THE ROLE OF BASIC FIBROBLAST GROWTH FACTOR (FGF-2) IN RHEUMATOID ARTHRITIS

by

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Contents

To My Uncle Wang Guozong

and

My Family -- My Paretns and My Sister.

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List of Abbreviations

CD	Cluster determinant.
CIA	Collagen-induced arthritis.
Col.	Collagen.
DJD	Degenerative joint disease.
FGF-2	Fibroblast growth factor-2 or basic fibroblast growth factor.
FN	Fibronectin.
GF	Growth factor.
GM-CSF	Granulocyte/macrophage colony-stimulating factor.
HLA	Human leukocyte antigen.
IFN-gamma	Gamma interferon.
IL	Interleukin.
Lam.	Laminin.
MCs	Mast cells.
MHC	Major histocompatibility complex.
MMP	Matrix metalloproteinase.
PDGF	Platelet-derived growth factor.
PG	Proteoglycans.
PMN	Polymorphonuclear neutrophils.
RA	Rheumatoid arthritis.
RF	Rheumatoid factor.
TGF-beta	Transforming growth factor beta.
TIMP	Tissue inhibitor of metalloproteinase.

Abstract

Rheumatoid arthritis (RA) is an immunologically mediated disease of unknown etiology characterized by chronic articular inflammation that leads to the destruction of the afflicted joint. It is one of the principal crippling joint diseases. Because of its tendency to disable without killing, it belongs at the top of the list of chronic diseases from the stand point of social and economic importance. The lack of knowledge about the etiology and disease mechanism(s) together with the heterogeneous nature of this disease impose tremendous difficulties in its diagnosis and treatment.

Increasing evidence has implicated polypeptide growth factors (GFs) and cytokines in RA. Prior to the start of my thesis work, our laboratory had demonstrated that cultured synovial fibroblasts synthesize one such GF, basic fibroblast growth factor (FGF-2) and proliferate in response to it. Based on this and other related observations, it was hypothesized that FGF-2, by the virtue of its potent angiogenic and mitogenic activities, plays a direct role in RA. This hypothesis is the subject of my thesis work. I have taken 3 main steps to test this hypothesis.

The first step was to detect FGF-2 and define its distribution in rheumatoid synovium. Using immunohistochemistry, immunoblot and Northern blot analyses, I showed that FGF-2 was expressed locally in synovial tissue and its distribution pattern was altered in a subset of rheumatoid synovium. The second step was to seek possible correlation between FGF-2 expression and pathological changes during articular inflammation. Using a well-established animal model, I was able to show that FGF-2 was localized at the site of joint destruction and exhibited tight temporal and spatial association with the disease course. These results strongly suggest that FGF-2 may be directly involved in the destructive articular inflammation. In the third step, an attempt was made to directly assess the role of FGF-2 during destructive articular inflammation in

the experimental animal model by modulating the disease processes with exogenous FGF-2 and a GF antagonist, suramin. This study has shown that administration of suramin exacerbated articular inflammation while continuous intravenous infusion of exogenous FGF-2 ameliorated the disease. Although the exact mechanism(s) underlying these changes await further elucidation, these observations directly implicate FGF-2 in the destructive articular inflammation.

As an extended part of the endeavor to understand the role of FGF-2 in RA, efforts have also been made to identify the cellular source(s) and potential cell target(s) of FGF-2. Two studies have been carried out to re-evaluate a fundamental question regarding the relative contribution of macrophage-like and fibroblast-like synoviocytes to synovial hyperplasia and joint destruction. Results from these studies revealed that synovial fibroblasts undergo active proliferation and contribute significantly to synovial hyperplasia, suggesting cellular responses to increased levels of GFs. The search for the cellular source of FGF-2 has led to a novel observation that mast cells serve as a major source of FGF-2 in several chronic proliferative diseases including RA, pulmonary fibrosis, nasal polyposis, and cutaneous hemangioma. A subsequent study using cultured murine mast cell lines has not only confirmed this observation but also presented evidence suggesting that FGF-2, despite the lack of a conventional sequence for secretion, can be released by mast cells without detectable cell injury or cell death.

I. Introduction and Background

I.-1. What is Rheumatoid Arthritis ?

Rheumatoid Arthritis is an Immunologically Mediated Disease

Rheumatoid arthritis (RA) is an immunologically mediated, chronic systemic inflammatory disorder of unknown etiology. It is characterized by chronic articular inflammation that leads to the destruction of cartilage and bone in the afflicted joint. Although research seeking a viral or bacterial agent that causes RA has failed to identify the etiology of RA, the attempt to seek correlation of such agent(s) with RA has provided insight to the mechanism of the pathogenesis of RA. There remains little doubt that RA is an immunologically mediated disease. Several lines of evidence support this notion:

Genetic:	Strong association with HLA-DR4, familial aggregation and concordance in monozygotic twins.
Pathologic:	Lymphoid cell infiltration and follicle formation in rheumatoid synovium.
Clinical:	General improvement following treatment with immune-suppressive drugs such as corticosteroid and methotrexate.
Immunologic:	Local synthesis of IgG rheumatoid factor (RF) by plasma cells; presence of IgG, IgM, and complement components in synovium and at the site of tissue damage; presence of antigen-antibody complexes in synovial fluid; frequent presence of autoantibodies to certain types of collagens.
Experimental:	Experimental arthritis resembling RA can be induced by a variety of non-infectious agents such as albumin and type II collagen, and the disease can be transferred to a naive recipient by cell or humoral components of the sensitized animal (see section II.-4).

Rheumatoid Arthritis is a Chronic Systemic Inflammatory Disorder

RA is recognized as a group of diseases that are heterogeneous in terms of the clinical manifestation, response to treatment and prognosis. In addition to articular destruction, RA exhibits other clinical features. One is its chronic course. Patients with RA exhibiting repeated cycles of remission and relapse over years to decades are not uncommon. The second is its systemic involvement. For example, rheumatoid vasculitis may occur in multiple organs and lead to ischemic necrosis such as gangrene of extremities. In addition, alveolar inflammation or fibrosing alveolitis, splenomegaly with neutropenia, abnormalities of liver function, and renal involvement are also encountered.

