

RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLASTS:
CYTOKINE-INDUCED INVASIVE PHENOTYPE AND ASSOCIATED
MECHANISM(S) OF TISSUE DESTRUCTION

by

Catherine Anne Frye

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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**Rheumatoid arthritis synovial fibroblasts: Cytokine-induced
invasive phenotype and associated mechanism(s) of tissue
destruction**

Frye, Catherine Anne, Ph.D.

The University of Arizona, 1994

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Catherine Anne Frye entitled Rheumatoid Arthritis Synovial Fibroblasts: Cytokine-Induced Invasive Phenotype and Associated Mechanism(s) of Tissue Destruction.

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DEDICATION

This work is dedicated to Jim and Sharon Cramer, for providing reason in the midst of pandemonium, and nonsense when it all got too serious.

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The invasive and destructive properties of pannus tissue contribute to the loss of cartilage and bone in rheumatoid arthritis (RA). To further analyze the process of cartilage degradation, we have developed an *in vitro* model which allows for observations of cellular degradation and invasion of a cartilage matrix isolated from porcine knee joints. This matrix contains collagen type II, proteoglycans and glycosaminoglycans, and is similar in composition to human cartilage. Synovial fibroblasts isolated from the pannus tissue of patients with aggressive RA, based on clinical evaluation, demonstrated a highly invasive phenotype in this *in vitro* model; whereas synovial fibroblasts isolated from pannus tissue of less aggressive RA, based on clinical evaluation, showed a less invasive phenotype. Further characterization of cell-cartilage matrix interactions reveal that RA and normal synovial fibroblasts adhere to a similar degree to cartilage matrix, but this adhesion can be inhibited with arginine-glycine-aspartic acid (RGD) peptides, thus implicating integrins in this attachment process. During the subsequent process of spreading on cartilage matrix, highly invasive RA synovial fibroblasts maintained a round phenotype for a longer duration than normal synovial fibroblasts. Furthermore, with respect to the degradative ability of these cells, the level of expression and secretion of key metalloproteinases was measured, and interstitial collagenase activity was shown to correlate with the RA phenotype, while no expression was detected in normal synovial fibroblasts. By comparison, there was no differential expression of stromelysin, gelatinase A and gelatinase B found in normal synovial fibroblasts versus RA synovial fibroblasts. Normal synovial

fibroblast invasion was augmented by culturing these cells in the presence of 5 U/ml IL-1 β or 18 U/ml TGF β ; whereas, PDGF at 100 ng/ml did not affect normal synovial fibroblast invasion. In addition, normal synovial fibroblasts cultured in 150 U/ml TNF α demonstrated a significant decrease in their ability to invade cartilage matrix. Collectively, these *in vitro* data suggest that highly invasive synovial fibroblasts correlate with advanced RA clinical disease. Moreover, this invasive phenotype may be due, in part, to decreased spreading ability on the cartilage matrix in addition to interstitial collagenase activity. This model allows for further molecular characterization of the invasive properties of the synovial fibroblast.

IV

BACKGROUND

A. Rheumatoid Arthritis Etiology and Epidemiology

Rheumatoid arthritis (RA) has been characterized as a chronic inflammatory disease of the joints. Patients present with symptoms reflecting the inflammatory component of the disease including malaise, fatigue, diffuse pain, stiffness after inactivity and loss of mobility in the diseased joint. Criteria for the diagnosis of RA have been based on clinical presentation and have defined the disease as classical RA, definite RA or probable RA (Condemni 1992). The clinical course of RA has varied from spontaneous remission in 5 years or less to progressive disease uncontrolled by drug therapy and, thus, has been considered a heterogeneous disease (Pincus et al 1993).

The etiological agent for RA has not been identified; although, evidence supporting the role of bacteria and autoimmunity has been prevalent. Animal models have demonstrated the arthritogenic properties of bacterial components such as *Lactobacillus casei* (Lehman et al 1983) and streptococcal cell walls in rat models of RA (Clark et al 1979). However, an organism has not been isolated from human RA joints. Interestingly, an animal model supporting the autoimmune nature of RA has been described in the MRL/l (lpr/lpr) mouse, which develops arthritis as well as other autoimmune phenomenon spontaneously (Koopman et al 1988).

RA as an autoimmune disease has been exemplified by the prevalence of some subtypes of the major histocompatibility complex (MHC), HLA-DR4, in RA patients. The known association of the MHC with T cells suggests an autoimmune component; however, a significant number of RA patients do not possess the HLA-DR4 allele (Nepom 1991). In an attempt to explain the non-

HLA-DR4 expressing RA patients, the shared epitope hypothesis was developed and suggests that different HLA molecules share regions of the molecule involved in ligand binding (Gregerson et al 1987). This idea has been shown to be a factor in RA, as 90% of RA patients contain a shared epitope of the DR4 allele, which is located in the ligand binding domain of the molecule (Nepom 1991). This evidence supports consideration of autoimmunity as a factor in the development of RA.

A number of recent reviews analyzing the pathogenesis of RA serve as strong support for the role of slow bacterial infections as a causative agent in the development of RA, particularly mycobacteria or a closely related organism (Rook et al 1992, Rook et al 1993, McCulloch et al 1993). Bacteria have been shown to cause arthritis in animals, as demonstrated by Barden and colleagues (1971) with *Mycoplasma hyorhinis* inducing an RA-like disease in swine. Immune reactions to bacterial stress proteins, specifically the 65 kDa mycobacterial heat shock protein (HSP), provide a separate line of evidence supporting a bacterial role in the pathogenesis of RA (Winfield et al 1991). Furthermore, an adjuvant arthritis model in rats indicates that a boost in immunization with a peptide from the 65 kDa mycobacterial HSP prior to inducing arthritis prevented the development of arthritis in these rats (Yang 1990). In the human system, T cells isolated from the synovial fluid of some patients with RA have been shown to proliferate *in vitro* in response to the mycobacterial HSP (Hill-Gaston et al 1989) or to a complex of mycobacterial antigens (Pope et al 1989). HSPs are conserved across species, and the immunological responses to the 65 kDa mycobacterial protein may result from

cross reactivity with host HSP released as a result of the joint damage in RA, rather than associated with the actual cause of the disease.

In an effort to characterize the etiological agent for RA, T cells from RA synovial fluid have been analyzed for clonal frequency. The results show that in a site of inflammation caused by a known antigen, antigen specific T cells range from 1:500 to 1:3000 T cells (Panayi 1993). By a variety of analyses, investigators have looked for restriction of T cell receptor (TCR) usage in both plasma and RA synovial fluid as a means of identifying T cells responding to the causative agent of RA (Marguerie et al 1992; Fischer et al 1991). To date, identification of specific TCR usage in RA has not been consistently identified in T cells derived from RA patients (Marguerie et al 1992, Panayi et al 1993, Dedeoglu et al 1993).

Although the inciting agent has not been identified, the resulting inflammation mediates the cartilage and bone damage observed in RA. The earliest histological changes in RA joint disease include: 1) synovial edema, 2) lining cell hyperplasia, 3) polymorphonuclear leukocyte invasion, and 3) fibrin deposition (Wortmann 1991). As a result of the inflammatory immune response, the normal synovial membrane proliferates and extends into the joint contacting bone and cartilage. This highly metabolic and proliferative tissue has been termed pannus tissue.

The synovial membrane consists of a 1-3 cell layer thick lining composed of macrophages (type A cells), fibroblasts (type B cells) and dendritic cells (Barland et al 1962). In fact, this membrane lines the inner surface of the joint capsule and comes into contact with bone and cartilage at specific attachment sites on the sides of the joint. The synovial lining secretes a fluid, known as

synovial fluid, which contains hyaluronic acid and provides lubrication in addition to nutrients for bone and cartilage. Under the inflammatory conditions of RA, the synovial membrane proliferates and becomes infiltrated with inflammatory cells including plasma cells, lymphocytes and macrophages (Kobayashi et al 1975). The proliferating synovial membrane (pannus tissue) extends into the joint contacting cartilage and bone at which sites cartilage and bone matrix dissolution has been evident by histological analysis of human joints (Fassbender 1983), as well as *in vitro* cultures of synovial tissue (Harris et al 1970).

Treatment of RA has been directed at controlling the immune response, with particular focus on the T cell, employing such agents as non-steroidal anti-inflammatory drugs and slow-acting anti-rheumatic drugs (Brooks et al 1993). T cells isolated from RA synovial fluid are positive for the activation antigen CD45RO, indicating that T cells are primed rather than unactivated cells (Matthews et al 1993). Moreover, RA has been demonstrated to be a T cell dependent disease in the streptococcal cell wall (SCW) rat model. SCW injected into susceptible rats causes the development of an arthritis that progresses clinically and histologically much like human RA (Clark et al 1979). In this SCW model, T cells were shown to be required for the fulminant development of RA, which was supported by injecting athymic and euthymic littermates with SCW and observing the development of arthritis in only the euthymic rats (Allen et al 1985).

Considerable attention has been directed at understanding the immunological aspects of RA, including T cells, endothelial cells, and macrophages with regard to understanding how these cells interact with each

other as well as the consequences of these interactions. It has recently been shown that the inhibition of T cell-B cell interactions, involving CD40 and gp39, blocked the development of arthritis in a collagen II induced arthritis model in mice. This emphasizes the importance of the immune response in the pathogenesis of RA (Durie et al 1993).

In essence, RA is an inflammatory joint disease with variable outcomes ranging from remission to loss of joint function requiring total joint replacement. Inflammation accompanied by the generation of pannus tissue provides the components needed for joint destruction. Studying the course of RA has focused on determining causative agents, developing biologically based treatment regimes and understanding mechanisms that contribute to RA as a disease.

B. Mechanisms of Cartilage Destruction:

The loss of cartilage in RA leads to a loss of joint space and joint function, which culminates in either disability or corrective surgery. Mechanisms mediating the loss of cartilage include: 1) the invasive and erosive growth characteristics of pannus; 2) macrophage and other inflammatory cell degranulation with concurrent release of lysosomal enzymes which damage joint matrices; and 3) the effect of inflammatory cytokines on chondrocytes, cells responsible for synthesizing and maintaining cartilage. Each of these components contributes to the loss of cartilage in RA.

Pannus tissue is a highly metabolic tissue which degrades cartilage at cartilage-pannus junctions (Woolley 1977, Kobayashi et al 1975). Kobayashi and Ziff analyzed sections of pannus and cartilage junctions by electron

microscopy and described three types of interactions: 1) pannus derived cell nests, including blood vessels invading into the cartilage; 2) pannus derived cell nests without blood vessels and invading into cartilage; and 3) acellular fibrous layer covering cartilage without an invasive nature. This report also proposed that cartilage pannus junctions 1 and 2 mediated damage via protease secretion while junction 3 starved the cartilage of nutrients, thus causing damage to the cartilage.

With respect to the involvement of proteases in joint destruction, matrix metalloproteinases (MMPs) are a family of matrix degrading enzymes which share several characteristics including: 1) zinc and calcium dependent enzyme activity; 2) secretion in zymogen form, requiring cleavage of the protein to the active form; 3) some degree of homology at the cDNA level; and 4) enzyme activity directed against a component(s) of the extracellular matrix (Woessner 1991). The MMP family can be broken down into three groups via the substrate(s) of the enzyme: collagenases, gelatinases and stromelysins (Matrisian 1992). Each of the proteins in the three classes of MMPs are encoded by separate genes and are under independent regulatory mechanisms (Unemori et al 1991, Sirum et al 1989). Active MMPs have been identified in synovial fluid collected from RA joints and culture supernatants of RA synovial fibroblasts (Sorsa et al 1992, Unemori et al 1991).

Interstitial collagenase was the first MMP identified with the enzymatic ability of degrading intact collagen fibers (Gross et al 1965).

Immunolocalization of active interstitial collagenase indicated that the enzyme was present in RA synovium primarily at sites of cartilage-pannus junctions, and these interstitial collagenase positive junctions were more of the type 2 class

described by Kobayashi and Ziff, consisting of pannus derived cells without blood vessels (Woolley et al 1977). It has been suggested that the synovial fibroblasts present in the pannus tissue secrete the interstitial collagenase, as synovial fibroblasts isolated from rabbits can be induced to secrete interstitial collagenase by exposure to proteases (Werb and Aggeler 1978) or to the tumor promoting phorbol diester, TPA (Aggeler et al 1984).

Another group of MMPs are the gelatinases, which degrade denatured collagen fibers as well as elastin (Matrisian 1992). Human RA synovial fibroblasts and normal dermal fibroblasts both express gelatinase A; whereas, RA synovial fibroblasts express gelatinase B, which can be induced in normal dermal fibroblasts by inflammatory cytokines (Unemori et al 1991).

The last class of MMPs are stromelysins, which degrade several extracellular matrix components including proteoglycans and fibronectin (Welgus 1991). Human RA synovial fibroblasts express the mRNA for stromelysin I, but not stromelysin II (Sirum and Brinkerhoff 1989). Stromelysin I enzyme activity has been demonstrated in RA synovial fluid (Walakovits et al 1992) and in the supernatants of cultured RA synovial fibroblasts (Case et al 1989). Synovial fibroblast secretion of matrix degrading MMPs contributes to the loss of cartilage in RA, yet it is not the only cell in pannus tissue responsible for cartilage loss in this disease.

Pannus tissue is an inflamed overgrowth of the normal synovial lining and is composed of inflammatory cells, such as macrophages and dendritic cells, and synovial fibroblasts. Degranulation of macrophage lysosomes releases mediators, including acid phosphatases, that damage cartilage (Lehman et al 1963). It has been proposed that activation of macrophages in

RA stimulates the production of fragile lysosomes, which are released from the cells contributing to the inflammatory immune response and cartilage damage. Subsequently, this results in further phagocytosis of particles, fragile vacuole formation, and the continuation of the inflammatory cycle (Weissman et al 1966). Microscopic evaluation of pannus tissue has verified that the macrophage-like cells in the proliferating pannus contain phagocytosed material including immune complexes in numerous vacuoles (Zucker-Franklin 1966). This observation supports the cycle of events proposed by Weissman and colleagues. Lysosomal degranulation of macrophages contributes to the continued inflammation of the RA joint and the release of factors which directly mediate cartilage damage.

Lastly, chondrocytes are responsible for synthesizing and maintaining cartilage. The main constituents of cartilage include collagen fibers and proteoglycans of which the latter are continuously degraded and replaced (Hardingham et al 1992). Inflammatory cytokines IL-1 β and TNF α are frequently found in RA synovial fluid (Yocum et al 1989, Moissec et al 1986), and TNF α has been shown to inhibit proteoglycan synthesis in porcine articular cartilage explant cultures (Hardingham et al 1992). Active depletion of matrix components in RA may be complicated by an inability of chondrocytes to replace lost proteoglycans, thus resulting in the loss of cartilage.

There are multiple mechanisms whereby cartilage can be damaged in the course of RA. While lysosomal degranulation and chondrocyte mediated damage are important contributors to cartilage loss, pannus tissue has primarily been considered responsible for the cartilage loss associated with RA.

Addressing some of the mechanisms associated with pannus mediated cartilage degradation has provided insight into the erosive nature of RA.

C. Inflammatory Cytokines in Rheumatoid Arthritis:

Inflammatory cytokines generated by the active immune response in RA affect resident cells in the joint, including chondrocytes (cells that synthesize cartilage), osteoblasts (cells involved in bone formation), and osteoclasts (cells involved in bone remodeling) as well as cells comprising the synovial membrane. Inflammatory cytokines contribute to the histological changes observed in RA, including conversion of the normal quiescent synovial membrane into the proliferative and invasive pannus tissue (Harris 1976; Hamilton 1983). Inflammatory mediators derived from T cells and macrophages have been identified in RA synovial fluid, including interferon gamma (IFN- γ), interleukin-2 (IL-2), tumor necrosis factor alpha (TNF α) and platelet derived growth factor (PDGF) (Miossec et al 1986, Koch et al 1992, Thornton et al 1991, Westacott et al 1990). Fibroblasts also produce mediators isolated from synovial fluid, including basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF β), granulocyte monocyte colony stimulating factor (GM-CSF), interleukin-1 beta (IL-1 β) and interleukin-6 (IL-6) (Bucala et al 1991, Alvaro-Garcia et al 1990). Some of these factors affect the growth characteristics and gene expression of fibroblasts, thus implicating the importance of these factors in the pathology of RA (Lafyatis et al 1989, Overall et al 1989, Dayer et al 1986).

Activated T cells infiltrate the synovium in RA, yet the concentrations of T cell derived cytokines in RA synovial fluid have been exceedingly low. In several studies, interleukin-4 (IL-4), IL-2, interleukin-3 (IL-3) or IFN- γ activity

was not detected in RA synovial fluid (Egeland et al 1987, Firestein et al 1990, Firestein 1987, Firestein et al 1988). However, mRNA for IL-2 and IFN- γ have been demonstrated in cells from synovial fluid and the synovial membrane (Buchan et al 1988a). The lack of detectable T cell derived cytokines in RA synovial fluid may be due to immediate consumption by target cells or to a short half life of the molecule(s) in synovial fluid (Ridderstad et al 1991). Another possibility is that T cells in RA synovium are nonresponsive or in a state of anergy, as demonstrated by T cells isolated from the peripheral blood of RA patients which were unable to perform the normal T cell function of regulating EBV-infected B cells (Depper et al 1981) .

