucchelli et al. coupled increased expression of IL-8 RNA with active inflammatory lesions within the bowel of patients suffering from Inflammatory Bowel Disease (Mazzucchelli et al., 1994). Seitz et al. (1991) as well as Koch et al. (1992) determined that IL-8 protein and mRNA was produced and localized to synovial tissue macrophages and that increased expression of this chemokine was indicative of a more severe arthritic lesion.

1.5.5.2. C-C Chemokines

The C-C chemokines, (also referred to as the β chemokines), specifically RANTES and MCP-1, are chemoattractants and activators of monocyte activity both in vivo and in vitro (Rollins et al., 1991). As well, memory T cell populations and both activated CD4 and

CD8 T cells are preferentially stimulated by various members of the β chemokines (Schall et al., 1990). Recently, RANTES has been documented to regulate the adherence of T cells to various components of the extracellular matrix as well as to recombinant human adhesion molecules (Lloyd et al., 1996).

With the expansion of specific C-C chemokine group members, recent studies have documented the presence of these mediators in inflammatory conditions. Luckacs et al (1994) using an in vitro model of hepatic granulomas demonstrated that MCP-1 RNA was constituitively expressed by intragranulomular fibroblasts and immunolocalization of the chemokine was also confirmed. As mononuclear cell infiltration is a hallmark feature of granuloma development, these authors suggest that MCP-1 may be an important mediator of this event. Loetscher et al (1994) have assessed the production of MCP-1 from rheumatoid synoviocytes in vitro and suggest that constitutive protein production is minimal; however, stimulated RA HSF do produce measurable MCP-1 in vitro. Schall and colleagues determined that RANTES mRNA is expressed within the synovial lining cells of arthritic joints (Schall et al., 1991). Similarly, Rathanaswami et al (1993) have shown that TNF/IL-1 activated HSF express increased RANTES protein and mRNA levels.

1.5.5.3. Modulation of Chemokine Expression

While a great deal of evidence has demonstrated that the chemokines are present within the inflamed joint, information regarding the regulation of chemokine production from stromal cells within the joint is relatively limited. As previously mentioned, Loecheter

et al (1994) illustrated that MCP-1 and IL-8 were produced by HSF derived from RA synovium. In addition, they also demonstrated that these same chemokines were inhibited by a variety of Disease Modifying Anti-Rheumatic Drugs (DMARD's) such as gold salts as well as steroids. In addition, this group examined the effect of a specific inhibitor of the arachidonic acid cascade, indomethacin, on IL-8 and MCP-1 production. According to their results, indomethacin did not directly effect the production of IL-8 or MCP-1 protein, similarly it had no effect on the IL-1-induced stimulation of MCP-1 or IL-8 release. These authors maintain that in terms of these chemokines, prostanoids have no effect on the regulation of their production or release. Rolfe and colleagues (Rolfe et al., 1992) analyzed the expression and regulation of MCP-1 from pulmonary fibroblasts of patients suffering from pulmonary fibrosis. The pulmonary fibroblasts were shown to produce MCP-1 mRNA and protein upon stimulation with IL-1 or TNF. In addition, this group examined the effectiveness of PGE and dexamethasone to modulate fibroblast derived MCP-1. They showed that both MCP-1 protein and mRNA was inhibited in a dose dependent manner by both immunomodulators.

As chemokines have been shown to be important mediators of adhesion, Mehindate et al (1994) demonstrated that the expression of MCP-1, RANTES and IL-8 was regulated by MHC II antigens. Their model examined the binding of HSF-derived MHC II molecules to various superantigens in vitro. Their data demonstrates that this process evokes an increase in the production of MCP-1, RANTES and IL-8 from HSF. This group maintains that the interaction between stromal cell populations and infiltrating immune cells is in some

ways self-perpetuating as this relationship potentiates the release of pro-adhesive/chemotactic modulators.

The increased presence of a variety of chemokines with diverse chemoattractant/pro-inflammatory properties within the inflamed joint earmark these low molecular weight mediators as important players in pannus formation within the synovial milieu. The regulation of HSF-derived chemokine production, in light of these important phlogistic characteristics, will become an important issue in further understanding the dynamic processes present within the arthritic lesion.

1.5.6. Matrix Metalloproteinases

The events involved in extracellular matrix (ECM) metabolism is a dynamic process which depends upon the activity of a variety of cells including fibroblasts, endothelial cells and many immune/inflammatory cells (Mauviel et al., 1993). One of the main physiological pathways utilized for ECM development, remodeling and pathological disassembly employs a family of enzymes known as Matrix Metalloproteinases (MMP) which contain zinc at their active site (Woessner et al., 1991). MMPs share significant structural homology but their substrate specificity is quite diverse. There are at least 11 endoproteinase members within the MMP gene family, all of which are formed as zymogens and subsequently activated once released into the extracellular environment (Matrisian, 1990). The pro-piece contains a cysteine residue which interacts with the active site and initially blocks activity. The zymogens can be activated by a variety of mediators including plasmin, MMP-3

(stromelysin) and a variety of mercuric agents (Docherty et al., 1990). These activators, either by removal of the pro-peptide or by altering the cysteine residue, allow access of the substrate to the active site. Homology between the family members lie within the hydrophobic signal peptide, the proline rich hinge element and a 200-amino acid vitronectin-like carboxyl terminal region (Emonard et al., 1990).

Regulation of MMP production is usually orchestrated by a host of soluble mediators including cytokines (Goetzl et al., 1996), growth factors (Brinckerhoff et al., 1990), PMA (Angel et al., 1987), and various secondary messengers such as cAMP (Takahashi et al., 1991). Many agents regulate MMP production at the DNA transcriptional level (Matrisian, 1991). Examinations of the 5' promoter/enhancer regions of genes have demonstrated that a variety of regulatory elements exist which, when activated, can effectively drive MMP transcription (Angel et al., 1987, Krane et al., 1990). Inhibition of MMP production can be regulated by repressive soluble signals. These include retinoic acid (Brinckerhoff et al., 1990), glucocorticoids (Jonat et al., 1990) and Transforming growth factor β (TGF) which can alter both the transcriptional regulation and cellular release of MMPs (Chandrasekhar et al., 1988). Direct inhibition of MMP activity is mediated by a family of naturally occurring inhibitors known as Tissue Inhibitor of Metalloproteases (TIMPs) (Denhardt et al., 1993). These proteins schiometrically bind to MMP in a 1:1 ratio rendering the protease inactive (Cottam et al., 1993). TIMP family members, in particular TIMP-1, are produced by many cell types including HSF and are themselves regulated by a variety of cytokines and growth factors (Dean et al., 1985, MacNaul et al., 1990). Net matrix metabolism/catabolism,

influenced by TIMP and MMP levels, may be a key element in pathological tissue destruction in conditions such as arthritis and cancer invasion and metastasis (Liotta et al., 1991, Cottam et al., 1993). Thus, while the MMP's have a vital role in normal embryological development and normal wound repair, inappropriate expression or regulation can lead to major alterations in tissue integrity and function.

1.5.6.1 Interstitial Collagenase (MMP-1)

Interstitial collagenase or MMP-1 was the first MMP family member to be discovered (Emonard et al., 1990). This enzyme is released as a 57 kDa protein in its latent form and is later cleaved into an active enzyme weighing 50-52 kDa. MMP-1 cleaves the triple helix collagen molecule and degrades collagen types I, II, III, VII and X as well as gelatin thus indicating its importance in tissue destruction and remodeling (Woessner, 1991).

Within the arthritic lesion, MMP-1 levels, as measured by Northern analysis of tissue derived from patients with RA and OA reveals an mRNA species of approximately 2.1 kb (McCachren et al., 1990, Gravallase et al., 1991, Firestein et al., 1991). Immunoreactive MMP-1 as assessed by ELISA as well as bioactive MMP-1 as assessed by zymographic analysis is also seen in increased amounts in the SF of affected patients as well as in the tissue culture media of HSF (Sorsa et al., 1992, Greis et al., 1994, Manicourt et al., 1995). Since the work of Harris et al, who showed that collagenase was present within the SF of RA patients, the cellular source of this destructive enzyme within the joint has been the subject of much investigation (Harris et al., 1970). Evanson et al (1968) determined that cultured

synovial cells could produce collagenase in vitro. Following this, Ridge et al (1980) determined that chondrocytes could also produce a collagen degrading substance which both groups hypothesized was MMP-1. It now appears that both type A and B synoviocytes, cartilage forming chondrocytes as well as infiltrating immune/inflammatory cells can and do produce increased levels of MMP-1 in the joint of patients with RA and, to a lesser extent, in patients suffering from OA (Clark et al., 1993, Lohmander et al., 1993). In addition, other studies have provided evidence that MMP-1 is increased in SF and serum of patients with RA which may suggest that RA may be systemic inflammatory lesion (Walakowitz et al., 1992, Clark et al., 1993). Not addressed in these reports however is the hypothesis that excessive spillage of MMP-1 from the joint(s) space into the circulation may skew MMP-1 results.

The regulation of MMP-1 production from HSF and other resident joint cells has recently been established. A great deal of work attempting to understand the transcriptional and post-transcriptional regulation of MMP-1 has focused on the effects of cytokines, particularly IL-1, in this process (Mauviel 1993). The regulation of MMP-1 transcription is mediated in part, by the activation *trans*-acivating binding proteins which interact with specific DNA motifs located within the 5' flanking regions of the MMP-1 gene. IL-1 as well as TNFα can stimulate the production of AP-1-specific DNA binding proteins (two oncogenes c-fos and c-Jun), which in turns stimulates MMP-1 transcription (Angel et al., 1987). Some growth factors, including transforming growth-factor-β and interferon-γ can inhibit the induction of MMP-1 from fibroblast with both transcriptional and post-

transcriptional regulation (Shapiro et al., 1990, Varga et al., 1990). DiBattista et al (1994) have recently investigated the effects of various species of prostaglandins on IL-1-induced MMP-1 production from normal HSF. They determined that prostaglandins could inhibit the IL-1-induced production of MMP-1 in vitro by enhancing cAMP levels and stimulating inhibitory pathways via the activation of protein kinase A.

Regulation of MMP-1 protein and mRNA production from HSF and other cells has also been shown to be affected by a variety of other stimuli; these include cell-matrix contact, various adhesion molecules lying on the surface of immune/inflammatory/stromal cells (Saarialho-Kere et al., 1993), Disease Modifying Anti-Rheumatic Drugs (DMARDs) including methotrexate (Firestein et al., 1994), and neuropeptides including Substance P and Vasacitve Intestinal Peptide (Lotz et al., 1988, Goetzl et al., 1996).

1.5.6.2. Other Matrix Metalloproteinases

Gelatinase (MMP-2) and Stromelysin (MMP-3) are two other MMP family members which are produced by HSF and are found to be present within the SM and SF of RA and OA joints (Firestein et al., 1992, Okada et al., 1989, Cawston et al., 1984). Stromelysin (MMP-3), with a molecular weight of 52-60 kDa, has a wider range of matrix substrates including gelatins, proteoglycans, laminin, fibronectin, and collagens III, IV and V (Matrisian, 1990). Another important characteristic of MMP-3 is its ability to cleave and activate pro-MMP-1 (Woesnner, 1991). In fact, MMP-3 is said to "superactivate" MMP-1 resulting in a 5 to10-fold increase in collagenase activity (Matrisian, 1990). In RA, an increased presence of immunoreactive/bioactive MMP-3 has been demonstrated in the SF (Manicourt et al., 1995).

In addition, in situ hybridization techniques have demonstrated that MMP-3 mRNA is present within the intimal layer of the SM in patients suffering from both RA and (to a lesser extent) OA (Firestein et al., 1992). As well, HSF in vitro produce substantial basal levels of MMP-3 in vitro as assessed by ELISA and zymography (MacNaul et al., 1990). The presence of MMP-3 in various skin tumors in both human and animal models has been linked to an increased risk in metastasis (Sato et al., 1992).

The generation of active MMP-3 is similar to MMP-1 in that they are expressed by the same cells and are stimulated and activated by similar mediators (Woessner, 1991). AP-1 binding sites exist within the MMP-3 promoter and c-fos and c-Jun protein up-regulation appears vital in MMP-3 synthesis (Woessner, 1991, deSouza et al., 1995). Initiators of this regulatory pathway include IL-1 and TNF-α in HSF and other cellular populations (Angel et al., 1987). TGF-β and glucocorticoids are potent inhibitors of MMP-3 production from a variety of cells including HSF derived from SM of patients with both RA and OA (Firestein et al., 1991, Unemori et al., 1994).

MMP-2 is also referred to as 72 kDa collagenase/gelatinase. This enzyme has a specificity which includes various types of collagen (IV, V and VII) as well as gelatins and fibronectin (Collier et al., 1988, Matrisian, 1990). In vitro studies examining the regulation of MMP-2 production from various cellular populations have determined that MMP-2, unlike MMP-1 and MMP-3, is not generally affected by IL-1-stimulation (Woessner, 1991, Takahashi et al., 1993). However, TGFβ does stimulate MMP-2 expression (Overall et al.,1991). In situ, inhibition of MMP-2 activity appears to be largely accomplished by TIMP-

2 (Fridman et al., 1992). MMP-2 protein levels, enzymatic activity and mRNA has been localized to cells and SF within the inflamed joint however its role in chronic inflammation of the joint is not fully understood (Hembry et al., 1995). In other organ tissues, where the demarcation of the basement membrane is apparent (such as the gastrointestinal tract, lung) and metastasis is a potential insult, MMP-2 production becomes an important parameter particularly in light of its fibronectin-degrading capabilities (Winberg et al., 1992, Hoyhtya et al., 1994). Other MMPs including 92kDa gelatinase (MMP-9) and the newly described collagenase-3 (Reboul et al., 1996) are found within inflammatory tissues however their role in arthritis is not well defined at present.

1.5.6.3. Tissue Inhibitor of Matrix Metalloproteinases (TIMP)

Normal processes of connective tissue morphogenesis, acute wound inflammation and repair require a coordinate balance between degradation and remodeling. With pathological states, particularly, degenerative joint disease (as well as metastasis) an imbalance in matrix breakdown and regeneration exists leading to tissue destruction and ultimately dysfunction (Krane et al., 1990, MacNaul et al., 1990). As MMPs are important modulators of this process, their naturally ocurring inhibitors, TIMPs, are of equal importance in altering matrix destruction in chronic inflammation. TIMP-1, the first and best characterized of these inhibitors, is a 29 kDa protein found in virtually all tissues (Matrisian, 1990, Carmichael et al., 1986).

A great deal of recent work has focused on the regulation of TIMP-1 since imbalances

in TIMP and MMP expression during chronic joint disease is evident and postulated to contribute to the lesion (Khokha et al., 1989, Liotta et al., 1991). Systemic administration of TIMP-1 protein to collagen-induced arthritic mice results in a lessening of joint swelling and other symptoms (Carmicheal et al, 1989). As well, decreased TIMP-1 expression is associated with an enhanced level of metastasis in a variety of animal models of tumor invasion (Khokha et al., 1989b). Therefore, manipulation of this MMP inhibitor may prove fruitful as a therapeutic for chronic inflammation or cancer treatment. A variety of mediators have been shown to alter TIMP-1 expression. In vitro analysis of TIMP-1 expression has shown that IL-6 (Lotz et al., 1991) , IL-1 and TNF-β (Overall et al., 1989) , epidermal growth factor and TGFβ (Edwards et al., 1987) can augment TIMP-1 expression in fibroblasts and HSF alike. More recent work by Richards et al has documented that oncostatin M, a member of the IL-6 family of cytokines, preferentially stimulates TIMP-1 expression to a greater degree then other members of this cytokine family in fibroblasts derived from the synovium and lung (Richards et al., 1993). In a variety of cells and tissues, TIMP-1 expression has been shown to be stimulated by various hormones (Ulisse et al., 1994) and prostaglandins (PG) (Roeb et al., 1993).

Other members of the TIMP family have recently been cloned including TIMP-2 which is a 22 kDa protein which is responsible for the neutralization of MMP-2 activity (Ward et al., 1991). In fibroblasts, TIMP-2 does not appear to be effected by cell stimuli, however, basal expression of TIMP-2 is noted within RA and OA-derived HSF (Hembry et al., 1995). TIMP-2 expression from other cell lines including rat hepatocytes, is stimulated

by LPS as well as by PGs in vitro (Roeb et al., 1995).

TIMP-3 is the latest family member of metalloproteinase inhibitors to be identified (Silbiger et al., 1994). It has been shown to be expressed in a variety of tissues (Leco et al., 1994) both normal and cancerous (Silbiger et al. 1994). Little to date is known about the regulation of TIMP-3 expression or whether it is involved in the arthritic lesion.

1.5.7. Prostaglandins

Prostaglandins (PGs), are one distinct member of the autocoid family derived from membrane phospholipids. Like most biological compounds, the activity of PGs was determined first and the actual purification of each family member occurred years later (Bergstrom et al., 1968). In most mammals, the precursor fatty acid arachidonic acid (eicosatetraenoic acid), is the most common polyunsaturated dietary fatty acid found in most cell membranes. Eicosanoid-synthesizing enzymes, namely, cyclooxygenase (COX) are the rate limiting factor ultimately leading to PG production (Needleman et al., 1986). There are seven family members within the PG arm of arachidonic acid metabolites. In tissue samples and serum, the most abundantly found PGs are prostaglandin E2 (PGE2) and PGI2 (prostacyclin) or its more stable derivative 6-keto PGF1α (Wolfe, 1982). Under normal physiological conditions, the biosynthesis of PGs is tightly regulated. A variety of autocoids, cytokines, growth factors, hormones and other physical/chemical stimuli which interact with cell membranes can enhance or diminish PG production (Raz et al., 1988, Raz et al., 1989).

PGs have numerous and diverse biological effects with a broad range of cellular/tissue targets. Within the cardiovascular system, PGs generally produce vasodilating effects (Needleman et al., 1986). Both PGE2 and PGI2 cause significant vasodilation and accompanying hypotension; in fact all vascular smooth muscle is relaxed by both prostanoids. In other smooth muscle preparations, including those within bronchial tree and the trachea, PGE2 causes relaxation of the smooth muscle layer (Spannhake et al., 1981). Within the gastrointestinal tract, members of the prostanoid family generally cause relaxation resulting in reduced intestinal transit times and significant diarrhea when administered orally (Wilson et al., 1981). Within the peripheral nervous system PGs have been shown to sensitize sensory nerve endings to the action of pain-inducing factors (Wolfe, 1982). In this sense, PGs are involved in the amplification of pain particularly during the inflammatory process.

The presence of PGE2 within the serum, tissue and biological fluids of patients suffering from arthritis, and in particular, RA has been verified (Salmon et al., 1983, Davies et al., 1984, Goldstein, 1988). The inflammatory significance of the enhanced PGE2 found in patients with arthritis is based on its described attributes from both in vivo and in vitro studies. In vivo instillation of PGE2 has been shown to increase vascular edema, exacerbate pain and prolong the arthritic lesion in a variety of animal models (Davies et al 1984). Ben-Av and colleagues (1995) have demonstrated that PGE2 stimulates the in vitro expression and production of vascular endothelial growth factor which is an important mediator in inflammatory angiogenesis as seen in the RA pannus. Other studies examining the effects

of PGE2 on bone metabolism/catabolism has documented that PGE2 can stimulate osteoclast activity and subsequent bone resorption and cartilage erosion (Robinson et al., 1975, Dayer et al., 1976). The increased presence of PGE2 within the arthritic lesion coupled with the documented effects of PG on bone resorption and cartilage catabolism suggested that PGE2 was a likely modulator of tissue breakdown, cartilage damage and associative joint pathologies in arthritis.

Subsequent studies have shown that PGs are released by a variety of cell types within the arthritic lesion. The type A (monocyte/macrophage) and Type B synoviocytes have both been shown to contain enhanced levels of COX protein in vitro and mRNA in situ in SM isolated from patients suffering from either OA or RA (Sano et al., 1992). A major stimulator of PGE2 release and of COX expression is IL-1 (Crofford et al., 1994). Dayer et al (1985, 1986) previously determined that IL-1 and TNF stimulated human synovial cells to produce markedly increased amounts of PGE2 in vitro. Subsequently, IL-1 has been shown to enhance the mRNA and protein expression of the inducible COX species from HSF both in vitro and in situ (Crofford et al., 1994). As an inflammatory marker, the presence of PGE2 in vivo or its release in vitro from HSF and other stromal cell populations has provided a benchmark for cellular activation and pro-inflammatory signaling.

While PGE2, based on its pro-inflammatory characteristics, undoubtably participates in the acute inflammatory process, its role during chronic inflammatory conditions is not fully understood. Skepticism in regards to PGs potentiating role in the chronic inflammatory process is based on the following parameters: a) PGE2 is a potent inhibitor of immune

function (Goodwin et al., 1977); b) exogenous PGs and their precursor molecules have been shown to effectively limit various clinical and physiological parameters of the arthritic lesion (Zurier et al., 1971, Leventhal et al., 1994) and c) various pro-inflammatory cytokines are inhibited in some systems by PGE2 in vitro (Kunkel et al., 1986).

Numerous studies examining the effect of PGE2 on human lymphocyte activation have documented that PGE2 inhibits T-cell mitogenesis (Minakuchi et al., 1990). The mechanism of action of PGs on T cells appears to be mediated by inhibition of IL-2 production through effectively altering the transcription of this T cell mitogen in vitro. Elevation of cAMP levels blocks IL-2 gene transcription and is likely the signal mediating PGE2 inhibition of IL-2 (Rincon et al., 1988, Paliogianni et al., 1993). In similar studies, various prostanoids including PGE2 were shown to inhibit the release of lymphocyte derived IL-4 (Strassmann et al., 1994). Other studies have also shown that PGE2 can effectively inhibit the other cytokines produced from T helper 1 (Th1) cells including interferon γ and TNF (Gold et al., 1994). In myeloid cells, a potent producer of PGs, recent work has shown that exogenous PGE2 can effectively inhibit the function of these important inflammatory cells. For instance, van der Pouw Kraan et al (1995) has documented that PGE2 can potently inhibit macrophage IL-12 production in vitro. As IL-12 is an important regulator of Th1 cytokines (Trinchieri, 1993) the modulatory effects of PGE2 on this population is important since Th1 cells aid in the activation of macrophages. The documented inhibitory effect of PGE2 on cytokines produced from T cells and macrophages is of interest with respect to the arthritic joint since these two cells are present within the pannus and are likely involved in

both the initiation propagation of the disease. Kunkel et al (1986) demonstrated that LPS stimulated murine peritoneal macrophages were inhibited in their capacity to produce IL-1 by exogenous PGE2 in a dose -dependent manner. Scales et al. (1989) demonstrated that PGE2 inhibited the synthesis and release of TNF in a similar system. The regulatory role of PGs on mesenchymally-derived cytokines has also recently been evaluated. In lung fibroblasts derived from patients suffering from pulmonary fibrosis, Rolfe demonstrated that fibroblast-produced MCP-1 was inhibited by PGE2 in a time and dose-dependent manner (Rolfe et al., 1992). In vitro, the suppression of synovial cell activities including proliferation by prostanoids have been documented (Newcombe et al., 1975). Subsequent studies by Baker et al demonstrated that the fatty acid dihomo-gamma-linolenic acid, a precursor to PGE2, suppressed synovial cell proliferation in vitro (Baker et al. 1989). Other studies examining the effect of PGE2 on fibroblast proliferation has garnered variable results.

ElAttar et al (1993) demonstrated that the proliferation of gingival fibroblasts derived from a normal individual was inhibited by exogenous PGE2. In addition, endogenous PGE2 as assessed by indomethacin stimulation, was also an important inhibitor of DNA synthesis. Gitter et al (1989) demonstrated that IL-1 and TNF stimulation of HSF resulted in an increase in proliferation. In their studies, the addition of indomethacin and other NSAIDs resulted in augmented proliferation suggesting that endogenous PGE2 were indeed inhibiting proliferation. In mouse fibroblasts, Durant et al (1989) demonstrated that PGE2 stimulated fibroblast proliferation and various NSAIDs did not alter the proliferative capacity of these cells. In Swiss 3T3 fibroblasts, cAMP enhancing mediators, including PGE2, increased

cellular proliferation in these cells. Hori et al (1991) demonstrated that in FS-4 fibroblasts, PGE2 antagonized proliferation induced by TNF in vitro. The variations seen within these studies may be due to the cellular source, culture conditions and species in question. Further studies addressing the in vitro ability of PGs to influence fibroblast/synovial cell proliferation, perhaps examining the molecular control over these events, appears necessary to better understand the dynamics of PGs effects. As previously mentioned, the in vitro effect of PGE2 on synovial fibroblast chemokine production (Loestcher et al., 1994) and GM-CSF release (Hamilton et al., 1992) is suggestive of an important role for this low molecular weight mediator as assessed by in vitro analysis of mesenchymal cell activity.