Rheumatoid Arthritis is a Major Crippling Disease

RA affects about 1% of the population and is found in all racial and ethnic groups. It may occur at any age and generally increases in incidence with advancing years. Women are affected two to three times more often than men. The peak incidence in women is between the third to fifth decades. Clinically, a typical case of RA would be a female patient in her forties presenting with morning stiffness, pain and swelling of afflicted joints in a symmetrical pattern. X-ray examination may show bone decalcification in the afflicted joint. Laboratory tests demonstrate "rheumatoid factor", autoantibodies that react specifically with Fc portion of IgG molecules, poor mucin precipitate, and an inflammatory effusion in synovial fluid. Symptomatically, the patient may respond to anti-inflammatory treatment. The course of RA remains unpredictable. But repeated relapse is the rule. The repeated remission and relapse ultimately lead to joint destruction and disable the victim. A significant percentage of patients with RA develop disability. RA is by far the leading cause of physical disability in people over 50 years of age, accounting for about 40% of all disabilities.

I.- 2. Etiology of Rheumatoid Arthritis

Inquiry into the etiology of RA started about half century ago with Schlesinger's research on the cause of acute rheumatic disease or rheumatic fever (1). Although streptococcal infection was later found to play a causal role in rheumatic fever, there is no evidence that RA is initiated by such infection. During the research on the origin of RA over the last 50 years, a variety of pathogens were sought as etiologic agents. Prominent candidates range from bacteria (such as diphtheroids, streptococcus and clostridium perfrigens) to mycoplasmas, to virus (such as herpes simplex virus, hepatitis B virus, EB virus, adenovirus, and retrovirus) (for review see (2)). Although studies of these pathogens were always initiated by some suggestive findings, extended research has invariably failed to establish their causal role in RA or an exclusive correlation with RA. To date, no live pathogen has been proven to cause RA. The failure to find a specific causal agent together with the autoimmune nature of RA suggests that RA is not caused by a single etiology. Instead, it is an outcome of a multifactoral process. Accordingly, the possible causal agents can be considered as "triggering events" that elicit RA in an immunologically susceptible host.

I.-3. Synovium Histology

Synovium, Synoviocytes and Synovial Fibroblasts

RA can affect any diarthrodial joint with hand, wrist, knee and foot joints as the most commonly involved. A typical diarthrodial joint consists of articular cartilage, joint cavity and a capsule. The capsule joins the ends of the bones and encloses the joint cavity that contains synovial fluid. The capsule is lined with soft connective tissue, synovial membrane. Histologically, normal synovium consists of 1-3 layers of surface lining cells, synoviocytes, and sublining connective tissue that contains synovial fibroblasts and blood vessels (for review, see (3)). The main function of synovium is to supply nutrients to articular cartilage and to provide lubrication for joint movement.

Ultrastructurally, two major types of synoviocytes are found in the surface, type-A and type-B synoviocytes (4). In resemblance to tissue macrophages, type A lining cells exhibit a dense nucleus, abundant cytoplasmic vacuoles, and a poorly developed rough endoplasmic reticulum. In addition, they express macrophage surface markers such as Fc-receptors, cluster determinants CD68, and CD14. They are, therefore, also referred to as macrophage-like synoviocytes. Type B synoviocytes exhibit ultrastructural characteristics of connective tissue fibroblasts and thus are referred to as fibroblast-like synoviocytes. They have a pale nucleus, relatively few vacuoles, a well-developed Golgi apparatus, and a prominent rough endoplasmic reticulum. A third type of synoviocyte with characteristics intermediate between macrophage or fibroblast has also been reported. The exact origin of synoviocytes is still a subject of controversy. Some macrophage-like synoviocytes may arise from the circulating bone marrow-derived monocyte pool. That synoviocytes may derive from a common unique precursor can not be ruled out either. It is estimated that approximately 2/3 of the lining synoviocytes in normal synovium are fibroblast-like and the remaining 1/3 are macrophages-like cells (4).

Synoviocytes are unique in that they form a lining surface layer, a function typical of epithelial cells, but they are not epithelial cells and thus lack a basement membrane to separate them from sublining connective tissue. The pathological implication of this feature is that the sublining connective tissue may contribute directly to changes in synovial surface that has direct contact with articular cartilage and bone tissues. One major component of the sublining connective tissue is synovial fibroblasts. Although the term "synovial fibroblast" is often used interchangeably with the term 'fibroblast-like synoviocytes", they may be used to define two distinct cell types with different morphology, tissue localization and, probably, origins. The relationship of these two types of cells remains unknown.

Synovium Plays a Central Role in Articular Inflammation

Although the devastating outcome in RA is the destruction of articular cartilage and bone that leads to disability, the earliest pathologic changes occur in the synovium. It is widely held that synovial pannus is the most important destructive element in RA and experimental arthritis. The synovial tissue in RA shows varying degrees of inflammatory changes with considerable hyperplasia and hypertrophy of synovial lining cells, increased vascularity, mononuclear cell infiltration, and focal aggregates of infiltrated lymphoid cells. Synovium with excessive growth and infiltration exhibits invasive behavior and causes erosion of articular cartilage and bone (see Section I.-4).

Several lines of evidence suggest that lining synoviocytes play a central role in initiating and sustaining articular inflammation during RA. First, synoviocyte hyperplasia is one of the earliest changes in RA. It often precedes clinical signs of RA (5). Second, lining synoviocytes function as a major source of GFs/cytokines that are important mediators of articular inflammation (6). They are also a source of proteinases that are directly responsible for joint destruction (7-9). Third, studies of experimental arthritis show that selective depletion of macrophage-like lining synoviocytes abolishes influx of inflammatory cells, indicating that they are required for the initiation of acute inflammation (10, 11).

I.- 4. Pathological Changes during Rheumatoid Arthritis

Nonspecific Inflammation Characterizes Histopatholgy of Rheumatoid Synovium

Pathological changes in RA lack unique disease-specific features. Typical inflammatory changes such as exudation, infiltration, cell injury as well as proliferation can all be found in RA. Since RA is a chronic disorder characterized by repeated remission and relapse, the dominant pathologic change may vary with disease stage and activity.

Exudation: Marked congestion and edema during the acute phase often leads to compact fibrin deposition onto the synovium surface and, to some extent, into the swollen sublining tissue. Such change is particularly prominent in areas close to the articular cartilage.

Infiltration: The principal infiltrating cells are lymphocytes and monocytes. Increased numbers of both T and B cells are found. The relative ratio of CD8+/CD4+ T cells varies with the stages of the disease. In long-standing RA, a large number of B/plasma cells is often present around blood vessels. These plasma cells synthesize rheumatoid factor. Increased numbers of mast cells are also observed (12, 13). Unlike other infiltrated cells that are mainly located superficially, mast cells are found throughout synovium and are abundant in core connective tissue where fibrosis is apparent.

Proliferation: RA is considered a proliferative disease characterized by synovial hyperplasia, neovascularization and villus/pannus formation (see below).