Monocyte/macrophage derived cytokines including IL-1 β , IL-6, TNF α , PDGF and TGF β have been shown to be expressed in RA synovial fluid (Yocum et al 1989, Brennan et al 1990, Firestein et al 1990, Buchan et al 1988a, Remmers et al 1991). Furthermore, TNF α , IL-1 β and IL-6 comprise a cytokine network in which TNF α stimulates IL-1 β production. IL-1 stimulates more TNF α production, while the two together stimulate IL-6. IL-6 completes the cycle by inhibiting production of IL-1 β and TNF α (Akira et al 1990). PDGF expression in the joint has been shown to be unique to inflamed joints as PDGF receptors are not present in normal synovium (Ridderstad et al 1991). TGF β activity has been demonstrated in RA synovial fluid (Lotz et al 1990) and has been shown to down-regulate matrix degrading enzyme production of RA synoviocytes (Wilder et al 1990). For the purpose of this review and germane to this dissertation, further discussion will be limited to IL-1 β , TNF α , TGF β and PDGF.

IL-1 β production has been associated with acute-phase responses in immunologic reactions and has primarily been associated with a mounting immune response rather than the maintenance of normal health (Dinarello 1992). Bacterial derived endotoxin has been shown to strongly stimulate the production of IL-1 β in blood derived monocytes (Demczuk et al 1987), which can be concurrently inhibited by prostaglandins (Knudsen et al 1986). IL-1 β production can be stimulated by cytokines including IL-1 β itself (Dinarello et al 1987) and TNF α (Dinarello et al 1986), and suppressed by other cytokines such as IFN- γ (Ghezzi et al 1988) and TGF β (Chantry et al 1989).

IL-1 β has been associated with the pathology of arthritis as infusions of IL-1 β into joints produce changes associated with arthritis (Feige et al 1989). Furthermore, *in vitro* exposure of RA synovial fibroblasts to IL-1 β can induce prostaglandin and collagenase production (Dayer et al 1986), in addition to bone resorption (Koenig et al 1988). IL-1 β administered with TNF α can induce protease production in chondrocytes (Dinarello 1992), thus IL-1 β probably contributes to the joint destruction associated with RA by affecting synovial fibroblasts as well as cartilage and bone.

TNF α is produced primarily by stimulated monocytes/macrophages and activated T lymphocytes (Rothe et al 1992) and stimulates bone resorption (Bertolini et al 1986) and proteoglycan resorption in cartilage (Saklatvala 1986). Synovial fibroblasts have been shown to produce collagenase and prostaglandins in response to TNF α *in vitro* (Dayer et al 1985). Furthermore, TNF α has been shown to be important in arthritis as anti-TNF α monoclonal antibody therapy significantly reduces the arthritis in a collagen-induced arthritis model (Williams et al 1992).

TGF β can be produced by most cells, which can also express receptors for this cytokine (Wakefield et al 1987). Platelets release TGF β in an inflammatory immune response (Wahl 1991), which is a potent chemoattractant for leukocytes (Wahl et al 1987) and can upregulate adhesion molecules associated with leukocyte-endothelial cell interactions (Ignatz et al 1989, Ignatz et al 1987). TGF β has been associated with the induction of matrix components, even to the extent of fibrosis formation in heart disease (Waltenberger et al 1993). In addition, TGF β can inhibit collagenase production and stimulate synthesis of tissue inhibitor of metalloproteinase (TIMP) and gelatinase A, thus demonstrating the role of this cytokine in matrix remodeling (Overall et al 1989).

Explants of synovial tissue secrete PDGF, which was immunolocalized to the macrophages in pannus tissue, and has mitogenic activity for RA synovial fibroblasts (Endresen et al 1992, Remmers et al 1991). In addition, PDGF supported anchorage independent growth of RA synovial fibroblasts (Lafyatis et al 1989), and thus is considered an important factor in the development and pathology of pannus tissue. Normal human skin fibroblasts secrete collagenase when exposed to PDGF (Bauer et al 1985). In addition, PDGF can induce proliferation of cells which leads to pathological conditions such as obliterative bronchiolitis, which is a fibroproliferative disorder that occurs after lung transplantation and leads to airway occlusion (Hertz et al 1992).

The inflammatory mediators present in a rheumatic joint affect the cells resident in the joint, including chondrocytes and synovial fibroblasts, as well as immune cells. Understanding how cytokines interact in a rheumatic joint, and how these combinations affect the gene expression of cells resident in the joint,

may provide avenues for controlling the inflammatory immune response through biologically based intervention.

D. *In vitro* Synovial Fibroblast Model of Invasion:

Pannus tissue has been described as a growth with malignant or transformed properties (Harris et al 1976). Implants of uncultured single cell suspensions of pannus tissue in nude mice form a mass that histologically appears much like pannus tissue, without the infiltration of leukocytes (Brinckerhoff et al 1981). A neoplastic phenotype was further associated with synovial fibroblasts isolated from RA pannus tissue in both human tissue (Lafyatis et al 1989) and tissue from SCW induced arthritis in rats (Yocum et al 1988), ascribing such characteristics as anchorage independent growth and formation of foci.

In a study by Lafyatis and colleagues (1989), the neoplastic characteristics of the RA synovial fibroblast were lost with successive *in vitro* passages, but could be supported by the addition of the inflammatory mediator, PDGF. These observations demonstrated the importance of paracrine derived growth factors in the generation of the neoplastic phenotype of synovial fibroblasts and indicated that the "transformation" of these cells was not permanent.

Transformed cell growth has frequently been associated with the expression of oncogenes responsible for uncontrolled proliferation. In a study by Anders and colleagues (1991), the expression of the proto-oncogenes, c-ras, c-src, c-fos and c-myb was shown to be unchanged between cultured RA

synovial fibroblasts and normal synovial fibroblasts isolated from cruciate ligament repair surgery. Expression of oncogenes c-mil, c-erbB-2, c-int-2 and c-sis was not detected. Although the expression of c-myc was shown to be higher in RA synovial fibroblasts, this difference was not significant compared with levels associated with the normal fibroblasts. Furthermore, oncogenes v-H-ras and myc were analyzed in early RA pannus tissue and shown to be expressed in proliferating synovial lining cells (Gay et al 1989). Oncogenes play a role in normal cell growth, and an upregulation of oncogenes or mutations of oncogenes resulting in unregulated cell growth leads to the generation of tumor cells. Some oncogenes are upregulated in RA synovium, yet *in vitro* cultures of RA synovial fibroblasts do not show tumorigenic properties when tested in nude mice (Brinckerhoff et al 1981). This indicates that the "transformation" of RA synovial fibroblasts is not a permanent phenotype, but rather a phenotype reflective of paracrine growth factors.

The contributions of the synovial fibroblast to pannus mediated destruction have been demonstrated with *in vitro* cultures of these cells. Hence, *in vitro* models for studying fibroblast mediated cartilage destruction have been developed to study of the mechanisms associated with the invasive phenotype.

Hamerman and colleagues (1967) demonstrated that *in vitro* cocultures of synovial fibroblasts with cartilage pieces depleted the cartilage of matrix components by 17-19 days, post-inoculation. Supernatants from the synovial cells did not result in loss of cartilage matrix, indicating that contact of the synovial cells with the cartilage was required for matrix depletion. During the 17-19 day incubation period, however, the synovial cells did not invade into the

cartilage. Hence, Hamerman's *in vitro* model showed the ability of fibroblasts to deplete cartilage but did not demonstrate invasive activity (Hamerman et al 1967). In retrospect, the coculture period of 17-19 days was in all probability not long enough for fibroblasts to invade intact cartilage, since a similar coculture *in vitro* model, developed by Harris and colleagues (1970), indicated fibroblast invasion required an incubation period of 5 weeks. Furthermore, electron microscopic analysis of these 5 week cultures of pannus tissue with autologous joint capsule revealed macrophages covering the surface of the capsule, while fibroblasts were seen deeper in the tissue (Harris et al 1970). The Harris model demonstrated the invasive nature of synovial fibroblasts; however, this assay did require a 5 week culture period, which was a time commitment that made characterization of the invasive process challenging.

In a recent publication, Janusz and Hare have introduced a coculture model of transformed cells (macrophages and fibroblasts) and cartilage pieces, similar in some aspects to Hamerman's model (Janusz et al 1993). In this later model, transformed macrophages alone or in combination with transformed fibroblasts mediated proteoglycan release from cartilage pieces. Janusz and Hare's model indicated that these transformed cell lines were much easier to obtain than cells from patient samples, yet correlating this model with RA would be difficult, as cells specifically associated with pannus tissue were not analyzed.

Although the *in vitro* models mentioned above have positive aspects, development of a more representative *in vitro* assay for analysis of synovial fibroblast invasion into cartilage requires several components. These include: 1) synovial fibroblasts from RA pannus tissue and normal synovial membrane;

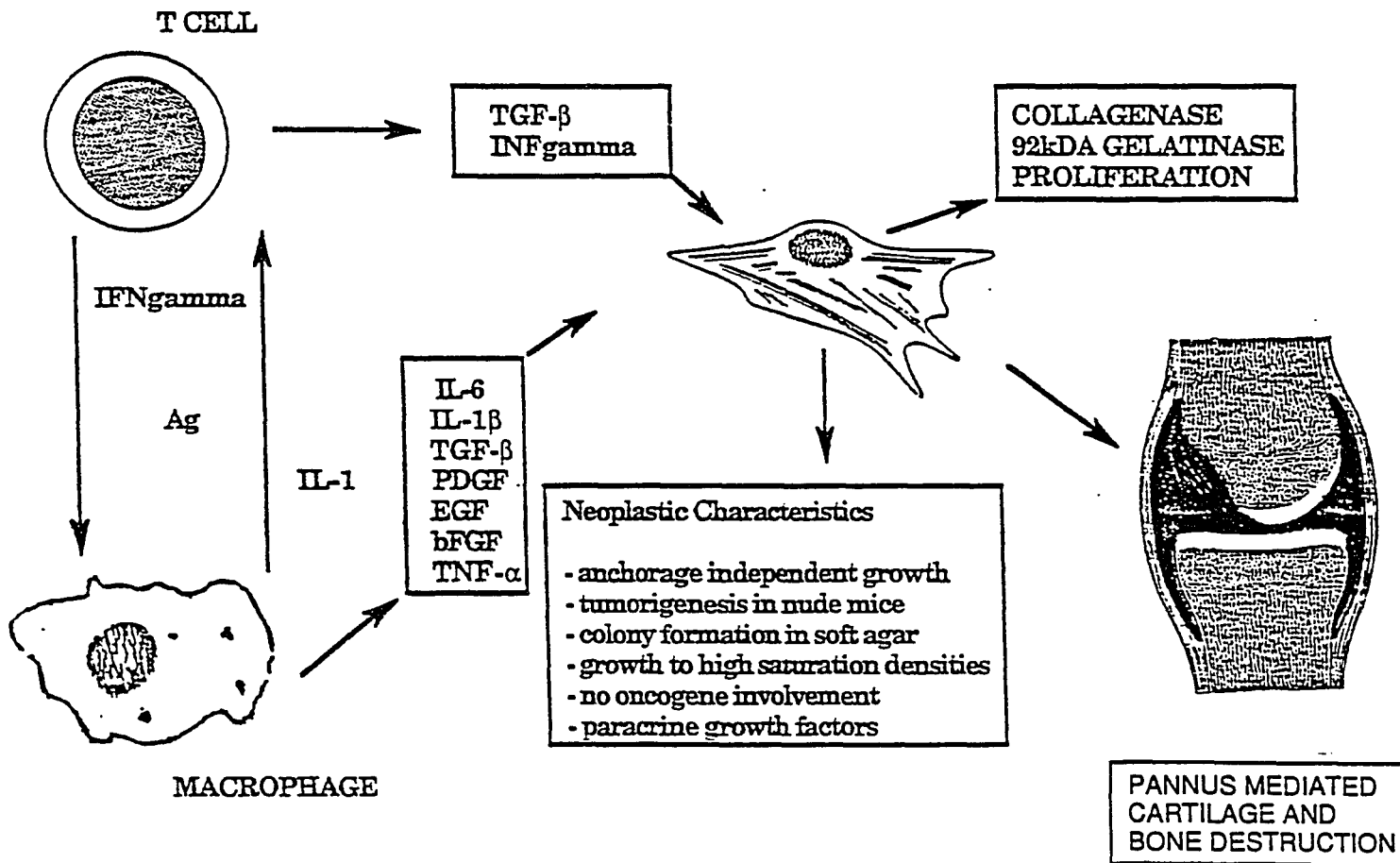
2) cartilage matrix; and 3) an assay period that would allow for reasonable manipulation of the system. Herein is described an *in vitro* assay for the study of RA synovial fibroblast invasion which incorporates all of these aforementioned components.

V. HYPOTHESIS STATEMENT

The overall goal of this project was to develop a reliable *in vitro* model that would allow observations related to the mechanism(s) underlying the ability of human synovial fibroblasts, isolated from rheumatoid arthritis pannus tissue, to invade and destroy cartilage matrix. This invasive phenotype is mediated in part by cell-matrix interactions, and in part by the upregulation of proteinases capable of degrading cartilage, which contributes to a catabolic state in the joint demonstrated as rheumatoid arthritis. As outlined in Figure 1, this project was designed to test the specific hypothesis that cytokines and growth factors, produced via the inflammatory immune response in rheumatoid arthritis patients, induce synovial fibroblasts to develop an invasive phenotype, which is demonstrable by the invasion and destruction of cartilage matrix.

Figure 1. Model diagram of hypothesis statement depicting the conversion of normal synovial fibroblasts to the RA invasive phenotype by exposure to inflammatory cytokines generated in the immune response associated with RA. The invasive phenotype is mediated in part by cell-matrix interactions and the upregulation of metalloproteinases capable of degrading cartilage.

Proposed Mechanism of Neoplastic-like Transformation of Synovial Fibroblasts



A. Isolation and characterization of synovial fibroblasts:

1. Isolation of cells: Synovial fibroblasts were isolated from rheumatoid arthritis pannus tissue and normal synovial membrane. Pannus tissue was obtained from patients with rheumatoid arthritis who were undergoing joint replacement surgery. Normal synovial membrane was obtained from patients undergoing joint arthroscopy, primarily for sports-related injuries, who did not have clinical evidence of rheumatoid arthritis. Fibroblasts were isolated as described previously by Yocum, et al. (1989). Tissue was minced to a fine consistency and washed in complete DMEM (Gibco BRL, Grand Island, NY) (10% bovine calf serum, 2% normal human serum, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin). Subsequently, the tissue was resuspended to a final concentration of 0.05 g/ml in complete DMEM containing 100 U/ml collagenase (Worthington Biochemicals, Freehold, NJ), and allowed to digest for 1-2 hours at 37°C in a 5% CO₂ humidified chamber. The partially digested tissue was washed in complete DMEM and inoculated as 0.2 gm in 4 ml into a T-75 cm² tissue culture flask (Costar, Cambridge, MA) and incubated without disruption for 7-10 days at 37°C in a 5% CO₂ humidified chamber. Synovial fibroblasts migrated from the minced tissue and grew as an adherent monolayer. Cells were collected with trypsin (Gibco BRL) and used prior to passage 6 (RA synovial fibroblasts) and passage 10 (normal synovial fibroblasts). Cultures were randomly demonstrated to be *Mycoplasma*-free by random testing with the Gen-Probe Rapid Detection System (Fisher, Pittsburgh, PA).

2. Characterization of synovial fibroblast cultures by immunofluorescence: Indirect immunofluorescence staining of vimentin was performed with DAKO mouse monoclonal anti-vimentin (diluted 1:20 in PBS) and rhodamine conjugated goat anti-mouse IgG (Cappel, Durham, NC). Synovial fibroblasts in complete DMEM were seeded at 50,000 cells per coverslip in a 6-well dish and allowed to adhere for 48 hr at 37°C in a 5% CO₂ humidified chamber. Coverslips were washed twice in room temperature phosphate buffered saline (PBS) and fixed in cold methanol for 5 min on ice, followed by air-drying and subsequent storage at -20°C. Coverslips were washed in PBS containing 0.5% BSA prior to overnight incubation with anti-vimentin antibodies at 4°C. These were then extensively washed in PBS containing 0.5% BSA, incubated with the secondary antibody, rhodamine conjugated goat anti-mouse IgG, for 1 hr in the dark, and washed in PBS containing 0.5% BSA.