While both the molecular and clinical role of exogenous and endogenous PGs in arthritis remains to be fully elucidated, some investigations have hinted at at protective effect of these molecules in vivo. A direct effect of prostanoids on the arthritic lesion in vivo was first proposed by Zurier and Quagliata. They provided evidence suggesting that prostanoids can effectively alter the arthritic lesion both in vivo and in vitro (Zurier et al., 1971). Using PGE1, a derivative of eicosotetranoic acid, they demonstrated that systemic in vivo instillation of this prostanoid significantly reduced the arthritic score and paw swelling associated with adjuvent-induced arthritis in rats. In another inflammatory disorder, Borak et al examined the role of exogenous PGs on idiopathic pulmonary fibrosis (IPF) and found that lung mesenchymal cell accumulation was limited by aerosol instillation of PGs (Borak et al., 1991). The pathology of IPF is characterized by the progressive accumulation of fibroblast-like cells and the apparent lack of PGE2 within the epithelial lining fluid. Re-

instillation of PGs was effective in limiting disease progression.

Taken together, these data suggest that fatty acid derivatives, and in particular PGs, can effect the chronic inflammatory response. PGE2 has been shown to have a variety of cellular targets (lymphocytes, macrophages and fibroblasts), all of which are important players within the arthritic lesion.

RESEARCH PROPOSAL

The synovial membrane is the focal point of inflammation in a variety of joint pathologies (Hamilton, 1983,Firestein et al, 1987, Harris, 1990). While the initiation signals for such chronic diseases like Rheumatoid arthritis are not well understood, previous investigations have implicated the SM and its Type A and Type B synoviocytes (HSF) as important driving forces in chronic tissue destruction (Firestein et al 1990). Firstly, the HSF has been shown to possess the gene expression and protein synthesizing attributes, both in vitro and in vivo, to influence its environment by producing and releasing pro-inflammatory mediators (Guerne et al 1989, Alvaro -Gracia et al 1989, Alvaro-Gracia et al 1990, Arend et al 1990, Alvaro Gracia et al. 1991) . Second the HSF is both a hyperplastic and hypertrophic cell enabling the entire SM to thicken and, coupled with the macrophage-like Type A- synoviocyte, can cause major remodeling of cartilage and eventually bone. Thirdly, in some forms of chronic inflammatory joint disease (particularly RA), the HSF appears to possess an altered phenotype and is often deemed to be a "transformed-like" cell. Its ability to grow in anchorage independent conditions coupled with its enhanced expression and production of various oncogenes and matrix-degrading enzymes aid in its ability to both orchestrate the destruction of joint tissue and potentiate the movement of the pannus out of its demarcated area (Hamilton, 1983, Lafyatis et al., 1989). It is clear that the resident fibroblast-like cell of the synovial membrane, once thought of as a structural component of the normal joint, possesses the potential to play a vital role in the cellular interactions during pathological states within the SM. However, a total understanding of the responses of the HSF to inflammatory stimuli within the joint is incomplete, as is the contribution which these

cells make to the inflammatory process. To this effect, we have been interested in determining the role of the HSF in chronic inflammatory joint disease.

The nature of this report is centered on the in vitro characteristics of the human synovial fibroblast and its responses to particular stimuli. We hypothesize that the HSF can act as an effector population within the SM milieu mediating both joint destruction and remodeling, by the production of intercellular mediators (cytokines), matrix degrading enzymes, and autocoids. In addition, through a significant contribution to the local cytokine/soluble mediator pool, we also hypothesize that HSF alter the morphology/pathology of the inflamed joint by initiating signals which effect the cellular constituents of the SM. We further suggest that the response of HSF to local mediators is in fact disease specific; that is, HSF derived from normal SM respond differently than HSF derived from chronically inflamed tissue (either OA or RA).

To approach these issues, we have utilized an in vitro system of activated HSF. Enzymatically dispersed synovial tissue originating from surgical specimens derived from joint replacement procedures have afforded researchers the opportunity to analyze the activity and nature of the cellular constituents of the SM. Cultures originating from OA and RA SM samples provide a relatively homogenous population of HSF after 2-3 passages of adherent cells in vitro. Studies examining the response of HSF to various cytokines and growth factors have been the basis of a variety of studies attempting to delineate the role of these cells within SM (Feldmann et al., 1996). Examination of culture medium after specific in vitro stimulation affords us the opportunity to better understand the contribution of the HSF

population to the inflammatory mediator pool. In addition, our system allows us to address the net in vitro effect of HSF soluble products/mediators on other cellular targets present within the SM to verify whether HSF do possess an important role in the dynamics of joint inflammation. The presence of other cells, particularly the Type A synoviocyte, as important players orchestrating the dynamic processes within the inflamed joint can not be ignored. However, this thesis will focus on the contribution of the HSF (Type B synoviocyte) and its role within the joint during the chronic inflammatory syndromes (see Figure 1).

In chapter two of this thesis, an analysis of HSF populations for in vitro cytokine production was undertaken. We proposed that the HSF could contribute to the local milieu by producing Granulocyte Macrophage (GM)-Colony-stimulating Factor (CSF) in vitro. As a pivotal cell within the proliferating pannus, we maintained that the HSF was a major source of this cytokine and the main contributor of GM-CSF to the local pool of inflammatory mediators. The production of the pro-inflammatory cytokine GM-CSF from SM-derived HSF has been previously reported (Alvaro-Gracia et al, 1989). This chapter confirms this finding and extends it by demonstrating that the HSF population not only makes this important cytokine but its expression is regulated by a novel locally-derived mediator (Substance P). A second premise to the hypothesis was the notion that chronically diseased tissue would react to inflammatory stimuli with heightened responses. It also shows that the production of GM-CSF by HSF derived from RA tissue is significantly higher than GM-CSF-derived from OA-derived tissue. In summary, it was established that HSF derived from RA SM produced more GM-CSF than that released from OA-derived HSF; that this cytokine was

regulated by a local inflammatory mediator; and that the HSF (either OA or RA-derived) was a major contributor of GM-CSF to the local environment of the joint.

The HSF has been shown to produce a plethora of soluble mediators which are found in the supernatants of in vitro-stimulated cultures (Richards et al, 1993). Coupling a biological effect to these soluble mediators usually comes from indirect documentation from the literature stating the many functions that such cytokines/autocoids/growth factors might have in vivo. In an attempt to examine the biological responses of HSF to modulators present within their local environment, the second paper presented addresses the biological significance of HSF produced GM-CSF. Since the hypothesis of this thesis maintains that HSF, in an apparent disease-specific manner, are an effector population, the second paper in Chapter 2 explores this sentiment by linking the finding of increased HSF production of GM-CSF to an important pathological parameter seen in the arthritic joint; lymphocyte retention. Firstly it is documented that GM-CSF is present in supernatants from HSF derived from OA and RA SM and that IL-1-stimulated RA-derived HSF produce more GM-CSF than OA-derived HSF. As well, it couples this finding with an important biological effect; HSF produced GM-CSF-mediated lymphocyte survival.

The contribution of the HSF and the SM to the pathogenesis of arthritis is recently becoming more clear (Haraoui et al, 1991). The pro-inflammatory gene products manufactured by HSF populations both in vivo and in vitro suggest that, locally, this contingent of cells may be a significant population to target for potential pharmacological therapies (Bandara et al., 1993). While new therapeutic strategies are being developed to

regulate the activity of stromal cell populations during various oncological and inflammatory diseases, one of the mainstay treatments for arthritis, remains Non-Steroidal Anti-Inflammatory Drugs (NSAID) therapy (Abramson et al., 1989). Inhibition of the arachidonic acid cascade limits the availability of prostaglandins which have been shown to significantly contribute to the pain, vascular edema, and potential loss of joint function associated with degenerative joint diseases, particularly OA and RA (Abramson, 1992). While the immediate benefits of NSAID use in arthritis are not disputable, long term disease modification from extended NSAID therapy has not been readiiy documented. In fact, it has recently been suggested that indomethacin, a potent NSAID not be used in patients with OA since it significantly contributes to the progression of the disease and limited patient compliance often renders it less effective (Scholes et al., 1995, Huskisson et al. 1995). This information coupled with the important data demonstrating that various species of prostaglandins, including PGE2 are immunosuppressive (Phipps et al., 1991) is suggestive of two hypotheses: i) NSAID therapy has a direct effect on joint cells other than the ability to inhibit cyclooxygenase products; and ii) The long term effects of endogenous prostanoids may actually benefit the joint by limiting the disease progression through the inhibition of various pro-inflammatory signals derived from local and distal forces in the host.

With our general interest in HSF biology, and other evidence in the literature which demonstrated that prostanoids could modulate fibroblast activity (Freundlich et al., 1986), we addressed this issue using a detailed analysis of the action of PGE2 and of one NSAID, indomethacin, on the responses of HSF in vitro. The first paper in this chapter further

extends the analysis of the production of GM-CSF as well as other cytokines which are important to the inflammatory lesion, IL-8 and IL-6, and examines the role which endogenous and exogenous PGE2 have on their production. While HSF production of IL-8 and IL-6 were stimulated by endogenous and exogenous PGE2, GM-CSF was inhibited. The second paper in Chapter 3 demonstrated that synovial and lung-derived fibroblasts were effected by PGE2 in synergy with another potentially important cytokine in inflammation, Oncostatin-M (OM), to increase the production of IL-6 at the protein and mRNA level. OM is a T-cell/monocyte-derived member of the IL-6 family of cytokines which has recently been shown to be an important mediator of the acute phase response and of the matrix metalloproteinase inhibitor Tissue Inhibitor of Matrix Metalloproteinases (TIMP) produced by HSF (Richards et al., 1993). This chapter suggested that HSF cytokine production was differentially regulated by prostanoids. IL-6, was stimulated by PGE2 while a potent pro-inflammatory mediator GM-CSF was significantly inhibited by prostanoids.

Most of the HSF populations we had addressed in Chapter 3 were derived from synovial tissue originating from OA patients, however an underlying theme was developing from some of our experiments; the cellular source of HSF (ie. RA or OA derived synovium) potentially effected the magnitude of the response to stimuli. With this in mind, we again examined the activity of human synovial fibroblast when stimulated by PGE2 in Chapter 4. Our hypothesis maintains that the response of HSF derived from differing disease states will respond to in vitro stimuli distinctly. With this, coupled with our previous findings that HSF respond to the local mediator PGE2 , we examined the effects of this mediator on three

relevant parameters inherent to the SM and to HSF activity; cellular proliferation, chemokine production and matrix metalloproteinase production. Our approach was to compare responses in three different tissue sources of HSF; normal, OA and RA-derived. This chapter determined that some response parameters of HSF biology are in fact tissue dependent. That is, the effect of PGE2 stimulation on HSF proliferation is different for cells derived from normal synovium than the effect on cells derived from OA or RA synovium. Normal HSF respond to PGE2 with inhibited proliferation whereas RA and OA-derived HSF respond to PGE2 with enhanced proliferation. In examining the effect of endogenous and exogenous PGE2 on chemokine production we show that MCP-1 and RANTES production are inhibited by PGE2 irrespective of the tissue source. Thirdly, this report documents the finding that HSF production of MMPs is not significantly effected by endogenous or exogenous prostanoids, suggesting that prostanoids may function as a homeostatic modulator within the diseased SM.

Chapter Two

This chapter is comprised of two papers; Paper 1: *Are lymphocytes the main target of Substance P neuromodulation in patients with Rheumatoid arthritis?* This paper details the finding of GM-CSF in supernatants from human synovial fibroblasts and lymphocytes citing the increased presence of GM-CSF from synovial fibroblasts derived from rheumatoid joint tissue as compared to synovial fibroblasts derived from osteoarthritic joint tissue. In addition, this was the first report published demonstrating that SP could stimulate fibroblasts to produce GM-CSF. All work in the manuscript was completed by the author of this thesis. Paper 2: *Synoviocyte-derived GM-CSF mediates human lymphocyte survival.* This work confirms the previous finding of increased GM-CSF from human synovial fibroblasts derived from joint tissue originating from patients suffering from rheumatoid and osteoarthritis. The novel finding in this paper was that HSF-derived GM-CSF could enhance lymphocyte survival. The significance of this finding is that the inflamed joint is maligned with increased numbers of lymphocytes wihich are thought to contribute to the pathology of the disease. All work performed in this chapter was done by the author of this thesis.

Are Lymphocytes a Target for Substance P Modulation in Arthritis?

By A. Agro and A.M. Stanisz

The contribution of the neuropeptide substance P to the pathogenesis of rheumatoid arthritis (RA) has recently been suggested. The presence of immunoreactive substance P in the serum and joint fluid of RA patients was significantly increased compared with age-matched control patients. To investigate the ability of substance P to alter lymphocyte activity during the disease, lymphocytes were isolated from the synovial fluid and blood of RA patients and their ability to respond to substance P as measured by [^{3}H]thymidine uptake was characterized. Upon exposure of RA synovial fluid and peripheral blood lymphocytes to various concentrations of substance P in vitro, no increase in proliferation was witnessed. To the contrary, control peripheral blood lymphocyte proliferation was significantly enhanced by various concentrations of substance P. However, synoviocytes from the joints of RA patients were responsive to substance P stimulation. These data suggest that substance P receptors may be desensitized on systemic and local lymphocytes in RA, or the proinflammatory activities of substance P may be mediated via the synovial membrane during chronic inflammation.

INDEX WORDS: Substance P; lymphocytes; rheumatoid arthritis; synoviocytes.

INTEREST IN THE interplay between the immune system and the nervous system is growing rapidly. Various neurally active products, including substance P (SP) and other neuropeptides, appear to markedly influence multiple parameters of immunity.[1-4] In part, this neuroimmunomodulation manifests itself in the process of inflammation by initiating, manipulating, and at times terminating various aspects of this process.[3]

Of particular interest is SP, which has been recently postulated to be a mediator of neurogenic inflammation.[5,6] Various groups have shown that SP can significantly influence neutrophil chemotaxis,[7] interleukin (IL)-1 and IL-6 release from monocytes,[8] and the release of oxidative enzymes from macrophages.[9] We and others have shown that lymphocyte proliferation and immunoglobulin synthesis, both in vitro and in vivo, are significantly enhanced by SP.[10-12]

The role of the nervous system, particularly SP, in the pathogenesis of rheumatoid arthritis (RA) is of growing interest. In experimental models of RA, Levine et al have shown that the presence of SP enhances synovitis.[13] Neural depletion of SP via the sensory nerve neurotoxin capsaicin eliminates inflammation within the inflamed joint. Increased levels of SP within the human inflamed RA joint have been documented, and the initiation of collagenase production, prostaglandin E_2 release, and IL-1 production from human synovial lining cells has been reported.[14]

RA is a chronic inflammatory disease[15] with increased numbers of T, B, and plasma cells within the synovial fluid and synovium.[15] The activity, distribution, and responsiveness of these cells have been studied. The proportion of $CD8^+$ cells is increased[16]; IL-2 receptor expression is increased[17]; and the response to various mitogens is limited (because of the proposed overstimulation of these cells within the joint).[18]

The purpose of this study was to establish whether lymphocytes are a target for SP modulation in RA. We examined the effect of SP on lymphocyte proliferation within the blood and synovial fluid of RA patients. We also observed the effects of SP on the release of the cytokine granulocyte-macrophage colony–stimulating fac-

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tor (GM-CSF) from lymphocytes because this cytokine is important in the pathophysiology of RA. In addition, we examined the effects of SP on synoviocyte production of GM-CSF to determine whether SP has a preferential cellular target. To our knowledge, this is the first study to analyze the effects of SP on systemic and local lymphocytes in a chronic inflammatory condition.

MATERIALS AND METHODS

Patients

Peripheral blood and synovial fluid were collected from consenting patients with rheumatoid or nonrheumatoid diseases and consenting volunteers. Twenty-two subjects participated in the study: 10 had RA based on the criteria developed by the American Rheumatism Association,[15] 5 were non-RA patients (4 had osteoarthritis [OA] based on radiography, and 1 had pseudogout based on urate crystal levels), and seven were normal volunteers who donated blood. It was not possible to measure each parameter in every patient. RA patients were receiving nonsteroidal antiinflammatory drug (NSAID) therapy alone except for 2 patients who also were receiving 10 mg of prednisone. Non-RA patients (5) also received NSAID therapy; laboratory volunteers received no medication.

Synoviocyte Isolation

Fragments of synovial tissues were obtained from RA or OA patients undergoing surgery for destructive joint disease. Extraneous connective tissue was removed, and the resulting preparation was finely minced and digested in Dulbecco's medium containing 1 mg/mL collagenase (Sigma Chemical Co, St Louis, MO) under serum-free conditions at 37°C for 3 hours. The digested synovium was then passed through a wire mesh, extensively washed, and cultured in Dulbecco's medium supplemented with 200 mmol/L L-glutamine (Gibco, Grand Island, NY), 25 mmol HEPES, 50 μg/mL gentamicin, and 10% fetal bovine serum (FBS; Gibco) at 37°C in 5% CO_2. After 24 hours, nonadherent cells were removed and fresh medium was applied. At subconfluence (4 to 5 days), the medium was changed and 10^{-8} mol/L SP was added. Cells were cultured for 48 hours, and supernatants were collected.

GM-CSF Assay

The presence of GM-CSF was assayed by an enzyme-linked immunosorbent assay (ELISA). (Genzyme Corp, Boston, MA). The linear portion of the standard curve corresponds to GM-CSF levels in the range of 4 to 250 pg/mL.

Lymphocyte Isolation

Synovial fluid and peripheral blood were collected in heparinized vacutainers and diluted 1:1 in 0.5 mol/L phosphate-buffered saline (PBS; pH 7.3). It was then layered over Ficoll-Hypaque (Pharmacia, Upsala, Sweden). Erythrocytes were lysed with cold isotonic NH_4Cl solution.[19] The preparation was centrifuged at 1,000*g* for 12 minutes, and the buffy coat was isolated and counted. Usual viability exceeded 90%, and lymphocyte purity after monocyte adherence[20] was greater than 95%.

Cultures

Isolated cells were resuspended in RPMI (Roswell Park Medical Institiute, Buffalo, NY) supplemented with 10% FBS, gentamicin (0.05 μg/mL), L-glutamine (10 n-mol/L), and phytohemoglutanin (PHA; 2 μg/mL). Cells were resuspended to a final concentration of 2×10^6 cells/mL. Cultures were set in 96-well plates (Corning, Boston, MA) and exposed to various dilutions of SP (Cambridge, Cambridge, England) ranging from 10^{-11} mol/L to 10^{-6} mol/L. These were incubated for 48 hours in 5% CO_2 at 37°C, after which [^{3}H]thymidine (1 μCi/well; specific activity, 6.7 mCi/nmol/L; New England Nuclear, Boston, MA) was added for an additional 24 hours. The cells were harvested and counted using scintillation counting. The results are presented as percentage change from the control assay and were calculated as follows:

$$\frac{(\text{cpm of Lymphocytes With SP}) - (\text{cpm of Lymphocytes Without SP})}{\text{cpm of Lymphocytes Without SP}} \times 100.$$

Radioimmunoassay

After aspiration, the synovial fluid was incubated with 1 mg/mL of hyaluronidase at 37°C under constant agitation. After 1 hour, the

synovial fluid was centrifuged at 12,000 rpm for 30 minutes and the supernatants were removed and stored at −70°C. Serum and synovial fluid samples were analyzed for SP using an inhibition-type radioimmunoassay using a polyclonal antibody to SP (ICN, St Paul, MN). All samples, antibodies, and SP had the endopeptidase inhibitor phosphoramidon (100 nmol/L; Sigma Chemical Co) added.

Statistics

All data were analyzed using Student's *t* test. The Bonnferoni correction was used where applicable.

RESULTS

Serum and Synovial Fluid Levels of SP

Isolated and purified synovial fluid and serum from RA patients and controls were assayed for the presence of SP. Levels of immunoreactive SP were significantly higher in the RA population than in controls (Fig 1). Mean serum levels in non-RA patients were 0.7 nmol/L compared with 12.2 nmol/L in RA patients (normal control levels ranged from 0.01 mol/L to 0.5 mol/L; data not shown) ($P > .001$). Levels in the synovial fluid were 1.5 nmol/L in non-RA samples and 18 nmol/L in RA patients ($P < .001$). The two patients treated with prednisone showed markedly lower SP levels in the serum synovial fluid.

Random samples were analyzed by high-performance liquid chromatography to verify that the entire SP molecule was been measured (data not shown).

In Vitro Lymphocyte Proliferation With SP

Normal peripheral blood mononuclear cell (PBMC) proliferation in the presence of PHA and SP for 48 hours is shown in Fig 2. SP caused a 75% increase in proliferation at 10^{-9} mol/L and a maintained 28% to 35% increase at concentrations ranging from 10^{-8} mol/L to 10^{-6} mol/L. PBMCs from non-RA patients were not significantly effected by SP in any concentration. Figure 2 also shows that SP caused a slight inhibition of [^{3}H]thymidine incorporation in RA patients at pharmacological concentrations (10^{-6} mol/L). Because the normal range of control proliferation is variable (up to 10% compared with unstimulated), the differences were not significant in non-RA patients, but proliferation was significantly lower in RA patients ($P < .01$). In vitro pulsing of RA synovial lymphocytes with SP had a significant inhibitory effect on proliferation (10^{-8} mol/L to 10^{-6} mol/L) (Fig 3). SP had no significant effect on non-RA lymphocytes although it was slightly stimulatory.

Effects of Substance P on Synoviocytes

The effect of 10^{-9} mol/L SP on synoviocyte production of GM-CSF is shown in Fig 4.

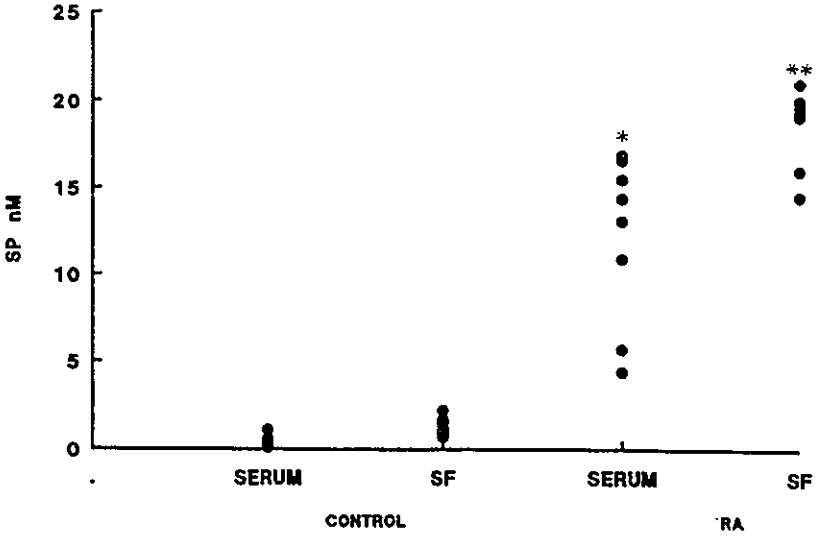

Fig 1: The presence of immunoreactive SP was measured by a radioimmunoassay in serum and synovial fluid of non-RA (control) and RA patients. *, RA patients who received prednisone before synovial fluid removal ($P < .001$ for both groups).**

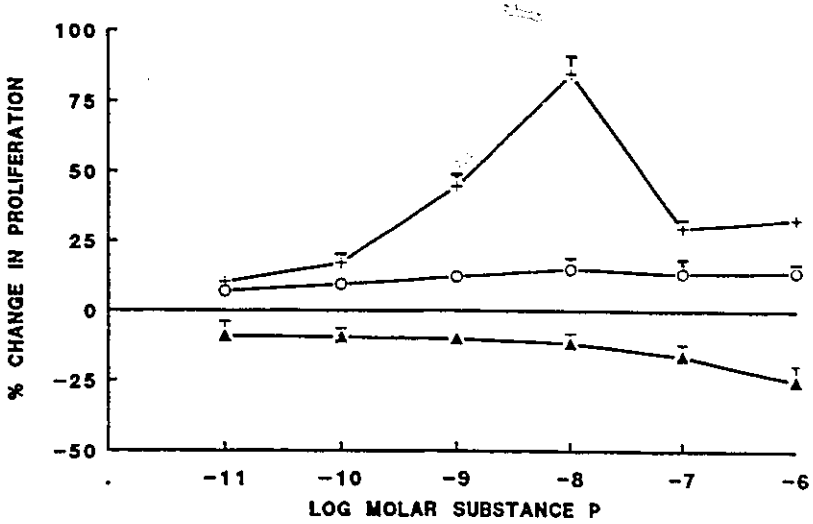

Fig 2: The response of mitogen-activated (PHA) normal PBMCs to SP is shown (+) and compared with the response of PBMCs from non-RA (O) and RA (▲) patients. The incorporation of [^{3}H]thymidine is measured and compared with unstimulated (no SP added) cells and expressed as a percentage. At peak physiological amounts of SP (10^{-9} mol/L), no response is seen in non-RA or RA PBMCs.

Baseline levels of GM-CSF ranged from 19 to 70 pg/mL for RA synoviocytes and from 10 to 17 pg/mL for non-RA synoviocytes. After SP stimulation, GM-CSF levels significantly increased in both RA (256 pg/mL) and non-RA (77 pg/mL) synoviocytes. Levels of GM-CSF from non-RA or RA lymphocytes were at the lower end of detection (4 pg/mL). Normal peripheral blood lymphocytes released 8 to 10 pg/mL of GM-CSF upon stimulation with SP (data not shown).