RA by its nature is a non-specific inflammation. Its pathologic changes lack unique disease feature. The lesions often lack histological specificity and vary from site to site within the joint. However, unlike a typical non-specific inflammation process such as that of normal wound repair, local proliferative change in RA is not self-limited and typically leads to invasive growth of a pannus that destroys bone, cartilage and tendon and ultimately disables the victim.

Synovial Hyperplasia is a Morphological Feature of Rheumatoid Synovium

Hyperplasia of the synovial lining cell layer is a morphologic feature of RA. A marked thickening of the lining layer of synoviocytes, which are often elongated and oriented in a closely arranged palisade perpendicular to the surface, characterizes synovial hyperplasia and is a hallmark of rheumatoid synovium. Despite that RA is considered to be a proliferative disease and synoviocyte hyperplasia is often regarded as a hallmark of rheumatoid synovium, macrophage-like cells of bone marrow origin have long been

considered as the major contributor to synovial hyperplasia (14) and the key destructive element of invading pannus (15, 16). Some evidence seems to support this notion. First, mitotic figures are rarely observed in the hyperplastic synovial lining cells. Second, immunohistochemical staining for Ki-67, a cell proliferation marker, is reportedly absent from the hyperplastic lining cells (17). Third, after lethal irradiation of mice and heterologous bone marrow transplantation, the synovial macrophages are gradually replaced by cells with genetic markers of the bone marrow donor strain (18). Fourth, synovial lining cells express a wide range of macrophage antigens, which favor a bone marrow origin (14). Results from these studies suggest that the hyperplastic lining layers of synoviocytes are derived from monocytes that have migrated from the blood, rather than from direct division of resident cells. It is only recently that studies by us and others have directed attention to synovial fibroblasts and the significance of the contribution by synovial fibroblast to RA has gradually been recognized(see Manuscripts 1 and 2). Evidence has now emerged that synovial fibroblasts play an important role in RA, especially in the destructive process.

Neovascularization is a Common Finding in Rheumatoid Synovium

As in granulation tissue formed during wound healing, the formation of a synovial pannus is accompanied by the ingrowth of a new vascular network. The increased numbers of blood vessels found in rheumatoid synovium correlate well with clinical synovitis (19). The change in microvasculature has been shown to be one of the earliest and most striking changes in the rheumatoid synovium (20). Angiogenesis appears to be an essential process during the development of RA. In an experimental animal model for RA, treatment with an anti-angiogenic compound greatly attenuates the articular inflammation (21). Thus, RA may be considered as an "angiogenesis-dependent" disease (for a review, see (22)). In addition to an increase in blood vessel number, other microscopic abnormalities are also present in the blood vessels in

rheumatoid synovium such as swelling of the endothelial cells and increased gaps between them (23) . It is known that by controlling the expression of adhesion molecules and by producing a variety of cytokines/growth factors, the endothelial cells also play an important role during the initiation and development of RA (for review see (23, 24)).

I.- 5. Pathogenesis of Rheumatoid Arthritis

Development of Rheumatoid Arthritis Involves a Sequence of Pathophysiologic Changes

As described in II.-4, the earliest pathologic changes of RA occur in the synovium. Rheumatoid synovium exhibits marked hyperplasia of lining synoviocytes, infiltration of inflammatory cells, and neovascularization. However, the exact sequence of these pathologic changes remains largely unknown. RA appears to undergo four pathophysiologic changes that may roughly reflect the sequence of pathogenesis: initiation, expansion, maintenance and destruction.

It is believed that the primary event in synovium during the development of RA is microvascular injury or activation that initiates an early inflammatory response such as exudation and cell infiltration (23). Synoviocytes also play an essential role during the initiation of articular inflammation, probably through GF/cytokine production (11). Upon the action of the triggering agent(s), GFs/cytokines such as IL-1 and TNF-alpha that are involved in the primary stage of RA are believed to act as chemoattractants and inducers of adhesion molecules to elicit exudation and infiltration of inflammatory cells into synovium. As a result, tissue damage, edema, and hypoxia may occur. Meanwhile these initiating factors may also induce expression of other GFs/cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-2, and thus trigger a cascade of cytokine activation.

The increased expression of GFs/cytokines not only potentiates the primary event but may also initiate new pathways leading to additional pathophysiologic changes such as activation of macrophages by GM-CSF as well as growth and differentiation of T and

B lymphocytes induced by IL-2. Thus, the scope of the inflammatory response is greatly expanded. In addition to GFs/cytokines themselves, other pathophysiologic factors may also induce and regulate the expression of GFs/cytokines. For example, rheumatoid synovium suffers severe hypoxia (25) which has been shown to be a micro-environmental factor in regulating FGF-2 and its receptor expression (26). Injury to endothelial cells during inflammation may also lead to an increased level of local FGF-2. Unfortunately, little is known about the regulatory mechanisms of expression of several major GFs that may play important roles in pannus growth and tissue destruction such as FGF-2, TGF-beta and PDGF.

The development of articular inflammation also seems to be a process by which a variety of interaction loops of GFs/cytokines are established. For example, Bomdara et al. (27) have reported that cell contact between T cells and synovial fibroblasts induced expression of adhesion molecules by synovial fibroblasts and expression of TNF-alpha by T cells. Moreover, TNF-alpha was essential for the induction of the adhesion molecules in this study. This suggests that expression of the adhesion molecules functions to recruit and "fix" more inflammatory cells which, by contact with the fibroblasts, may produce more cytokines. An interaction loop may thus be formed. The interaction loops of GFs/cytokines appear to be critical for local cell activation in rheumatoid synovium and for sustaining the articular inflammation. It has been demonstrated that production of metalloproteinase by cultured synovial fibroblasts gradually decreases in culture. However, it can be restored by adding activated monocytes back to the culture (28, 29), indicating that cell interaction is essential for these key mediators of joint destruction.

The sustained inflammatory processes and continuous expansion of inflammatory response may lead to exaggerated local cell activation characterized by increased cell

proliferation, GF/cytokine expression and proteolytic enzyme production. As a result, invasive growth of inflamed synovium occurs and joint destruction ensues.

Two Major Disease Processes Lead to Joint Destruction

Two major ongoing changes in rheumatoid synovium play important roles during the pathogenesis of RA. The first involves immune complex formation and activation of the complement system. These interconnected processes trigger a sequence of reactions that leads to increased vascular permeability, inflammatory cell infiltration, and augmented proteolytic activity. In addition to immunoglobulins (especially IgG), constituents of articular tissue such as type II collagen are also targeted as antigens by autoantibodies. An increase in proteinase levels and activity may result from 1) influx of polymorphonuclear neutrophils (PMN) that secrete a variety of proteolytic enzymes including collagenase and elastase (see below); and 2) activation of plasminogen which in turn, may lead to a proteinase activation cascade (for review see (30, 31)).