Direct immunofluorescence staining of HLA-DR and CD11b was performed with Becton-Dickinson phycoerythrin conjugated anti-human HLA-DR (diluted 1:10 in PBS) and CD11b (diluted 1:10 in PBS). Human blood polymorphonuclear leukocytes (PMNs) were used as positive controls for surface molecules HLA-DR and CD11b. PMNs were plated on coverslips in 6-well dishes, and allowed to adhere for several hours. Synovial fibroblasts in complete DMEM were seeded at 50,000 cells per coverslip in a 6-well dish and allowed to adhere for 48 hr at 37°C in a 5% CO₂ humidified chamber. Coverslips were washed twice in PBS at room temperature, fixed for 10 min at room temperature in 3.7% formaldehyde/PBS, washed three times in PBS and stored in the final wash at 4°C. Coverslips were then washed in PBS containing

0.5% BSA prior to incubation with anti-HLA-DR or anti-CD11b antibodies overnight at 40C.

All coverslips were mounted on glass microscope slides with gelvatol mounting medium, and observations were photographed with a Zeiss standard 18 fluorescence microscope at various magnifications with an automatic rhodamine filter set and Zeiss 35 mm camera attachment using the manual exposure mode of the camera.

3. Cell Morphology: Coverslips in a 6-well dish were coated with cartilage matrix and allowed to air-dry. Synovial fibroblasts were seeded (50,000 cells in DMEM containing 10% NuSerum [Collaborative Research, Bedford, MA] per coverslip) and incubated 24 hr at 37⁰C in a 5% CO₂ humidified chamber. Cells were observed with a Wild M40 inverted light microscope and photographed with a Pentax 35 mm camera attachment at a final magnification of 125X.

B. Isolation and characterization of porcine cartilage matrix:

1. Extraction of hyaline cartilage: Hyaline cartilage was isolated from the joints of the front and rear legs (from the hip to the hoof) of 12-16 week old conventional Yorkshire cross pigs obtained from the Wilcox hog farm (Wilcox, Arizona). Cartilage scrappings were defatted in 1:1 (v:v) chloroform-methanol at a ratio of cartilage to solvent of 1:5 (w:v) for 2 hr at room temperature and air-dried to constant weight overnight to remove the solvent. Cartilage was extracted in 10 volumes of 4 M guanidine chloride (Sigma, St. Louis, MO), 0.05 M Tris (pH 7) and 5 mM each of iodoacetic acid (Sigma) and EDTA, pH 7, for 3 days at 40C with constant stirring. The extract was filtered through cheesecloth

and the insoluble portion re-extracted with the guanidine chloride solution twice over the next two days. The extractions were pooled, cleared by centrifugation and dialyzed against 10 mM ammonium bicarbonate and subsequently lyophilized and stored at -20°C until use (Gwande et al 1990, Syttestand et al 1984).

2. Biochemical analysis of cartilage matrix components: Cartilage matrix was resuspended in PBS and diluted in standard Laemmli gel sample buffer (Laemmli 1970) and electrophoresed in a 12.5% SDS polyacrylamide gel. The gel was stained with Coomassie blue (0.25% Coomassie blue R235, 25% isopropanol, 10% glacial acetic acid) for approximately 30 min and destained in 10% acetic acid, 10% methanol until the color stopped leaching from the gel. Proteoglycans were extracted from the cartilage extract by adjusting the solution to 5.2 M cesium chloride and centrifuged in a Beckman Ultracentrifuge rotor 41 at 35,000 rpm for 72 hr (Heinegard et al 1986). The gradient was divided into 4 equal fractions and electrophoresed in a 1.2% polyacrylamide-0.6% agarose gradient polyacrylamide gel and stained with Toluidine blue to detect proteoglycans (Carney 1986, Dische 1947, McDevitt et al 1971).

3. Morphological assessment of cartilage matrix:

a) Light microscopy: Cartilage matrix was spread on a 35 mm petri dish and allowed to air-dry. The matrix was stained with Coomassie blue and destained in 10% acetic acid, 10% methanol. The matrix was observed with a Wild M40 inverted light microscope and photographed with a Pentax 35 mm camera attachment at 125X final magnification.

b) Scanning electron microscopy: Cartilage extract was spread on a glass coverslip and allowed to air dry. The matrix was fixed in cacodylate-

buffered Karnovsky's fixative (Karnovsky 1965) followed by dehydration using a series of ascending grades of ethanol. The critical point drying method was used to dry the matrix (Porter et al 1973). The matrix was coated with gold palladium and observed in an Etec Autoscan electron microscope, operating at an accelerating voltage of 20 Kv.

C. Description of synovial fibroblast invasion model:

1. MICS chamber: The Membrane Invasion Culture System (MICS) assay was performed as previously described with slight modifications (Hendrix et al 1987). The chamber was composed of 14 contiguous wells that are traversed by a polycarbonate filter. The cartilage matrix was spread over each 10 μm pore polycarbonate filter (Poretics, Inc., Livermore, CA) and allowed to air-dry prior to assembling the chamber. The chamber was assembled by filling the lower wells to a positive meniscus with DMEM containing 10% NuSerum, covering the lower wells with the polycarbonate filter, positioning the filter matrix side up and then attaching the upper half of the chamber with screws. The upper wells contained 1 ml of DMEM containing 10% NuSerum. Synovial fibroblasts were seeded (1×10^5 cells per well) in the upper wells and the chamber incubated for 48 hr at 37°C in a 5% CO₂ humidified chamber. Fibroblasts which had invaded through the matrix-coated filter(s) were collected from the bottom chamber using trypsin and the side sampling ports in the chamber, which allowed direct access to the lower well. The collected cells were stained sequentially with Eosin stain and Wright's stain and counted using a Zeiss light microscope as previously described (Hendrix et al 1987). Each

culture of synovial fibroblasts was run in triplicate by inoculating into three or more wells of the chamber, and each experiment repeated at least twice.

Migration assays were performed as described for the invasion assays except the polycarbonate filters were soaked in a 0.1 mg/ml gelatin solution overnight prior to assembling a 48 well chamber. Selected cells were allowed to migrate for 6 hr; then the cells on the upper side of the filter that did not migrate were removed, the filter stained and migrated cells on the undersurface of the filter were counted as described for the MICS chamber.

2. Statistical analysis: Invasion is expressed as the percentage of cells seeded that were able to invade through the matrix. Statistical analysis consisted of standard T tests, standard error and standard mean calculated using the AbStat program for statistical analysis. Where indicated, normal synovial fibroblast invasion is arbitrarily set at 100%, and test samples are expressed as a percentage of normal or control values. In addition, the range of actual invasion values are provided when appropriate.

D. Evaluation of clinical activity:

Patients were scored blindly for the severity of their disease activity. This was based upon the primary rheumatologist's evaluation of each patient's clinical history and radiographic evaluation. The scoring system used is described as follows:

- 0 Very benign, slowly progressive course.
- +1 A slowly progressive course, with occasional episodes of inflammation.
- +2 A moderately aggressive course, requiring disease modifying drugs, but slowly progressive over 10 years or greater.

- +3 An aggressive course requiring disease modifying drugs early in the illness, but relative unresponsiveness to such treatment.
- +4 A very aggressive course in which all drugs have failed to induce remission, and the disease has been progressive over a period of 5 years or less with significant joint destruction.

The radiological score was based upon the viewing of X-rays taken prior to surgery. The scoring system developed was as follows:

- 0 A degenerative arthritis picture with spur formation and cartilaginous loss, but not bony erosions.
- +1 A degenerative process with evidence of spur formation, marked cartilaginous loss and very minimal erosions.
- +2 Clear cut erosive disease.
- +3 Moderate amount of erosion with periarticular osteopenia and a significant degree of destruction.
- +4 Extensive erosive disease with marked destruction of the joint as well as periarticular erosion and marked loss of cartilage.

E. Analysis of cell adhesion and spreading on cartilage matrix:

1. Adhesion assay: Bacterial 96-well plates (Costar) were incubated with 150 μ l of cartilage matrix per well overnight at 40C and subsequently washed 3 times in 200 μ l of PBS containing 1% BSA. Synovial fibroblasts (150,000) in DMEM containing 10% NuSerum were incubated in 500 μ l of 1 mg/ml peptide (glycine, arginine, glycine, glutamic acid, serine, proline [RGE] or glycine, arginine, glycine, aspartic acid, serine, proline [RGD] containing peptides) or

media alone as a control for 30 min. Each sample was divided into 3 wells so that each well contained 5×10^4 synovial fibroblasts. The 96-well plate was incubated for 1 hr at 37°C in a 5% CO₂ humidified chamber and extensively washed in PBS. The wells were stained with Toluidine blue for 1-4 hr and washed repeated with 200 µl aliquots of PBS until the color stopped leaching from the plate. The final wash of PBS was left in the wells and the plate analyzed on a Titertek Multiscan ELISA reader at 620 nm. Percent inhibition of binding was calculated by arbitrarily setting the ELISA value corresponding to the adherent cells of the media control at 100 and comparing with RGD and RGE incubated cells. Standard mean, standard error and student T-tests were performed using the AbStat program.

2. Spreading assay: Petri dishes (35 mm) were coated with the cartilage matrix and allowed to air-dry prior to inoculation of 50,000 synovial fibroblasts and subsequently incubated at 37°C in a 5% CO₂ humidified chamber. Representative fields were photographed with a Wild M40 inverted light microscope and a Pentax 35 mm camera attachment beginning with 30 min and ending at 24 hr post-seeding. The percentage of round cells was calculated by counting the number of cells maintaining a round morphology and dividing by the total number of cells in that field. A minimum of 17 cells per field were analyzed.

F. Analysis of proteolytic activity of synovial fibroblasts on cartilage matrix:

1. SDS-substrate polyacrylamide gel electrophoresis: Cell-free culture supernatants were obtained by inoculating 200,000 synovial fibroblasts in 500 µl of DMEM containing 10% NuSerum into a 24-well dish pre-coated with the

cartilage matrix. As a control, 500 μ l of DMEM containing 10% NuSerum were also cultured on the cartilage matrix without the addition of cells. The dishes were incubated at 37°C in a 5% CO₂ humidified chamber for 48 hr, the supernatants harvested and centrifuged to remove cells and cell debris. The supernatants were stored at -80°C until analysis. Cell-free culture supernatants were diluted 2:1 in sample Laemmli buffer without beta-mercaptoethanol (Laemmli 1970). Samples were not heat denatured since this assay depends on enzyme activity, and were electrophoresed in a 10% SDS polyacrylamide gel containing either 0.5 mg/ml alpha casein (Sigma) or 1 mg/ml gelatin (BioRad, Richmond, CA). Stromelysin activity was measured on gels containing alpha casein as the substrate while gelatinases A and B and interstitial collagenase activity were detected on gels containing gelatin as the substrate. The gels were electrophoresed in standard running buffer (0.1% SDS, 25 mM Tris, 192 mM glycine). Subsequently, the SDS in the gel was exchanged with Triton X100 by washing the gel twice for 15 min in 50 mM Tris, 2.5% Triton X100 (w:v), pH 7.4, to allow the proteins in the gel to renature. The gel was incubated for 24 hr at 37°C in activation buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl₂, pH 7.5), stained in Coomassie blue, and destained in 10% acetic acid and 10% methanol until the blue color stopped leaching from the gel. The presence of clear bands denoted active enzyme as the Coomassie blue detects undegraded substrate as a dark blue color.

2. Probe isolation: Plasmids were isolated using the Maxi and Mini Plasmid prep kits from Promega (Madison, WI). Plasmid pStrH13' (a kind gift from Dr. Lynn Matrisian, Vanderbilt University) contained a 600 bp *EcoRI/BamHI* insert of the human stromelysin cDNA (Muller et al 1988).

Plasmid pCollH3' (a kind gift from Dr. Lynn Matrisian, Vanderbilt University) contained a 407 bp *Xba*I insert of human interstitial collagenase cDNA (Muller et al 1988). Plasmid pH3a (a kind gift from Dr. William Stetler-Stevenson, National Cancer Institute, NIH) contained a 1.1 kpb *Eco*RI insert of the human gelatinase A (72 kDa gelatinase) cDNA (Levy et al 1991). Inserts were purified by DNA gel electrophoresis in 1% agarose in TBE buffer to separate inserts from vectors. Inserts were electrophoresed onto NA-45 membrane (Schleicher and Schuell) and rinsed in NET buffer (0.15 M NaCl, 0.1 M EDTA, 20 mM Tris, pH 8.0) to remove residual agarose. The insert was released from the membrane by incubation in high salt buffer (1 M NaCl, 0.1 M EDTA, 20 mM Tris, pH 8.0) for 2 hr at 68°C. The solution was pipeted away from the membrane and DNA was isolated by adding 1 volume of water and 1 volume of ethanol and precipitating at -20°C overnight. The precipitate was isolated by centrifugation for 15 min, washed in 70% ethanol, and air-dried. The DNA pellet was resuspended in 50 µl or less of TE and quantified by electrophoresis in 1% agarose in TBE and comparing the ethidium bromide stained band to a known concentration gradient of DNA. Inserts were radiolabelled via nick translation (Gibco BRL), and labelled insert was purified by loading the reaction mixture onto a G-50 resin column and the labelled insert peak isolated from the column by monitoring with a Geiger counter.

3. Northern blot analysis: Sixty mm dishes were coated with the cartilage matrix and allowed to air-dry. Subsequently, 1×10^6 synovial fibroblasts were added and the dishes were incubated for 48 hr at 37°C in a 5% CO₂ humidified chamber. The cells were lysed in 1.8 ml of 8 M guanidine chloride, 10% sarcosine solution, the lysate scraped into a corner of the dish

with a rubber policeman and collected using a 25 gauge needle and syringe. The lysate was divided equally between two eppendorf tubes and each sheared 3 times with the 25 gauge needle and syringe. An 125 μ l aliquote of 3 M sodium acetate, pH 5.2, was added to each eppendorf tube and the sample sheared 3 times with the 25 gauge needle and syringe. The sample was centrifuged at top speed for 20 min at 4⁰C, the supernatant collected (900 μ l from each tube) and combined with a half volume of ethanol (450 μ l). The sample was incubated for a minimum of 40 min at -20⁰C, then centrifuged for 30 min at 4⁰C to collect the nucleic acids. The sample was reextracted in 500 μ l of 8 M guanidine chloride, 3 M NaOAc pH 5.2 at 68⁰C. A half volume (250 μ l) of cold ethanol was added and the sample held at -20⁰C for a minimum of 40 min. The RNA was collected by centrifugation at top speed for 30 min at 4⁰C and washed in 70% ethanol for 5 min followed by a wash in 100% ethanol for 5 min. The sample was rehydrated in formaldehyde loading buffer (Sambrook et al 1989) and loaded onto a 1% agarose formaldehyde gel (0.75 gm agarose, 65 ml DEPC H₂O, boil and cool, 7.5 ml 10 X MOPS, 3.8 ml formaldehyde). The samples were electrophoresed at 100 V until the dye marker reached 80 cm from the wells and the gel was washed twice for 20 min in 10 X SSC prior to transfer in 10 X SSC to the nylon membrane GeneScreen Plus. The RNA was transferred by capillary transfer overnight in 10 X SSC and the membrane subsequently baked at 80⁰C for 1 hr to reverse the formaldehyde reaction, followed by UV crosslinking for 1200 sec.

The membrane was prehybridized in 50% formamide, 750 mM NaCl 75 mM NaCitate, pH 6.4, 5 X Denhardts, 50 mM NaPO₄, pH 6.5, 0.2% SDS, 250 μ g/ml ssDNA at 42⁰C overnight. Probes were incorporated into the

hybridization buffer (750 mM NaCl, 75 mM NaCitrate, pH 6.4, 1 X Denhardt's, 20 mM NaPO₄, pH 6.5, 0.2% SDS, 100 µg/ml ssDNA) as 5 x 10⁶ cpm per ml of hybridization solution. Hybridization was done overnight at 42°C with constant agitation. The blot was washed in 3 separate steps: 2-15 min washes in 2 X SSPE (0.3 M NaCl, 20 mM NaH₂PO₄, 2 mM EDTA, pH 7.4) room temperature, 2-45 min washes in 2 X SSPE, 1% SDS at 65°C, 2-15 min washes in 0.1 X SSPE at room temperature. Radioactivity was measured by exposing the blot to X-ray film (Konica Corporation, Tokyo, Japan) for 2-5 days with an intensifying screen.

F. Cytokine modulation of invasion:

1. Cytokine treatment of normal cells: Normal cells were removed from liquid nitrogen and inoculated into T-75 tissue culture flasks (Costar) and allowed to grow to 95% confluence and removed with trypsin. The cells were washed and inoculated equally between four T-75 tissue culture flasks and cultured with specific concentrations of selected cytokines or in complete DMEM as a control. TNF α (Genzyme, Cambridge, MA) was cultured with normal synovial fibroblasts at 10 U/ml and 150 U/ml. TGF β 1 (Genzyme) was used at 0.5 U/ml, 3 U/ml and 18 U/ml. IL-1 β (Genzyme) was used at 5 U/ml and 25 U/ml. PDGF was a kind gift from Amgen (Thousand Oaks, CA) and was used at 30 ng/ml and 100 ng/ml. The media was replaced every 3 days until the cells reached 80-90% confluence. Cells were harvested with trypsin for further analysis.