DISCUSSION

The presence of immunoreactive SP in the inflamed joint in RA patients has been documented recently.[21,22] Additionally, SP-containing as well as calcitonin gene-related peptide–containing nerves are present within normal

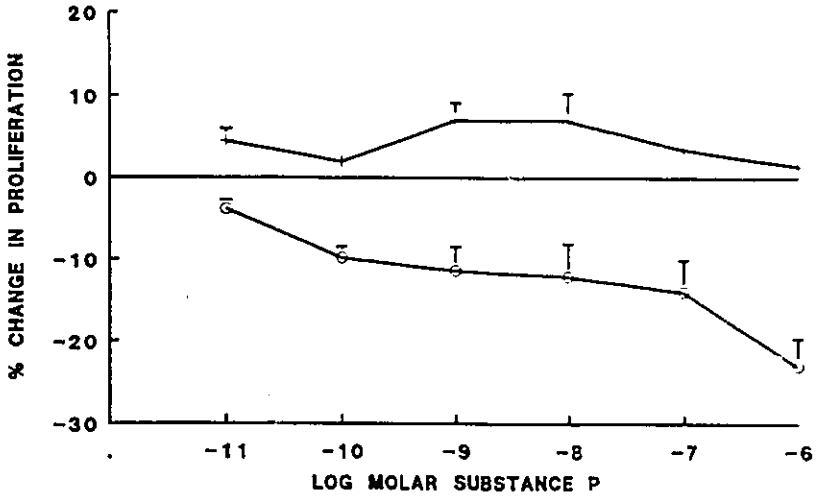

Fig 3: Synovial fluid lymphocytes were isolated from non-RA (+) and RA (O) patients and exposed to mitogen and SP. In RA patients, SP caused a significant inhibition of [^{3}H]thymidine incorporation, whereas in non-RA it had little or no effect.

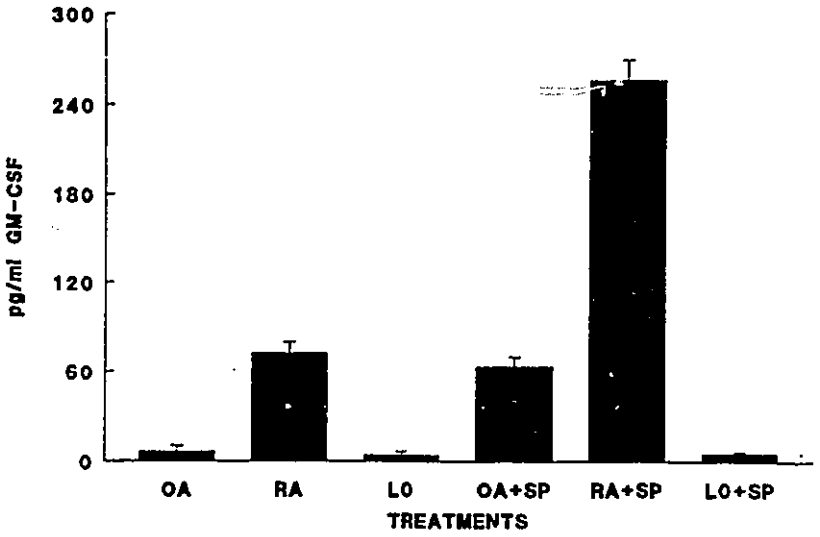

Fig 4: The level of GM-CSF in SP-stimulated synoviocyte cultures was assessed. An ELISA technique shows that 10^{-9} mol/L SP significantly increased the release of GM-CSF from RA and non-RA synoviocytes and not from RA lymphocytes (LO) from the synovial fluid ($P < .05$).

and arthritic joints in animal and human studies.[23,24] The role of nerves as mediators of inflammation has been chronicled, but the effects of their products, particularly neuropeptides, on lymphocytes during inflammatory distress have not been investigated. In light of studies showing that neuropeptides significantly alter various immunological parameters in nonpathological states, it may be surmised that disruption of the neuroimmune axis during disease may, in part, contribute, initiate, or exacerbate the inflammatory response.

In this study, increased levels of SP within the serum and synovial fluid of RA patients are associated with a decrease in lymphocyte responsiveness to exogenous SP as measured by [^{3}H]thymidine incorporation. We also show that SP alters the production of GM-CSF from synoviocytes but not from lymphocytes.

The effects of SP on human lymphocyte proliferation were first documented in 1983.[25] Receptors for SP were characterized on human lymphocytes; functionally, SP caused an increase in the uptake of [^{3}H]thymidine in mitogenically stimulated lymphocytes.[26] Within the joint, lymphocytes are relatively activated and therefore not responsive to various mitogens.[18] In this study, synovial lymphocytes responded to PHA activation with a slight increase in proliferation (data not shown), yet SP did not further enhance this state or act in a mitogenic fashion. Even at physiological levels of SP (which cause maximal stimulation in normal lymphocytes), synovial-isolated lymphocytes were not affected. Interestingly, PBMC lymphocytes also were not responsive to SP at any concentration while levels of SP were significantly increased in the serum. Based on various experimental protocols in which PBMCs are used as a source of internal control cells (because they reportedly act with a "normal" response to mitogen[16-18]), these results are significant and may be explained by the following. First, lymphocyte receptors for SP may be desensitized because of large amounts of the peptide found in situ. Repeated stimulation with SP causes a typical tachyphylaxis in many systems,[27] and receptor kinetics and binding are altered during repeated administration.[28] Various animal models of inflammation show similar findings with lymphocytes, SP levels, and other cellular populations.[29-32]

However, upon analyzing the binding properties of SP to RA lymphocytes from synovial fluid and the periphery, we saw that receptor numbers were only slightly decreased but affinity remained the same (A. Agro, A.M. Stanisz; manuscript in preparation). Because the receptor kinetics appear to be unaltered on lymphocytes, we suggest that these events may occur at a later stage than receptor-ligand binding, such as cytokine production or synthesis.

A second explanation may be that lymphocytes are not the direct targets of nervous innervation and SP stimulation during RA. We show here that GM-CSF release is enhanced by

SP in synoviocytes and not in lymphocytes. GM-CSF is present in increased amounts in the joints of RA patients and appears to have a paracrine role in promoting angiogenesis as well as upregulating the expression of major histocompatibility complex class II antigens on various populations of synoviocytes.[33] Synovial fibroblasts stimulated by SP produce increased amounts of collagenase and prostaglandin E_2 as well as a number of cytokines, including IL-1 and IL-6.[14,33] SP has also been shown to enhance synoviocyte proliferation in RA.[14] These products, in turn, can have profound effects on lymphocyte activity, and therefore SP could bypass a direct innervation of lymphocytes and act through a more susceptible target.

The stromal-cell population of synoviocytes, which has been shown to respond to SP in recent studies[14] and is now considered an essential component of the local inflammatory response, appears to be a potentially important cell affected by SP.

It is hoped that new information about the interaction between nerves, neuropeptides, and immunity in chronic inflammatory states will lead to some novel therapeutic approaches to the management of inflammatory conditions.

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Synoviocyte Derived Granulocyte Macrophage Colony Stimulating Factor Mediates the Survival of Human Lymphocytes

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Abstract. **Synoviocytes have been shown to be effector cells capable of synthesizing and secreting a variety of cytokines and growth factors. We demonstrate here that synoviocyte derived conditioned medium has immunoregulatory properties as it enhances human peripheral blood lymphocyte survival in a dose dependent manner *in vitro*. The effect elicited by synoviocyte derived conditioned medium from patients with rheumatoid arthritis (RA) was greater than that induced by synoviocyte derived conditioned medium from patients with osteoarthritis. Granulocyte-macrophage colony stimulating factor (GM-CSF) was found in synoviocyte derived conditioned medium with significantly higher levels present in synoviocyte derived conditioned medium from patients with RA. Recombinant human GM-CSF induced survival of human lymphocytes *in vitro* and a monoclonal antibody to human GM-CSF fully abrogated synoviocyte derived conditioned medium induced survival. Our results demonstrate that synoviocyte derived GM-CSF may be important in the retention of lymphocytes, which is a central pathological characteristic of the rheumatoid joint.** (*J Rheumatol 1992;*19:1065–9)

Key Indexing Terms:
SYNOVIOCYTE LYMPHOCYTE SURVIVAL GM-CSF

Rheumatoid arthritis (RA) is a relapsing chronic inflammatory disease of unknown etiology[1]. Although many organs may be involved, synovitis, leading to joint destruction is the prime cause of the pain and functional impairment which characterize the disease[2-5]. The typical hyperplasia of the synovial membrane is accompanied by an increase in the number of inflammatory cells in the synovial fluid (SF) as well as within the membrane itself. Increased levels of T lymphocytes of the helper/inducer CD4 phenotype as well as a variety of class II possessing antigen presenting cells within the area suggests that the disease is antigen dependent[6-9]. Although a number of possibilities have been investigated, the so called RA antigen remains elusive[9-11].

The inflamed joint represents a microenvironment rich in inflammatory and immunomodulatory factors[1,2,12-14]. While the source of these factors has been traditionally ascribed to infiltrating immune/inflammatory cells, it has recently become clear that the synovial membrane itself contribute to the local cytokine content[2]. Indeed, synoviocytes ha been shown to produce a variety of cytokines and grow factors *in vitro* including interleukin (IL)-1, IL-6, IL-8, tra forming growth factor β_1, platelet derived growth factor an granulocyte-macrophage colony-stimulating factor (G CSF)[2,12-18]. With regards to GM-CSF, there is evidence t synoviocytes contribute to the local content of this cytoki some 5-fold more than do the adjacent immune cells wit the SF or synovial membrane[2,13]. These cytokines are c able of modulating a broad spectrum of activities on infla matory cells and thereby they may contribute to the perpe ation of the inflammatory response. Our purpose wa investigate whether cytokines derived from human syno cytes are involved in regulating the retention of lymphocy in the joint.

We show here that conditioned medium of synoviocyt derived from patients with RA (RA-SCM) markedly i creases the survival of normal peripheral blood lymphocyt *in vitro* compared to fully supplemented culture medium, a that this RA-SCM induces a greater effect in comparison wi conditioned medium of synoviocytes obtained from patien with osteoarthritis (OA-SCM). The increased lymphocyt survival seen under these conditions represents true survival as SCM had only a small effect on lymphocyte proliferation as assessed by ^{3}H-thymidine incorporation. Additionally, we have established that RA-SCM contains greater amounts

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of GM-CSF compared to OA-SCM. Finally, we show that the preincubation of SCM with a monoclonal neutralizing rat antihuman GM-CSF antibody fully abrogated the survival of lymphocytes.

Together, our results show that synoviocyte derived GM-CSF may play an important role in the accumulation of lymphocytes within the joint infrastructure in RA.

MATERIALS AND METHODS

Synoviocytes. ARA classified RA and OA tissue was obtained at the time of surgery from patients undergoing joint replacement procedures. Tissue was processed within 20 min of removal and a section of the tissue was dissected for histological examination. Synoviocytes were isolated using described methods[13]. Briefly, tissue was finely minced in sterile conditions and digested for 3–4 h in Dulbecco's medium containing 1 mg/ml collagenase (Sigma Co., St. Louis, MO) and 0.5 mg/ml hyaluronidase (Sigma Co.). The resulting material was filtered through a wire mesh and the cell suspension was then plated in 35 mm flasks (Corning Glass Co., Corning, NY) at a density of 2×10^5/ml in Dulbecco's medium supplemented with 10% fetal bovine serum (FBS: Gibco, Grand Island, NY), 200 nM L-glutamine, 50 μg/ml gentamicin and 25 mmol/l Hepes buffer (Boehringer Mannheim Canada Ltd., Dorval, PQ) herein called complete medium. Cells were kept in a humidified atmosphere at 5% CO_2 and 37°C. After 24 h, nonadherent cells were removed and fresh complete medium containing 1% FBS was added. Supernatants were collected from fresh isolates after 96 h of culture. RA-SCM was pooled from 6 primary RA cell cultures and OA-SCM was pooled from 7 primary OA cell cultures. Supernatants were collected from single passaged cells only.

Lymphocytes. Thirty ml of blood were collected in heparinized tubes from various laboratory volunteers. Blood was diluted 1:1 in 0.8 M NaCl and layered over a Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden). The buffy coat of mononuclear cells was removed and plated in 35 mm Petri dishes in RPMI 1640 medium supplemented with 10% FBS, 200 nM L-glutamine, 25 mmol/l Hepes buffer, 50 μg/ml gentamicin and 1 μg/ml phytohemaglutin (Pharmacia) for 3 h. Nonadherent cells were removed and counted using trypan blue. Viability always exceeded 96%. Lymphocyte purity was greater than 95% as assessed by the Diff-Quik modification of the May-Giemsa technique and nonspecific esterase staining (American Scientific Products, McGraw Park, IL). Cells were then plated in 96 well plates (Corning) at a concentration of 2.5×10^6/ml.

Cytokines and antibodies. Recombinant human (rh) GM-CSF (specific activity 9.3×10^6 U/mg) was obtained from Genetics Institute (Boston, MA). A rat antihuman monoclonal neutralizing anti-GM-CSF antibody was generously provided by Dr. John S. Abrams (DNAX Research Institute, Palo Alto, CA). This antibody is capable of neutralizing up to 5 U/ml recombinant GM-CSF (10 pg/ml). SCM was incubated for 1 h with anti-GM-CSF before being added to the lymphocyte cultures. The content of GM-CSF in both RA and OA-SCM was measured by using a commercially available specific immunoassay (Genzyme Corp. Boston, MA) with a limit of sensitivity of 4 pg/ml. Human affinity purified IgG was used as control serum.

Assessment of lymphocyte survival and proliferation. SCM was added to lymphocyte cultures at various dilutions and the number and viability of lymphocytes were evaluated at Days 2, 4, 6, and 8. Survival was assessed by trypan blue exclusion and was carried out in 3 separate wells for each concentration at every time point. All cells were removed from each well and survival was expressed as:

$$\frac{(\text{Total \# of cells retrieved}) \times (\% \text{ of cells excluding trypan blue})}{(2.5 \times 10^5 \text{ total cells plated})} \times 100$$

Proliferation. To assess proliferation, lymphocytes were seeded in 96-well plates (Corning) at a concentration of 1×10^5/well in complete medium with or without PHA (1 μg/ml) with or without SCM at various dilutions This was followed 48 h later by the addition of ^{3}H-thymidine (6.7 mmol/Ci. NEN Co. Boston, MA) for 24 h. At this time, cells were harvested and ^{3}H-thymidine incorporation measured via scintillation procedures described elsewhere[19].

Statistics. Data were compared using a Students t test. A p level above 0.0 was deemed nonsignificant.

RESULTS

Lymphocyte survival. After the isolation of synoviocytes and their subsequent *in vitro* culturing for 72 h, supernatants were collected and tested for survival promoting properties. Figure 1a shows that RA-SCM promotes the survival of lymphocytes in a dose dependent fashion. A 0.1% dilution of this SCM maintained lymphocyte survival for 8 days above 25% whereas survival in the control cultures (RPMI media alone) was only 0.5% at Day 8 ($p < 0.001$). A 0.0015% dilution was not significantly different from control. Survival of lymphocytes was also enhanced by OA-SCM (0.1% dilution) although to a lesser extent than that induced by RA-SCM (18–25%) at Day 8. Figure 1b demonstrates the effect of

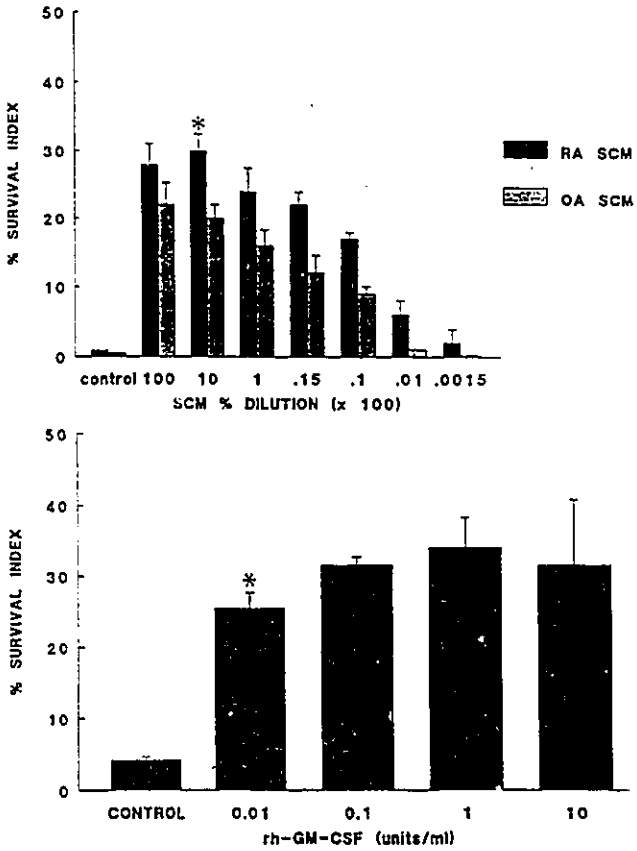

Fig. 1. Dose response of lymphocyte survival induced by RA-SCM and OA-SCM on Day 8 (1a). At a 0.1% dilution, RA-SCM has a significantly greater effect on survival than OA-SCM ($p < 0.01$). Both conditioned media induced a significantly greater level of survival than control medium at Day 8 ($p < 0.001$). Figure 1b illustrates the effect of recombinant human GM-CSF on lymphocyte survival at Day 8. A 0.01 U/ml dilution of rhGM-CSF significantly enhanced peripheral blood lymphocyte survival (24%) above control (3.5%) medium. * ($p < 0.01$).

rhGM-CSF on survival at Day 8 where 0.01 units/ml (1 pg/ml) maintained survival at 24%.

Time course. Figure 2 demonstrates the effect of time on lymphocyte survival. A 1:1000 dilution of RA-SCM was sufficient to maintain survival at levels significantly greater than control on each of the days examined. Again, RA-SCM was more effective than OA-SCM in maintaining lymphocyte survival on each day studied but did not reach significance until Day 6 ($p < 0.05$).

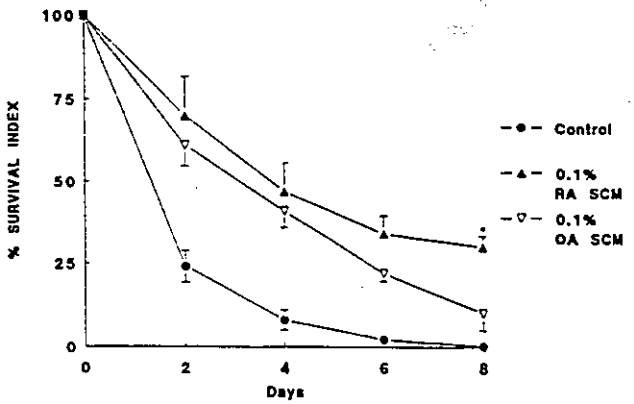

Fig. 2. Lymphocyte survival induced by SCM over time. At days 2, 4, 6 and 8 a 0.1% dilution of RA and OA conditioned medium caused an increased level of survival compared to that induced by control ($p < 0.01$). At Day 8, the effect induced by RA-SCM was significantly greater than OA-SCM. Diff-Quik staining at each time point demonstrated that over 95% of the cells were lymphocytes. * ($p < 0.01$).

GM-CSF levels. The presence of GM-CSF in culture supernatants of synoviocytes has been established[20,21]. In Table 1 we confirm that GM-CSF was present within our system by illustrating that GM-CSF was found in both RA and OA-SCM. Though the variation of GM-CSF levels between patients in each disease group was high, OA levels never overlapped with levels found in RA-SCM, which were significantly higher than OA levels ($p < 0.01$). The range of GM-CSF in RA samples was from 17 to 256 pg/ml and in OA samples fr[illegible]e lower end of detection (4 pg/ml) to 8.7 pg/ml. Co[illegible] medium, (with 1% FBS) contained minimal levels of GM-CSF.

Anti-GM-CSF neutralization. Figure 3 shows the effect of various dilutions of anti-GM-CSF on lymphocyte survival at Day 4 using a 0.1% dilution of RA-SCM. A 1:50 dilution of the neutralizing antibody was sufficient to reduce lymphocyte survival at Day 4 to control values (cells cultured

Table 1. *GM-CSF levels in RA SCM and OA SCM (ELISA)*

	GM-CSF (pg/ml)
RA Pooled (n = 7)	69* (17–256)
OA Pooled (n = 8)	6.4 (0–8.7)
1% Dulbecco's medium	< 4.0

* RA GM-CSF content vs OA GM-CSF content, $p < 0.01$.
Range of GM-CSF in parentheses.

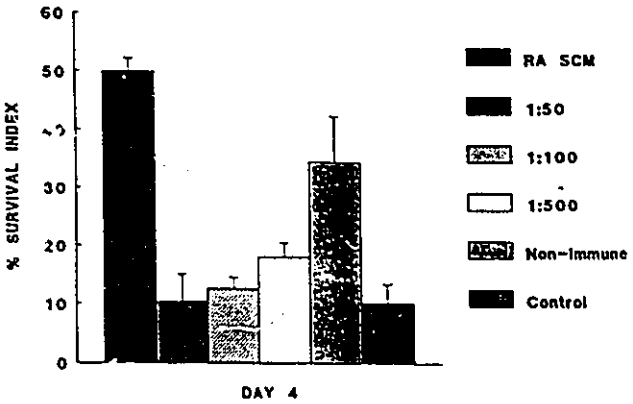

Fig. 3. Effects of a human monoclonal anti-GM-CSF neutralizing antibody on SCM induced survival. A 1:50 dilution of the antibody preincubated with 0.1% RA-SCM significantly inhibited survival into the range of nonsignificance versus control. Nonimmune human IgG was not significantly different from RA-SCM.

in RPMI alone). There was no significant difference between control nonimmune serum and 0.1% RA-SCM.

Lymphocyte proliferation. We next examined the effect of OA and RA-SCM as well as rhGM-CSF on lymphocyte proliferation. Lymphocytes incubated with either SCM did not significantly enhance ^{3}H-thymidine uptake over media alone after 72 h of culture (Table 2). rh-GM-CSF caused a marginal increase in lymphocyte proliferation after 72 h (Table 2). No mitogenic activity of either SCM was seen (data not shown).

Table 2. *Lymphocyte proliferation in response to SCM and rhGM-CSF*

Media	CPM (SD)	n
Control (Dulbecco's)	123218 (20517)	10
RA SCM (0.1%)	98037 (16006)	10
OA SCM (0.1%)	101546 (20106)	10
rhGM-CSF	112909 (11991)	8

DISCUSSION

Recent evidence indicates that the inflamed joint is, particularly in RA, a microenvironment rich in cytokines[2,12,18,20]. A number of immune and inflammatory cells that are capable of producing these factors reside in the inflamed joint. However, it has recently become apparent that synoviocytes, the heterogenous population of cells within the intimal layer of the synovial membrane, can also synthesize the majority of these mediating molecules[2,20]. For example, IL-1, IL-6 and IL-8 have all been found in increased amounts both in the SF of patients with RA as well as within the synovium[17,18,21,22]. GM-CSF, in addition to inducing differentiation of hemopoietic progenitors, regulates a wide spectrum of biologic activities in mature cells[2,23,24]. Firestein, *et al* showed that the content of this cytokine in the SF of patients with RA is increased and also demonstrated

that human synoviocytes synthesize this molecule[2,13,20]. Furthermore *in vitro* studies suggest that a substantially greater component of GM-CSF content in the SF originates from synoviocytes rather than lymphocytes[2,19], suggesting that synoviocytes are in fact effector cells themselves which could, therefore, play a major role in the regulation of the joint inflammatory response.

The retention of otherwise short lived lymphocytes within the synovial space in an activated phase is a hallmark of the inflammatory response characteristic of RA. Though a variety of mechanisms have been proposed for this phenomenon, the molecular regulation remains to be fully elucidated. It has recently been argued that the prolongation of lymphocyte survival could contribute to the accumulation of these cells in the joint cavity. A recent study by Scott, *et al* has shown that conditioned medium from synovial fibroblasts supports the viability of an activated T cell clone. However, the cytokine(s) responsible for this effect were not identified but the data in that study suggest that the interleukins 1 through 7 were not involved[25]. On the basis of this observation, the authors put forward the hypothesis that the surrounding stroma may induce T cell memory rather than continuing antigenic stimulation.

In our study, we found that SCM conditioned medium from humans markedly promotes the survival of naive human peripheral blood lymphocytes. In addition, SCM has a rather negligible effect on lymphocyte proliferation as assessed by thymidine incorporation. We also show that SCM contains GM-CSF and that rhGM-CSF promotes lymphocyte survival. Similar concentrations of rhGM-CSF (0.01 U/ml = 1 pg/ml) and SCM derived GM-CSF (0.1% = 0.7 pg/ml by ELISA) maintained lymphocyte survival above 20%. Finally, we demonstrate that preincubation of the SCM with a monoclonal neutralizing antihuman GM-CSF antibody fully abrogates the effect elicited by the conditioned medium. Thus, we conclude that synoviocyte derived GM-CSF promotes the survival of lymphocytes *in vitro*. There are quantitative differences with regards to the extent of the survival enhancing effects between our study and that of Scott, *et al*[25]. The most likely reason for this apparent discrepancy is that our results are expressed as "survival" rather than "viability." In this and other studies[23] we elect to assess survival in the way described in the Materials and Methods section because it takes into account both the total number of cells remaining in the culture as well as the proportion of these cells that are viable. Clearly, examination of cell viability alone has much less biological significance.