The second arm of the pathogenic pathway in RA is characterized by invasive growth of pannus formed by synovial tissue, the most important destructive element in RA. Active cell proliferation that leads to neovascularization and synovial hyperplasia associated with increased proteolytic activity plays the key role during joint destruction. The outgrowing panni invade and replace articular cartilage, subchondral bone, and adjacent tendon with granulation tissue. Although it appears to be a reasonable assumption that joint destruction in RA is a direct result of the immune complex formation and inflammatory cell infiltration, studies suggest that invasive growth of synovial pannus plays a major role in the destructive process. For example, despite that increased number of PMN are found in synovial fluid from patients with active RA and PMN-related proteinases, especially elastase, have been implicated in tissue destruction, articular cartilage in contact with the synovial fluid remains intact. In contrast, over forty percent joint destruction takes place at the areas near synovium-cartilage junction that are

not exposed to joint cavity (32). Moreover, synovial fibroblasts rather than the infiltrated inflammatory cells such as macrophages or lymphocytes have been shown to be the major source of proteinases. Some researchers even suggest that joint destruction can occur in the absence of inflammatory cell infiltration (33). Thus the destructive process in RA is considered by some as an analogy of tumor invasion. It is hypothesized that synovial cells undergo tumor-like transformation during RA (34). In support to this hypothesis are two findings: 1) increased expression of several oncogenes were reportedly detected in rheumatoid synovium (35); and 2) synovial cells from both patients with RA and Lewis rats with streptococcal cell wall-induced arthritis exhibit anchorage-independent growth in culture (36). However, fundamental difference exists between the tissue behavior of synovial pannus and that of malignant tumors. Synovial pannus is only locally invasive and never metastasizes. Although the mechanism(s) underlying such invasive nature of rheumatoid synovium, a non-tumor soft tissue, remains largely elusive, it is now widely held that polypeptide growth factors play important roles (see section II.-7).

Proteolytic Enzymes are the Key Mediators of Joint Destruction

There is little doubt that proteolytic enzymes are key mediators of the destruction process in RA. The involvement of proteinases in RA is 3-fold. First, they directly cause tissue destruction by degrading proteoglycans, collagens and other extracellular matrix components. Second, they are required for angiogenesis, a prominent process in rheumatoid synovium that is essential for invasive growth of the inflamed synovium. Third, they play critical roles in tissue remodeling related to the formation of pannus and villus.

Proteinases include a variety of enzymes from a wide range of cell types that may be involved in RA. Of the several groups of proteinases listed, matrix metalloproteinases (MMPs) are of particular importance. Synovial fibroblasts and articular chondrocytes

express collagenase and stromelysin in response to GFs/cytokine in vitro (37-39). Several members of the MMP family have also been localized in rheumatoid synovium by immunohistochemistry and in situ hybridization (7-9, 40-42). The importance of MMPs during RA is further underscored by the finding that corticosteroid-induced improvement of RA is accompanied by a significant decrease in collagenase expression (43). Active MMPs can be specifically inhibited by a group of natural endogenous inhibitors, tissue inhibitors of MMPs (TIMPs) (for review, see (44)). It appears that an imbalance between MMPs and TIMPs may be held accountable for excessive tissue destruction during RA (8, 42). However, the attempt to inhibit joint destruction with TIMPs only met limited success. This may be attributed to their low specificity for tissue substrate targets and low selectivity of activators (for review, see (45, 46)). Thus, control of their expression regulation may be more effective as a potential therapeutic strategy.

With neutrophils and macrophages as two exceptions, MMPs are not stored in most cell types, nor are they synthesized and secreted until a clear signal arrives that they are needed. GFs/cytokines have been shown to play an important role in regulating MMP production in vitro. GFs/cytokines that directly regulate MMP expression include IL-1, FGF-2, PDGF-BB, IFN-gamma, and TGF-beta. They exert their regulatory effect via several mechanisms (pathways). First, they may trigger MMP synthesis by upregulating proto-oncogene products such as c-fos, c-jun and ETS (for review see (47, 48)). Most MMP family members contain a responsive DNA sequence element or TRE at the 5'-flanking region of their gene that is essential for induction of MMP expression. C-fos and c-jun are the protein components of the activator protein-1 (AP-1) that bind to this site. Studies have shown that synovial fibroblasts express several MMPs in response to GFs/cytokines and other inflammatory mediators through this pathway (38, 48). The second pathway that leads to MMP expression may involve other cellular responses to GFs/cytokines. For example, cell proliferation in response to GFs may also

trigger the expression of MMPs. Although transcriptional regulation is the major mechanism governing MMP expression, regulation at post-transcriptional levels may also occur. However, the mechanism(s) has not been well elucidated.

I.- 6. Polypeptide Growth Factors/Cytokines and Rheumatoid Arthritis

Polypeptide Growth Factor/Cytokines are Involved in RA

There is a large body of evidence indicating that GFs/cytokines play important roles in RA. First, a variety of GFs/cytokines such as transforming growth factor beta (TGF-beta) (49), tumor necrosis factor alpha (TNF-alpha) (50, 51), platelet-derived growth factor (PDGF)-B chain and acidic fibroblast growth factor (52) have been localized in rheumatoid synovium and joint tissues of experimental arthritis. Second, the disease processes in arthritis can be altered by administration of exogenous GFs/cytokines (53-55). Along the same line, GFs/cytokine antagonists can ameliorate experimental arthritis (56, 57). Third, transgenic mice carrying human TNF-alpha transgenes show deregulated patterns of TNF-alpha expression and develop chronic polyarthritis. Treatment of these mice with an antibody against TNF-alpha completely prevents the arthritis (58).