2. Analysis of cytokine receptors by immunofluorescence: Synovial fibroblasts (50,000 cells) were plated on glass coverslips and allowed to attach

and spread for 24-48 hr in complete DMEM with the appropriate cytokine(s). Coverslips were washed 3 times in PBS and fixed for 5 min in 3.7% formaldehyde/PBS. Subsequently, the coverslips were washed 3 times in PBS and stored in the last wash at 40C until use. Receptors for the specific cytokines were measured using indirect immunofluorescence microscopy. Polyclonal rabbit anti-human TGF β receptor type II (UBI, Lake Placid, NY), and monoclonal rat anti-human TNF α receptor, p80 (Genzyme) were used as primary antibodies. Secondary antibodies tagged with fluorochromes were used to detect primary antibody binding, namely, FITC-F(ab)'₂ fragment of rabbit anti-rat (Zymed, South San Francisco, CA) and R-PE-F(ab)'₂ fragment of goat anti-rabbit IgG (Zymed). Coverslips were washed in PBS containing 1% BSA, incubated with primary antibodies to cytokine receptors overnight at 40C, washed extensively in PBS containing 1% BSA, and incubated with the appropriate secondary antibody for several hours in the dark at 40C. The coverslips were observed with a Zeiss standard 18 fluorescence microscope at various magnifications with an automatic rhodamine filter set and photographed with a Zeiss 35 mm camera attachment using the manual exposure mode of the camera.

VII.

RESULTS

A: Development of an *in vitro* Rheumatoid Arthritis Invasion Model:

1. Isolation and Characterization of Synovial Fibroblasts:

Synovial fibroblasts were derived from rheumatoid arthritis pannus tissue and normal synovial membrane, which was collected from patients undergoing orthopedic surgery for joint replacement. Normal synovial lining was isolated from patients undergoing arthroscopic surgery for joint injury, and this tissue did not show any indications of arthritis. Isolation of synovial fibroblasts was performed as described in Materials and Methods. Adherent cells reached confluence roughly 3 weeks after plating the tissue in tissue culture flasks, and subsequent fibroblast cultures were used after passage 2 to insure the culture was devoid of macrophages, dendritic cells and inflammatory cells, but not after passage 6 to avoid problems with phenotypic drift.

To demonstrate the lack of immune cells in the cell cultures, immunofluorescence staining with antibodies to selected cell markers, vimentin (a cytoskeletal intermediate filament which is indicative of mesenchymal cell lineage), HLA-DR and CD11b (markers associated with immune cells and macrophages) was performed. Fibroblasts isolated from rheumatoid arthritis pannus tissue and normal synovial membrane stained positive for the intermediate filament vimentin and negative for HLA-DR expression as well as CD11b, as shown in Figure 2. As a positive control, blood derived polymorphonuclear leukocytes (PMNs) stained positive for HLA-DR and CD11b (data not shown). As a negative control, cells were stained with fluorescent tagged secondary antibodies only and were negative (Fig. 2).

To determine if the isolated cells were synovial fibroblasts, cell samples were analyzed morphologically on a cartilage matrix by inverted light microscopy, as shown in Figure 3. The cells grew as an adherent monolayer, and contained abundant cytoplasm and several nucleoli. Furthermore, there was no morphological difference between cells grown on plastic and cells seeded on cartilage matrix (data not shown).

2. Characterization of Cartilage Matrix:

To analyze the interaction of synovial fibroblasts with cartilage, a cartilage matrix invasion barrier was developed. Hyaline cartilage isolated from porcine joints was extracted in a 4 M guanidine hydrochloride solution and dialyzed against ammonium bicarbonate and subsequently lyophilized. The powder was resuspended in PBS for further analysis. As shown in Figure 4, collagen type II and other proteins can be detected on polyacrylamide gels stained for proteins with Coomassie blue. Dr. Rocky Tuan analyzed the cartilage extract for proteoglycans which was shown to contain 13.6% proteoglycans as determined from the uronic acid quantification. Further analysis of the proteoglycan content of the cartilage extract can be visualized in Figure 5.

To demonstrate the architectural "matrix" forming properties of the cartilage extract, a petri dish was coated with the extract, air dried and the morphology observed with light and high resolution scanning electron microscopy. Observations recorded with an inverted light microscope demonstrated the fibrillar nature of the matrix, as shown in Figure 6. Enhanced

resolution of the matrix with scanning electron microscopy (Fig. 6), demonstrated an array of interlocking and interweaving meshwork of fibrils.

3. Correlation of *in vitro* Synovial Fibroblast Invasion of Cartilage Matrix with Clinical Progression of Rheumatoid Arthritis:

To investigate the ability of synovial fibroblasts to invade the cartilage matrix, the Membrane Invasion Culture System, (MICS) (Hendrix et al 1987) was adapted to incorporate both the cartilage matrix and synovial fibroblasts. In this model, normal and RA synovial fibroblasts were able to invade through the cartilage matrix-coated filters in a 48 hr assay, as shown in Table 1.

To determine if a correlation existed between the *in vitro* synovial fibroblast invasion of the cartilage matrix and the patients' clinical progression, and medical histories were evaluated for RA disease progression as well as the extent of bone erosions at the time of surgery. According to this assessment, RA patients can be divided into low invasive and high invasive groups, which correlate with a mild form of RA and aggressive RA, respectively, as shown in Figure 7.

B: Mechanisms of Synovial Fibroblast Invasion of Cartilage Matrix:

1. Synovial Fibroblast Attachment and Spreading on Cartilage Matrix:

To determine if integrins were involved in synovial fibroblast attachment to cartilage matrix, an adhesion assay on cartilage was performed incorporating peptide inhibitors of integrin mediated attachment. Integrins are molecules which have the ability to mediate cell matrix interactions and require an arginine-glycine-aspartic acid (RGD) sequence in their ligands for binding to the

substrate (Hynes 1987). Peptides incorporating the RGD sequence compete with the ligand for binding and thus prevent cell association with the matrix. In this set of experiments, the ability of integrins to mediate synovial fibroblast association with the cartilage matrix was assayed using peptide inhibitors. As shown in Figure 8, peptides containing an RGD sequence inhibited both normal and RA synovial fibroblast attachment to cartilage matrix by 70%.

To analyze the ability of synovial fibroblasts to spread on the cartilage matrix, both normal and rheumatoid arthritis derived synovial fibroblasts were seeded on the cartilage matrix and photographed at specific time points post-plating. A representative of 2 experiments is shown in Figure 9, at 30 min post-plating, most cells have attached to the cartilage matrix; however, the majority of cells maintain a round morphology. After 2 hr post-plating, normal synovial fibroblasts are well into the spreading process, as indicated by the presence of dendritic-like processes extended by the cells. Rheumatoid arthritis synovial fibroblasts at 2 hr post-plating are less spread and maintain a more round morphology with 29% of RA-02 and 23% of RA-01 cells maintaining a round morphology compared with 17% of the normal synovial fibroblasts.

2. Analysis of Metalloproteinase Activity of Synovial Fibroblasts:

A: Zymography of Enzyme Activity:

To investigate the ability of synovial fibroblasts to secrete active matrix degrading metalloproteinases, cell free culture supernatants were measured by SDS-substrate gel electrophoresis. Gelatin containing gels demonstrated gelatinase A (72 kDa gelatinase) and gelatinase B (92kDa gelatinase) activity in supernatants from both normal and RA synovial fibroblasts, as shown in

Figure 10. Interstitial collagenase activity was observed in supernatants only from RA synovial fibroblasts (Fig. 10). Casein containing gels showed stromelysin activity in both normal and RA synovial fibroblast supernatants as shown in Figure 11.

B: Northern Blot Analysis:

To examine the level of gene expression of metalloproteinases in a quantitative assay, Northern blot analysis was performed. As shown in Table 2, both normal and RA synovial fibroblasts express stromelysin and gelatinase A, with no significant difference between the level of expression. Interstitial collagenase expression was restricted to RA synovial fibroblasts, though not all RA samples were expressing interstitial collagenase (i.e. RA-01) and it is worth noting that the low invasive RA sample, RA-03, produced less interstitial collagenase mRNA than the highly invasive RA-02.

C: Cytokine Modulation of Normal Synovial Fibroblast Invasion of Cartilage

Matrix:

1. Effect(s) of Specific Cytokines on Synovial Fibroblast Invasion:

To determine if specific cytokines contained in RA synovial fluid, can modulate the invasive ability of normal synovial fibroblasts, cells were cultured for 2 weeks in the presence of specific cytokines and subsequently analyzed in the invasion assay. The cytokines analyzed included IL-1 β , TNF α , TGF β and PDGF in concentration ranges found in RA synovial fluid. As shown in Figure 12A, IL-1 β at 5 U/ml and above increased the invasive ability of most normal synovial fibroblasts, and TNF α at 150 U/ml significantly reduced the invasive ability of normal synovial fibroblasts (Fig. 12B). TGF β at 18 U/ml enhanced the

invasive phenotype of normal synovial fibroblasts, and PDGF at either 30 ng/ml or 100 ng/ml did not modulate the invasive ability of most normal synovial fibroblasts, as shown in Figure 13.

To further analyze the effects of cytokines on the invasive ability of normal synovial fibroblasts, specific combinations of these cytokines were incubated with normal synovial fibroblasts. Table 3 shows that the IL-1 β and TGF β combination enhanced the invasive ability of one of the normal synovial fibroblast samples analyzed, while the TNF α and IL1 β combination inhibited the invasive ability of normal synovial fibroblasts. In contrast, the TNF α and TGF β combination had no effect on the invasive phenotype of the normal synovial fibroblasts.

2. Localization of Specific Cytokine Receptors on Synovial Fibroblasts:

To demonstrate the presence of cytokine receptors on normal synovial fibroblasts cultured in the presence of specific cytokines, immunofluorescence microscopy was performed. As shown in Figure 14, cells cultured in TNF α stained for TNF α receptors, and cells cultured in TGF β stained for TGF β receptors. Normal synovial fibroblasts stained positive for the same receptors; however, the cells cultured with cytokines stained more intensively.

Figure 2. Indirect immunofluorescence microscopy of representative staining of vimentin intermediate filaments in normal, N-03 (A), and RA synovial fibroblasts, RA-01 (B). Cells stained with only rhodamine conjugated IgG secondary antibody serve as a negative control for N-03 (C) and RA-01 (D). (Final magnification 3150X).

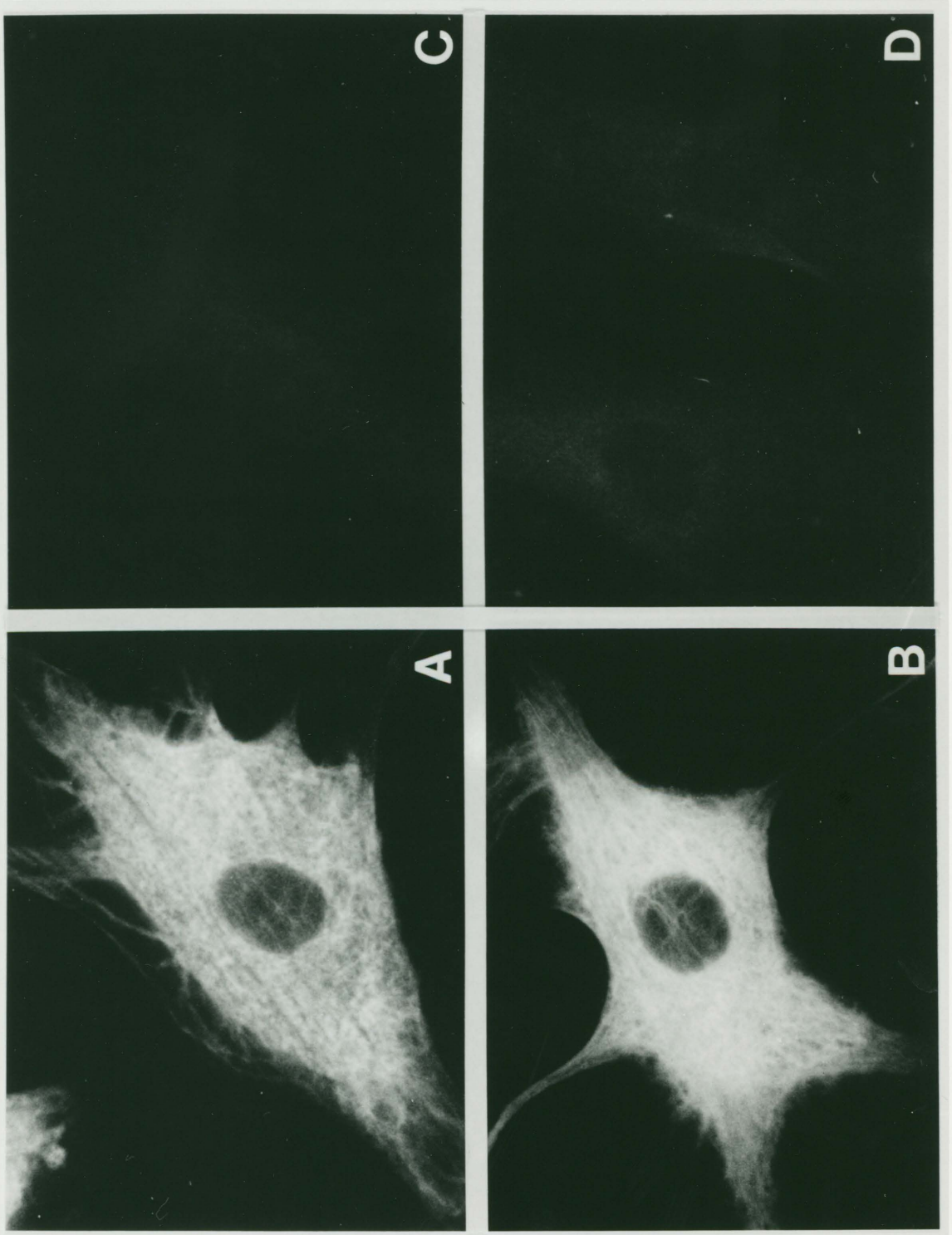


Figure 3. Inverted light microscopy photograph of normal synovial fibroblasts seeded on cartilage matrix. Cells were allowed to attach and spread for 24 hr on cartilage matrix. (Final magnification 500X)

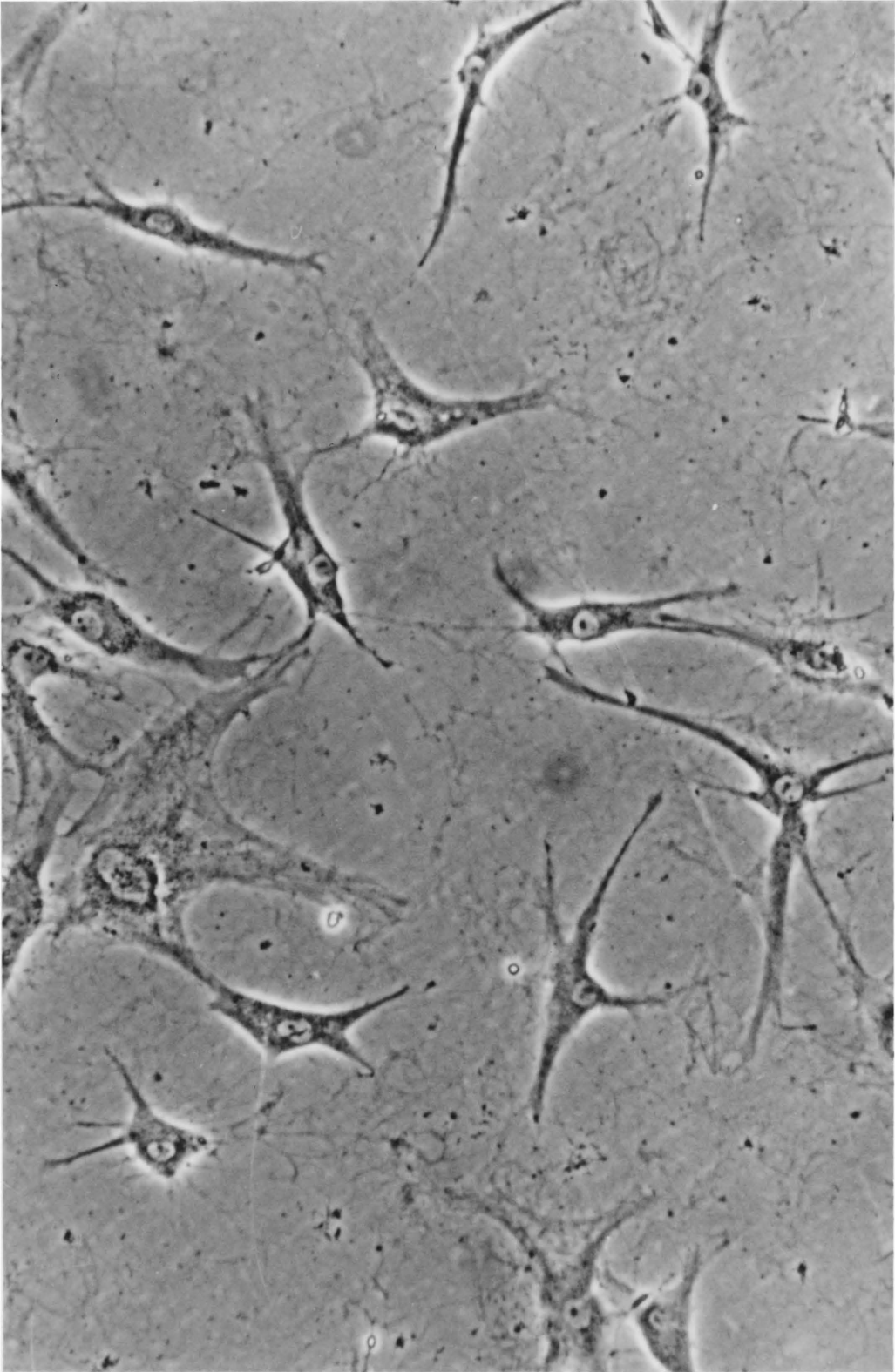


Figure 4. Coomassie blue stained 12.5% polyacrylamide gel of cartilage extract. Cartilage extract at a starting protein concentration of 1 mg/ml was serially diluted such that each lane contained a dilution of sample to loading buffer of: A) 1:1, B) 1:2, C) 1:3, D) 1:4, E) 1:5, F) 1:6, G) 1:7 and H) 1:8. The gel was electrophoresed under standard conditions containing SDS. Molecular weight size markers are labelled in kDa. The collagen alpha chain doublet is indicated by an arrow.