GM-CSF is involved in the survival of a number of inflammatory cells. For example, we have shown that human lung fibroblast derived GM-CSF fully mediates the survival of human eosinophils, and Markowicz, *et al* have shown that rhGM-CSF enhances the survival of peripheral blood dendritic cells[26]. In addition, we have recently documented that GM-CSF together with M-CSF derived from human airway fibroblasts and epithelial cells strikingly promotes the survival and differentiation to macrophages of human peripheral blood monocytes[27]. It is likely but not yet documented that synoviocyte derived GM-CSF regulates other lymphocyte activities in addition to survival. Indeed, as it has been indicated by Firestein and Zvaifler, GM-CSF may play an important autocrine and paracrine role in the RA synovium[2].

Our data also show that RA-SCM promoted lymphocyte survival to a greater extent compared to OA-SCM, and this is consistent with the finding that RA-SCM contained 10-fold higher levels of GM-CSF. Differences in cell number between RA and OA synoviocyte cultures were negligible at 72 h and therefore cannot explain these observations (data not shown). Since cells from all tissues were isolated and treated in an identical manner, we propose that our findings suggest that the effector behavior expressed by RA synoviocytes *in vitro* reflects activation of these cells *in vivo* as it is consistent with our previous observations using lung fibroblasts[23].

To conclude, our data and that of others stresses the effector potential of synoviocytes. Clearly, their contribution to inflammation in general, and to the regulation of immune and inflammatory cell function cannot be dismissed. Additional investigations into the role of the synoviocytes' effector function may be important in understanding the mechanisms involved in chronic inflammation and may suggest novel intervention targets.

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Chapter Three

The work in this chapter continues investigations into the biology of the human synovial fibroblasts by illustrating that prostaglandins can effectively modulate the production of cytokines produced by activated HSF in vitro. The title of the first paper in this chapter is: *Prostaglandin E enhances IL-8 and IL-6 but inhibits GM-CSF production by IL-1stimulated human synovial fibroblasts* in vitro. This work was the first to demonstrate that PGs could alter HSF-derived cytokine production at both the protein and mRNA level. All work presented in the paper was completed by the author of the thesis. The second paper in this chapter demonstrates that HSF are responsive to PGE2 in combination with another potentially important inflammatory cytokine, Oncostatin M (OM). This work demonstrates that PGE2 can synergize with OM to further induce IL-6 protein and mRNA from HSF. The title of this paper is: *Interactions between oncostatin M, interleukin-1 and PGE2 in the induction of IL-6 expression in human fibroblasts.* The author of the thesis was responsible for the setup and completion of experiments that utilized PGE2 including: IL-6 assays and Northern blot analysis.

Prostaglandin E_2 Enhances Interleukin 8 (IL-8) and IL-6 but Inhibits GMCSF Production by IL-1 Stimulated Human Synovial Fibroblasts *in Vitro*

ALBERT AGRO, CARRIE LANGDON, FRANK SMITH, and CARL D. RICHARDS

ABSTRACT. ***Objective.*** To examine *in vitro* the effect of prostaglandin E_2 (PGE_2) on synovial cell cytokine production.

Methods. Human synovial fibroblasts were stimulated with PGE_2 alone or PGE_2 in combination with interleukin 1α (IL-1α) (5 ng/ml) and/or indomethacin (10^{-6} M) and assessed for the production of IL-8, IL-6, and granulocyte macrophage colony stimulating factor (GMCSF) at the protein and messenger RNA (mRNA) levels.

Results. PGE_2 alone had little detectable effect on IL-8 or GMCSF; however, a small enhancement of both IL-6 mRNA and protein levels was seen. While all cytokines were markedly stimulated by IL-1α, coaddition of the cyclooxygenase inhibitor indomethacin enhanced IL-8 and GMCSF levels, but caused a reduction in IL-6 expression. The addition of PGE_2 to cultures stimulated with IL-1α and indomethacin resulted in increases in IL-6 mRNA and protein expression while causing a concomitant reduction in GMCSF protein and mRNA expression. PGE_2 and illoprost (PGI_2 analog) enhanced IL-8 production in stimulated cells.

Conclusion. While PGE_2 alone has limited effects on synovial cell production of IL-8 and GMCSF, its effects are significant in context of IL-1α stimulation; endogenous PGE_2 may alter cytokines secreted by mesenchymally derived cells. PGE_2 may be an important modulator of cytokine driven inflammation. (*J Rheumatol 1996;23:862–8*)

Key Indexing Terms:
PROSTAGLANDINS CYTOKINES FIBROBLASTS ARTHRITIS

The inflammatory process is a homeostatic mechanism orchestrated by both infiltrating mononuclear cells and mesenchymally derived local cells. While cell to cell contact is an important aspect of inflammation, cell derived soluble factors, including cytokines and low molecular weight mediators, appear to be vital in mediating the process[1]. Macrophage/monocyte derived cytokines including interleukin (IL) IL-1 and tumor necrosis factor (TNF) have been shown to be important contributors to the inflammatory process. However, more recent data suggest that local stromal cell populations, such as synovial fibroblasts, are also significant contributors to the local cytokine pool seen in inflammatory pathologies such as arthritis[2,3]. Granulocyte macrophage colony stimulating factor (GMCSF), granulocyte colony stimulating factor, IL-6 and the chemokine IL-8, and arachidonic acid metabolites including prostaglandins (PG), leukotrienes, and other lipoxygenase/cyclooxygenase dependent mediators are all major products of fibroblasts and are found in inflamed tissues in various systems[4–9]. While the presence of such mediators within the inflamed joint is indicative of an activated system, their precise role in potentiating the arthritic lesion is still under scrutiny.

Traditionally ascribed a proinflammatory role, low molecular weight arachidonic metabolites, particularly PG, are implicated in the acute regulation of vascular alterations during inflammatory reactions[8,10]. PG production is mediated by the oxidation of arachidonic acid by 2 isoforms of cyclooxygenase, a constitutive form (COX-1) and an inducible form (COX-2)[11]. COX-2 is regulated by various cytokines including IL-1 and TNF[12] and appears to be expressed in increased amounts during arthritis[13]. An inhibitory effect of PG on immune cell function has been documented[9]. PGE_1 and PGE_2 can inhibit the release of IL-1 and TNF from myeloid cells. In their study on mesenchymal cell regulation, Hamilton, *et al* showed that fibroblast derived colony stimulating factors are modulated by endogenous and exogenous prostanoids at the protein level[14]. Therefore, a link between PG and stromal cell cytokine release has been established.

Increased expression of the low molecular weight

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chemokine IL-8 is seen within the rheumatoid synovium and is believed important in the recruitment of neutrophils and other inflammatory cells to this area[15,16]. On the other hand, IL-6, present in increased amounts within the joint of patients with various arthritides[17], may act as a homeostatic cytokine via its induction of the acute phase response by the liver[18] and by its inhibitory effects on monocyte production of TNF and IL-1[19]. While its exact role during chronic inflammation is unclear, IL-6 may also act as a protective factor locally through induction of tissue inhibitor of matrix metalloproteinase (TIMP-1) expression[20].

We examined the effects of PG on basal and IL-1 induced expression of IL-8, IL-6, and GMCSF in human synovial fibroblasts *in vitro*. We found PG upregulates production of both protein and mRNA for IL-8 and IL-6, while simultaneously inhibiting GMCSF expression. We also provide evidence that endogenous production of arachidonic acid metabolites are important regulators of these cytokines *in vitro*, as the introduction of a cyclooxygenase inhibitor alters the expression of the various cytokines measured.

MATERIALS AND METHODS

Synovial cell cultures. Human synovial cultures were established from 7 nonrheumatoid donors undergoing total joint replacement surgeries at Chedoke-McMaster Hospitals, as described[5]. There were 4 male and 3 female patients ranging in age from 53 to 72 years. In brief, the synovial intimal layer was dissected, finely minced, and enzymatically dispersed using 0.5 mg/ml hyaluronidase and 1 mg/ml collagenase (Sigma Chemical Co., St. Louis, MO, USA) for 3–4 h in RPMI medium at 37°C. The resulting material was filtered through a sterile wire mesh and the cell suspension was plated in 75 mm flasks (Corning Glass Co., Corning, NY, USA) for 24 h in RPMI containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 50 µg/ml gentamycin, and 50 µg/ml amphotericin B (Fungizone). Cultures were passaged at 80% confluence and maintained in a humidified tissue culture incubator at 37°C. Cells were routinely used before Passage 7. We could not determine a difference in the responses of human synovial fibroblasts from earlier passages to any stimulators. As well, ideal growth kinetics (viability, survival, doubling time, etc.) was best seen in cells used at this passage. Later time points (after Passage 10) often gave spurious results. For ELISA studies, adherent human synovial fibroblasts were plated in 24 well plates at a concentration of 1×10^4 cells/well in RPMI medium containing 10% FBS (Gibco) overnight, washed, and replenished with RPMI containing 2% FBS with the appropriate stimulus for 18 h. For RNA studies, the fibroblasts were seeded into 150 mm flasks (Corning Co.) in RPMI (10% FBS), allowed to reach 80% confluence, washed, and the media was replaced with RPMI (2% FBS) overnight. Flasks were stimulated on the next day with the indicated stimuli for 18 h.

ELISA, GMCSF, IL-8, and IL-6 bioassay. Supernatants collected from stimulated and control synoviocyte cultures were assayed for GMCSF and IL-8 using commercial ELISA kits (Biotrak, Amersham, Amersham, UK) with a limit of sensitivity of 4 and 50 pg/ml, respectively. Samples were run in duplicate in the ELISA and experiments shown are representative of the cell lines tested. IL-6 was assessed using the B9 hybridoma proliferation assay as described[21]. Data was assessed for significant differences by one way analysis of variance. Multiple comparisons of mean values at each treatment point were subjected to Newman-Keuls' *post hoc* multiple comparison tests. P values are reported in the figure legends.

RNA isolation. After appropriate stimulation regimes, cells were trypsinized and pelleted at 200 g and RNA was isolated according to the method of Chomczynski and Sacchi[22]. After quantitation by optical density at 260 nm, samples were run in formaldehyde denaturing agarose gels, blotted onto nylon membrane (Biotrans), and fixed by standard techniques. Blots were then probed with cDNA fragments or oligonucleotide sequences of human IL-6, GMCSF, and IL-8. cDNA was labeled using random primers, Klenow, and ^{32}P-CTP (Amersham). The IL-8 oligonucleotide probe has the sequence 5'-GTT-GGC-GCA-GTG-TGG-TCC-ACT-CTC-AAT-CAC-3' and is specific for human IL-8 mRNA[23]. Blots were washed at 55°C with 1 × SSC in 1% SDS for 30 min and exposed to Kodak X-AR x-ray film.

Reagents. Human recombinant IL-1α was a gift from Dr. M. Widmer (Immunex, Seattle, WA, USA) and PGD_2, $PGF_{2\alpha}$ illoprost (PGI_2 analog), PGE_2 and indomethacin were purchased from Cayman Chemical. cDNA for human GMCSF was a kind gift from the Genetics Institute (Boston, MA, USA). cDNA for TIMP-1 was a kind gift from A.J.P. Dougherty (Celltech, UK).

RESULTS

PGE_2 alone enhances production of IL-6 but not GMCSF or IL-8. Table 1 shows production of GMCSF, IL-6, and IL-8 protein obtained from a representative cell line stimulated with 10^{-7} M PGE_2 or IL-1α for 18 h. There was no detectable effect of PGE_2 alone (tested at 10^{-6}–10^{-10} M) on IL-8 or GMCSF levels in 6 cell lines tested; however, IL-6 levels were slightly increased after 18 h stimulation. IL-1 stimulation resulted in high levels of GMCSF, IL-8, and IL-6 in culture supernatants. Thus, we could not detect any significant action of PGE_2 alone on the production of GMCSF or IL-8 by these cells, even though they responded by increasing IL-6 output (albeit at low levels compared to response to IL-1). These results were consistently observed in all other cell lines tested. To ensure this was not due to nonspecific activation, we examined IL-6 mRNA. Since the effect of cyclic AMP inducing agents, including PGE_2, on early (1–2 h) enhancement of the IL-6 mRNA has been documented in skin fibroblasts[6], we examined mRNA levels in synovial fibroblasts at various time points after PGE_2 stimulation. Specific RNA signals for IL-6 were evident at 1 h after stimulation and decreased thereafter. mRNA signals for GMCSF and IL-8 were very low in untreated cells and PGE_2 stimulation had no measurable effect (data not shown). Human synovial fibroblasts have been shown to constitutively produce TIMP-1 mRNA. In our hands, PGE_2 had no effect on TIMP-1 mRNA expression over the time

Table 1. GMCSF, IL-6, and IL-8 protein levels in human synovial fibroblasts stimulated with PGE_2 or IL-1.

Time	GMCSF (pg/ml)	IL-6 (ng/ml)	IL-8 (ng/ml)
Control 18 h	ND*	4.6 (0.97)	ND
PGE_2 18 h	ND	12.2 (3.74)	ND
IL-1 18 h	4064 (338)	44 (9)	39.6 (6)

Data shown is from 1 fibroblast cell line (human synovial fibroblast 11) in duplicate. Results are representative of other lines tested in such a time course. PGE_2 concentration was 10^{-7} M.

* Not detectable within detection limits of ELISA (for IL-8 < 150 pg/ml and GMCSF < 40 pg/ml) or B9 hybridoma bioassay (for IL-6 < 10 pg/ml) (see Materials and Methods).

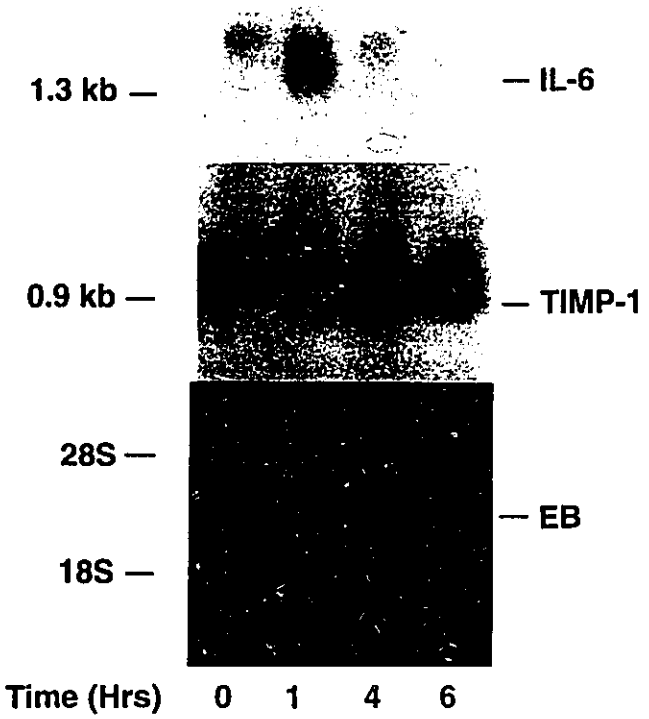

Figure 1. Effect of PGE_2 on human synovial fibroblast production of IL-6 at various time points. Fibroblasts were incubated in 75 mm^2 flasks (Corning Co.)-with 10^{-7} M PGE_2 for 0, 1, 4, and 6 h in media containing 2% FBS. RNA was collected (see Materials and Methods). Hybridization to the 1.3 kb species of human IL-6 is shown (top panel). TIMP-1 hybridization was used as a control (center panel). Ethidium bromide stained gel demonstrates equal loading of RNA. Densitometry analysis was used to equalize loading differences (bottom panel).

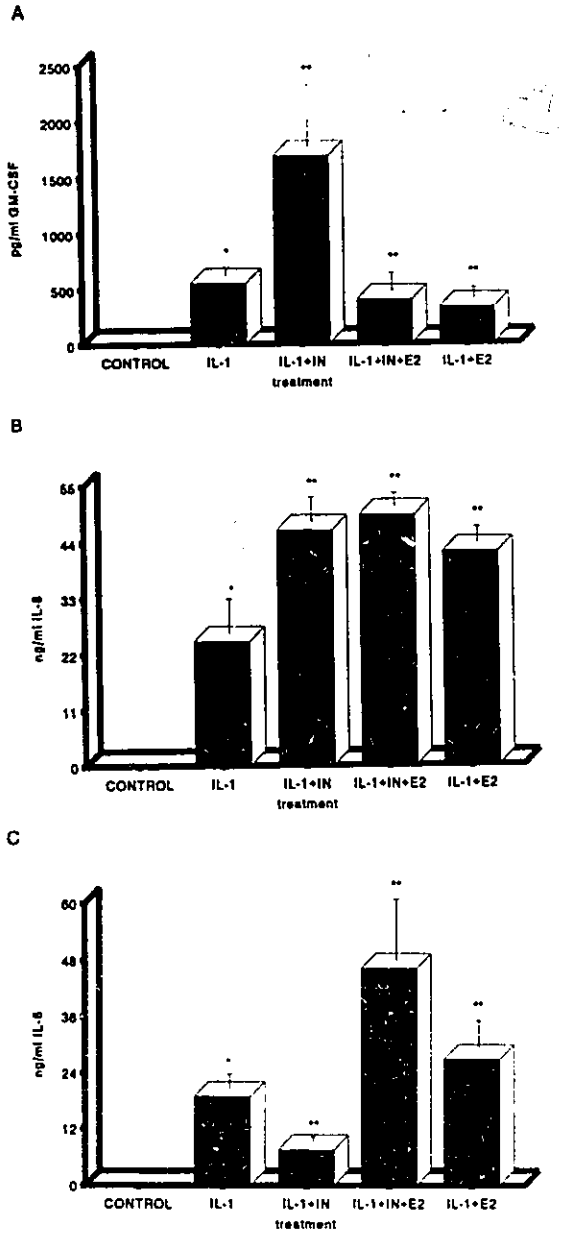

Figure 2. GMCSF, IL-8, and IL-6 production from human synovial fibroblasts *in vitro:* Effect of PGE_2, IL-1, and indomethacin. (For details see Materials and Methods). Data are mean values of 4 experiments in duplicate on human synovial fibroblast 12 (n = 8). *A significant difference from control fibroblasts ($p < 0.001$ for each cytokine stimulated with IL-1). **A significant difference ($p < 0.005$) from IL-1-treated fibroblasts (IN: indomethacin).

assessed (Figure 1). Phosphorimagery of specific signals showed that PGE_2 stimulation did not alter TIMP-1 mRNA levels at various time points after stimulation; however, IL-6 mRNA signals were clearly detectable at 1 h but not thereafter (3-fold increase compared to background). We detected no signal for GMCSF or IL-8 on reprobing the same blots.

IL-1 induced GMCSF, IL-6, and IL-8 levels. Since PGE_2 did not significantly affect the basal expression of IL-8 and GMCSF, we addressed whether endogenous and exogenous PGE_2 could be involved in the IL-1 regulation of these mediators. Production of GMCSF, IL-6, and IL-8 by human synovial fibroblasts stimulated *in vitro* with combinations of IL-1 (5 ng/ml), PGE_2 (10^{-7} M), and indomethacin (10^{-6} M) are shown in Figure 2 and Table 2. The data in Figure 2 are derived from 4 separate experiments using one cell line (human synovial fibroblasts 12) done in duplicate and are representative of the 6 cell lines tested. Here we show that 18 h stimulation with IL-1 significantly enhances GMCSF release (Figure 2A) from fibroblast cultures derived from OA tissue (541 pg/ml). Indomethacin (10^{-10}–10^{-6} M) had no detectable effect on GMCSF production in the absence of IL-1 stimulation (data not shown). The addition of indomethacin to IL-1 stimulated cells caused a further 3-fold increase in GMCSF production (1640 pg/ml), suggest-

Table 2. Change from control in GMCSF, IL-8, and IL-6 protein production from various human synovial fibroblast cell lines.

Treatment	HSF 4	HSF 6	HSF 11
	GMCSF		
Control	100	100	100
IL-1	350	210	340
IL-1+Indo	1375	290	1400
IL-1 + Indo + PGE_2	270	75	510
IL-1 + PGE_2	200	110	290
	IL-8		
Control	100	100	100
IL-1	950	600	1300
IL-1 + Indo	1350	700	1800
IL-1 + Indo + PGE_2	1150	1100	3250
IL-1 + PGE_2	1100	1595	2600
	IL-6		
Control	100	100	100
IL-1	2800	1100	1900
IL-1 + Indo	1200	500	750
IL-1 + Indo + PGE_2	4100	3200	5250
IL-1 + PGE_2	3750	1540	3330

Values represent change from control for 3 additional cell lines. Each point represents the mean of a single experiment measured in duplicate. Control values were set at 100% (see Materials and Methods). HSF: human synovial fibroblasts.

ing that an endogenous prostanoid may be involved in the inhibition of GMCSF protein production. The addition of PGE_2 to IL-1 and indomethacin treated cultures was able to totally abolish the indomethacin dependent increase in GMCSF. As well, exogenous PGE_2 also inhibited GMCSF production from IL-1 stimulated fibroblasts (down to 249 pg/ml) in the absence of indomethacin.

IL-1 stimulated fibroblasts produced significantly more IL-8 (24.9 ng/ml) than control samples (below limit of detection), as shown in Figure 2B. The addition of indomethacin caused an increase in the IL-8 production from fibroblasts (24.9–47.5 ng/ml), similar to effects on GMCSF. In contrast, the addition of PGE_2 to IL-1 and indomethacin treated cultures did not cause inhibition, but rather caused a marginal increase in IL-8 levels in this cell line. In addition, cultures stimulated with IL-1 and PGE_2, showed a significant increase in IL-8 production (41 ng/ml) above IL-1 alone (24.9 ng/ml).

When tested alone, PGE_2 stimulation caused a small increase in IL-6 release from fibroblasts derived from osteoarthritic patients (Table 1). Figure 2C shows that, as expected, IL-1 stimulation resulted in a marked enhancement of IL-6 production. The addition of indomethacin inhibited IL-6 production, and further addition of PGE_2 resulted in an increase in IL-6 levels above IL-1 stimulated cultures.

Table 2 summarizes GMCSF, IL-8, and IL-6 protein data obtained from 3 additional cell lines tested. Data are expressed in terms of change compared to controls due to the variation existing between the various cell lines tested. It is evident that endogenous prostanoid products are important in regulating the production of GMCSF, IL-8, and IL-6. As well, it appears that PGE_2, the major arachidonic acid metabolite released from fibroblasts, can significantly inhibit GMCSF (in all lines examined), while it enhances IL-6 and IL-8. One cell line (human synovial fibroblast 4) did demonstrate an unusual response to indomethacin and PGE_2, with a slight decrease in IL-8 production. This could be attributed to tissue culture variation, since this was the only line of 6 examined that responded in this manner.

IL-1 induced GMCSF, IL-6, and IL-8 mRNA levels. To test whether the effects of PGE_2 were also detectable at the mRNA level, Northern analysis of the fibroblast cultures stimulated for 18 h with various combinations of IL-1, PGE_2, and indomethacin was performed. Figure 3 shows that the 0.9 kb GMCSF message was induced by IL-1 stimulation. Costimulation with IL-1 and indomethacin resulted in a further increase in mRNA levels. Similar to the protein expression of GMCSF (Figure 2), the addition of PGE_2 to IL-1 and indomethacin treated fibroblasts resulted in a significant decrease in the GMCSF mRNA signal. Figure 3 shows that the mRNA for IL-8 was enhanced by IL-1 treatment (8-fold over basal by phosphorimagery, corrected for densitometric differences in ethidium bromide stained gels). Addition of IL-1 and indomethacin resulted in a 14-fold increase of the IL-8 message. The further addition of PGE_2 resulted in a 16-fold change from basal levels in mRNA for IL-8. IL-6 mRNA was enhanced by IL-1 stimulation (Figure 3). The addition of indomethacin to IL-1 stimulated cultures resulted in slight reduction of the IL-6 message, reflected in the B9 assay of protein activity (Figure 2). Further addition of PGE_2 to IL-1/indomethacin treated cultures resulted in a significant increase in IL-6 mRNA signal.