Although a variety of GFs/cytokines may be involved in articular inflammation and some of them exhibit similar biological activities in vitro, each GF/cytokine may have its own functional repertoire. GFs/cytokines can be pro- or anti-inflammatory and, therefore, can be classified accordingly. Typical pro-inflammatory GFs/cytokines include TNF-alpha, IL-1, IL-2 and IL-8. The main putative functional activities of these pro-inflammatory cytokines include 1) inducing the expression of adhesion molecules; 2) acting as chemoattractants for inflammatory cells; and 3) increasing vascular permeability. GFs/cytokines that often exhibit anti-inflammatory effects in experimental arthritis include TGF-beta and IL-10. It is interesting that some of the GFs/cytokines also have naturally present antagonists. Examples include IL-1 and IL-1 receptor antagonist, TNF-alpha and

soluble receptors for TNF-alpha (for a review, see (59)). However, it is unclear whether this is a general rule or whether this only applies to a limited number of GFs/cytokines. Studies have shown that some exogenous GFs/cytokines can have both pro- and anti-inflammatory effects on experimental inflammatory diseases including arthritis, depending on the time and route of administration and on other GF/cytokines present or administered concomitantly. TGF-beta and IL-1 beta are two good examples. Intraarticular administration of TGF-beta elicits acute articular inflammation (54, 55), while systemic administration of TGF-beta suppresses acute and chronic arthritis in experimental animals (56, 60). In type II collagen-induced experimental arthritis in rats, treatment with human recombinant IL-1 for 5 days starting one day before the collagen inoculation significantly delayed the articular inflammation whereas the same treatment starting from day 6 to day 10 enhanced the inflammation (61). Thus, it seems naive to classify GF/cytokine simply into pro- or anti-inflammatory categories. Moreover, it is not known whether the pro- or anti-inflammatory effects of the exogenous GF/cytokine accurately reflect the pathophysiologic functions of their endogenous counterparts during articular inflammation.

An alternative way to look at the functional aspects of GFs/cytokines is their involvement in a particular disease process or stage (see Table II). Although a particular GF/cytokine may be involved in different processes or stages of articular inflammation, its role and relative importance may vary accordingly. This is due not only to its intrinsic bioactivities but also to the stage-dependent differences of the tissue microenvironment associated with different batteries of GFs/cytokines. In other words, it is not the bioactivities or effects of a single GF/cytokine but a combination of bioactivities of a battery of GFs/cytokines in a coordinated manner that determines the pathophysiologic changes and outcome of each stage in RA.

Functional Interaction of GFs/Cytokines is Important during Articular Inflammation

RA like other types of inflammation is an intricate disease process that may be mediated by a complex network of cytokines and polypeptide growth factors (6, 62). Results from studies of experimental arthritis have strongly indicated that functional collaboration of GFs/cytokines plays an important role during the initiation and development of articular inflammation. For example, intraarticular injection of IL-1 alone induces acute articular inflammation but does not cause bone erosion (63), while co-injection of IL-1 and FGF-2 leads to joint destruction (64). Intraarticular injection of TGF-ß induces neutrophil recruitment to synovial tissues (54, 55). However, administration of TGF-ß via the same route attenuates IL-1-induced destruction of articular cartilage (65). Moreover, systemic administration of TGF-beta significantly ameliorates collagen-induced polyarthritis in rats (56, 60). The exact modes of the functional interaction of GFs/cytokines vary. At least four different mechanisms can be depicted. First, a GF or cytokine may directly regulate the expression of another cytokine by a paracrine fashion. Second, a GF or cytokine may regulate expression of receptors for another cytokine on the target cell. Cellular response to the second cytokine is thereby potentiated. FGF-2, for instance, upregulates IL-1 receptor on chondrocytes and potentiates IL-1-induced synthesis of metalloproteinases by chondrocytes (66, 67). Third, two or more GFs/cytokines exert effects coordinately (synergistic or antagonistic) on the same disease process through independent mechanisms. TNF-alpha/IL-1 offer an good example of such functional interaction. TNF-alpha exhibits a bioactivity profile very similar to that of IL-1 (68). Several studies have shown that TNF-alpha does not, however, appear to act through induction of IL-1; yet actions of TNF-alpha and IL-1 are additive at all concentrations in a variety of bioassays in vitro (for review, see (24)). In an experimental arthritis model, co-injection of IL-1 and TNF-alpha elicits a greater inflammatory response than is observed with either of them alone (69). Fourth, the

interaction between a GF/cytokine and its natural antagonist(s) may also modulate its bioactivities or function(s).

GFs/Cytokines in Chronic Inflammation

Studies of several pro-inflammatory cytokines such as IL-1 and TNF-alpha have provided insight about early disease processes during inflammation. RA, however, is characterized by its chronic course and destructive nature. Little is known about the mechanisms underlying such chronicity and invasiveness. Several fundamental questions have yet to be addressed: 1) what contributes to sustaining the articular inflammation? 2) what GFs/cytokines are accountable for invasive growth of rheumatoid synovium? 3) what is or are the tissue sources of these GFs? 4) what type(s) of cells mediate joint destruction induced by GFs? Extensive studies of these pro-inflammatory cytokines such as IL-1 and TNF-alpha indicate that they may play critical roles in initiating and establishing the inflammation. However, results from these studies do not seem to offer satisfactory answers to these questions. GFs with the potential to induce active proliferation of residential cells and angiogenesis are more likely to be involved in the chronic phase of RA and to be accountable for the invasive growth of rheumatoid synovium. FGF-2 characterized by its potent mitogenic and angiogenic activities is a candidate as one of the potential mediators involved in sustained inflammation and invasive growth of synovium.

I.- 7. Basic Fibroblast Growth Factor (FGF-2) and Rheumatoid Arthritis

FGF-2 is a Multifunctional Polypeptide Growth Factor

FGF-2 is a member of a heparin-binding growth factor family (fibroblast growth factor family) that includes at least 10 members sharing 20 to 40% homology in their gene structure. Important features of members of this growth factor family include 1) high affinity to heparin; 2) mediation of their biological functions through the same

membrane receptors; and 3) requirement of heparan sulfates for binding to their receptors (for review see (70, 71)).

FGF-2 is a potent mitogen for a variety of normal diploid mammalian cell types from mesoderm and neuroectoderm lineages (70, 71). It induces DNA synthesis, promotes G0 to G1 progression, and stimulates cell division in soft agar. The mitogenic effect of FGF-2 is extremely potent. When tested on vascular endothelium, for example, it triggers cell proliferation at concentrations as low as 1 pg/ml (Gospodarowicz, 1985). Among the broad spectrum of target cell types are endothelial cells, fibroblasts, chondrocytes and osteoblasts. All of these types of cells are actively involved in RA.

FGF-2 is also a potent angiogenic factor. It induces angiogenesis by 1) stimulating the proliferation of endothelial cells; 2)) enhancing endothelial cell migration; and by 3) increasing production of plasminogen activator and collagenase by capillary endothelial cells (72); FGF-2 has been shown to induce neovascularization *in vitro* (73) as well as *in vivo* (74).

FGF-2 is encoded by a single gene with multiple translation initiation sites that gives rise to different forms of FGF-2 ranging from 18 to 32 kD (75-77). In most tissues the 18 KD is the dominant form. Subcellular localization of different forms of FGF-2 appears to be different, but their functional differences remain unknown. FGF-2 is characterized by its high affinity to heparin. It has been shown that extracellular matrix components such as proteoglycans play important role in regulating the bioactivities of FGF-2. However, unlike other polypeptide GFs that exert their biological effects through paracrine mechanism, FGF-2 lacks a conventional secretory sequence in its structure, and its release mechanism is still a subject of controversy. Its lack of a conventional secretory peptide, together with its increased extracellular level following cell injury, has led to the hypothesis that compromised plasma membrane integrity is the mechanism for FGF-2 release.