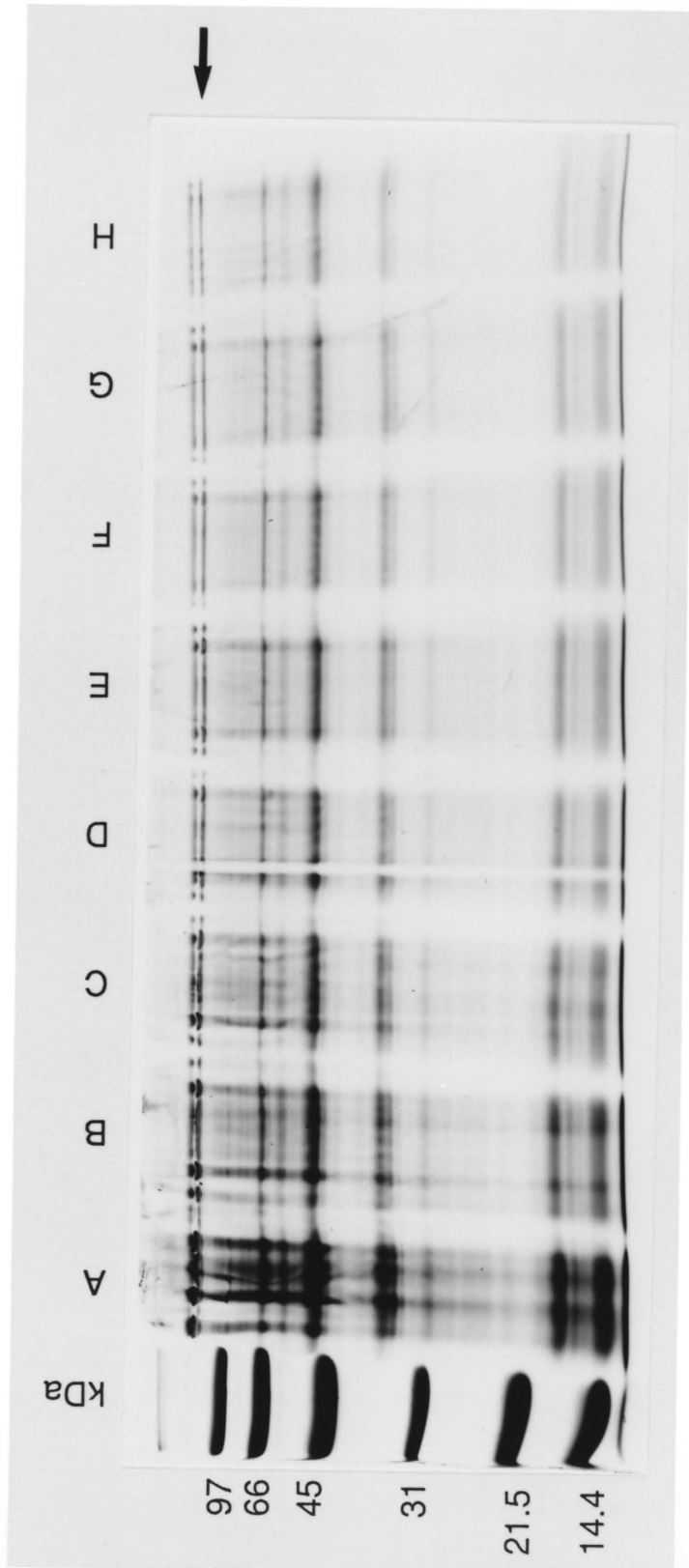


Figure 5. Toluidine blue stained 1.2% polyacrylamide-0.6% agarose gel of proteoglycans in CsCl density gradient fractionated cartilage extract. The CsCl fractions were dialyzed against NH_4CO_3 , lyophilized, subjected to uronic acid determination and adjusted to 1 mg uronic acid equivalent per lane. Lanes represent the four fractions of the cartilage extract: a) least dense CsCl fraction collected from the top of the gradient; b) directly below fraction a; c) directly above fraction d; and d) most dense CsCl fraction collected from the bottom of the gradient. Lane x represents a standard of bovine cartilage extract.

a b c d e f g h x

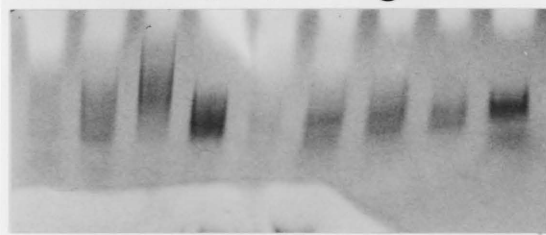
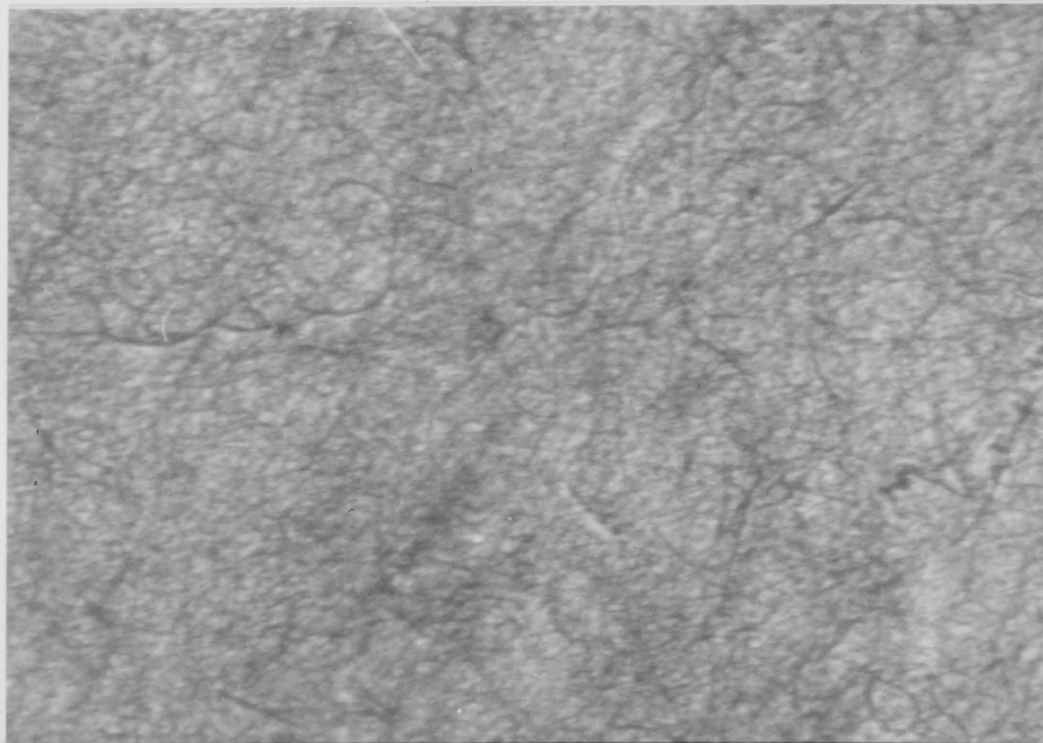


Figure 6. Morphological analysis of cartilage matrix. Inverted light microscopy of cartilage matrix coated onto a petri dish and subsequently stained with Coomassie blue (A). (Original magnification 125X) Scanning electron micrograph of a cartilage coated glass coverslip (B). (Final magnification 4375X).

A



B

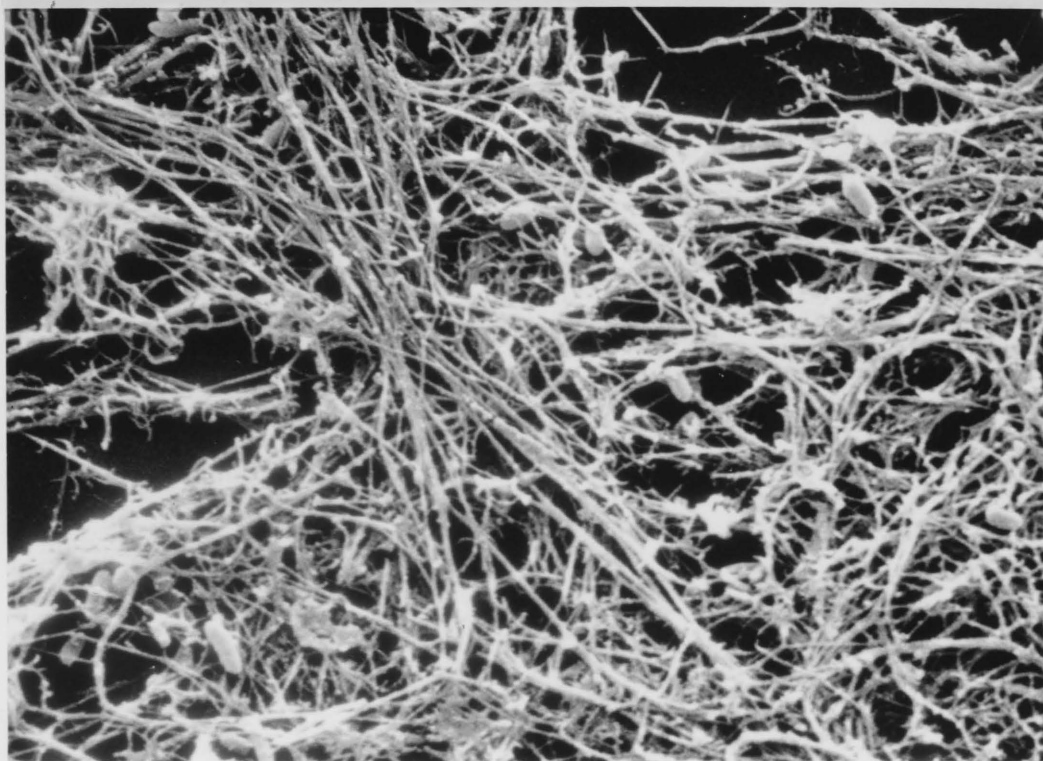


Table 1: Compilation of *in vitro* Invasion Data of Rheumatoid Arthritis (RA) vs Normal (N) Synovial Fibroblasts with Respect to Scores Derived from Associated Patient Clinical History.

Invasion through cartilage matrix was measured using the MICS assay in which RA and N synovial fibroblasts were analyzed for their ability to penetrate a cartilage-coated polycarbonate filter, containing 10 μm pores. Error is expressed as standard error of the mean of n=3 wells minimum of a representative experiment. Invasion rates are expressed as the percentage of cells loaded compared with the post-invasive cells collected from the undersurface of the filter. Scores were derived from patient evaluation(s) for clinical progression of disease and bone involvement at the time of surgery. Scores range from 0, mild disease activity and little bone involvement, to +4, aggressive disease with extensive bone involvement. Statistical analysis was performed by comparing the RA invasiveness to a paired normal synovial fibroblasts invasion.

* $p < 0.05$

** $p < 0.01$

A SAMPLE INVASION SCORE

RA-01	5.9%±0.4% ^{**}	3-4+ clinical 2-3+ bone
RA-02	5.8%±1.2% [*]	2+ clinical 2+ bone
RA-03	0.3%±0.2% ^{**}	1+ clinical 0 bone
RA-04	1.4%±0.2% ^{**}	0-1+ clinical 0 bone
RA-05	1.1%±0.1% [*]	high clinical 0 bone
RA-06	3.2%±0.2%	1-2+ clinical 3-4+ bone
RA-07	2.2%±0.3%	3+ clinical 3+ bone
RA-08	1%±0.07% ^{**}	Data not available
RA-09	1.4%±0.2% ^{**}	1+ clinical 0-1+ bone
N-01	3.4%±0.4%	Normal Tissue
N-02	2.7%±0.3%	Normal Tissue
N-03	2.2%±0.2%	Normal Tissue

Figure 7: Scatter graph of the RA synovial fibroblast invasion rates and combined score of clinical and bone evaluations as described in Table 1. (Statistical analysis by Spearman's Rank, $r= 0.795$).

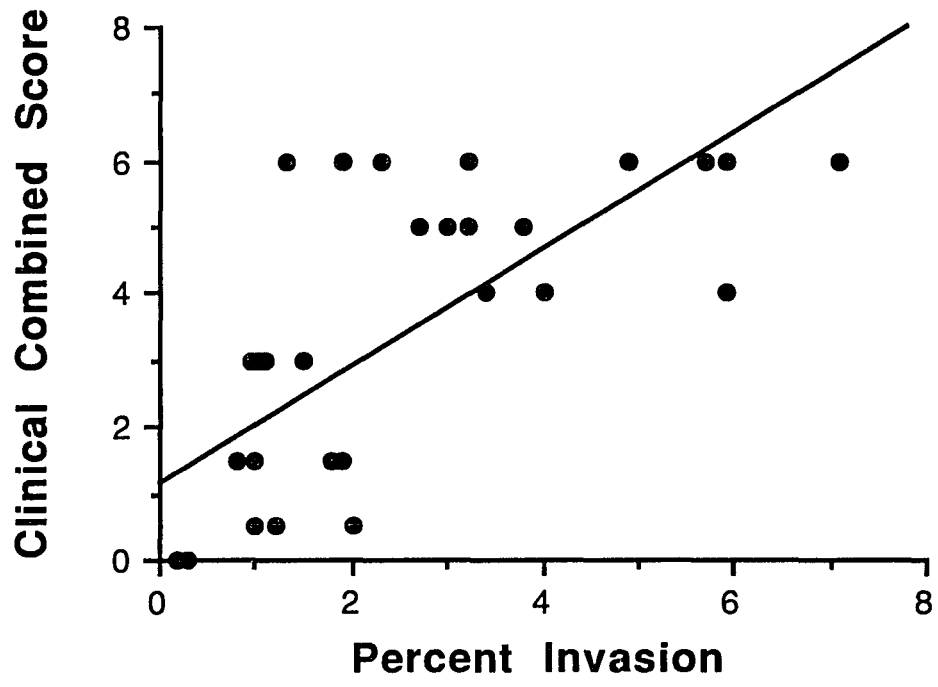


Figure 8: Bar graph demonstrating the effect(s) of RGD containing peptides on the ability of both normal and RA synovial fibroblasts to attach to cartilage matrix. Cells were incubated for 30 min in 1 mg/ml peptide prior to seeding onto the cartilage matrix for 1 hr. Unattached cells were washed away, and the attached cells stained with Toluidine blue and quantitated on an ELISA reader. Attachment values of control cells incubated with media alone were arbitrarily set at a value of 1, and the test values expressed in comparison to this control. RGE peptides were used as a control for peptide binding. Statistical analysis was performed by comparing the peptide incubated cells with the media control.