Effect of other prostanoids on GMCSF, IL-6, and IL-8 production. Since indomethacin inhibits the production of other COX dependent products, we chose to examine the effects of several different prostanoids in the same system. To test whether other cyclooxygenase products are instrumental in regulating IL-8 expression in IL-1 treated cultures, we stimulated cells with several different prostanoids that might have regulatory function. Table 3 shows the effects of other prostanoids on fibroblast production of GMCSF, IL-6, and IL-8. Synoviocytes stimulated with PGD_2, $PGF_{2\alpha}$, or illoprost alone showed no detectable effect on basal cytokine production (data not shown). In cultures stimulated with IL-1 and indomethacin, only those further stimulated with PGE_2 demonstrated significant changes in IL-6 and GMCSF production. To determine which prostanoid (if any) could be an inhibitor of IL-8 in IL-1/indomethacin treated cultures, we tested these same prostanoids on human synovial fibroblast 12. Table 3 shows that while PGE_2 slightly enhanced

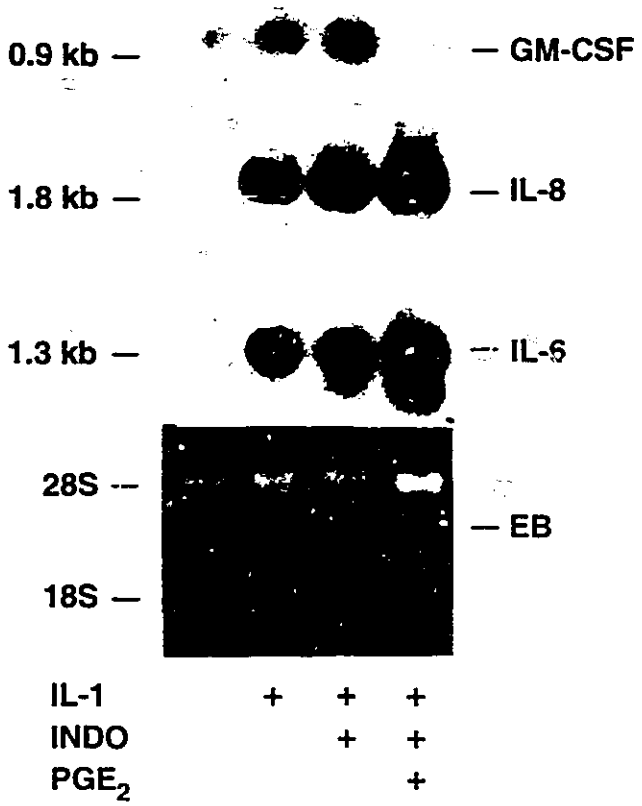

Figure 3. GMCSF, IL-8, and IL-6 mRNA regulation in human synovial fibroblasts: Effect of PGE_2, IL-1, and indomethacin. Northern blot analysis of stimulated fibroblasts. The human GMCSF cDNA hybridizes to a 1.0 kb, IL-8 oligonucleotide to a 2.1 kb, and IL-6 cDNA fragment to a 1.6 kb species of RNA. Lane 1, control; Lane 2, IL-1 (5 ng/ml); Lane 3, IL-1 + indomethacin (1 μM); Lane 4, IL-1 + indomethacin + PGE_2 (0.1 μM). Bottom panel: Ethidium bromide stained gel to verify equal loading of RNA. As well, densitometry was used to equalize loading discrepancies.

Table 3. Effect of other prostanoids on synoviocyte production of GMCSF, IL-6 and IL-8.

	GMCSF (pg/ml)	IL-6 (ng/ml)	IL-8 (ng/ml)
Control	ND	ND	ND
IL-1	4495 (649)	39 (6.8)	33 (6)
IL-1+ Indo	7956 (683)	25 (2.1)	39 (6.7)
IL-1+Indo+PGE_2	4253* (570)	42* (4.4)	52* (5.9)
IL-1+Indo+$PGF_{2\alpha}$	7411 (665)	28 (9.9)	41 (3.5)
IL-1+Indo+PGD_2	6604 (740)	30 (1.5)	36 (9.9)
IL-1+Indo+Illoprost	7120 (852)	30 (5)	53* (1.4)

Data represent mean values obtained from one experiment in triplicate using a single cell line (human synovial fibroblast 12). Numbers in parentheses are standard deviations. ND: nondetectable. Protein levels were determined by ELISA for GMCSF and IL-8 and the B9 hybridoma growth assay for IL-6 (See Materials and Methods).
Statistical comparison of IL-1+Indo groups and IL-1+Indo+prostanoids was assessed using Newman-Keuls' test.
* $p < 0.001$.

IL-8 release from IL-1/indomethacin stimulated cultures, illoprost (the stable prostacyclin analog) had an effect similar to PGE_2 on IL-8 protein production. The effect of both PGE_2 and illoprost on IL-1/indomethacin stimulated IL-8 release was deemed significant in this and other cell lines tested (Table 3).

DISCUSSION

Our report demonstrates that specific prostanoids can upregulate IL-8 production in synovial fibroblasts in culture. IL-8 is a member of the chemokine family of low molecular weight cytokines instrumental in proinflammatory signaling during both acute and chronic inflammation[5,16]. Its presence during RA is increased both in joint fluid and cells of the pannus[17]. IL-8 expression is enhanced by IL-1, TNF, and lipopolysaccharides (LPS) in a variety of cellular systems including synovial fibroblasts. We detected no measurable levels of IL-8 in OA derived cultures and no effect of PGE_2 on IL-8 expression alone. The effect of endogenous prostanoids on IL-1 induced IL-8 protein expression is evident (Figure 2 and Table 2). Unlike the effect seen with GMCSF, addition of PGE_2 to IL-1 stimulated — and less so IL-1/indomethacin treated — cultures caused an increase in the expression and release of IL-8 from synovial fibroblasts. This appears to be relatively unique to synovial fibroblast populations, since PGE_2 causes inhibition of IL-8 protein and mRNA from IL-1 or LPS stimulated monocytes[24]. To determine whether another endogenous prostanoid was responsible for inhibiting IL-8 production, we examined other PG (Table 3). While none demonstrated inhibiting qualities, illoprost, the stable analog of prostacyclin (PGI_2), acted similarly to PGE_2 in enhancing IL-8 production. The effect of prostacyclin on cytokine production/release, particularly from stromal cells, during inflammatory pathologies, to our knowledge has not been investigated. However, given that prostacyclin and its stable analog illoprost act similarly to PGE_2 in terms of vascular permeability, leukocyte infiltration, and pain modulation, it is possible that these mediators work via similar receptor mechanisms[10,25].

The effect of IL-1 on synovial fibroblast GMCSF production is well documented[4,5,14]. IL-1 caused significant enhancement of GMCSF, which was further enhanced by addition of indomethacin, suggesting that an important cyclooxygenase dependent inhibitor of GMCSF is induced by IL-1 [indomethacin had no effect alone (data not shown)]. Co-culturing IL-1 and indomethacin with PGE_2 resulted in marked reduction in GMCSF protein and mRNA expression in all cell lines tested. This is consistent with others[14] at the protein level in human fibroblasts and suggested that GMCSF was regulated by both endogenous and exogenous PGE_2. Other prostanoids had no effect on GMCSF production (Table 3). Hamilton, *et al* provided protein evidence from a single cell line grown from explanted tissue[14]. Our studies on GMCSF expression, both at the protein and

mRNA level (Figures 2 and 3; Table 2), confirm and extend previous data. PGE_2 had no detectable effect on basal expression of GMCSF produced from OA synovial fibroblasts. This is not surprising since we and others have shown that the level of GMCSF protein produced by unstimulated OA synovial fibroblasts is undetectable by ELISA[5,14]. We have observed that human synovial fibroblasts derived from patients with RA produce measurable levels of GMCSF protein[3,5] which, in our hands, are not affected by PGE_2 alone.

The role of IL-6 during chronic inflammation is controversial. The levels of both protein and mRNA are significantly enhanced in various inflammatory diseases including RA, yet a role for this cytokine in perpetuating the lesion has been evasive[7,17,26,27]. In RA it can be argued that the B cell stimulating ability of IL-6 is responsible for the increased presence of rheumatoid factor, so prevalent during the disease. However, within synovial fluid, few activated B cells are seen[2,28]. On the other hand, IL-6 and other members of the IL-6 family of cytokines (oncostatin M, IL-11, leukemia inhibitory factor) induce the expression of TIMP-1, the endogenous matrix metalloproteinase inhibitor, as well as homeostatic protease inhibitors from the liver (acute phase proteins), which suggests a protective role for this cytokine in tissue destruction during arthritis[20,21,29]. We show that PGE_2 is able to induce the expression and release of IL-6 and enhance IL-1 stimulated IL-6 levels. It is also evident that endogenous prostanoids regulate IL-1's ability to enhance IL-6, since indomethacin was able to significantly inhibit IL-1 upregulation of IL-6. This was unique to PGE_2, since $PGF_{2\alpha}$, PGD_2, and illoprost had no such effect.

While nonsteroidal antiinflammatory drugs (NSAID) appear to be a necessary therapy to manage pain and relapsed inflammation during RA[30,31], the longterm effect of this treatment must be carefully scrutinized. While indomethacin has been shown to affect the cytokine profile derived from mononuclear cells[24], fibroblast responses to indomethacin alone, in terms of cytokine production, appear to be minimal. We have found that indomethacin does not alter IL-6, IL-8, or GMCSF protein levels in synovial fibroblasts directly (data not shown). However, in an activated system (i.e., IL-1 stimulated human synovial fibroblasts), indomethacin has significant effects on GMCSF, IL-6, and IL-8[14]. In contrast to our findings, Loetscher, *et al* showed that indomethacin had no effect on IL-8 release from IL-1 stimulated human synovial fibroblasts[32]. They used the fibroblasts derived from patients presenting with RA, which in our hands respond differently to many stimuli when compared to fibroblasts derived from patients with osteoarthritis[5]. Second, the deleterious acute activity of PG, both endogenously and exogenously, must be carefully compared to the apparent longterm benefits of these molecules through altering the inflammatory process *in vivo* and *in vitro*. Zurier, *et al* have demonstrated that a variety of PG and arachidonic acid metabolites inhibit many of the clinical signs and symptoms of inflammation associated with various forms of arthritis[9,33,34].

Taken together, these results demonstrate that PGE_2 is an important endogenous modulator of IL-1 induction of IL-8, GMCSF, and IL-6 from human synovial fibroblasts. They confirm and extend studies on the effect of PGE_2 on GMCSF protein and mRNA regulation[14]. Furthermore, our data suggest that PGE_2 is important both endogenously and exogenously in the pathology of arthritis, as it is able to induce IL-8 and IL-6 mRNA and protein and simultaneously negate GMCSF mRNA and protein from the same population of mesenchymal cells.

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INTERACTION BETWEEN ONCOSTATIN M, INTERLEUKIN 1 AND PROSTAGLANDIN E_2 IN INDUCTION OF IL-6 EXPRESSION IN HUMAN FIBROBLASTS

C.D. Richards and A. Agro

The role of Oncostatin M (OM), a monocyte/macrophage and T-cell product, in regulating IL-6 expression in fibroblasts of lung or synovial origin was examined in vitro. Although by itself OM had a minimal effect on enhancing IL-6 production by fibroblasts, in combination with IL-1α or PGE_2, OM addition resulted in a dose-dependent synergistic enhancement of IL-6 production. This synergistic effect with either IL-1α (5 ng/ml) or PGE_2 (10^{-7} M) was clearly evident at concentrations of OM of 10, 20 or 50 ng/ml. Levels of IL-6 resulting from OM and IL-1α stimulation could be reduced by indomethacin (10^{-6} M) and restored again by also adding PGE_2. Northern blots probed for IL-6 mRNA showed cooperative enhancement of steady state levels at 18 hours of stimulation by OM and IL-1α, or OM and PGE_2. Probing for mRNA of the metalloproteinase inhibitor TIMP-1 showed that stimulation by OM, IL-1α or PGE_2 enhanced TIMP-1 levels. However, OM (alone) or PGE_2 or both combined did not elevate the metalloproteinase stromelysin-1 mRNA signals. Analysis utilizing a rat IL-6 promoter-luciferase reporter gene construct showed that OM stimulation resulted in activation of transcription that synergistically enhanced IL-1-induced levels of reporter gene expression. These results show that although OM has minor effects on IL-6 production alone, the combination of OM and other mediators result in markedly enhanced IL-6 production by fibroblasts in vitro.

The process of inflammation involves interaction between numerous cell types at the local tissue site that is mediated primarily by cell-derived factors. Various bone-marrow derived cells that are present (histiocytes, macrophages, mast cells) or infiltrate the tissues (monocytes, lymphocytes and granulocytes) produce many of these mediators that include cytokines, enzymes and smaller molecular species. In addition, local cells of connective tissue such as fibroblasts express numerous products upon activation, and thus provide an important additional source of mediators at sites of inflammation. The pro-inflammatory cytokines IL-1 and TNF can initiate the activation of many fibroblast functions including a marked elevation of production of cytokines and prostaglandins,[1-6] modulation of proliferation,[7-9] and production of matrix metalloproteinases.[5,6,10,11] Acute responses to tissue damage very clearly show enhanced IL-6 protein levels in plasma or serum[12,13] and chronically inflamed tissues such as the rheumatoid arthritic joint also produce high amounts of IL-6.[14] These levels result in part from production by monocyte/macrophage and connective tissue/stromal cells activated at the site of tissue inflammation.

Oncostatin M (OM) is a product of activated T cells and monocytes[15,16] and thus can be produced by cells which may infiltrate sites of inflammation. This cytokine has a number of bioactivities in vitro including modulation of growth of a number of tumour cell lines,[17,18] differentiation of the M1 cell line,[19] up-regulation of LDL receptors on human hepatoma HepG2 cells,[20] and stimulation of acute phase protein synthesis by hepatocytes;[21] we have recently shown that OM regulates the expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) in fibroblasts and HepG2 cells.[22,23] Brown *et al.*[24] have also shown that OM induces IL-6 expression in endothelial cells, and

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we became interested in whether OM contributes to control of local connective tissue/stromal cell production of IL-6. In this study we have examined the role of oncostatin M in modulating IL-6 expression in human lung and synovial fibroblasts. We show here that unlike its action on endothelial cells, OM alone did not significantly increase IL-6 expression. However, OM acted synergistically with either IL-1α or PGE_2 to enhance IL-6 expression in vitro in these cells. Furthermore, OM acted in additive fashion with IL-1α or PGE_2 to enhance TIMP-1 but not stromelysin mRNA levels. We also demonstrate that OM stimulation of fibroblasts resulted in increased transcription of genes downstream from the rat IL-6 promoter. These results suggest that OM may be an important regulator of local production of mediators such as IL-6 at tissue sites of inflammation.

RESULTS

The production of IL-6 in human lung fibroblast cell lines was examined in response to human recombinant IL-1α and Oncostatin M. Table 1 shows that at 5 ng/ml, IL-1α induced 22-fold increases in IL-6 bioactivity (as measured in the B9 proliferation assay) in supernatants of cells stimulated for 18 h whereas OM alone at 20 ng/ml induced approximately 2-fold enhancement of basal levels over the same time of treatment. OM did not produce any greater enhancement of IL-6 at higher concentrations. Costimulation with both IL-1α and OM, at these same concentrations, acted synergistically to produce 75-fold increase in IL-6 content of supernatants over control cell cultures. These results were averaged from four separate experiments using two different normal human lung cell lines. The variation observed reflects differences in IL-1-induced levels of IL-6 production between different cell lines. However the relative fold increases were consistent. To examine this in more detail, OM was added at increasing concentrations alone or in combination with IL-1α to individual cell lines of human lung fibroblasts (Fig. 1A) or human synovial fibroblasts (Fig. 1B). IL-1α alone caused elevation of IL-6 from undetectable to 180 ± 20 ng/ml in lung fibroblast supernatants and from undetectable to 54 ± 6 (1 ng/ml IL-1α stimulation) or 72 ± 7 ng/ml 5 ng/ml IL-1α stimulation) in synovial fibroblast supernatants. A dose-dependent enhancing effect of OM was evident in both cell lines when added with IL-1α. A synergistic effect was clearly evident at 10, 20 or 50 ng/ml concentrations of OM when added with 5 ng/ml IL-1α.

TABLE 1. IL-1α and OM regulation of IL-6 production from lung fibroblasts

	IL-6 activity ng/10^6 cells	Fold induction
Control	5.8 ± 2.5	1
IL-1α	126 ± 41	22
OM	9.8 ± 4.5	2
OM + IL-1α	434 ± 135	75

Lung fibroblast cell lines were cultured in Corning petri dishes (100 mm). Confluent cells (10^6 cells/dish) were then stimulated in medium containing 2% FCS for 18 h with IL-1α (5 ng/ml), OM (20 ng/ml) or the combination. Supernatants were then taken and assayed for IL-6 bioactivity and compared to standard curve of known amounts of recombinant IL-6. The results are expressed as the means ± standard deviation of four separate sets of cultures representing two separate cell lines derived from normal lung tissue.

IL-1 in both α and β forms has potent effects on many secreted products of fibroblasts in culture.[6,25–28] These include products of eicosanoid metabolism and PGE_2 is the major product produced in these cell cultures as well as the major eicosanoid found in body fluids due to inflammation in vivo. Furthermore, cAMP-inducing agents including PGE_2 have been shown to regulate IL-6 transcription.[29] We thus examined the role of PGE_2 in combination with OM, in enhancing production of IL-6 by lung and synovial fibroblasts of human origin. While PGE_2 had minimal effects over a wide concentration range, co-addition of 20 ng/ml OM enhanced IL-6 production. Figure 2A shows that 10^{-7} or 10^{-6} M PGE_2 in combination with 20 ng/ml OM resulted in 4- and 6-fold (respectively) elevation of IL-6 detected in supernatants. Figure 2B shows that while OM alone (50 ng/ml) was able to induce a maximal 2-fold increase in IL-6 production, the addition of PGE_2 at 10^{-7} molar enhanced IL-6 release by 4–5-fold at OM concentrations of 10, 20 and 50 ng/ml. Thus, PGE_2 and OM costimulation act synergistically in causing these fibroblasts to produce IL-6. It is noteable however that the maximal IL-6 output produced by the PGE_2/OM combination was significantly lower than that produced by IL-1/OM costimulation (compare maximal levels in Fig. 1 versus Fig. 2).

To assess the contribution of IL-1-induced endogenous PGE_2 to the synergistic effects of IL-1/OM combination, fibroblasts were treated with indomethacin (10^{-6} Molar) to block PGE_2 synthesis and then stimulated with IL-1α and OM (Fig. 3). Indomethacin completely blocked PGE_2 release in response to IL-1α (data not shown). When indomethacin was added the synergistic action of OM and IL-1 was partially inhibited, and exogenously added PGE_2 (10^{-6} M) restored high levels of IL-6 production by these cells. Similar effects were observed in three separate experiments. This suggests that an indomethacin-sensitive

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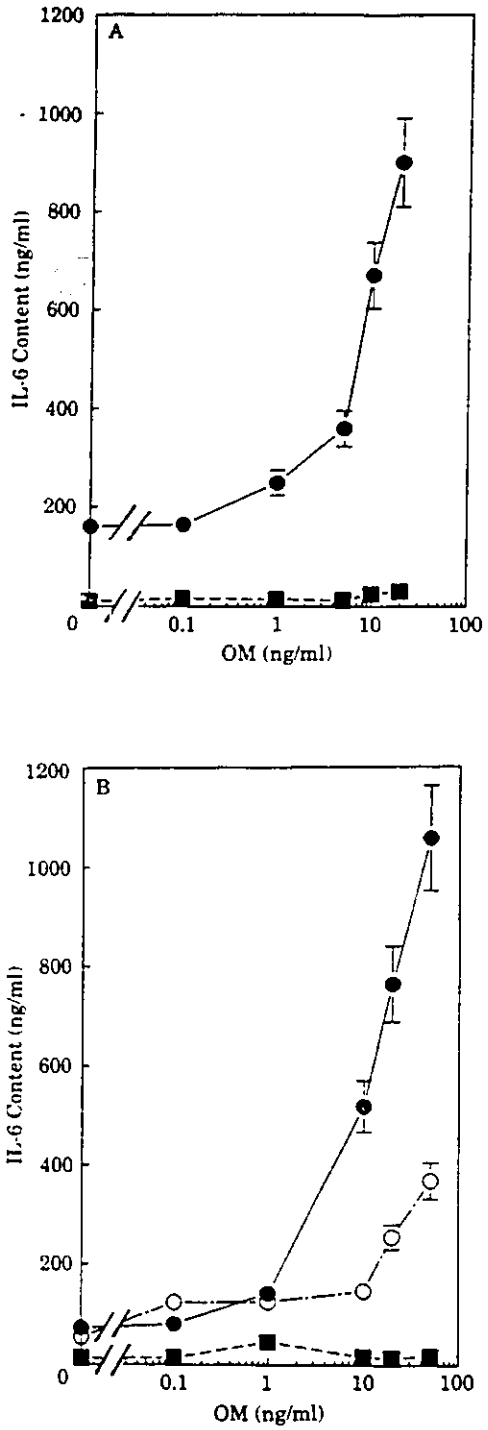

Figure 1. OM synergizes with IL-1α to induce IL-6 release.

Fibroblasts were seeded in 24-well costar plates (10^5 cells/well), allowed to recover for 1–2 days and then were stimulated for 18 h in 2% FCS-containing medium with the indicated concentrations of OM (X-axis) and IL-1α at 0 (■), 1 (○) or 5 (●) ng/ml. Shown are representative experiments on human lung fibroblasts (A) and human synovial fibroblasts (B). IL-6 content of the supernatants were assayed in the B9 proliferation assay and amount of IL-6 in ng/ml was calculated from a standard curve of known human recombinant IL-6. Error bars represent the standard error of the mean of triplicate analysis.

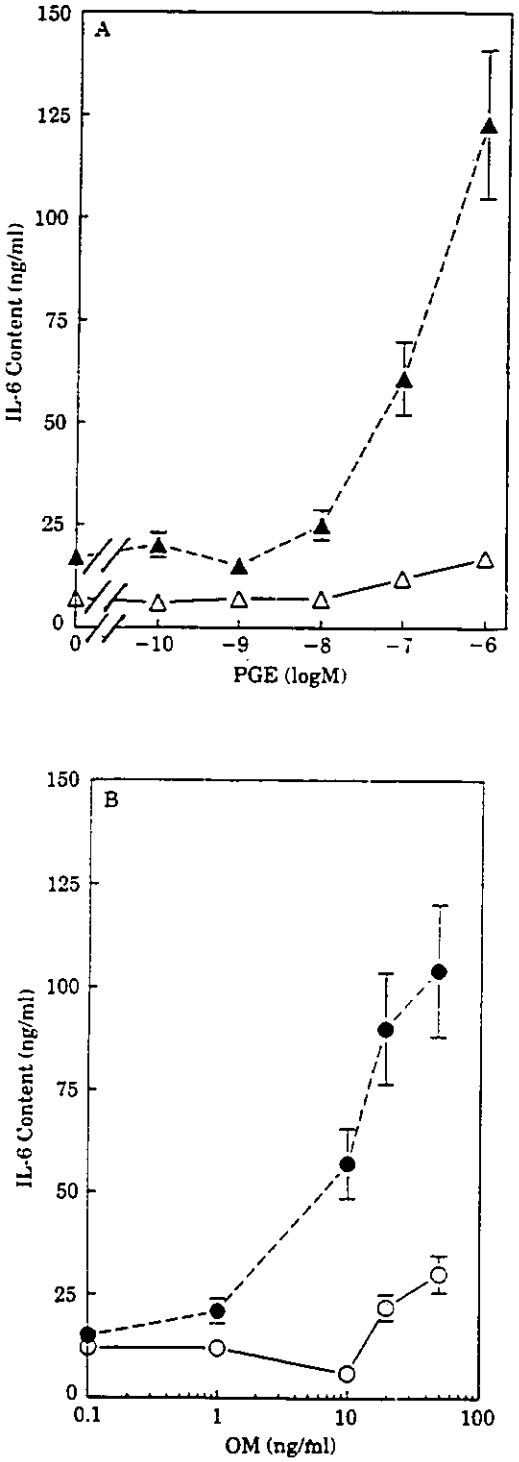

Figure 2. OM and PGE_2 synergize to induce IL-6 release.

Confluent fibroblasts were cultured and treated as in Fig. 1 but with different stimuli. (A) PGE_2 was added at time zero to final concentrations as indicated in the absence (△) or presence (▲) of 20 ng/ml OM added at time 0. (B) Oncostatin M was added at the indicated concentrations in the absence (○) or presence (●) of addition of 10^{-7} M PGE_2 at time 0. Supernatants were collected after 18 h and assayed for IL-6 activity as described in Fig. 1 and Methods. Error bars represent the standard error of the mean.

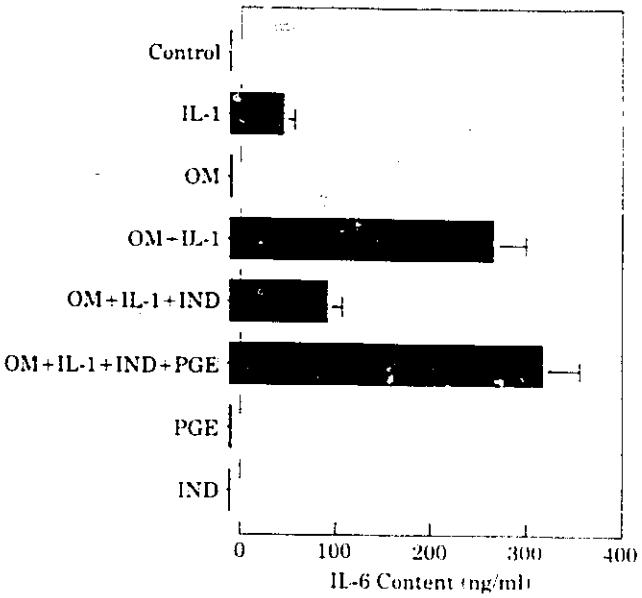

Figure 3. Indomethacin inhibits OM/IL-1 effects on IL-6 production.

Lung fibroblasts were cultured in 2% FCS-containing medium and the indicated reagents at final concentrations of 20 ng/ml OM (OM), 5 ng/ml IL-1α (IL-1), 10^{-6} M indomethacin (IND) and 10^{-6} M PGE_2 (PGE). Eighteen hours after addition of these stimuli, supernatants were collected and measured for IL-6 content. Error bars represent the standard error of the mean. This is one representative experiment of three separate cell cultures that show a similar effect.