Involvement of FGF-2 in Rheumatoid Arthritis -- A Hypothesis to be Tested

As discussed in previous sections, although several pro-inflammatory cytokines such as IL-1 and TNF-alpha may play critical roles in initiating and establishing articular inflammation in RA, GFs such as FGF-2 with potent angiogenic and mitogenic activities that target a wide range of cell types are more likely to be involved in the destructive process associated with the established disease. Several lines of evidence suggest that FGF-2 may play a direct role in RA. First, FGF-2 is a potent mitogenic and angiogenic factor. Excessive cell proliferation and neovascularization are often observed in active RA characterized by on-going joint destruction. Second, FGF-2 is also a potent inducer of proteinase production by fibroblasts (38, 78, 79). Increased expression of several proteinases in rheumatoid synovium has been well documented (7, 8, 40). Third, FGF-2 induces proliferation of synovial fibroblasts *in vitro* (80). Fourth, FGF-2 is required for bone erosion associated with IL-1 induced articular inflammation (64). FGF-2 is also involved in articular wound repair. For example, FGF-2 enhances and facilitates cartilage and bone repair (81) and functions as an osteogenic factor in vitro as well in vivo (82, 83). Based on these observations, a hypothesis that FGF-2 plays a direct role in RA was formulated. However, results from most of these studies have only indirectly implicated FGF-2 in RA. Attempts have thus been made to test the hypothesis by directly determining the involvement of FGF-2 in RA (see Section II.)

II. Rationale and Study Design

II.- 1. Questions and Rationales

To test the hypothesis that FGF-2 plays a direct role in RA, three questions have to be answered:

1) is FGF-2 present in and expressed by the normal or diseased synovium?

2) are FGF-2 levels and distribution patterns in RA different from that in normal synovium and correlated with disease processes (e.g. synovial hyperplasia and joint destruction)? and
3) could the disease processes of RA or experimental arthritis be altered by exogenous FGF-2 or its antagonist?

Accordingly, several experiments have been designed to address these questions (see II.-3). The rationale behind these studies is that if FGF-2 plays a direct role in RA, it should be present in the inflamed joint tissue; its tissue distribution and/or expression should be correlated with the disease process; and modulation of FGF-2 level and/or activity should alter the disease process. The outline of these experiments and pertinent findings is presented in the following section (II.-3.). In addition, if FGF-2 were found in rheumatoid synovium and played a role during RA, it is reasonable to assume that certain type(s) of cell in synovium may produce FGF-2 while others may respond to it as its targets. Four extended studies have thus been carried out in an attempt to identify the cellular source of FGF-2 and its potential target cells in rheumatoid synovium (see II.-4).

II.- 2. Selection of "Control" for the Study of RA in Patients

Study of a disease in patients is often complicated by multi-factorial influence on the course and clinical picture of the disease. Factors such as age, sex, duration of the disease, medication are always encountered. This is particularly true in the study of a chronic disease with immunologic features such as RA. Since it is impossible to control the influence from these factors, the best solution to obtain valid data appears to be study of a large number of patients. This requires tremendous amount of time and persistent efforts. Another main difficulty in conducting research involving human subjects is to obtain an appropriate control group for certain aspects of a disease being investigated. It is especially difficult when invasive procedures are involved in the study. To circumvent this problem in studies presented in this thesis, I have selected the disease condition,

osteoarthritis (OA) or degenerative joint disease (DJD), as an alternative control. DJD is an inherently non-inflammatory disorder of movable joints characterized by deterioration of articular cartilage, as well as by formation of new bone at the joint surface. Since it exhibits several features contrasting those of RA in the aspects to be investigated (see Table III) and surgical tissue specimens are frequently available, it has been used in the present studies and studies by others.

II.- 3. Selection of Animal Models for the Study of Rheumatoid Arthritis

As described above, age, sex and medication are all known influencing factors in RA. The multi-factorial influence on the course and clinical picture of RA in patients imposed great difficulty in interpreting data collected during the study. In addition to examining a large number of patients, an alternative is to use an experimental animal model. Experimental arthritis has been developed in rats, mice, rabbits, dogs and pigs. The list of animal models of arthritis is long. In most models, however, synovial hyperplasia, infiltration of inflammatory cells, and joint destruction are readily observed. Although each model may mimic certain aspects of RA, no experimental animal model of arthritis developed so far closely resembles RA in all aspects. Therefore, the choice of animal models in efforts to understand the mechanisms of RA depends largely on which aspects of RA are the focus of the investigation. The most widely used animal models are type II collagen-, Streptococcal cell wall-, and adjuvant--induced polyarthritis in rodents (for a review, see (84)).

Type II Collagen-Induced Arthritis in Rats: Polyarthritis induced by inoculation of native type II collagen (CIA) was first described in 1977 by Trentham et al. (85). Immunohistochemical characterization of the afflicted joint with CIA demonstrates many similarities to the situation described previously in human RA and in adjuvant arthritis (for review see (86)). Type II collagen-induced arthritis model exhibits several important

advantages over adjuvant arthritis model in terms of resemblance to human RA. Following inoculation, susceptible rat strains develop both cell and humoral immune response to collagen, measurable serum level of "rheumatoid factor", and polyarthritis with chronic features if homologous type II collagen is used. However, a low incidence (approximately 60%), extremely variable latency (2 weeks to 2 months), and limited availability of native homologous type II collagen limits its application.

Streptococcal Cell Wall-Induced Arthritis: Intraperitoneal injection of sonicated cell walls from group A streptococci induces acute polyarthritis in susceptible rat strains. The arthritis exhibits a two-phase nature: the acute inflammation occurs within 72 hours, subsides in 4 to 5 days and is followed by chronic erosive changes that last 10 to 12 weeks in most animals (for review see (87)). The chronic course is the major feature of this model. Other features of this model that are similar to human RA include complement dependence, and the remitive and relapsing nature of the lesion.