* $p < 0.05$

** $p < 0.01$

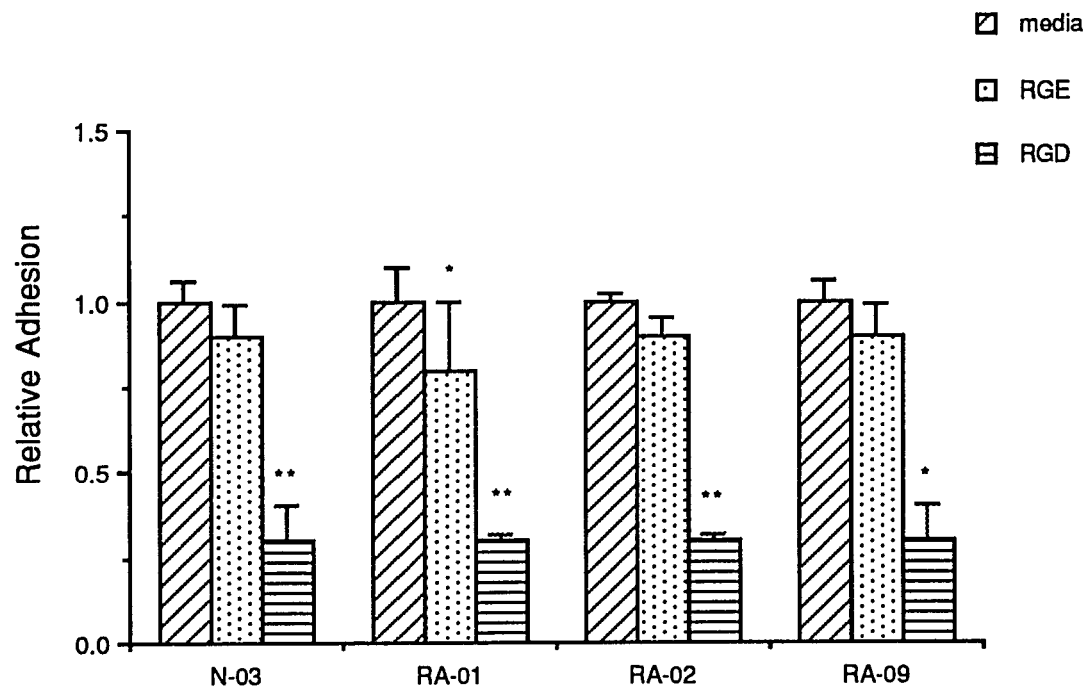


Figure 9: Morphology of normal (N-03) and rheumatoid arthritis (RA-01, RA-02) synovial fibroblasts on cartilage matrix. Phase-contrast micrographs of synovial fibroblasts after 30 min and 2 hr of attachment and spreading on cartilage matrix are compared. At 30 min all samples are composed of 100% round cells. At 2 hr, 17% of N-03 are round, 29% of RA-02 are round, and 23% of RA-01 are round, as determined by counting the number of round cells in two fields and dividing by the total number of cells. A minimum of 17 cells per sample were counted and the experiment repeated with similar results. (Final magnification 250X).

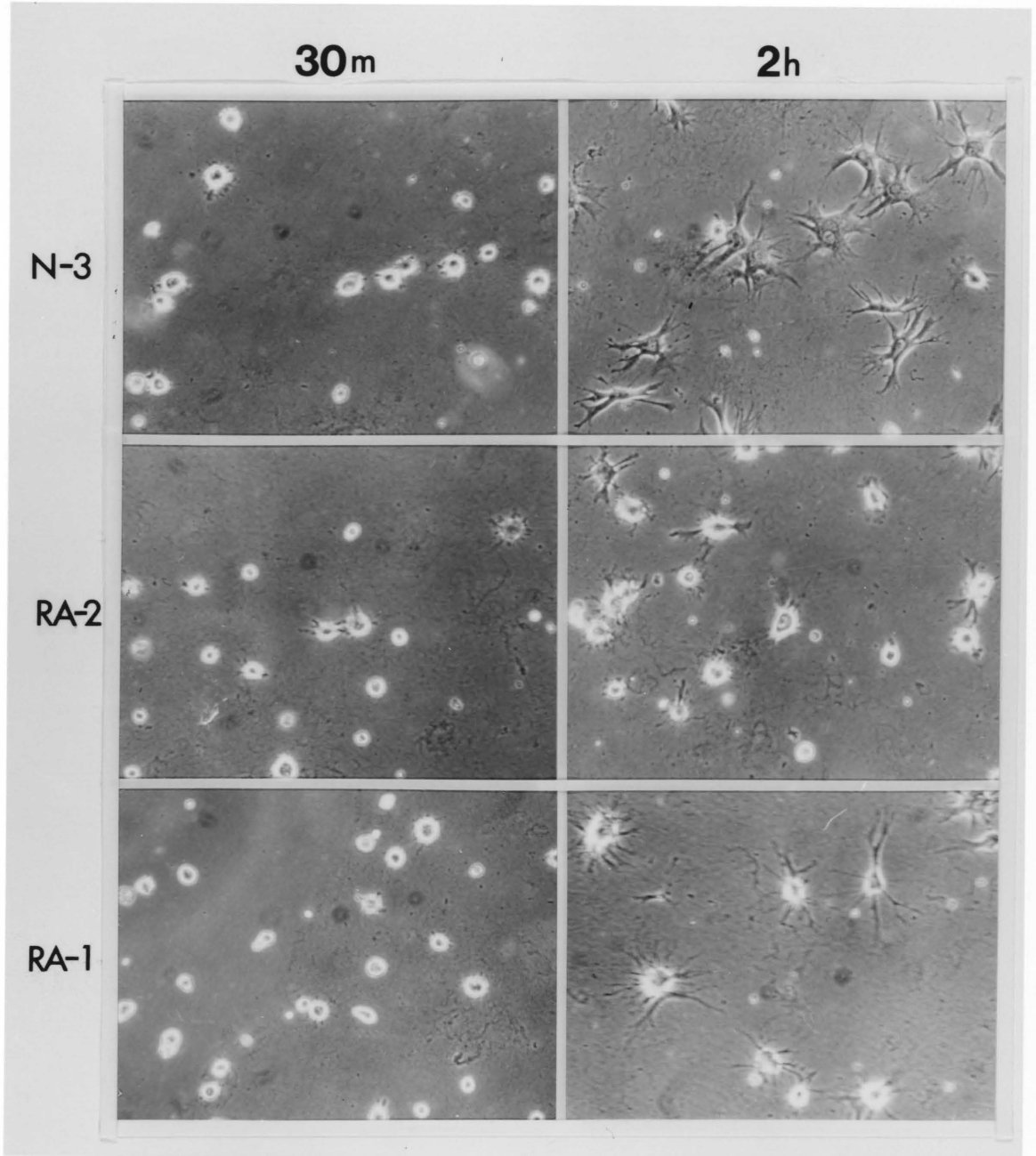


Figure 10: Zymographic analysis of gelatinolytic activity of gelatinase A (A), gelatinase B (B), and interstitial collagenase (C) from 48 hr supernatants of normal and RA synovial fibroblasts cultured on cartilage matrix. Control consists of media supernatant from cartilage matrix without cells. Enzyme activity is denoted by clear zones where the substrate contained in the gel has been degraded and is not stained by Coomassie blue.

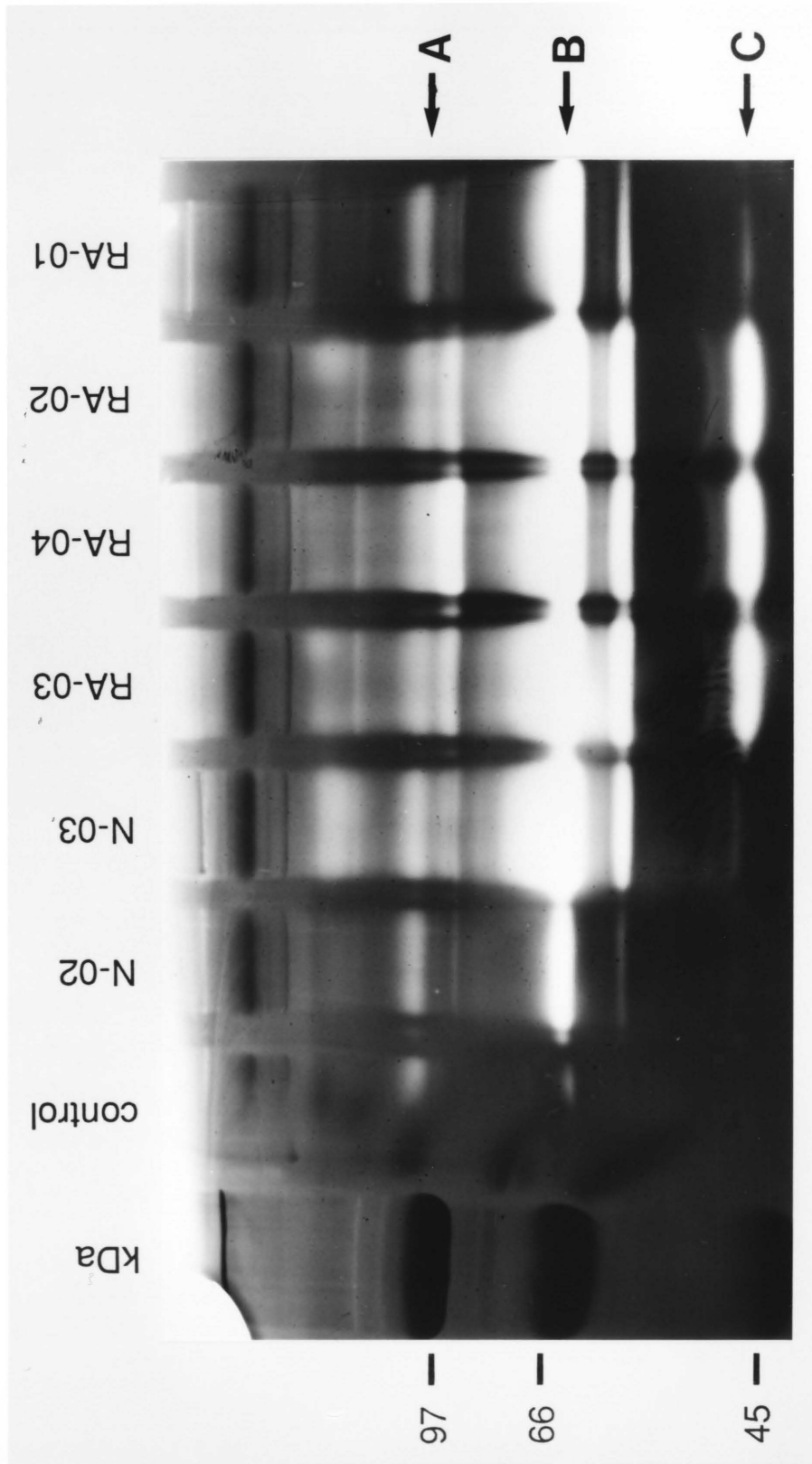


Figure 11: Zymographic analysis of stromelysin activity, indicated by an arrow, from 48 hr supernatants of normal and RA synovial fibroblasts cultured on cartilage matrix. Control consists of media supernatant from cartilage matrix without cells. Enzyme activity is denoted by clear zones where the substrate contained in the gel has been degraded and is not stained by Coomassie blue.

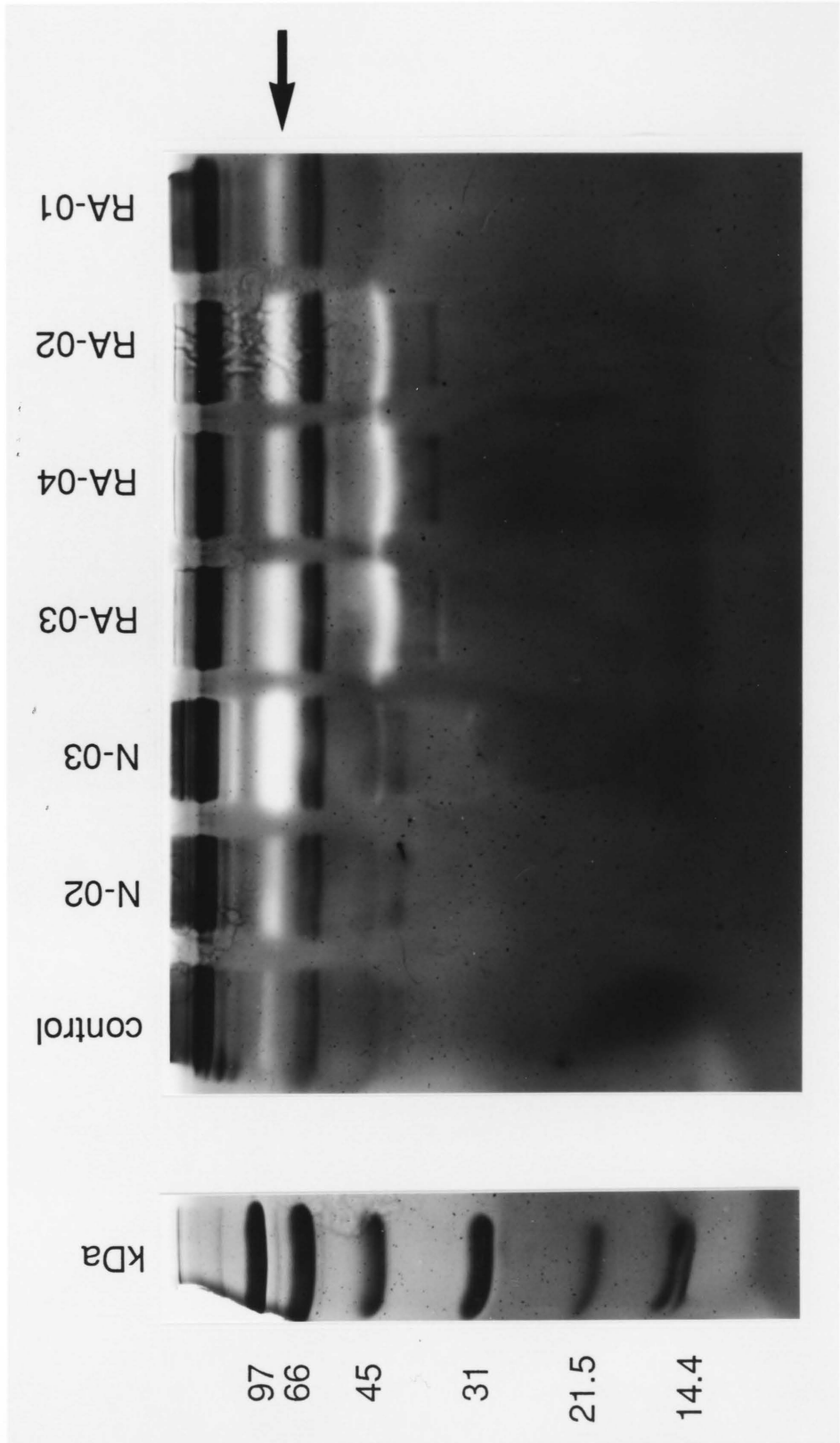


Table 2: Densitometric Scanning of Northern Blot Analysis of Metalloproteinase Gene Expression.

Densitometric scanning analysis of Northern blots of gelatinase A, interstitial collagenase and stromelysin gene expression in normal and RA synovial fibroblasts. RNA was isolated from 48 hr cultures of cells grown on cartilage matrix and fractionated in a formaldehyde gel, capillary blotted to nylon membranes and probed with ³²P-labeled probes for each of the metalloproteinase genes. Relative values derived from densitometric scans are expressed as a ratio of metalloproteinase signal to glyceraldehyde dehydrogenase (GAP) signal as a measure of equal loading. ND = not done

SAMPLE	INTERSTITIAL COLLAGENASE	GELATINASE A	STROMELYSIN I
RA-01	-0-	5.14	ND
RA-02	0.81	4.47	0.96
RA-03	0.51	ND	0.92
N-01	-0-	ND	0.92
N-02	ND	3.06	0.79
N-03	ND	4.11	0.88

Figure 12: Bar graphs demonstrating percent invasion of normal synovial fibroblasts incubated with varying concentrations of IL-1 β (A) and TNF α (B) measured in the MICS invasion assay during a period of 48 hr using a cartilage matrix. Standard error bars are expressed as standard error of the mean of n=3 wells minimum per sample. The minimum number of cells counted per bar graph was above 150. Statistical analysis was performed by comparing the cytokine incubated cells with untreated cells.