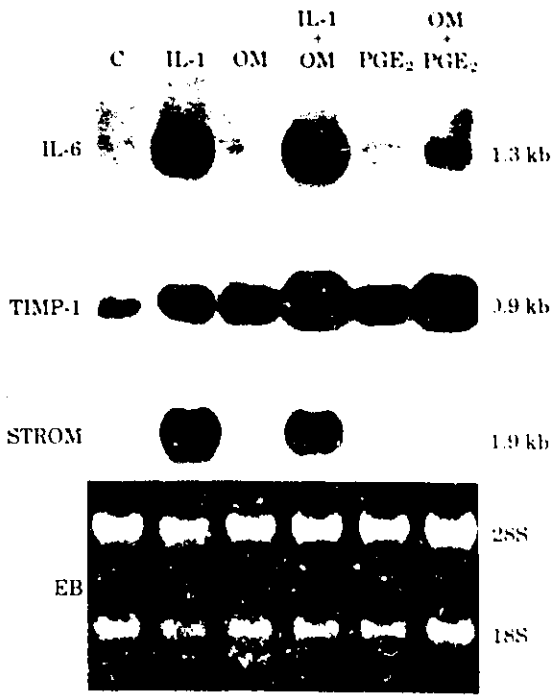

Figure 4. Expression of IL-6 mRNA.

Confluent fibroblasts were treated with the indicated mediators for 18 h with final concentrations of: IL-1α, 2.5 ng/ml; OM, 20 ng/ml; PGE 10^{-6} M. RNA was harvested at this time point and examined for mRNA expression using Northern Analysis. The blotted RNA was stained with ethidium bromide (bottom panel, EB) and shows equivalent loading of total RNA per lane. The blot was probed sequentially with cDNA encoding human IL-6, TIMP-1, and stromelysin-1 (STROM). The approximate size of the signals are shown at right.

product is involved in the IL-1/OM cooperative effects.

To assess whether the IL-6 bioactivity readout was reflected in IL-6 mRNA levels, Northern analysis was performed on normal lung fibroblast cells treated with the various stimulatory agents (Fig. 4). The transferred RNA, stained with ethidium bromide on the Nylon blot, was photographed and shows equivalent loading of total RNA in each lane (lower panel, EB). At 18 h of stimulation, steady state levels of the 1.3 kb signal, corresponding to IL-6 mRNA, were markedly enhanced by 2.5 ng/ml IL-1α. OM (20 ng/ml) alone did not significantly effect the IL-6 signal, however in combination with 2.5 ng/ml IL-1α, resulted in a further elevation in IL-6 signal (33-fold by densitometry) over that of IL-1α alone (26-fold) at the 18 h time period. While PGE_2 (alone) or OM (alone) addition did not enhance the IL-6 signal, OM and PGE stimulation together increased the mRNA levels resulting in a 4.4-fold elevation over untreated cells.

We have recently shown that OM is a potent stimulator of TIMP-1 mRNA and protein production by fibroblasts and HepG2 cells in culture.[22,23] To test whether TIMP-1 gene expression was affected by the coaddition of IL-1α or PGE_2, Northern blots were probed for TIMP-1 mRNA levels (Fig. 4, 2nd panel). OM alone caused a 3-fold elevation in signal detected at 0.9 kb and IL-1α alone induced a 2.4-fold increase. Interestingly, PGE_2 stimulation alone caused an increase (2.6-fold) in TIMP-1 mRNA. Costimulation with OM and either IL-1α or PGE_2, enhanced TIMP-1 mRNA levels 5.7-fold (OM/IL-1) and 5-fold (OM/PGE) over control levels as assessed by densitometry scans of autoradiographs. Reprobing the same blots with cDNA for the matrix metalloproteinase stromelysin-1 showed a marked elevation of the 1.8 kb signal by IL-1α (140-fold), but a reduction (105-fold) by addition of OM to IL-1α. OM or PGE_2 alone, or OM and PGE_2 combined did not elevate stromelysin mRNA signal above control. These results show both up- and down-regulation of certain products by OM, suggesting it has selective effects on gene expression.

IL-1 induction of IL-6 expression in fibroblasts has been shown to result largely from transcriptional regulation.[30] Human, mouse and rat IL-6 genes have been cloned and sequenced revealing numerous potential transcriptional control elements in the 5′

promoter region[31] which appear to be involved in responses to various stimuli. The rat IL-6 promoter is highly homologous to those in human and mouse, and contains serum response elements with homology to the c-fos promoter, cAMP response elements (CRE), AP-1 binding sites, IL-1 response elements and NFkB response elements.[32–35] In order to assess the effect of OM on IL-6 gene transcription, we analysed luciferase expression in human lung fibroblasts stably transfected with a rat IL-6 promoter-luciferase construct.[36] This chimera (rIL-6p5) contained the sequence from −585 to +14 of the rat IL-6 gene attached to the firefly luciferase reporter gene. After splitting the cells to 24-well costar plates, the cells were stimulated as usual in 2% FCS-containing medium. Analysis of luciferase activity in the cell lysates (Table 2) showed that

TABLE 2. OM enhances transcription in pRIL-6-luc transfected lung fibroblasts

Treatment	Luciferase activity (units/10 μg)	
	Experiment 1	Experiment 2
Control	18 (1)	157 (1)
IL-1	49 (2.7)	316 (2.0)
OM 50 ng/ml	59 (3.3)	62 (0.4)
IL-1 + OM (1)	240 (13.3)	689 (4.4)
IL-1 + OM (10)	433 (24)	955 (6.1)
IL-1 + OM (20)	545 (30)	1241 (7.9)
IL-1 + OM (50)	425 (23.6)	798 (5.1)

The stably transfected human fibroblast cell line (RonP5) was split and seeded at 10^5 cells/well in 24-well costar dishes and then allowed to recover for 24 h (experiment 1) or 48 h (experiment 2) in 10% FCS-containing medium. The medium was then replaced with 2% FCS-containing medium with the indicated cytokines (IL-1 at 5 ng/ml, OM at 1, 10, 20 or 50 ng/ml). After another 24 h, cell lysates were prepared, luciferase activity assayed and expressed as units per μg protein in the lysate. Numbers in parenthesis represent the fold change relative to luciferase activity in untreated (control) cell lysates.

OM addition resulted in a dose-dependent enhancement of reporter gene expression. Addition of 1, 10, 20 and 50 ng/ml of OM with 5 ng/ml IL-1α resulted in approximate fold increases of 12, 23, 35 and 27 (respectively) of luciferase expression in experiment 1, and 4.4, 6.1, 8 and 5 (respectively) in experiment 2. These results suggest that OM can cause activation at the transcriptional level to enhance IL-1-induced IL-6 expression in a dose-dependent fashion.

DISCUSSION

Fibroblast-derived cytokines such as IL-6, IL-8, GM-CSF, G-CSF, stem cell factor and TGF-β, among others, may control multiple aspects of inflammatory responses. The regulation of cytokine production by fibroblasts is dependent on the concentration and combination of mediators present in culture or at local sites of inflammation. In examining IL-6 expression in lung and synovial fibroblasts in vitro, we found that simulation with human recombinant OM resulted in minimal increase in IL-6 production. However, we also show that Oncostatin M induces markedly greater levels of IL-6 production in fibroblasts in combination with other mediators such as IL-1α (Fig. 1). Brown *et al.*[24] have shown that OM alone can induce significant IL-6 expression in endothelial cell cultures, but the fibroblast cell line HepM responded poorly by comparison. OM was shown to act synergistically with IL-1 or TNF in IL-6 production by endothelial cells, and we now show a more pronounced synergism in fibroblasts. In addition, our analysis of transcription responses with the rat IL-6 promoter showed that OM stimulation resulted in synergistic enhancement of an indicator gene (luciferase) expression in IL-1α-stimulated cells (Table 2). This suggests that the regulation of IL-6 by OM is due at least in part to transcriptional activation. Tissue-specific differences in receptor expression, signal transduction or IL-6 gene regulation may explain differential effects on endothelial versus fibroblast cells.

OM also acted synergistically with PGE_2 to enhance IL-6 production (Fig. 2) and mRNA levels (Fig. 4) but to a lower maximal amount compared to IL-1α stimulation. OM (alone) or PGE_2 had minor effects on IL-6 protein production and neither product altered levels of IL-6 mRNA at 18 h (Figs 2 and 4). PGE_2 stimulation of cAMP elevation was previously shown to increase IL-6 expression in foreskin fibroblasts;[29] however, we found only minimal effects in our system unless either OM or IL-1α was present. This may reflect the different cell types used or conditions of the assays. Synergy in OM/IL-1 induction of IL-6 may thus involve PGE_2 which is endogenously produced after cells are stimulated by IL-1α. Our results with cells treated in the presence of indomethacin show that one or more endogenous eicosanoid products plays a role in the IL-1/OM synergy, and that exogenous PGE_2 can reverse the inhibition by indomethacin (Fig. 3). Thus, endogenous PGE_2 may play an important part in the IL-1/OM synergistic effects on IL-6 secretion, although we have not yet defined this eicosanoid product as solely responsible for this effect.

The synergism of OM on IL-6 production suggests that mechanisms that result from OM/receptor interaction are distinct from those invoked by IL-1α or PGE_2. This is not surprising since OM has been shown to act at specific receptors[37] composed of a complex containing the gp130 signal-transducing molecule[38–40] and a second presumed OM specific recep-

tor chain. Tyrosine phosphorylation of gp130 may then be involved[41,42] in subsequent signalling, and OM has been shown to activate specific tyrosine kinase activity in endothelial cells.[43] The mechanism by which OM modulates IL-6 is unknown and may be similar to those involved in OM-induced TIMP-1 regulation, and/or involve early responsive genes such as EGR-1 or c-jun.[44] On the other hand, IL-1α and β both act through an 80 kdalton IL-1 receptor on fibroblasts[45,46] and involve phosphorylation events due to serine/threonine kinases.[47,48] Activation and binding of the nuclear factors NFIL-6 (α and β) to IL-1 response elements appear to regulate IL-6 gene transcription,[34,49] although NFkB[50–52] or other nuclear factors may also be involved. Moreover, post-transcriptional modifications such as mRNA stability may be involved in IL-1α regulation of IL-6 synthesis, since elevation of luciferase activity in our system did not directly parallel IL-6 mRNA elevation. PGE_2 is thought to act by increasing levels of intracellular cAMP and may thus act through mechanisms that compliment those of IL-1 or OM. The enhancement of TIMP-1 mRNA by PGE_2 may also be due to cAMP elevation or its sequalae.

The regulation of expression of cytokines, proteinase inhibitors (TIMP-1) and metalloproteinases by various mediators suggests that a complicated orchestration of control mechanisms occurs at sites of inflammation and ECM remodelling. Our results suggest that OM may have important modulatory effects on local production of IL-6, which in turn contributes to plasma levels of IL-6. This may serve to further enhance systemic responses and activities of IL-6 in inflammatory states. In addition, OM showed cooperative effects with IL-1α or PGE_2 in enhancing TIMP-1 expression. The matrix metalloproteinases and their specific inhibitors (TIMPs) play a prominent role in local extracellular matrix metabolism in normal tissue remodelling as well as chronic inflammation and tumour invasion and metastasis.[53–56] Thus OM by itself or with other inflammatory mediators may play a role in altering the balance of local enzymatic activity, and modulate connective tissue breakdown.

MATERIALS AND METHODS

Reagents

cDNA and genomic clones for OM were previously isolated and characterized.[15] Human recombinant OM was expressed in CHO cells[18,57] and purified by reverse-phase HPLC. Human recombinant IL-1α and IL-6 were provided by Dr S. Gillis (Immunex Corp., Seattle). cDNA clones for human TIMP-1 and stromelysin were provided by Dr A.J.P. Docherty (Celltech Ltd, Slough, UK).

Fibroblast culture and stimulation

Cultures of human synovial fibroblasts were derived from biopsy of inflamed synovium from patients undergoing joint replacement surgery. Synovium was removed from extraneous tissue, washed in serum free medium (DMEM), minced finely and digested with 1 mg/ml collagenase (Sigma) and 0.4 mg/ml hyaluronidase (Sigma). Digestions proceeded at 37°C with gentle agitation on a rotator for 3–4 h. The suspension was then filtered through a sterile wire mesh, centrifuged at 200 g and the pellet resuspended in 10% FBS-containing DMEM. Cells were counted and seeded at 2×10^6 cells/20 mls/75 cm^2 tissue culture flasks, allowed to adhere overnight, washed and replenished with fresh medium. Synovial fibroblast cultures were then grown and passaged by standard culture techniques. Human lung fibroblasts were cultured as explants from normal lung tissue[7] that was finely minced and allowed to incubate overnight at 37°C, 5% CO_2, 100% humidity. Debris was then washed off and fibroblast cultures were passaged by standard techniques.

IL-6 bioassay

Confluent flasks were split and seeded in 24-well COSTAR dishes (10^5 cells/well), then allowed to recover for 1–2 days in 10% FCS containing medium. Cells were then stimulated with the indicated cytokines in 2% serum-containing medium for 18 h after which conditioned medium was assayed immediately or stored at −20°C for future analysis. IL-6 was assayed as previously described[58] using the B9 hybridoma proliferation assay. Briefly, the IL-6-dependent B9 cells (provided by Dr L. Aarden) were plated into 96-well microtitre wells in 100 μl of MEM 10%, and 100 μl of various dilutions of test sample supernatant. After incubation for 3 days at 37°C, 5% CO_2 and 100% humidity, the proliferation of cells were measured using the MMT assay as described by Mosmann *et al.*[59] Units of IL-6 activity were compared to a standard preparation of human recombinant IL-6, and expressed as ng/ml IL-6 produced in culture supernatants over 18 h in culture. The variability in this assay has a coefficient of variations of <5% within an assay, and <10% between separate assays.

RNA analysis

Total RNA was prepared from fibroblasts at 18 h post-stimulation and isolated using the method of Chomczynski and Sacchi.[60] Northern blots were prepared by standard techniques and probed with cDNA probes representing the entire cDNA sequence of human TIMP-1, human stromelysin-1 or the coding region of human IL-6 labelled by the random primer technique. Preliminary experiments suggested that the housekeeping gene GAP-3DH mRNA was significantly altered by some combinations of these cytokines when compared to the ethidium bromide stained 28S and 18S bands on Northern blots. Thus the intensity of signals were compared using densitometry analysis and nor-

malized to the intensity of ethidium bromide stained 18S and 28S bands analysed on negatives of photographs. The results were expressed as fold increases from control levels.

Transcriptional analysis

A stably transfected cell line (RonP5) courtesy of Dr J. Gauldie and Astra Pharmaceuticals was used to assay OM effects on IL-6 promoter activity. This cell line was produced from human lung fibroblasts by cotransfecting pRSVneo with a rat IL-6 promoter-luciferase gene chimeric clone (rIL-6p5), and subsequent selection for resistant cells with G418 (250 μg/ml for 2 days, 75 μg/ml for 15 days). rIL-6p5 contains sequences from −585 to +14 of the rat IL-6 promoter inserted upstream of the luciferase gene in p19luc.[36] Colonies were pooled, split, divided and grown to confluence before assay. Cells were then split, seeded at 2×10^5 cells/well in 24-well COSTAR dishes, allowed to recover overnight and then stimulated with cytokines in 2% FCS-containing medium for 24 h. Cell lysates were prepared from pelleted cells by repeated freeze–thaw in 100 μl of 100 mM KP04, 1 mm DTT as described.[36] The lysates were centrifuged at 12 000 rpm for 15 min and the supernatants were assayed for protein content (Biorad) and luciferase activity.[36] Activity (light units) were corrected for any slight variation of protein content of the lysates before calculations of fold change relative to unstimulated cell lysates.

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Chapter Four

To further demonstrate the activity of the HSF to modulators within its environment, this chapter continues to address the role of PGE2 on HSF biology. HSF proliferation, chemokine production and matrix metalloproteinase mRNA production were examined in our in vitro system. Of significance is the finding that HSF derived from synovial membranes originating from normal individuals and from patients suffering from RA and OA respond uniquely to PGE activation. The title of this manuscript is : *Human synovial fibroblast activation by prostaglandin E: effects on proliferation and cytokine and matrix metalloproteinase production.* This is the first report demonstrating that HSF derived from normal SM responded differently to Pgs than HSF derived from inflamed SM. The author of the thesis was responsible for all the experiments performed in this manuscript.

PROSTAGLANDIN E_2 MODULATES PROLIFERATION, RANTES AND MCP-1 PRODUCTION BY HUMAN SYNOVIAL FIBROBLASTS: PROLIFERATION, BUT NOT CYTOKINE RESPONSE, DIFFERS IN CELLS DERIVED FROM NORMAL AND ARTHRITIC SYNOVIUM

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ABSTRACT

Prostaglandin E_2 (PGE_2) is a major product found in inflamed joints and can affect a number of cell types found in joint tissue. We have examined the effects of PGE_2 on human synovial fibroblasts (HSF), an important cellular constituent of normal and inflamed synovium. Exogenous PGE_2 caused an inhibition of HSF proliferation in fibroblast cultures derived from normal synovium. In contrast, HSF derived from the synovium of patients suffering from osteoarthritis (OA) or Rheumatoid arthritis (RA) responded to PGE_2 (10^{-10} to 10^{-6} M) with an increase in proliferation as measured by ^{3}H-thymidine incorporation. HSF production of two members of the chemokine family, monocyte chemotactic peptide (MCP-1) and RANTES was markedly stimulated upon stimulation with IL-1α. The addition of indomethacin (10^{-6} M) to IL-1-stimulated cells resulted in a significant increase in the production of RANTES, MCP-1 and GM-CSF. Further stimulation with PGE_2 caused a significant decrease in RANTES, MCP-1 and GM-CSF production from all cell lines, irrespective of the patient tissue source. IL-1α stimulated MMP-1 and less so TIMP-1 mRNA levels in HSF. The addition of indomethacin to IL-1-stimulated cultures had a minor inhibitory effect on MMP-1 and TIMP-1 mRNA at 18 hours. Further addition of PGE_2 had a small stimulatory effect on MMP-1 mRNA from OA but not normal-derived HSF, however MMP-1 or TIMP-1 protein levels in supernatants were not affected at this time point. These results show that PGE_2 can modulate HSF activation by markedly affecting the IL-1-induced production of chemokines, but modulates proliferation in opposite fashion in HSF derived from normal (inhibition), than its stimulatory effect on HSF derived from OA or RA synovium.

INTRODUCTION

While the initiating factors involved in chronic inflammatory joint disease are not well understood, the lesion which develops within the synovial membrane has been described in some detail (1). In rheumatoid arthritis (RA), the synovial lining thickens due to the influx of activated macrophages and the local accumulation of fibroblast-like synoviocytes (1,2). An important effector cell within the synovial membrane is the synovial fibroblast. Its use in in vitro studies have enabled researchers to gain a better understanding of the intricacies involved in chronic joint diseases. A variety of pro-inflammatory soluble mediators including Interleukin-1 (IL-1) and tumor necrosis factor (TNF) can be found in the joint space and have been shown to be produced by cells within the synovial membrane itself (3,4). Both IL-1 and TNF induce synovial fibroblast production of matrix degrading enzymes and other pro-inflammatory cytokines and mediators and it is believed that these molecules are in part responsible for perpetuating the destruction of joint tissue seen in both osteoarthritis (OA) and RA (3,4).

Activated human synovial fibroblasts (HSF) produce a number of cytokines which affect the inflammatory process. These include the chemokines monocyte-chemotactic peptide (MCP-1), RANTES and Interleukin-8 (IL-8) which contribute to control of leukocyte cell infiltration; granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage-CSF (GM-CSF) which activate phagocytic cells and prolong survival of mature hemopoetic cells, and IL-6 which can act on monocytes, lymphocytes and local connective tissue cells as well as distant targets such as hepatocytes with systemic effects (5-8). In addition, the proliferation of synovial fibroblast cells, which can be enhanced by growth factors such as PDGF and TGF-β, will contribute to the pathogenesis of inflammation (9). HSF are also believed to be a major source of matrix

metalloproteinases (MMPs) (2). Degradation of the extracellular matrix (ECM) is dependent on the activity of at least three subclasses of MMPs produced by fibroblasts; collagenase (MMP-1), gelatinase (MMP-2) and stromelysin (MMP-3) (10-12). The activities of MMPs are regulated by a family of tissue inhibitors of metalloproteinases (TIMPs) which are also expressed by HSF and other cells within the synovial membrane (13).

Another major product synthesized by fibroblasts during chronic inflammatory conditions is the arachidonic acid metabolite prostaglandin E_2 (PGE_2). PGE_2 possesses a number of important biological functions which contribute to a variety of local and systemic effects. This as well as other small molecular weight mediators are thought to be important modulators of the inflammatory processes within the inflamed joint. The basis of widely used therapeutic intervention for arthritis is to inhibit the production of the key enzyme responsible for the liberation of PGE_2 (cyclooxygenase; COX) through the action of non-steroidal anti-inflammatory drugs (NSAIDs) (14). However, some evidence suggests that prostanoids may have beneficial effects in models of inflammation and more recently, more specific effector functions of PGE_2 have been indicated. We have previously shown that PGE_2 can modulate HSF production of the cytokines GM-CSF, IL-8 and IL-6 (15). As well, others have shown that PGE_2 can affect macrophage production of IL-6, IL-8, MMPs and TIMPs (16-18).

To sort out the relationship between PGE_2 and synovial fibroblast activation, we performed a detailed study analysing the modulation by PGE_2 of HSF proliferation, cytokine synthesis and MMP and TIMP production in vitro. We have examined the effects of both endogenous and exogenous PGE_2 on IL-1-induced levels of MCP-1, RANTES and GM-CSF secretion as well as expression of MMP-1, and TIMP-1 mRNA levels. Our data suggests that the

activity of PGE_2 in regulating HSF proliferation is dependent upon the source of HSF tissue; that is, HSF derived from normal joints respond differently than do HSF originating from patients suffering from OA and RA. However, we also show that PGE_2 inhibits RANTES and GM-CSF markedly and MCP-1 less markedly in all HSF cell lines irrespective of the synovial tissue of derivation.

MATERIALS AND METHODS

Reagents

PGE_2 and indomethacin were purchased from Caymen Chemical Co. IL-1α was kindly provided by Dr. M. Widmer (Immunex Co. Seattle, WA). All other reagents were purchased from Sigma Co. St. Louis, MO .

Human Synovial Cell Cultures

Primary cell lines of human synovial fibroblast (HSF) were derived and isolated from patients suffering osteoarthritis(OA) or Rheumatoid arthritis (RA) undergoing total joint replacement as previously described (7). Normal HSF lines were derived in the same fashion from synovium of trauma/amputee patients. After mincing and digestion of synovium with collagenase and hyaluronidase, cells were plated and grown in RPMI medium containing 10% FBS (Gibco, Grand Island, NY) supplemented with μ50/ml of fungizone and gentamycin. Normal HSF were supplemented initially with 20 % FBS. All cells were grown in 75 mm^2 flasks (Corning Glass Co. Corning, NY). Cells were grown to 80 % confluence before being passaged and were routinely used for all studies between passage 5 and 7. For RNA studies, cells were plated in 150 mm^2 flasks in medium containing 10% FBS (20% for normal HSF), allowed to reach 80% confluence, washed and the medium was replaced with RPMI (2% FBS) overnight. Stimuli were then added the following day for the indicated time.

^{3}H-Thymidine Proliferation Assays

HSF cultures were resuspended in RPMI medium (10% FBS) and seeded in 96-well plates

(Corning Co.) at a concentration of 2.5×10^3 cells/well. Cells were allowed to adhere to the wells, were then washed and stimulated with various concentrations of PGE_2 (with 12 replicates of each point) in RPMI medium containing 10% FBS. These were cultured for 18 hours after which [^{3}H]-thymidine 0.5μCi/well (specific activity 53mCi/mmmol; NEN, Boston, MA) was added for an additional 6 hours. 96 well dishes were than placed in -20^{0}C freezers until scintillation counting was performed. Plates were then freeze-thawed four times to dislodge any remaining adherent cells and harvested using previously described methods (19). Data represents 4 experiments on at least 2 HSF cell lines from each of normal, OA or RA derivation .

mRNA Isolation and Northern Analysis

Total RNA was prepared from HSF using the method of Chomczynski and Sacchi (20). Briefly, 80% confluent HSF were washed and replenished in medium containing 2% FBS. Various stimuli were then added and cultures were further incubated for 18 hours before RNA isolation. Northern blots were prepared by standard techniques and probed with cDNA probes representing the coding region of human Collagenase (MMP-1), Gelatinase (MMP-2) and TIMP-1. These probes were provided by Dr. A.J.P. Docherty (Celltech, Ltd, Slough, UK). The intensity of ethidium bromide stained 18S and 28S bands analysed on negatives were used to assess equal loading of RNA. As well, quantification of signals were compared by assessing the intensity of autoradiographed gels via spectrophotometry and phosphorimagery analysis.

Elisa Analysis of Protein Levels in Supernatants

HSF from various cell lines were subcultured in 24 well dishes (Corning) at an initial concentration of $5x10^3$ cells/well in medium containing 10% FBS. 12 hours before stimulation,

cells were washed in sterile PBS and replenished with medium containing 2% FBS. Cells were stimulated with IL-1, PGE_2 and indomethacin in triplicate as indicated for 18 hours. Supernatants were collected and stored at -20°C until used for ELISA. RANTES, MCP-1 and GM-CSF ELISA kits were purchased from R& D Systems (St. Paul, MN) and the samples were assayed according to the specifications of the manufacturer. HSF supernatants used for chemokine analysis were also subjected to MMP-1 and TIMP-1 ELISA determination. ELISA kits were obtained from Amersham (Boston MA). Data represents the mean values of triplicate cultures and SD.