Adjuvant Arthritis of Rat: Adjuvant arthritis (also referred as adjuvant polyarthritis) induced by a single injection of Freund's adjuvant containing bacterial components was first described in detail by Pearson in 1956 (88). Ten to 14 days following the foot pad or tail injection of killed mycobactieria in mineral oil, susceptible strains of rats develop an inflammatory polyarthropathy which is characterized by the abrupt onset of acute or subacute inflammation affecting the ankles, wrists, tarsal, and interphalangeal joints. Synovial hyperplasia and bone destruction are prominent. It increases in severity to a peak at 20 to 25 days after the adjuvant injection and progresses to irreversible joint destruction and ankylosis. This model is highly reproducible, and its disease course as well as its severity are fairly predictable. Immunohistologically, adjuvant polyarthritis in rats shares many similarities with human RA (for review see (89). Joint tissues of afflicted animals exhibit increased expression of MHC class II antigens, accumulation of activated macrophages, and predominant infiltration of CD4 T lymphocytes. However,

B/plasma cells are usually absent. The induction of the inflammation by complete Freund's adjuvant, the latent period between inoculation and clinical expression of the disease, and the induction of the disease in naive recipients by passive transfer of sensitized T lymphocytes, but not serum fractions, indicate that adjuvant arthritis is due to a delayed immune response to an unknown antigen. Several other features of this model that lead to its widespread use include 1) technical ease of induction; 2) reproducibility and predictable clinical course in susceptible rat strains; and 3) the availability of reproducible objective quantification of the severity of joint inflammation. It is also for these reasons that this model was selected for this thesis work.

II. - 4. Outline of the Thesis Work

Step 1. Detection of FGF-2 and its Distribution Patterns in Rheumatoid Synovium.

Specific Aim: To detect FGF-2 and define its distribution patterns in rheumatoid synovium.

Rationale: If FGF-2 plays a direct role in the joint inflammation, it should be localized in the afflicted joint tissues.

Experimental Design: Human synovial tissues from normal subjects and patients with RA were collected and examined for FGF-2.

Methods: Immunohistochemistry, immunoblot, Northern blot and RNase protection assay.

Major Findings: FGF-2 is expressed locally in synovium and is closely associated with vascular cells and mast cells. The distribution pattern of FGF-2 is altered in a subset of rheumatoid synovial tissues in which increased FGF-2 staining was found in hyperplastic lining synoviocytes and synovium-cartilage interfaces. However, immunoblot and mRNA analyses revealed no consistent quantitative changes of FGF-2.

Status: Completed.

Reference: Manuscript 1 (see page 44). (Lab. Invest., in press 1995).

Step 2. Correlation of FGF-2 Expression with Pathologic Changes of Arthritis.

Specific Aim: To determine if FGF-2 distribution patterns and expression levels are correlated with disease stage and pathological changes.

Rationale: If FGF-2 is implicated in the disease, its tissue distribution and/or expression would be correlated with pathological changes in the joint tissue at different stages of the disease. However, results from rheumatoid synovial samples from different patients are inconsistent (step 1). This may be attributed to the heterogeneous nature of this disease, stage, activity and duration of the disease as well as age, sex and, especially, medication that is known to modulate growth factor expression. Therefore, a well-established, and highly reproducible animal model has been used to circumvent these problems.

Experimental Design: polyarthritis was induced in rats and FGF-2 expression in the joint tissues was examined at different time points.

Methods: Animal model, immunohistochemistry and immunoblot.

Major Findings: FGF-2 was localized at the site of joint destruction and exhibited tight temporal and spatial association with the disease course. In contrast, another growth factor, PDGF-A, exhibited a different association.

Status: Completed.

Reference: Manuscript 2 (see page 73). (Published in Am. J. Pathol., 145:1127-1139, 1994)

Step. 3. Modulation of Articular Inflammation with Exogenous FGF-2 and an Antagonist

Specific Aim: To test effects of FGF-2 and its antagonist on articular inflammation.

Rationale: If FGF-2 plays a significant role in articular inflammation, modulation of FGF-2 level and/or activity at an appropriate time would alter the disease process.

Experimental Design: FGF-2 or a growth factor antagonist (suramin) were given to rats during the development of adjuvant arthritis, and the degree of joint inflammation was examined.

Methods: Drug delivery, vascular cannulation, and X-ray radiography.

Major Findings: Surprisingly administration of suramin exacerbated articular inflammation while continuous intravenous infusion of exogenous FGF-2 starting prior to onset of arthritis ameliorated the disease.

Status: Completed.

Reference: Manuscript 3 (see page 110). (Submitted for publication).

II.- 5. Outline of Related and Extended Studies

Study 1. Characterization of Cell Proliferation in Rheumatoid Synovium

Specific Aim: To test the hypothesis that local cell proliferation contributes significantly to the hyperplasia of rheumatoid synovium.

Rationale: It is a commonly held belief that hyperplasia of synovial lining cells results from migration of monocytes from blood rather than from direct division of resident cells. However, it is the fibroblast-like synoviocytes that have been shown to have invasive potential and ability to proliferate in response to growth factors including FGF-2 in vitro. Based on these findings and morphological observation, I postulated that synovial fibroblasts may proliferate and contribute to synovial hyperplasia upon stimuli from polypeptide growth factors such as FGF-2.

Experimental Design: Immunolocalization of cell type-specific and cell proliferation markers in synovial samples from patients with RA and trauma.

Major findings: Increased cell proliferation of fibroblast-like synoviocytes occurs locally and contributes to synovial hyperplasia.

Status: Completed.

Reference: Manuscript 4 (see page 130). (Published in: Arthritis Rheum. 37:212-220, 1994).

Study 2. Identification of Proliferating Cells in Rheumatoid Synovium

Specific Aim: To determine the proliferative activity of synovial fibroblasts and their possible direct involvement in synovial hyperplasia and joint destruction.

Rationale: FGF-2 is a potent mitogenic factor for fibroblast and endothelial cells. The presence of FGF-2 in rheumatoid synovium may lead to increased cell proliferation of its tenable target cells, such as fibroblasts and endothelial cells.

Experimental Design: Immunohistochemistry with specific antibodies to cell type-specific and proliferation markers was used to identify the growth fraction and patterns of synovial fibroblasts in synovial samples from patients with RA.

Method: Immunohistochemistry.

Major Findings: Active proliferation and altered distribution patterns of synovial fibroblasts were found in a subset of rheumatoid synovial samples.

Status: Compete.

Reference: Manuscript 5 (see page 156). (Submitted for publication).

Study 3. Identification of the Cellular Source of FGF-2 during Chronic Inflammation

Specific Aim: To identify the cellular source of FGF-2 in rheumatoid synovium.

Rationale: In the process of characterizing FGF-2 distribution in synovium, I noticed the intracellular localization of FGF-2 in a group of tissue cells that exhibit morphology and distribution pattern similar to that of mast cells (see Manuscript 2.). This observation suggests that mast cells may express FGF-2 and serve as a source of FGF-2.