* p<0.05

** p<0.01

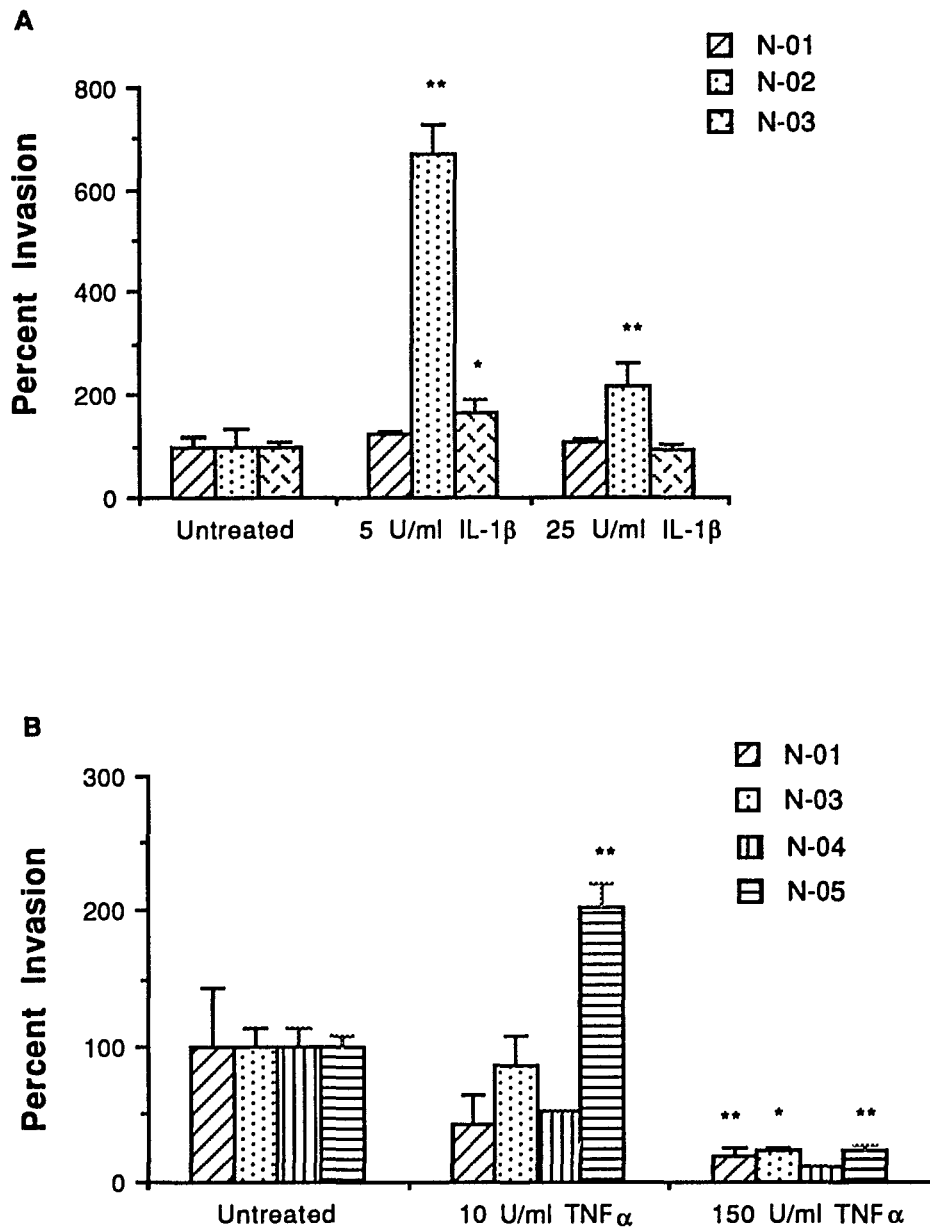


Figure 13: Bar graphs demonstrating percent invasion of normal synovial fibroblasts incubated with varying concentrations of TGF β (A) and PDGF (B), measured in the MICS invasion assay during a period of 48 hr using a cartilage matrix. Standard error bars are expressed as standard error of the mean of n=3 wells minimum per sample. The minimum number of cells counted per bar graph was above 150. Statistical analysis was performed by comparing cytokine incubated cells with the untreated control.

* p<0.05

** p<0.01

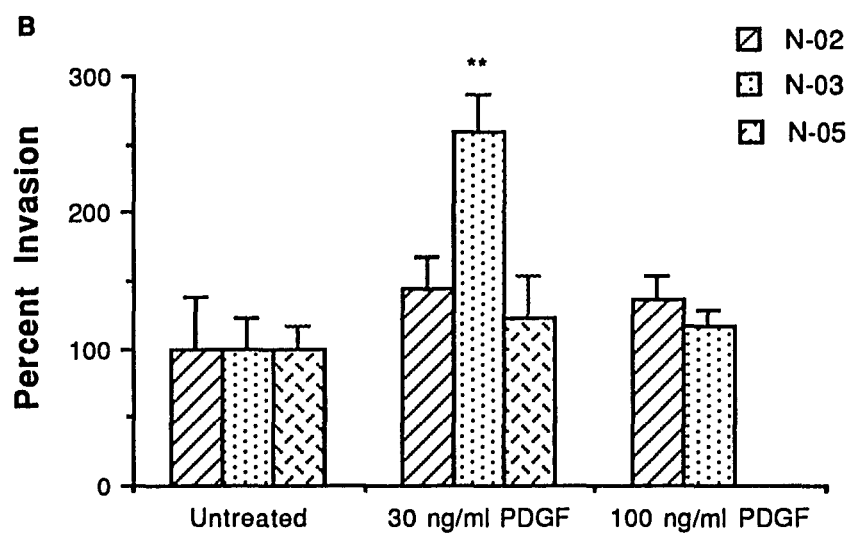
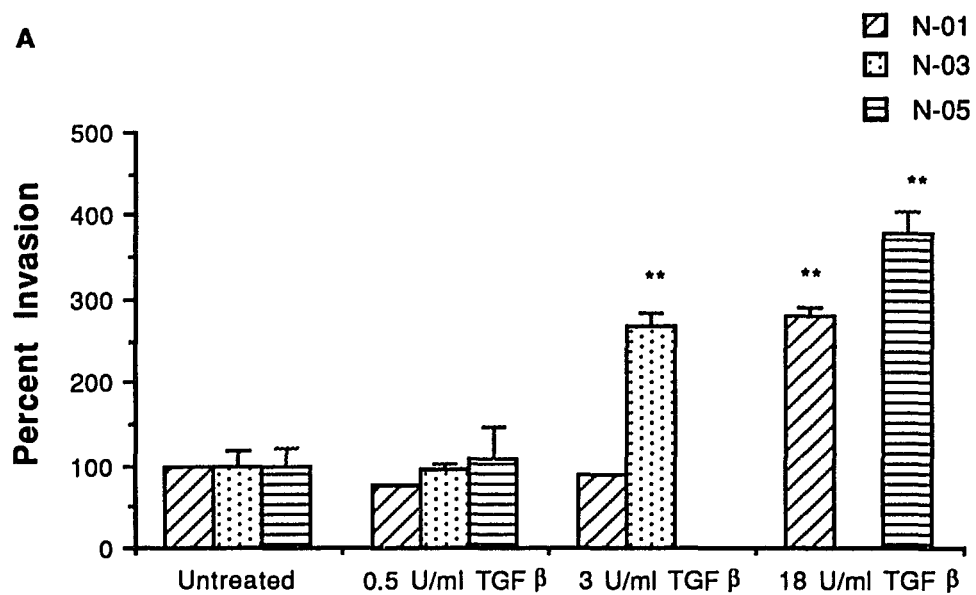
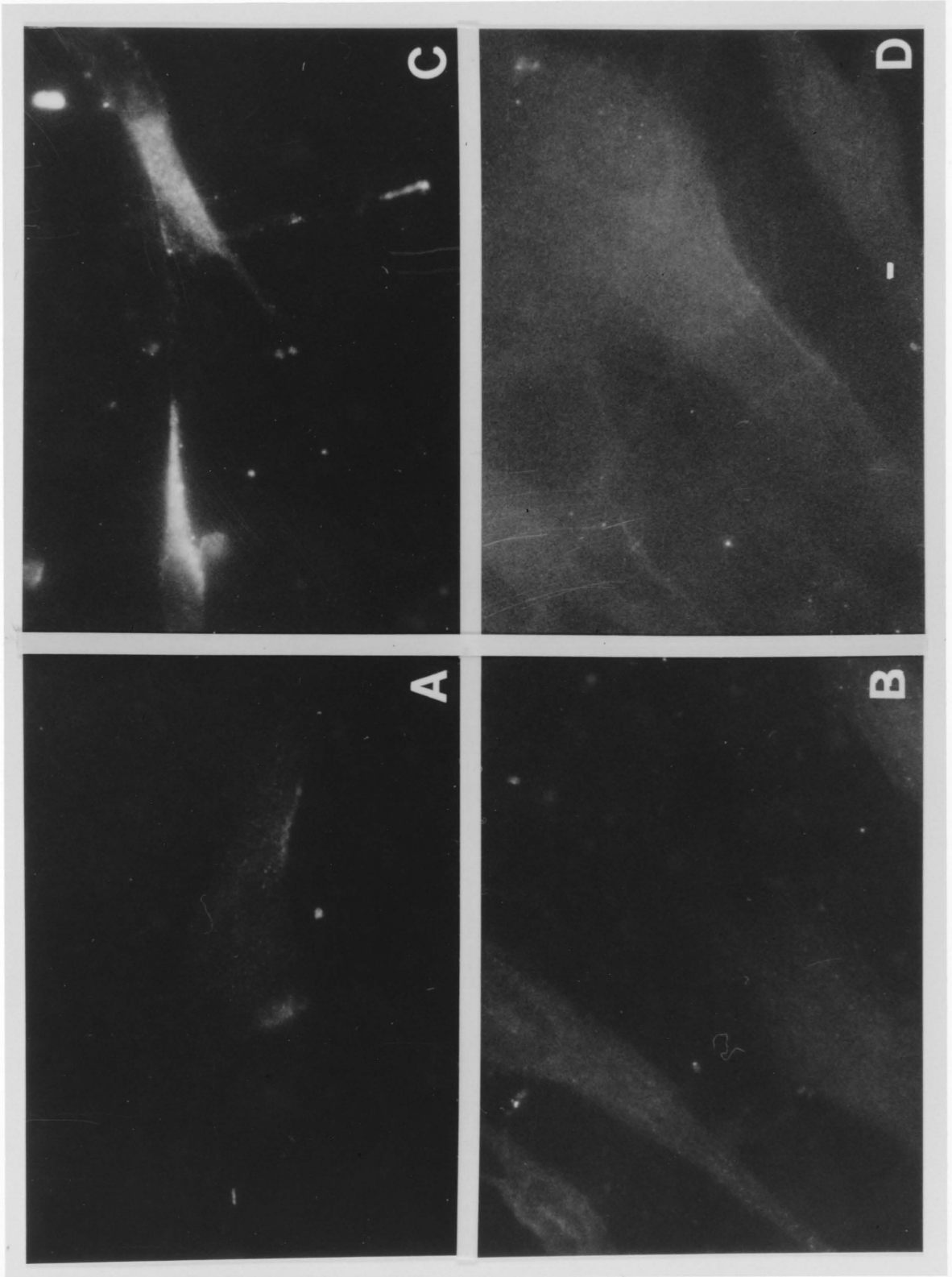


Table 3: Cytokine Combination Effects on Normal Synovial Fibroblast Invasion

Compilation of the percent invasion of normal synovial fibroblasts, incubated with specific combinations of cytokines, measured in the MICS invasion assay during a period of 48 hr using a cartilage matrix. Untreated normal synovial fibroblast invasion was arbitrarily set at a value of 100, and the test values compared to this control. Error is expressed as standard error of the mean of n=3 wells minimum per sample. ND = not done

Percent Invasion			
COMBINATION	N-05	N-03	N-01
18 U/ml TGF 5 U/ml IL-1	81	189 ± 31	ND
150 U/ml TNF 5 U/ml IL-1	13	47	44
18 U/ml TGF 150 U/ml TNF	75 ± 17	67 ± 15	ND
UNTREATED	100 ± 8	100 ± 19	100 ± 19

Figure 14: Indirect immunofluorescence microscopy of cytokine receptors on normal synovial fibroblasts; stained for TGF β receptors (A) or TNF α receptors (B). Normal synovial fibroblasts cultured with 18 U/ml TGF β ; stained for TGF β receptors (C), or cultured with 150 U/ml TNF α and stained for TNF α receptors (D). (Final magnification 2380X).



VIII.

DISCUSSION

Pannus tissue has been shown histologically to degrade and invade cartilage and has *in vivo* growth characteristics described as tumor-like (Harris 1976). Synovial fibroblasts isolated from pannus tissue also maintain characteristics associated with neoplastic cells *in vitro* (Lafyatis et al 1989). This dissertation describes the development of a clinically relevant, *in vitro* model which is representative of the pannus-cartilage junctions *in situ*. Specifically, biological observations can be made related to cell-matrix interactions and secretion of metalloproteinases which may contribute to the invasive nature of RA synovial fibroblasts. Furthermore, the role of inflammatory cytokines in the conversion of quiescent normal synovial fibroblasts into the invasive RA synovial fibroblast phenotype was also investigated.

The *in vitro* model is composed of synovial fibroblasts, cartilage matrix, and the Membrane Invasion Culture System (MICS), originally developed for the analysis of tumor cell properties associated with the metastatic phenotype (Hendrix et al 1987). The cellular component of the model, synovial fibroblasts, were isolated from normal synovial membrane and rheumatoid arthritis pannus tissue, and demonstrated morphological characteristics of synovial fibroblasts isolated by other investigators including a large cytoplasm, dendritic-like processes and nucleoli (Fig. 3) (Fassbender 1983, Trabandt 1992). In addition, synovial fibroblasts used in this study expressed markers associated with mesenchymal lineage cells, and did not express surface markers associated with immune cells, as demonstrated by immunofluorescence microscopy (Fig.

2). Based on these observations, the cells isolated and cultured from pannus tissue and synovial membrane contained virtually 100% synovial fibroblasts.

The matrix component of the invasion model involved the isolation and characterization of a cartilage extract from porcine hyaline cartilage. Polyacrylamide gel electrophoresis of the cartilage extract demonstrated the presence of the major constituents of cartilage; collagen II and proteoglycans (Figs. 4 and 5) (Heinegard et al 1987). Quantification of uronic acid, which is a measure of proteoglycans, indicates the extract contains 13.6 % proteoglycans, which correlates with intact cartilage that contains 5-10 % proteoglycans (Heinegard et al 1989). Cartilage is composed of a number of constituents in addition to collagen type II and proteoglycans including; link protein, biglycan, decorin, fibronectin (Heinegard et al 1989) as well as multiple types of collagen such as type IX (Ye et al 1991) and XI (Mendler et al 1989). Hence, the cartilage extract used in this study is very similar in composition to intact cartilage, with the exception of the lack of collagen type X.

In the development of the RA model, it was essential to show that the cartilage extract has matrix forming properties. The morphological characteristics of the cartilage extract were investigated with light microscopy and scanning electron microscopy, which revealed an interweaving and interlocking array of microfibrils (Fig. 6). This woven matrix was subsequently incorporated into the invasion assay as a cartilage matrix barrier.

Multiple *in vitro* invasion assays have been developed incorporating a myriad of matrices, and are associated primarily with the analysis of tumor cell invasiveness (Hendrix et al 1987, Siegal et al 1993). For example, the matrix barriers used in tumor cell invasion assays reflect the extracellular matrix

involved in tumor cell extravasation from blood vessels and frequently involves the use of Matrigel (commercially available through Collaborative Research), a reconstituted basement membrane matrix secreted by Engelbreth-Holm-Swarm (EHS) murine tumor cells (Kleinman et al 1986). An *in vitro* assay system described by Hendrix and colleagues (1987) incorporates a Matrigel matrix in a 72 hr assay analyzing melanoma tumor cell invasion. A similar matrix derived from human amnions from the placenta, Amgel, has also been incorporated in *in vitro* invasion assay systems and provides the advantage of being derived from a human source (Siegal et al 1993). Collagen I gels containing tissue specific cells can also be used to analyze tumor cell invasion. In this later study, human lung fibroblasts were incorporated into a collagen I gel with eosophageal tumor cells, and the subsequent invasion by tumor cells into the gel was analyzed by sectioning the gel and quantifying the degree of invasion by tumor cells (Doki et al 1993).

Each of the systems identified above incorporates matrices or host factors similar to those encountered by tumor cells *in vivo*. In a similar vein, this study has generated a cartilage matrix which is reflective of cartilage joint tissue *in vivo* and allows for the analysis of RA synovial fibroblast invasion of this matrix *in vitro*.

Cartilage matrix and synovial fibroblasts were incorporated in the *in vitro* invasion assay, MICS, and both normal and RA synovial fibroblasts were invasive across the cartilage barrier (Table 1). The ability of normal synovial fibroblasts to invade through cartilage matrix was not an unexpected result since fibroblasts have been shown to have matrix modifying characteristics that would enable these cells to degrade matrix components (Murphy et al 1990).