RESULTS

HSF Proliferation

The dose effect of PGE_2 on HSF proliferation is shown in Figure 1A. The data shown is from a single representative experiment with 12 replicates for each point. The figure shows that proliferation of HSF derived from normal joints is inhibited by PGE_2 in a dose dependent fashion. The peak effect of inhibition seemed to occur at 10^{-7}M PGE_2. HSF-derived from patients suffering from joint disease (either osteoarthritis (OA) or rheumatoid arthritis (RA)) however, were stimulated to proliferate by PGE_2. The peak stimulatory effect was seen at 10^{-8}M for RA-derived HSF and 10^{-7}M for OA-derived HSF. Figure 1B demonstrates that dexamethasone caused a dose-dependent inhibition of HSF proliferation irrespective of the disease state from which the HSF were derived. At all doses tested, PGE_2 was not toxic to cells as viability of HSF populations was maintained as assessed by exclusion of Trypan Blue staining (data not shown).

A similar result was seen in all other cell lines tested. Figure 2 illustrates the effect of PGE_2 on proliferation in a series of cell lines tested as change versus control. The proliferation of HSF derived from RA synovial cell lines significantly increased versus control when stimulated by PGE_2 at 10^{-10}M (an average increase to 199% of control). The peak effect occurred when RA cell lines (n=3) were stimulated with 10^{-8}M PGE_2 (397% change). The proliferative response of HSF derived from OA synovium (n=4) to PGE_2 was significantly increased at 10^{-8}M PGE_2 (average of 215% of control) and remained consistent at higher concentrations up to 10^{-6}M PGE_2. While cells derived from inflamed synovium (RA or OA) responded to PGE_2 by increases in proliferation as measured by ^{3}H-thymidine incorporation, HSF derived from normal synovium were inhibited by

PGE_2. A significant decrease in proliferation versus control was seen when normal HSF (n=3) were stimulated with 10^{-8}M PGE_2 (61% of control). This inhibition of normal HSF proliferation peaked at 10^{-7}M PGE_2 (47%) and continued to be significant at 10^{-6}M PGE_2. Indomethacin, at concentrations of 10^{-10} to 10^{-6} M, had no measurable effect on the proliferation of any of the cell lines tested regardless of HSF source (data not shown).

MCP-1 and RANTES Production

Having determined that the proliferative responses of HSF to PGE_2 differed according to source of synovium, we were interested in determining if any other parameters of HSF activation, such as cytokine synthesis responded in a similar manner. Figure 3 shows the response of the various populations of HSF to IL-1α, PGE_2 and indomethacin treatment in terms of RANTES (Figure 3A), MCP-1 (Figure 3B) and GM-CSF (Figure 3C) production. The basal release of RANTES was not detectable in the supernatants of HSF derived from RA, OA or normal synovium. IL-1α (5 ng/ml) stimulation caused an increase in RANTES production from all lines tested. The further addition of indomethacin to IL-1-stimulated HSF resulted in a clear increase in RANTES production in RA derived (246 to 449 pg/ml), OA derived (127-198 pg/ml) and normal derived (109-229 pg/ml) HSF cell lines. Since indomethacin inhibits, the endogenous production of PGE_2, we hypothesized that the inhibiting cyclooxygenase product might well be PGE_2. When IL-1 and indomethacin treated cultures were stimulated with 10^{-7}M PGE_2, a dramatic decrease in RANTES production as assessed by ELISA was evident, from 449 to 85 in RA-derived HSF, from 198 pg/ml to 49 pg/ml in OA-derived HSF, and from 229 pg/ml to 63 pg/ml in normal HSF. This correseponds to decreases to 19, 24 and 28% of RANTES levels.

ELIZA analysis of MCP-1 (Figure 3B) determined that both OA (410 pg/ml) and RA (1194pg/ml)-derived HSF produce basal amounts of this C-C member of the chemokine family. Stimulation with IL-1α (5 ng/ml) resulted in a significant increase in the production of MCP-1 with RA-derived HSF (7348 pg/ml) showing higher levels than OA-derived HSF (5405 pg/ml) and normal-derived HSF (4866 pg/ml). The addition of indomethacin to IL-1-stimulated cultures resulted in a significant increase in MCP-1 production in both RA (9816 pg/ml) and normal (7647 pg/ml)-derived HSF and smaller increased in OA-derived HSF. Addition of PGE_2 to IL-1/indomethacin stimulated HSF cultures resulted in a significant reduction in MCP-1 production in all lines tested. This response occurred at both 10^{-7}M and 10^{-6}M PGE_2. Futhermore, addition of exogenous PGE_2 to IL-1α-stimulated cells (in the absence of indomethacin) also resulted in the reduction of RANTES and MCP-1 production from RA and normal-derived HSF supernatants (data not shown).

These same supernatants were examined for the presence of GM-CSF. We have previously shown that IL-1-stimulated GM-CSF production by OA-derived HSF is inhibited by PGE_2 (15). Figure 3C confirms this and shows that this same phenomena occurs in RA-derived and normal-derived HSF. IL-1 caused a significant increase in GM-CSF levels in all lines tested, and the the addition of indomethacin resulted in further increases in GM-CSF levels in RA (2190 to 5678 pg/ml) and OA (1636 to 2835 pg/ml) normal (719 to 1663 pg/ml) and derived cells. The further addition of PGE_2 resulted in a marked significant decrease in GM-CSF levels to 12%, 63% and 20% of IL-1/indo-induced levels in RA, OA and normal-derived HSF respectively.

Regulation of TIMPs and MMPs

We have previously found that PGE_2 could stimulate TIMP-1 mRNA in human lung fibroblasts (21), and thus examined synovial fibroblasts in response to PGE_2. In figure 4, we found that PGE_1 or PGE_2 stimulation (10^{-6}M) did not regulate TIMP-1 (0.9 kb), TIMP-2 (3.5 kb) or TIMP-3 (2.2 and 2.5 kb) mRNA signals in human synovial fibroblasts. Oncostatin M stimulation, and less so IL-1 stimulation, enhanced TIMP-1 signals but not TIMP-2 or TIMP-3 signals. Responses were similar in normal and RA-derived HSF (not shown).

Since PGE_2 has been shown to modulate the cytokine-induced expression of MMP and TIMPs in various inflammatory cell populations (22-25), we next examined the effects of PGE_2 in this system. To examine this at the mRNA level, we completed Northern blots of stimulated cells (Figure 5). We could not detect any effect of PGE_2 or indomethacin alone on MMP-1, mRNA production from HSF derived from normal, OA or RA-derived joint tissue (data not shown). All populations of HSF stimulated for 18 hours with IL-1α responded by demonstrating an increase in mRNA, and the addition of indomethacin to IL-1 treated HSF resulted in a slight decrease in MMP-1 mRNA levels after 18 hours. The further addition of PGE_2 to IL-1/indomethacin stimulated cells, slightly enhanced the mRNA levels of MMP-1 in OA-derived HSF but not HSF derived from normal synovium. In the same blots reprobed for TIMP-1, we observed a low but consistent elevation upon IL-1α stimulation. The co-addition of indomethacin did not have notable effects and further stimulation with PGE_2 caused a slight decrease in TIMP-1 signal in all cell lines. Basal expression of MMP-2 (gelatinase) mRNA in normal and OA HSF was not affected by PGE_2 at various concentrations (data not shown). The level of MMP-2 mRNA expression was not significantly affected by IL-1, indomethacin, PGE_2 or any combination thereof

in any of the cell lines tested (Figure 5). This was consistent with previous reports showing that MMP-2 is not responsive to IL-1 stimulation (10,12).

ELISA data examining the protein production of MMP-1 and TIMP-1 is shown in Table 1, which demonstrates that basal levels of TIMP-1 from HSF derived from all lines tested was measurable. The level of TIMP-1 protein expression from medium containing 0% FBS was not significantly different than supernatants isolated from cells grown in 2% FBS suggesting that the levels and changes seen are indicative of the activation state of the cells (data not shown). IL-1-stimulation resulted in a approximately a 2 fold increase in TIMP-1 protein levels in all lines tested. The addition of indomethacin or PGE_2 to IL-1-stimulated cultures did not significantly alter the TIMP-1 levels in any of the lines tested. Also shown in Table 1, the basal production of MMP-1 protein for cells derived from normal synovium was minimal (below the level of detection for the assay), but RA-derived HSF produced higher basal level of MMP-1 (2.7 ng/ml). IL-1 stimulation caused a significant increase in MMP-1 production, and the further addition of indomethacin or PGE2 did not significantly alter the level of MMP-1 in any of the cell lines tested.

DISCUSSION

The effects of PGE in chronic inflammation of the joint are not completely understood. Evidence exists for both "pro-inflammatory" (vascular effects, pain, bone resorption) and "anti-inflammatory" effects (chondroprotection, lymphocyte inhibition) of PGE. We have here examined the human synovial fibroblast derived from normal, OA and RA patient synovium, and responses to PGE_2. In arthritis, the synovial fibroblast cells acquire a matrix degrading phenotype probably due to an interaction with the host of cytokines and other modulators present in the local milieu (4,7,11). This phenotype has been well characterized to include: enhanced cellular proliferation, increased protease synthesis and cytokine release and increased expression of HLA markers involved in the perpetuation of the disease (27). The present results demonstrate that 1) PGE_2 can effectively decrease RANTES, GM-CSF and MCP-1 from all IL-1 stimulated HSF tested, and 2) PGE_2 decreases proliferation of normal-derived HSF, but enhances proliferation of OA and RA-derived HSF.

Fibroblast proliferation is a key event in several normal processes including wound healing (28). However, aberrant cell proliferation leading to fibrosis is a hallmark feature in a variety of disease states including later stages of arthritis (1,27). The effect which prostanoids have on fibroblast proliferation has previously shown varying results. Nolan et al suggested that the stimulatory effect of Epidermal growth factor on murine fibroblasts was via PGE_2 (29). Hori et al., using the human foreskin fibroblast cell line FS-4 demonstrated that prostanoids including PGE_2, are responsible for TNF-induced inhibition of fibroblast proliferation (30). Here, we have provided detailed evidence that HSF derived from patients suffering from either osteoarthritis or rheumatoid arthritis are directly stimulated to proliferate in response to PGE_2. However, in HSF

derived from normal synovium, PGE_2 causes a dose-dependent inhibition of proliferation. Butler et al. have previously documented that the presence of indomethacin in IL-1-stimulated explant-derived synovial fibroblasts (OA) results in enhanced HSF proliferation (31), but the study did not examine the direct effects of exogenous PGE_2. While we feel it is appropriate to compare HSF derived in a similar manner (we use the collagenase digest technique), it is less suitable to compare between HSF derived by different techniques (such as explants in ref. 31), which may lead to seperate HSF phenotypes being selected in culture. It may be of interest to determine whether variations in gene and protein expression are effected by the method of synoviocyte isolation used. In our hands, indomethacin had no effect on proliferation itself (data not shown). Dexamethasone however, in all lines tested, caused a marked inhibition of proliferation. These results strongly suggest that both OA and RA synovium yield cells which are uniquely altered by PGE_2 whereas response to glucocorticoids is similar to normal cells. The basis of this is unknown, and could be due to differences in type of PGE receptor expression that predominates, or to an indirect effect such as altered growth factor responses to PGE_2 in arthritis cells.

The chemokine family of cytokines are low molecular weight structurally related proteins important in the chemotaxis and migration of immune and inflammatory cells. MCP-1 and RANTES are members of the C-C chemokine family and are generally chemotactic for mononuclear cells including monocytes, basophils and memory T lymphocytes (5). IL-8, on the other hand belongs to the C-X-C family and is generally considered intimately involved in neutrophil chemotaxis and angiogenesis (32,33). MCP-1 and RANTES have been found in the synovial fluid and in the cells of the synovial membrane of patients with inflammatory joint diseases (5,34).

To our knowledge, this is the first report which assesses the effect of endogenous and exogenous PGE_2 on the HSF expression of RANTES. To this end, this data may have relevance in vivo since the expression of RANTES may be affected by NSAIDS in therapeutics. Loetscher et al. examined the effects of indomethacin on MCP-1 and IL-8 production from rheumatoid synovial fibroblasts and determined that both were not affected by indomethacin stimulation (34). In our hands, indomethacin significantly enhanced MCP-1 and RANTES production in IL-1α-stimulated HSF derived from normal, OA or RA synovium. The addition of PGE_2 then markedly decreased this effect which Loetscher et al did not find. The reason for the incongruity between our results may be due to methodological differences. Loetscher et al utilized slightly different isolation techniques, and different stimulation protocols, with the addition of indomethacin and IL-1 at one hour prior to further stimulation with PGE_2. Of interest, our results demonstrate is that modulation of chemokine secretion by PGE_2 does not appear to depend on tissue origin, since normal, OA and RA-derived HSF respond similarly to endogenous and exogenous PGE_2. Thus, the regulation of chemokines likely employs similar mechanisms in all the HSF, and proliferation responses must some how diverge in the OA and RA-derived cells from normal.

Collagenase (MMP-1) production by the synovial membrane has been shown to be intimately involved with the matrix degradation seen in arthritis (10-12). As well, it has recently been suggested that the expression of MMP-1 protein levels are higher in diseased tissue (RA or OA) than in control tissue (35). We show here that PGE has relatively minor effects on TIMP-1 and MMP-1 levels in untreated and in cells treated with IL-1 and indomethacin. The effect of endogenous and exogenous prostanoids in terms of MMP-1 production has been examined by DiBattista et al (23). They demonstrated that normal HSF production of MMP-1 is inhibited by

prostanoids in cells stimulated with IL-1β (0.1 to 0.2 ng/ml). We used IL-1α at considerably higher molar concentrations (IL-1α at 5 ng/ml) that induced marked cytokine production, and also examined indomethacin effects, thus our experimental conditions were somewhat different. It may be that PGE is more effective at modulating MMP induced by lower concentrations of IL-1(α or β).

In part, these results help support the importance of PGE_2 in regulating the dynamics of cellular activity of fibroblasts derived from the synovial membrane in vitro. Here the effect of PGE_2 on proliferation was shown to be unique and dependent on the disease state of originating synovial membrane. Whether this functional difference in response exists in HSF in situ or in vivo is not clear. Certainly other responses to PGE_2 are not differentiated as dramatic effects of PGE_2 on RANTES, or on GM-CSF was noted in normal, OA and RA derived HSF. Expansion of HSF phenotyes with altered responses to PGE_2 could play a role in arthritis disease.

FIGURE LEGENDS

FIGURE 1 Effect of Prostaglandin E_2 and Dexamethasone on Human Synovial Fibroblast Proliferation:

HSF were seeded into 96 well plates at a concentration of 2.5×10^3 cells/well and stimulated with various cencentrations A) PGE_2, B) dexamethasone; for 18 hours. Cultures were then pulsed with 0.5uCi/well of ^{3}H-thymidine for 6 hours and scintillation counting was performed. Error bars represent SD from the mean of 12 replicates. Each cell line was tested 4 times. This is a representative of 4 seperate experiments of each cell line that showed consistent results. Normal HSF (+), OA (_) and RA (O). *represents $p<0.01$ based on the *Students t Test.*

FIGURE 2 Effect of Prostaglandin on Proliferation of multiple cell lines.

Seperate cell lines including normal (solid, n=30), OA (small hatched, n=4) and RA (hatched, n=3) derived HSF populations were compared in parallel over a range of PGE_2 doses. Twelve replicates were averaged and data expressed as percentage change versus control (unstimulated). *$p<0.01$

FIGURE 3 Human Synovial Fibroblasts Production of (A) RANTES, (B) MCP-1 and (C) GM-CSF as Assessed by ELISA.

Normal (solid), OA (small hatched) and RA (hatched)-derived HSF were stimulated with IL-1 (5 ng/ml), 10^{-6}M indomethacin (IN), and PGE_2 at two concentrations (10^{-6}M and 10^{-7}M) as indicated. Supernatants were taken 18 hours later, stored frozen (-20°C) and then analyzed by specific ELIZA. Data is representative a single experiment done in triplicate on each of two cell lines for normal, OA and RA-derived HSF.

*represents a significant difference from IL-1 ($p<0.001$)

**represents a significant difference from IL-1+indo ($p<0.005$)

FIGURE 4: TIMP 1, 2 and 3 mRNA levels in synovial fibroblasts stimulated by PGE_2

Human synovial fibroblasts derived from OA tissue were stimulated for 18 hours in 2% FCS.1=control; 2= with PGE1 (10^{-6} M); 3= PGE_2 (10^{-6} M); 4= PGE_2+ PGE_1 ; 5 = IL-1 (5 ng/ml); 6= OSM (20 ng/ml). RNA was extracted and analyzed by Northern Blots (as in methods) using cDNA probes for TIMP-1 and TIMP-2 (A) and TIMP-3 (C). Gels stained with ethidium bromide (EB) served to assess equal loading of RNA (B).

FIGURE 5: MMP expression in normal and OA-derived HSF: effect of PGE_2

Expression of MMP-1, MMP-2 and TIMP-1 mRNA from Normal (A) and OA (B)-derived HSF were analysed by Northern blots. HSF cultures were grown in $75mm^2$ to subconfluence in medium containing 10%FBS. Cultures were then serum depleted overnight and stimulated with various combinations of PGE_2 ($10^{-7}M$), IL-1 (5 ng/ml), $10^{-6}M$ indomethacin (IN) for 18 hours after which the mRNA was isolated and examined using Northern blots (as in methods). Differences in lane loading were compared using ethidium bromide stained 18S and 28S RNA (EB).

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		C	IL-1	IL-1+IN	IL-1+IN+PGE2
TIMP-1	NORMAL	20.2 (4.2)	42.3 (6)	48.2 (6)	49.3 (5.4)
(ng/ml)	RA	48.8 (2.7)	75.7 (4.8)	83.9 (2.7)	83.9 (2.7)
		C	IL-1	IL-1+IN	IL-1+IN+PGE2
MMP-1	NORMAL	0.82 (0.05)	2.01 (0.11)	2.13 (0.47)	1.62 (0.29)
(ng/ml)	RA	2.69 (0.064)	22.58 (1.22)	21.90 (2.31)	18.34 (1.20)

TABLE 1: Normal and RA-derived HSF MMP-1 and TIMP-1 Production: effect of PGE_2

HSF derived from tissue secured from normal and RA joints were cultured (as in methods) with the inicated stimuli including IL-1α (5ng/ml), 10^{-6}M indomethacin (IN) and 10^{-6} PGE2. Supernatants were obtained after 18 hours of stimulation and subjected to specific ELISA analysis for MMP-1 and TIMP-1. Data represents results mean of triplicate cultures and numbers in parentheses are SD.

FIGURE 1

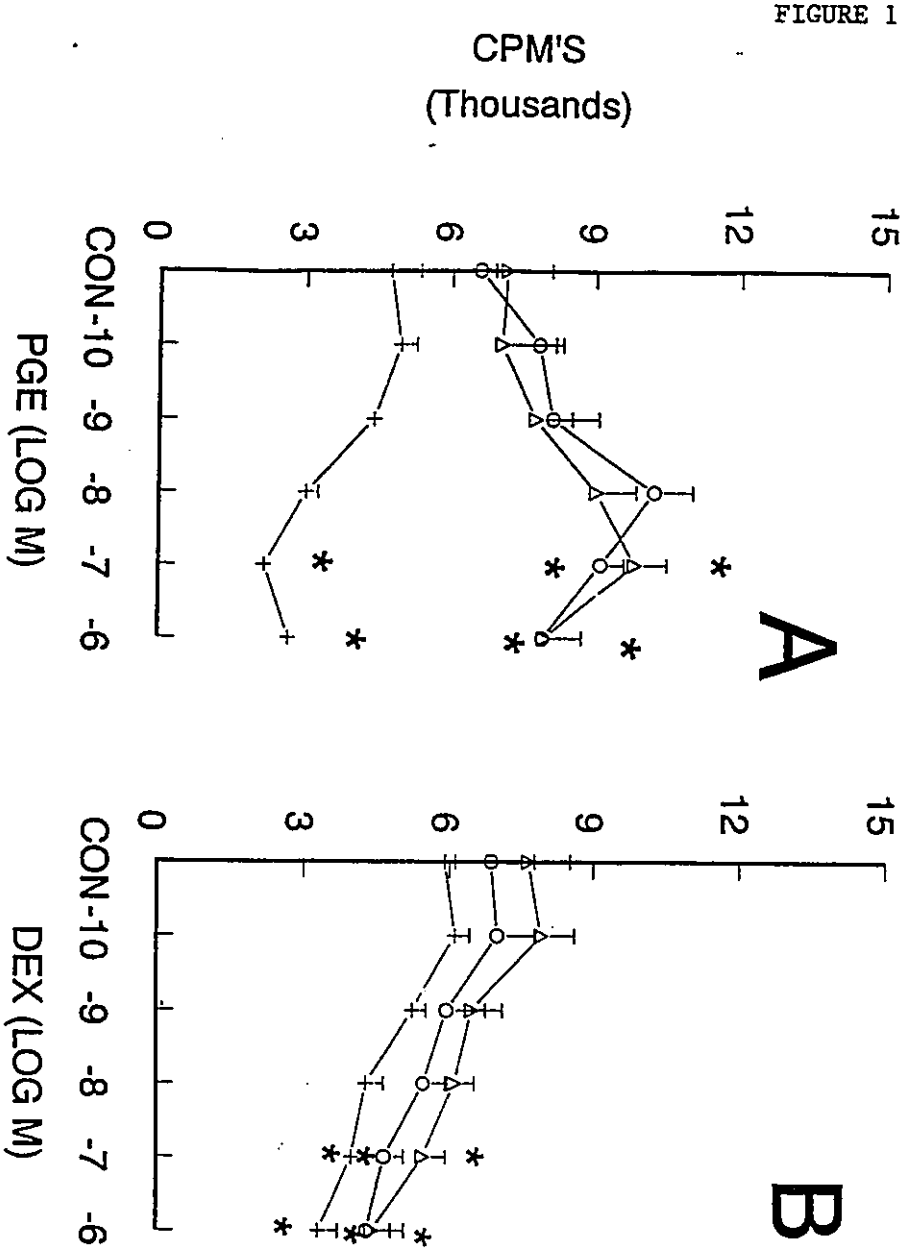

CPM'S
(Thousands)
0
3
6
9
12
15
CON-10 -9 -8 -7 -6
PGE (LOG M)
A
CON-10 -9 -8 -7 -6
DEX (LOG M)
B