Based on the invasion analysis, RA synovial fibroblasts can be divided into roughly two groups; cells of low invasive potential versus high invasive potential. A clinical evaluation of both patient history and bone involvement indicated that the highly invasive RA samples correlated with both an aggressive clinical disease and extensive bone involvement, as demonstrated by RA-01 and RA-02, while a low invasive rate correlated with a mild form of RA (e.g. RA-04). However, this clinical correlation was not 100%, as indicated by RA-07, which exhibited a mid-low range of invasion ($2.2 \pm 0.3\%$) *in vitro*, while the clinical evaluation indicated a fairly aggressive disease with mid-range bone involvement. One explanation for this lack of correlation includes a phenomenon known as "burn-out". In this study, synovial fibroblasts were isolated from end stage disease in which the joint has been extensively damaged by RA with the deformity resulting in loss of motion. At the time surgery, the inflammation may be completely gone, as the disease has culminated in joint destruction (Fassbender 1983). Without the stimulating effects of the inflammatory immune response, the aggressive nature of pannus tissue can be obliterated. Synovial fibroblasts isolated from such "burn-out" tissue may reflect this condition by exhibiting a lower invasive potential. This hypothesis could be addressed by obtaining tissue from recently diagnosed RA patients, where the pannus tissue is metabolically active and would be far from the terminal stage of "burn-out".

This *in vitro* invasion model was developed to analyze synovial fibroblast mediated cartilage destruction. Other components of the pannus tissue or rheumatoid joint, including cells such as macrophages or T cells and inflammatory mediators, were not present in this assay system; however, this

relatively simple model could be adapted to incorporate other cell types and/or inflammatory mediators. These modifications, in essence, would create an *in vitro* joint for further investigation into the pathology of RA and the invasive nature of pannus tissue.

The process of invasion has been described and partially characterized in relation to tumor cell metastasis, and can be considered as a series of steps; beginning with attachment to the basement membrane, followed by dissolution of the membrane, and culminating in cellular movement through the degraded matrix (Liotta et al 1991). Applying these basic principles governing tumor cell invasion to the study of synovial fibroblast invasion provided a framework for determining key factors underlying RA joint destruction. Specifically, the steps of adhesion, spreading and production of matrix degrading enzymes were examined in relation to normal and RA synovial fibroblast invasion through cartilage matrix.

In the initial step of adhesion, integrins were considered, which are cell surface glycoproteins demonstrated to mediate cell-matrix interactions (Postigo et al 1993). In this study, integrins were shown to be involved in synovial fibroblast-cartilage interactions by incorporating a peptide inhibitor of integrin activity in a cartilage matrix adhesion assay. Both normal and RA synovial fibroblast adhesion to cartilage matrix was inhibited by peptide inhibitors of integrins (RGD containing peptides) while and RGE containing peptide had no effect. This indicated that both cell types use integrins in their adhesion to cartilage matrix (Fig. 8).

Several cell adhesion molecules have been shown by immunohistochemical staining to be upregulated in the RA synovium, including

CD31 (PECAM), CD44 (hyaluronate receptor) and the $\beta 3$ integrin subunit (Johnson et al 1993). RA synovial fibroblasts stained positive for CD44, which functions as a hyaluronate receptor and may be important in synovial fibroblast adhesion to cartilage which contains hyaluronic acid. Lymphocytes, endothelium and synovial lining cells from RA synovial tissue express the $\beta 1$ integrin subunit in association with VLA antigens (El-Gabaway et al 1993), which bind to several components of the extracellular matrix (Ruoslahti et al 1989) and have also been associated with lymphocyte association with endothelial cells (Schimizu et al 1992).

Cell adhesion molecules LFA-3 and ICAM-1 have been shown by immunohistological staining to be present on synovial fibroblasts (Hale et al 1989); however, these adhesion molecules are associated with cellular interactions involving immune cells rather than matrix components. RA and normal synovial fibroblasts express the $\alpha 6$ integrin subunit and possibly the $\beta 1$ subunit, which together can form a laminin receptor which could bind laminin, a component of the extracellular matrix, which may be important in cellular interactions in the normal synovial membrane (Nikkari et al 1993).

Several cell adhesion molecules have been identified on the RA synovial fibroblast; however, it is unclear at this time how these molecules contribute to the fibroblast interaction with cartilage. In this study, it was shown that integrins in general are involved in the synovial fibroblast-cartilage matrix interaction.

An analysis of the subsequent process of cell spreading showed that normal and RA synovial fibroblasts maintain different spreading kinetics on cartilage matrix. At 30 min post-plating, normal and highly invasive RA synovial fibroblasts have attached to the matrix, but both cell types maintain a round

morphology. By 2 hr post-plating, normal synovial fibroblasts were well into the spreading process, as demonstrated by the extension of pseudopodia, while the highly invasive RA synovial fibroblasts had a greater percentage of round cells (Fig. 9). Maintaining a round morphology has been associated with a more invasive phenotype, as demonstrated by B16-F1 melanoma cells in which cells grown in a spherical morphology demonstrated more lung metastasis than cells grown as a flat monolayer (Raz et al 1983). In addition, cells expressing dual intermediate filaments have been shown to maintain a more round morphology, which correlates with enhanced migration and invasion through an extracellular matrix barrier (Chu et al 1993). Maintaining a round morphology may be an important mechanism for highly invasive RA synovial fibroblast invasion.

While both normal and highly invasive RA synovial fibroblasts were able to attach to the cartilage matrix through integrins, as well as non-integrin type receptors, the subsequent process of spreading on the cartilage matrix indicates the two cell types may display either different profiles of adhesion molecule(s) receptors or variations in receptor clustering that mediate the spreading process or receptor concentration. Further investigation into the types of adhesion molecules on normal and RA synovial fibroblasts may prove helpful in identifying molecules involved in the adhesion of pannus to cartilage.

Another step in the invasion process includes the degradation of a matrix barrier. In the study of tumor cell metastasis, the barrier consists of extracellular matrix components; however, in this invasion model, the barrier consisted of cartilage matrix. Metalloproteinases are secreted enzymes capable of degrading the extracellular matrix components and have been shown to contribute to tumor cell metastasis (Ossowski 1992). In this study,

specific metalloproteinases including interstitial collagenase, stromelysin, gelatinase A and gelatinase B, were investigated in relation to synovial fibroblast invasion of cartilage matrix. Normal and RA synovial fibroblasts plated on the cartilage matrix produced the matrix degrading metalloproteinases; stromelysin, gelatinase A and gelatinase B, while interstitial collagenase expression was associated solely with RA synovial fibroblasts (Figs. 10 and 11). Normal synovial fibroblast expression of matrix metalloproteinases was not surprising since fibroblasts are matrix modifying cells and matrix metalloproteinases serve a normal function (Murphy et al 1990).

Interstitial collagenase activity of pannus tissue was associated with disease activity in a study of 24 RA patients (Lazarus et al 1968). In the present study, interstitial collagenase enzyme activity, as measured by SDS-substrate gels and Northern blot analysis, was associated with the RA phenotype, which is in agreement with Lazarus and colleagues. Interstitial collagenase activity did not correlate with the invasive phenotype of the synovial fibroblast, as both low and high invasive RA derived cells produced interstitial collagenase. Northern blot analysis indicated that highly invasive RA-02 expressed more interstitial collagenase mRNA than the low invasive RA-03, yet this difference is not significant (Table 2). In addition, the highly invasive RA-01 did not produce detectable levels of interstitial collagenase message or enzyme activity. Hence, the role of interstitial collagenase in synovial fibroblast invasion was unclear from this study, but may contribute to the RA phenotype. Further study needs to be done to clarify this interesting observation.

Matrix metalloproteinase production has been associated with signal transduction through the cell adhesion molecules, integrins. Rabbit synovial fibroblasts have been shown to upregulate expression of interstitial collagenase and stromelysin by cross-linking of the fibronectin receptor (an integrin molecule that binds to fibronectin, an ubiquitous component of the extracellular matrix) with antibodies or fibronectin fragments (Werb et al 1989). In addition, melanoma tumor cells have been shown to express gelatinase A in response to antibody mediated activation of the vitronectin receptor (integrins are involved in binding to the extracellular matrix component vitronectin), which subsequently augmented their invasiveness through a basement membrane barrier (Sefter et al 1992). It is interesting to note that the extracellular matrix and fragments thereof, mediate gene expression in cells and may be an important aspect in the production of matrix metalloproteinases in RA. However, further studies are necessary to address this speculation.

The process of RA synovial fibroblast invasion is a complex series of steps. A specific factor associated with one of these steps cannot be attributed to the highly invasive RA synovial fibroblasts. Cell spreading on the cartilage matrix indicated that highly invasive cells maintained a more round morphology on cartilage matrix than less invasive normal synovial fibroblasts. This difference in spreading suggests a difference in molecules involved in cell-matrix interactions; however, this area has remained uninvestigated. Enzyme production has been shown to be important for matrix dissolution, but is not the only component involved in the invasive process in this *in vitro* assay. Previous investigators have made the connection between adhesion molecules and the secretion of metalloproteinases involved in the invasive process. Further

investigation into the ability of cartilage to induce expression of metalloproteinases, and specifically fragments of cartilage components, may provide insight into the regulation of enzyme production involved in RA. The process of invasion involves a series of steps, and differences in several of these steps may translate into a phenotypic difference measured as a more invasive cell.

The *in vitro* invasion model allows for analysis of factors important in the generation of the RA invasive phenotype, specifically the role of inflammatory cytokines present in the rheumatic joint (Westacott et al 1990). In this study, whether the specific inflammatory cytokines, IL-1 β , TNF α , TGF β and PDGF, could convert normal synovial fibroblasts to the highly invasion RA synovial fibroblast phenotype was investigated. The data revealed that IL-1 β , TNF α , TGF β and PDGF alone and in combination affect normal synovial fibroblast invasion of cartilage matrix. IL-1 β (Fig. 12A) and TGF β (Fig. 13A) alone induced invasion, TNF α (Fig. 12B) suppressed the invasive phenotype of normal synovial fibroblasts, while PDGF (Fig. 13B) had no effect on the invasive ability.

Previous studies demonstrated that IL-1 β can induce collagenase production in human RA synovial fibroblasts (Dayer et al 1986), which may contribute to the invasive nature of normal synovial fibroblasts exposed to IL-1 β . However, the present studies were unable to detect an increase in interstitial collagenase enzyme activity in cell free culture supernatants of normal synovial fibroblasts cultured with IL-1 β using SDS substrate gel electrophoresis (data not shown). The mechanism for IL-1 β modulation of invasion is unclear at this time but may involve metalloproteinase production.

TGF β induced an invasive phenotype in normal synovial fibroblasts. Previous work has strongly associated TGF β with the production of matrix components, even to the detriment of the host, as in scar formation or fibrous tissue formation (Waltenberger et al 1993, Border et al 1992), and has been shown to repress the production of collagenase in cytokine stimulated cells (Edwards et al 1987). Conversely, TGF β induced expression of gelatinase A in human gingival fibroblasts (Overall et al 1989) and contributes to the pathology of RA, as demonstrated by intra-joint injections of TGF β promoting the inflammatory conditions in the SCW rat model (Wahl et al 1991). In this study, TGF β promotion of the invasive phenotype of normal synovial fibroblasts supports the previous observations of Wahl and colleagues in the SCW rat model.

Normal synovial fibroblasts cultured with high concentrations of TNF α were much less invasive than the untreated normal synovial fibroblasts. In light of previous work in which antibodies to TNF α significantly reduced the severity of collagen-induced arthritis in mice (Williams et al 1992), these results were somewhat surprising. TNF α has also been shown to induce collagenase production in RA synovial fibroblasts (Dayer et al 1985), which might contribute to an invasive phenotype. On the other hand, the role of TNF α in suppressing tumor growth and metastasis has been established in a study of tumor cells genetically altered to express TNF α , which formed small tumors and no metastases (Oliff et al 1987), thus suggesting a role for this cytokine in the suppression of metastatic behavior. The role of TNF α in enhancing the immune response is well characterized as T cells demonstrate an enhanced proliferative response to IL-1 β when exposed to TNF α (Beutler et al 1989), and TNF α

enhances the T cell response to antigenic challenge (Troppmair et al 1988). TNF may contribute to the inflammation of RA, thus generating other factors involved in the generation of the invasive pannus tissue.

In this study, PDGF did not enhance the invasive phenotype of normal synovial fibroblasts. PDGF has been associated with the induction of proliferation of fibroblasts (Endresen et al 1992, Remmers et al 1991). It is important to note that proliferation and invasion are very different processes and that proliferating cells may not necessarily be very invasive. Proliferation is important in the development of pannus tissue but may not contribute to the invasive characteristics of this tissue. PDGF did not stimulate invasiveness in glioblastoma tumor tissue (Engebraaten et al 1993), which correlates with the observations that normal synovial fibroblasts incubated with PDGF do not demonstrate an invasive phenotype on cartilage matrix.

A preliminary analysis of combinations of cytokines indicate that the suppressive effects of $\text{TNF}\alpha$ on invasion dominate the stimulating effects of $\text{IL-1}\beta$ with an overall decrease in the invasive ability of normal synovial fibroblasts cultured in $\text{TNF}\alpha$ and $\text{IL-1}\beta$ (Table 3). Synovial fluid is composed of a variety of cytokines, and observations of cytokines acting alone or in limited combinations does not reflect the *in situ* environment of an inflamed joint. On the other hand, it is important to note how the combinations affect cell behavior and it would be interesting to further investigate the addition of other cytokines to this combination and observe the resulting invasive phenotype of normal synovial fibroblasts. It is not unexpected that cytokines in combinations will affect normal synovial fibroblast differently than cytokines alone.

The effect of the $\text{TNF}\alpha$ and $\text{TGF}\beta$ combination treatment suggests that the cytokines were antagonistic with respect to the invasive phenotype with the end result of no modulation (Table 3). This observation was not unexpected as $\text{TGF}\beta$ has been shown to suppress the activity of other cytokines (Edwards et al 1987) and has shown suppressive effects on inflammation as demonstrated in mice containing a null mutation of the $\text{TGF}\beta$ gene, which concurrently suffered excessive inflammation and early death (Kulkarni et al 1993). It is hypothesized that the signals transduced in the normal synovial fibroblasts by the $\text{TNF}\alpha/\text{TGF}\beta$ combination were antagonistic with the end result being little modulation of invasion.

Both $\text{IL-1}\beta$ and $\text{TGF}\beta$ induced invasion when cultured separately with normal synovial fibroblasts, and a combination of these cytokines appeared to also induce invasion, but not in a synergistic manner (Table 3). It was expected that this cytokine combination would strongly promote the invasive ability of normal synovial fibroblasts. It can be postulated that the mechanisms involved in the induction of invasion by the separate cytokines actually antagonize each other, as the cytokine combination did not change the invasive ability of normal synovial fibroblasts. Synovial fluid is composed of many inflammatory mediators in varying concentrations. It is important to remember that the assays incorporated in this study create a somewhat artificial environment by simplifying the synovial fluid to just a few factors. However, these analyses provide insight into the effects of cytokines alone and can be complicated by the incorporation of multiple factors that may be more reflective of synovial fluid. For instance, the combination of $\text{TNF}\alpha$, $\text{IL-1}\beta$ and $\text{TGF}\beta$ may modulate the

invasive ability of normal synovial fibroblasts differently than the cytokines alone or in pairs.

It would be expected that inflammatory mediators would not only promote the invasive activity of synovial fibroblasts, as demonstrated with IL-1 β and TGF β , but would also down regulate the invasive nature of the pannus tissue, as for TNF α . An analysis of cytokine receptors indicated that normal synovial fibroblasts maintain a low level of cytokine receptors on the cell surface; however, these receptors stained more intensely after the cells were exposed to cytokines, indicating a cellular response to inflammatory mediators (Fig. 14). It would not be surprising that the damage to cartilage by the pannus tissue invokes cytokines important in down regulating the invasive process. It has been shown that specific cartilage related molecules can be isolated from the blood of patients with RA and that these degradation products of cartilage can be associated with the degree of joint inflammation in these patients (Poole et al 1990). Understanding how these peptides affect the active immune response in the rheumatic joint may provide insight into how cells respond to the degradation of cartilage and other factors important in the regulation of the invasive pannus tissue.

It is important to remember that RA is a disease process that takes years to culminate in joint destruction. This work allows for further investigation with earlier RA tissue to elucidate both mechanisms of invasion as well as factors important in the generation of the pannus tissue. This study describes the development and utilization of a clinically relevant invasion assay reflecting the *in situ* environment of pannus-cartilage junctions. An analysis of mechanisms associated with the process of invasion revealed that cell-matrix interactions

involved in the adhesion and spreading of synovial fibroblasts on cartilage matrix may contribute to the highly invasive RA phenotype. A further analysis of metalloproteinase production indicated both normal and RA synovial fibroblasts secreted metalloproteinases, and that specifically interstitial collagenase may contribute to RA joint destruction. In addition, the invasive ability of normal synovial fibroblasts was modulated by specific cytokines known to be present in the synovial fluid of RA patients. This model allows for further analysis of the invasive phenotype of synovial fibroblasts and the mechanisms associated with cartilage destruction in this disease.

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