FIGURE 2

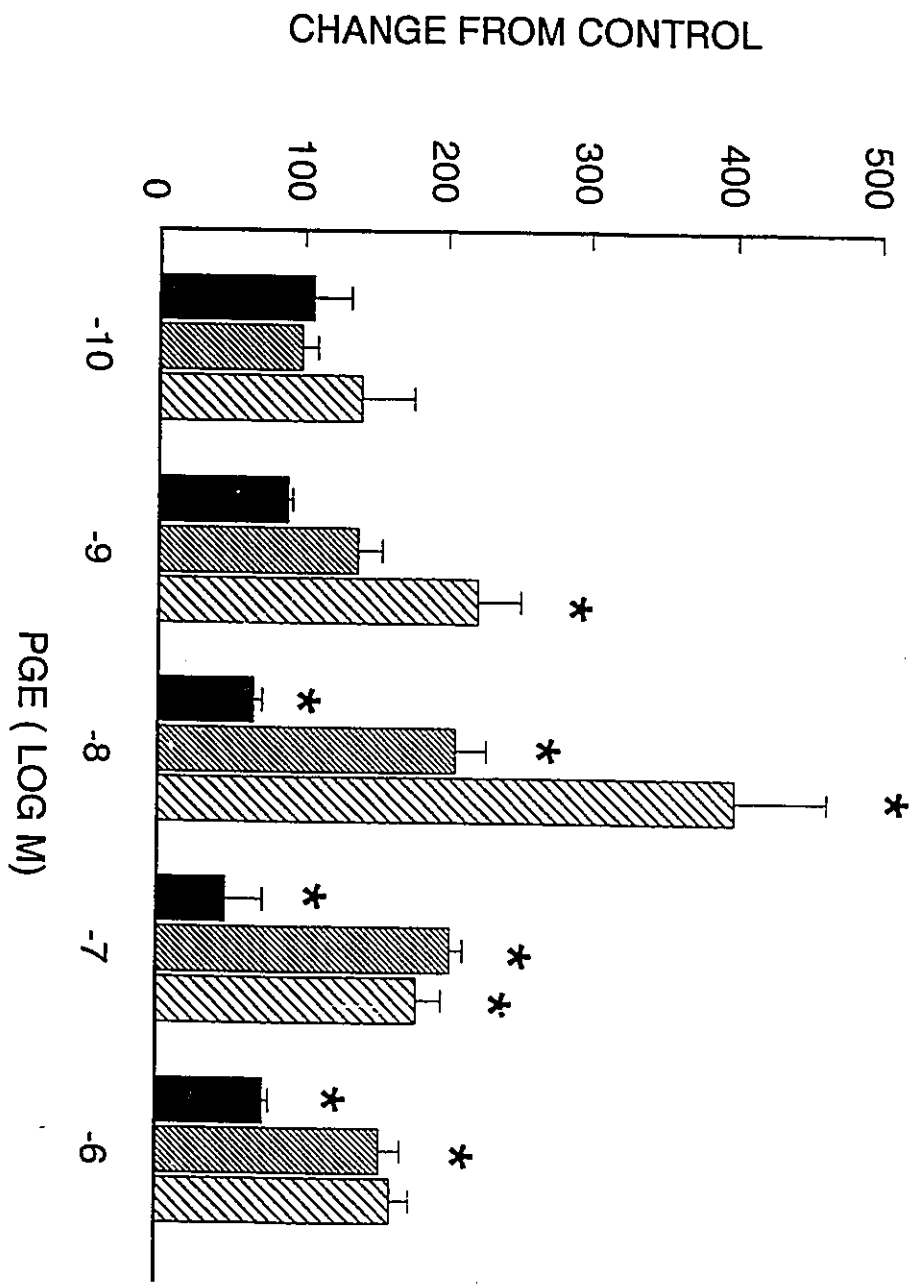


FIGURE 3

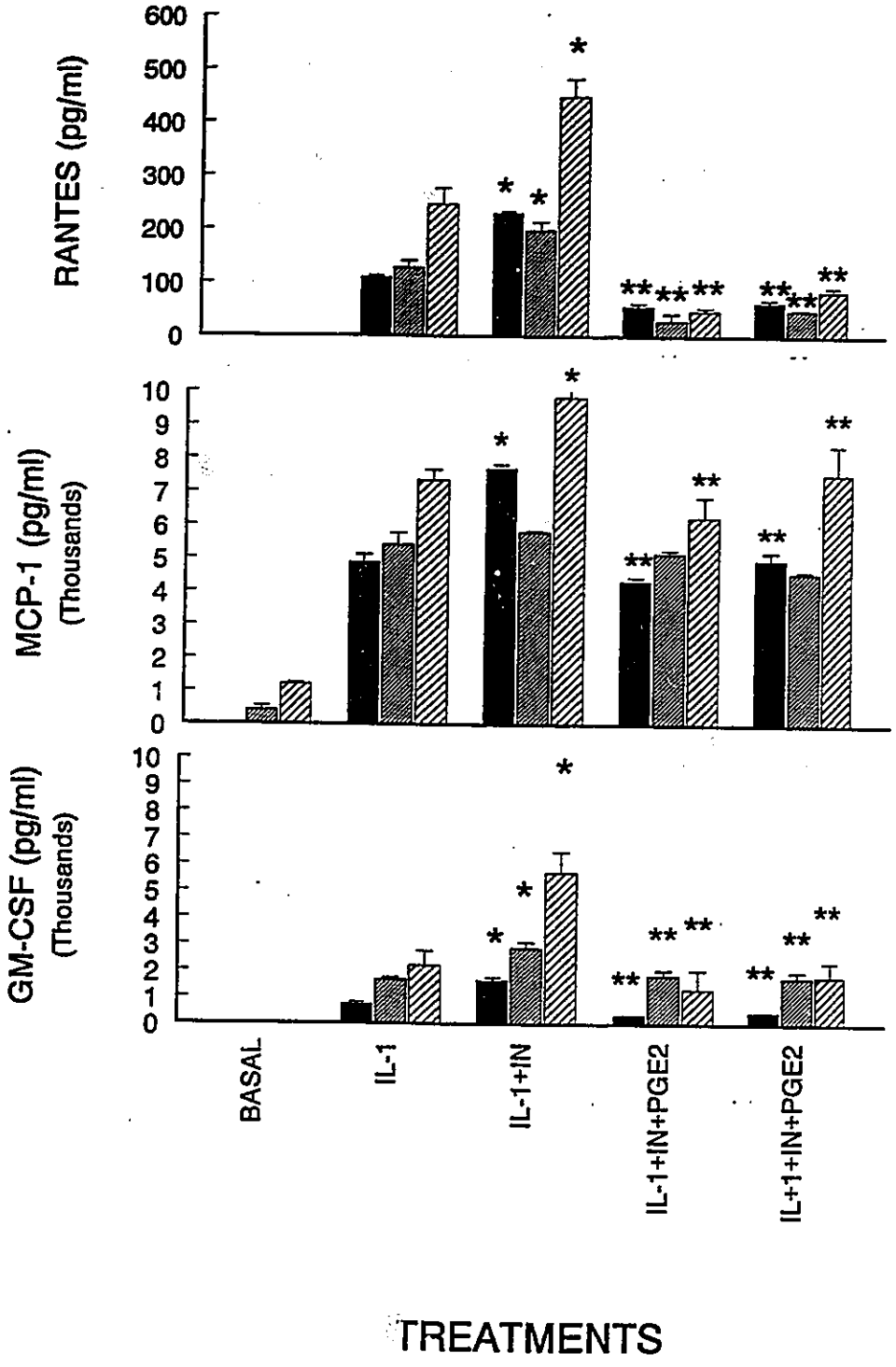

RANTES (pg/ml)
600
500
400
300
200
100
0
MCP-1 (pg/ml)
(Thousands)
10
9
8
7
6
5
4
3
2
1
0
GM-CSF (pg/ml)
(Thousands)
10
9
8
7
6
5
4
3
2
1
0
BASAL
IL-1
IL-1+IN
IL-1+IN+PGE2
IL+1+IN+PGE2
TREATMENTS

Figure 4

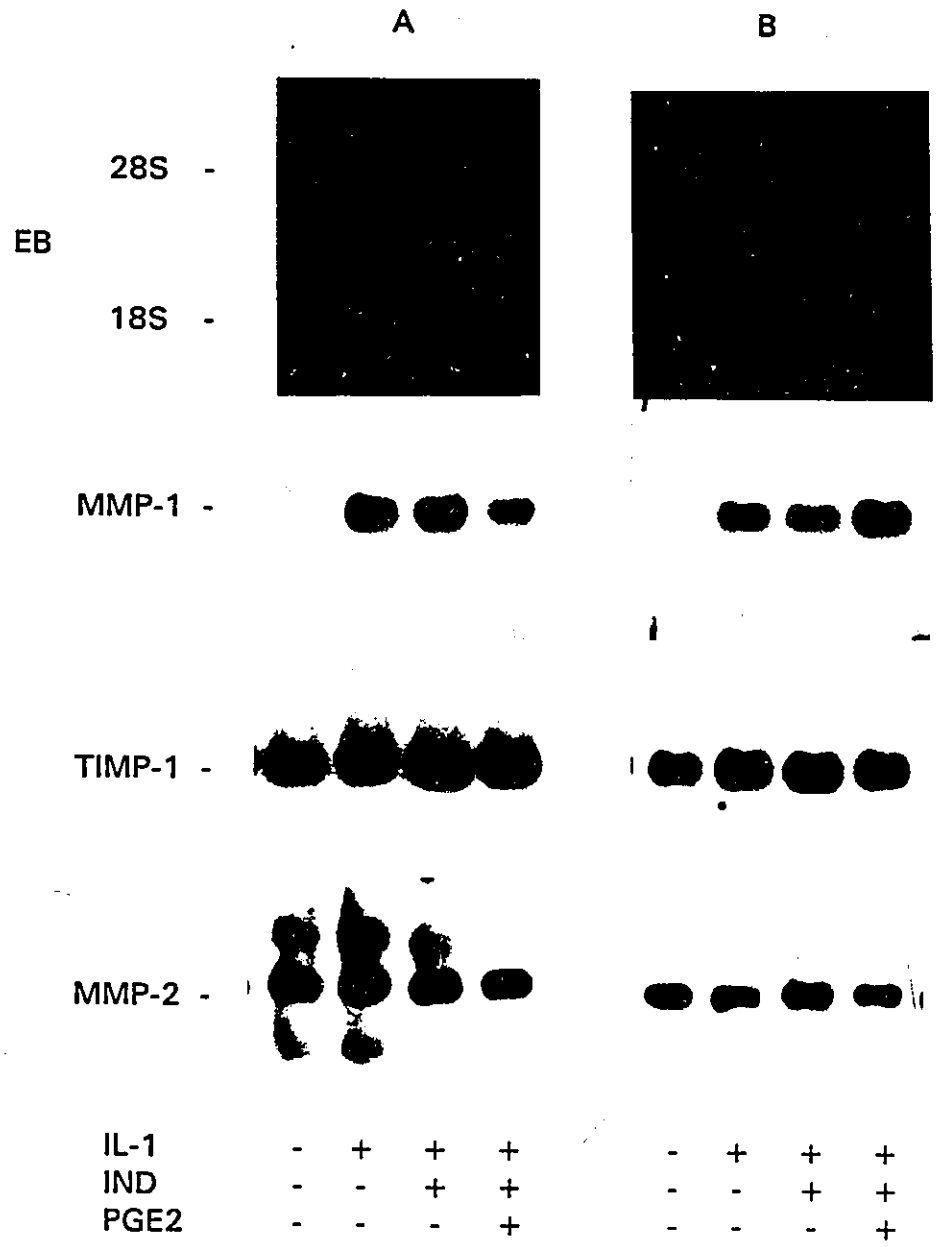
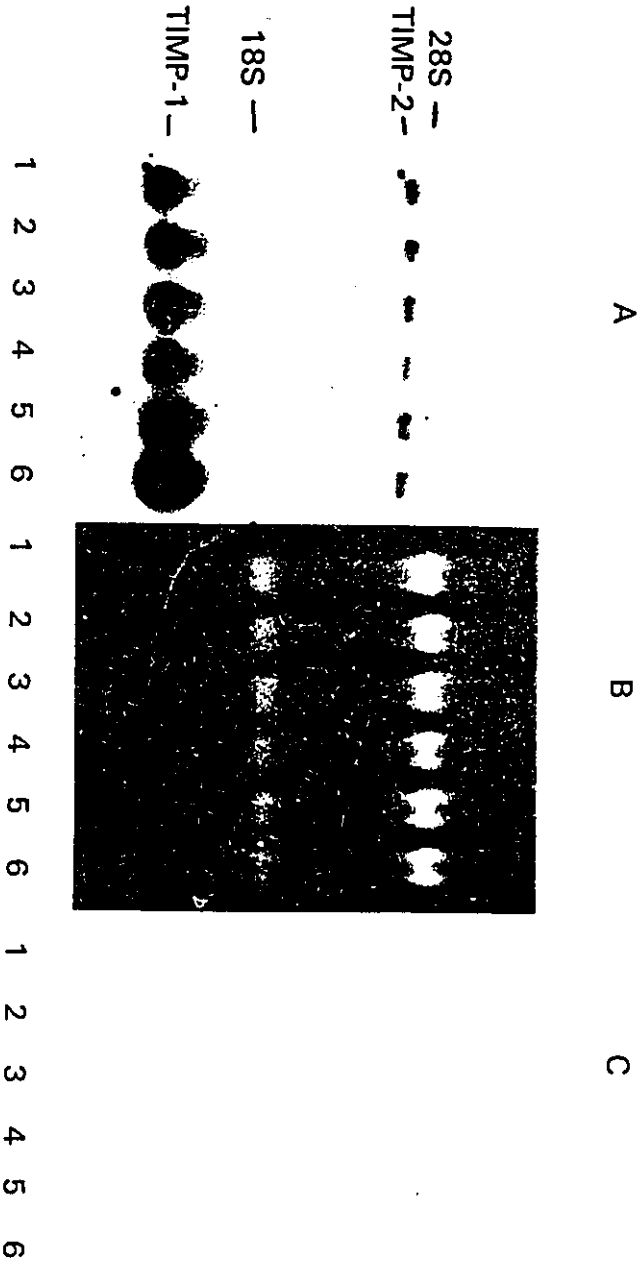


Figure 5



Chapter Five

SUMMARY

The synovial membrane is a dynamic tissue lining the capsule of diathroidal joints. Its cellular constituents, resident Type A and B synoviocytes along with other local stromal and immune cells, are responsible for maintaining homeostasis within the environment with which they exist. In pathological states, both the morphology and activation state of the entire membrane and its components is significantly altered. Grossly, the membrane swells due in part to the local proliferation of microvessels, the expansion of the fibroblast population within the intimal layer of the membrane and the infiltration of monocytes/macrophages from the surrounding tissues and periphery. The influx and enhanced proliferation within the cellular compartment coupled with the increased accumulation of various synovial fluid constituents contributes significantly to joint swelling.

Elucidation of cellular and molecular events underlying the visible gross changes are critical to understand the intricacies of the disease process. At the cellular level, recent work has suggested that the local compartment within the inflamed joint houses the necessary resident and infiltrating cells and soluble mediators to initiate and drive the pathological lesion. This thesis hypothesizes that the Type B synoviocyte (human synovial fibroblast) is a central cell involved in arthritic disease progression. As illustrated in Figure 1, the HSF, by producing and releasing a variety of soluble mediators and responding to the activation signals initiated by other cells is suggestive that the HSF has multiple roles in affecting the local environment. The work of Soden et al. (1989) probably best exemplifies the importance of the HSF in synovitis. This group, who bilaterally biopsied the SM of patients presenting with idiopathic unilateral joint pain, demonstrated that, even in clinically

uninvolved joint of patient who are diagnosed early with inflammatory joint disease, there existed synovial lining hyperplasia. While not all of these patients were later diagnosed with RA, the data is suggestive that HSF expansion and activity may be a predictive diagnositic tool for chronic synovitis.

This thesis has presented evidence that demonstrates that: a) the human synovial fibroblast is able to contribute to the cytokine pool within the joint milieu; b) HSF-derived cytokines can significantly affect the activity of other cells present within the joint and that levels of cytokine release (basal or stimulated) is dependent upon the patient source of HSF (OA or RA); c) exogenous and endogenous prostaglandins, produced by Type A and B synoviocytes, can effectively modulate the production of HSF-derived cytokines alone and in combination with other potentially important soluble mediators; and d) some of the effect of prostaglandins on HSF biology, particularly proliferation can be dependent on the source of synovial tissue; that is HSF derived from tissue obtained from patients suffering from OA and RA can respond differently to PGE than do HSF derived from normal synovial membrane.

The importance of the cytokine network and its role in orchestrating inflammatory responses is well accepted, however the complexities and interactions within local sites such as the arthritic lesion remain unclear (Arend et al., 1995). In chapter two, data is presented which both corroborates existing information and adds new data demonstrating that GM-CSF is produced by cells of the SM and can alter the phenotype of other cells (namely lymphocytes) within the local environment. This data suggests that the human synovial

fibroblast contributes a large share of the manufacturing and secreting of GM-CSF into the joint space. As well, we demonstrated that the level of HSF-derived GM-CSF produced in vitro from cells derived from tissue originating from RA synovium was higher than levels found in cultures originating from OA synovium. This finding is not unique to GM-CSF as others have shown that rheumatoid joint is rich in cytokines/growth factors usually with levels greater than that seen in osteoarthritis (Lafyatis et al., 1989). The regulation of GM-CSF production was also addressed here by demonsrating that SP, a putative pro-inflammatory neuropeptide, stimulated its release. This was the first report demonstrating that SP could enhance fibroblast-derived GM-CSF. As SP levels have been shown to be increased in arthritis (Marabini et al.,1991) the ability for this peptide to modulate the production of GM-CSF is a further indication that SP is working within the joint as a pro-inflammatory meidator. The functional activity of the enhanced production of a pro-inflammatory mediator such as GM-CSF was also assessed in this chapter. GM-CSF derived from HSF was shown to be able to support the survival of naïve peripheral blood lymphocytes in vitro. This effect was inhibited by specific antibodies to GM-CSF and recombinant material used in similar in vitro studies garnered the same results. It can be presumed that the in vivo consequence of this activity of HSF-derived GM-CSF is increased lymphocyte retention; a hallmark feature of the SM in rheumatoid arthritis (Harris, 1990). Of added importance was the finding that the ability of HSF-derived GM-CSF to support lymphocyte survival in vitro was more pronounced in cells isolated from SM derived from patients with RA then OA. As noted, microscopic examination of the synovial membrane

in rheumatoid arthritis shows a pronounced tissue leukocytosis whereas in OA, the SM and surrounding tissue does not have as conspicuous an infiltration (Altman, 1995). The effect on lymphocytes of GM-CSF derived from normal HSF has yet to be investigated however it would be expected that its effects on lymphocyte survival may be less than that seen from diseased tissue since we have shown in chapter 4 that the levels of GM-CSF derived from IL-1 activated normal HSF is lower than that seen in HSF derived from OA or RA tissues.

Chapters three and four of this thesis deal with the responsiveness of various populations of HSF to in vitro stimulation with cytokines and prostaglandins. The response of HSF to prostaglandins is of particular interest in light of the putative pro-inflammatory characteristics which PGEs possess during the acute inflammatory response (Goldstein, 1988). A great deal of data supports the notion that PGEs involvement within the inflamed joint is a detriment. Significantly increased levels of PGE in joint tissues coupled with the evidence supporting PGEs ability to exacerbate the pain response, stimulate edema, vascular permeability (Goldstein, 1988) and potentially stimulate bone resorption (Robinson, 1975) provides sufficient evidence for the therapeutic inhibition of PGE during arthritic disease. However, the long term effectiveness of NSAID therapy and PGE inhibition on both rheumatic joint disease and osteoarthritis has been recently questioned. In fact, recent studies have determined that indomethacin, a commonly used NSAID, should not be used to treat OA since it increased the rate of radiographic deterioration of affected joints (Huskisson et al., 1995). This relevant clinical information coupled with the data supporting the immuno/inflammatory-inhibiting properties of PGE helped form the basis for our

investigations into the activity of prostanoids on HSF biology.

With the hypothesis that HSF can modulate their local and peripheral environment in mind, we demonstrated that IL-1-activated HSF populations were responsive to both endogenous and exogenous PGE stimulation. The advent of NSAIDs has given researchers the ability to effectively eliminate endogenously produced prostanoids. Using the COX-inhibiting NSAID indomethacin, chapter three illustrates that the cytokines GM-CSF, IL-6 and IL-8 are all partially regulated by endogenous products of the arachidonic acid cascade and namely, PGE. In light of the pro-inflammatory characteristics which PGE generally possess, our data, in human synovial fibroblasts, demonstrated that exogenous PGE could in fact significantly inhibit mRNA and immunoreactive GM-CSF in vitro. The effect of GM-CSF on HLA activation, monocyte chemokine synthesis and apoptosis (Firestein et al., 1993, Takahashi et al., 1993, Gehrmann et al. 1995) and its effects on neutrophil synthesis of chemokines and arachidonic acid metabolites (Takahashi et al., 1993, Pouliot et al . 1994) are well recognized. Based on these pro-inflammatory and immuno-activating properties of GM-CSF along with the present data demonstrating GM-CSFs effect on lymphocyte survival is suggestive that GM-CSF has an important role in perpetuating and exacerbating the arthritic lesion. Therefore it can be reasoned that early inhibition of its activity within the inflamed joint by PGE may limit the progressive nature of the lesion.

The net effect of PGE on HSF-derived IL-6, a potentially protective cytokine, was stimulatory. The evidence supporting a protective role for IL-6 and other members of this cytokine family is growing. IL-6 may effect the balance of catabolism and anabolism as

orchestrated by the synovial fibroblast by stimulating TIMP expression and having little effect on any MMP release (Lotz et al., 1992). As well IL-6 and other members of the IL-6 family have been shown to stimulate the acute phase response thought to be important in the initial elements of wound healing. In addition, in vivo evidence has been presented which documents an anti-phlogistic effect of IL-6 in both animal models of arthritis and in human clinical trials (Dasgupta et al., 1992, Wood et al., 1992). PGE2 was also shown to enhance IL-8 production from IL-1-stimulated HSF in vitro. These results suggest that the apparent protective effects which PGE2 stimulation had in terms of IL-6 stimulation and GM-CSF inhibition were not true for IL-8. One explanation of these results may be that PGE2 enhancement of IL-8 production by HSF may be an acute response of the HSF to IL-8. A major role of IL-8 in arthritis appears to be neutrophil chemotaxis and angiogenesis of the SM. It can be postulated that the acute increase in angiogenesis and leukocyte chemotaxis may be benefit the joint infrastructure at an early stage by allowing the immediate influx of inflammtory/immune cells to properly eliminate a foreign body and/or mediate wound repair. In this light, the stimulation of IL-8 by PGE2 may be acutely protective, however, as the chronicity of the disease becomes more established by the enhanced activation of HSF and Type A synoviocytes by other cytokines, the morphological/spatial changes induced by acutely active chemokines and PGE2 within the synovium become detrimental to the joint. Recent work by Fuller et al (1995) has suggested that IL-8 suppresses the resorption of isolated rat osteoclasts. These results suggest that, while generally pro-inflammatory, this family of chemokines possess multifunctional attributes which must be further studied to

gain a complete understanding of their biological significance.

Chapter four goes on to further characterize the effect of PGE on other parameters of HSF biology including cellular proliferation, MMP release and chemokine production. As well, the response to PGE was addressed in HSF derived from varying tissue sources. HSF were isolated from SM derived from patients suffering from RA, OA or from non-diseased (normal) joints. In terms of HSF proliferation, PGE caused a pronounced stimulation of thymidine incorporation in HSF derived from inflamed joints (RA or OA). However, in HSF originating from normal SM, PGE markedly inhibited proliferation. The novel examination of the effectiveness of PGE to modulate the two chemokines, RANTES and MCP-1 demonstrated again that both endogenous and exogenous PGE inhibited the production of each protein from IL-1 stimulated HSF in vitro regardless of tissue source. In general, there was no significant effect of PGE on MMP or TIMP-1 production in vitro. These results signify that; i) HSF can respond to local mediators produced potentially by themselves or typically by Type A synoviocytes within the joint; ii) the inflammatory significance of enhanced PGE, particularly in terms of their ability to effect cytokine/chemokine modulation should be re-addressed since some effects of these low molecular weight modulators is not necessarily detrimental to the surrounding tissue; and iii) the responsiveness of HSF to PGE appears to be significantly dependent on the tissue source from which the SF were derived.

The effectiveness of prostanoids to alter the activity and phenotype of Type B human synoviocytes suggests that a reassessment of PGEs activity as a pro-inflammatory mediator of tissue damage in arthritis could be considered. Using Hollander's (1991) criteria, a close

examination of the characteristics of PGE is suggestive that this mediator may not fill all the necessary requirements. The availability of PGE to the joint infrastructure by the activation of COX and the ultimate liberation of arachidonic acid stores as well as findings that demonstrate that PGE is significantly increased within the actively inflamed lesion satisfy the first two criteria. As for criterion 3, which implies that the pro-inflammatory mediator must act on normal tissue giving an effect which resembles the damage seen in the joint, PGE, in our hands, does not significantly alter the phenotype of normal HSF in a manner which mimics the activity on HSF derived from diseased tissue. In fact, data in chapter 4 has shown that PGE inhibits proliferation in normal HSF in vitro, however, a hallmark feature of the diseased joint is a proliferating pannus with significantly enhanced synovial expansion (Zvaifler et al., 1994). In our hands, PGE has minimal effect on the production of MMPs from normal HSF which, in the diseased joint in both RA and OA, are significantly increased. In addition, GM-CSF release in vitro from IL-1-stimulated normal HSF cultures is inhibited by PGEs. This again demonstrates that the active mediator (PGE) does not always elicit a pro-inflammatory signal from normal tissue. As for criteria 6, the progression of tissue damage is not necessarily prevented by pharmacological inhibition of PGE in rheumatoid arthritis and may actually exacerbate radiological changes seen in the lesion in OA (Huskisson et al., 1995). In summary, apart from being present in high amounts in diseased tissue, PGE does not conform to all the characteristics of a mediator inducing tissue destruction within the diseased joint. While the stringency of these criterion may be too severe and are based, in part, on in vitro results, characterization of potential pro-

inflammatory mediators must be carefully scrutinized to avoid unnecessary pharmacological/scientific investigation into every mediator deemed to have "some" potential therapeutic benefit. Of further significance is the realization that singling out a specific mediator as a potential pro- or anti-inflammatory agent may be rash since the effects of many mediators (for example, the effect of PGE and OM on IL-6 release as presented in chapter 3) are dependent upon the action of other mediators to exhibit their full potential effect.

In conclusion, evidence has been provided which suggests that the human synovial fibroblast can significantly contribute to the dynamic processes going on within its immediate and perhaps, its more distant environment. Data has been presented which demonstrates that HSF are affected by locally-derived mediators including prostanoids. To further address the issue of PGE modulation of HSF biology it may be prudent to undertake investigations into the expression of receptorsfor PGE in these processes. As shown in chapter 2 and 4, the responsiveness of HSF to various stimuli often differs depending on the tissue source of the isolated synovial fibroblasts. Experimentation into prostanoid receptor status is a burgeoning area with the recent cloning of the various subtypes of prostaglandin E (EP) receptors. It appears that there are at least 4 classes of EP receptors which utilize unique signal transduction pathways (Coleman et al., 1994). The expression of these classes of EP receptors have yet to be fully examined on fibroblast populations. In light of the varying effects of PGE on HSF biology, which appears to be dependent on tissue source and readout gene analyzed, it may be sensible to investigate the type and activity of PGE

receptors on synovial fibroblasts. Results from such studies may further our understanding of both human synovial fibroblast biology and prostaglandin kinetics with respect to inflammatory joint disease.

Chapter 6

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