Experimental Design: Specific cell markers in conjunction with anti-FGF-2 were used to identify these FGF-2 positive cells by immunohistochemistry. Cultured human mast cells were stimulated and examined for FGF-2.

Methods: Immunohistochemistry, cell culture, immunoblot, RT-PCR.

Major Findings: 1) Mast cells serve as a major source of FGF-2 in human tissues characterized by hyperplasia, fibrosis, and angiogenesis. 2) Cultured human mast cells express FGF-2 at both protein and mRNA levels.

Experiment Status: Completed.

Reference: Manuscript 6 (see page 178). (Submitted for publication).

Study 4. Examination of Possible Mechanism of FGF-2 Secretion

Specific Aim: Determine if FGF-2 can be released by mast cells.

Rationale: The altered distribution pattern of FGF-2 without concordant changes in FGF-2 levels observed in Step 1 and 2 suggest local release. Since mast cells may serve as a major source of FGF-2 and mast cells release several bioactive products including cytokines, they may also release FGF-2.

Experiment Design: Four cultured murine mast cell lines were examined for release of FGF-2 following stimulation by agents that induce degranulation.

Methods: Cell culture, ELISA, Northern blot, immunocytochemistry and immunoblot assays.

Major Findings: Three of four mast cell lines tested express and release FGF-2 into culture medium. Both constitutive and inducible release were observed. The magnitude of FGF-2 released following induction could not attributed to cell injury or death.

Status: In progress.

Reference: Manuscript (abstract) 7 (see page 209).

Table I. Common Proteinases and Matrix Substrates

Enzymes	Matrix Substrates	Cellular Sources	GFs/Cytokine Inducers
Serine proteinases			
Plasmin	Gelatin, FN,	Liver cells	Plasminogen activator.
Elastases	Col. I, III, IV	PMN, macrophages	IL-1, IL-8
Cathepsin G	Col. I, III, IV.	Neutrophils	
Metalloproteinases			
Interstitial collagenase	Col. I, II, III, VII, X.	Synovial fibroblasts	IL-1, TNF-α, FGF-2,
MMP-1			
Fibroblast collagenase			
Neutrophil collagenase	Col. I, II, III.	Neutrophils	
72-kD gelatinase	Col. IV, V, VII; FN, gelatin.		
92-kD gelatinase	Col. IV, V; GT.		
Stromelysin	Col. III - V; PG, Lam, FN, gelatin.	Synovial fibroblast	IL-1, TNF-α, FGF-2, PDGF.
MMP-3			
Transin			
Proteoglycanase			

Table I. (Continued)

Enzymes	Matrix Substrates	Cellular Sources	GFs/Cytokine Inducers
Stromelysin-2	Col. III - V; FN, gelatin.	Fibroblasts	
Transin-2			
MMP-10			
Uterine MMPs	Col. I, III, IV, V; PG, FN.		
MMP-7			
PUMP-1			
Cysteine proteinases			
Cathepsins B.	Col. I, III, gelatin, FN, GA.	Synovial fibroblasts	IL-1, GM-CSF
Cathepsin L	Col. I-V, IX, XI.	Synovial fibroblasts	EGF, PDGF, TNF-α

* Sources (see (46, 95, 96)).

+ Abbreviations: FN, fibronectin; Col., collagen; Lam., laminin; PG, proteoglycan.

Table II. Involvement of Growth Factors/Cytokines in Rheumatoid Arthritis

Initiation	
IL-1	Chemoattraction, vascular injury
TNF-alpha	Chemoattraction, induction of adhesion molecules.
IL-8	Chemoattraction
Expansion and Maintenance	
TNF-alpha	Stimulates production of other cytokines.
IL-1	Augmentation of T and B cell function.
IL-2	Promotes growth and differentiation of T and B lymphocytes.
GM-CSF	Activation of antigen-presenting cells.
FGF-2	Angiogenesis.
IL-6	Autoantibody, such as RF, production.
Destruction	
FGF-2	Proteinase production, pannus growth.
IL-1	Proteoglycan degradation, collagenase production.
PDGF-BB	Proteinase production, cell proliferation.
TGF-beta	Pannus growth.
Repair	
FGF-2	Angiogenesis, osteogenesis, cell proliferation.
PDGF-A	Maturation of osteogenic tissue
TGF-beta	Collagen and fibronectin production.

Table III. Contrasting Features of RA and DJD

	RA	DJD
Clinic Features		
Age (year)	≥ 40	≥ 60
Sex	Female/male = 2-3.	Mainly male
Genetic	DW4/DR4 positive	No such link
Afflict Joints	Peripheral joints	Single large joint
Disease Feature		
Nature of Disease	Inflammatory	Degenerative
	Autoimmune	Mechanical
Involvement	Systemic	Articular only
Rheumatoid Nodule	Yes (25%)	No
Pathology		
Synovial Hyperplasia	Prominent	Usually minimal
Cell infiltration	Prominent	Minimal
Angiogenesis	Apparent	Absent
Cartilage Damage	Marginal erosion	Central eburnation
Bone Erosion	Prominent	Less severe
Laboratory		
Rheumatoid Factor	Positive (70%)	Negative
Synovial Fluid	Indicative of inflammation	No apparent change
X-ray		
Joint Surface	Marginal erosion of cartilage	Loss of cartilage
Subchondral	Osteoporosis	Sclerosis and cyst
Osteogenesis	No	Yes (osteophyte)

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III. Manuscripts

Expression of Basic Fibroblast Growth Factor in Synovial Tissue from Patients with Rheumatoid Arthritis and Degenerative Joint Disease†

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Running Title: **Basic FGF in Rhuematoid arthtis.**

Abstract

Background: Recent studies have implicated polypeptide growth factors in the development of rheumatoid arthritis (RA) which is characterized by synoviocyte hyperplasia and neovascularization. One such polypeptide, basic fibroblast growth factor (bFGF) is of particular interest due to its potent mitogenic and angiogenic activities. We have previously reported that cultured human synoviocytes synthesize and bind bFGF, and also proliferate in response to it (1). Recently, we found a close association between increased bFGF expression and destructive changes in arthritic joints from rats (2). Now we extend our study by detecting *in vivo* expression of bFGF in human synovial tissues obtained from patients with RA.

Experimental design: Human synovial tissues from patients with RA, degenerative joint disease (DJD) and trauma were collected during joint surgery. The expression of bFGF protein and mRNA by the synovia was examined by immunolocalization, Western blot, Northern blot and RNase protection assays. Synovium from patients with DJD and trauma was used to compare with rheumatoid synovium. Double immunostaining with cell-type-specific antibodies was carried out to identify cellular sources of bFGF.

Results: 1.) Both polypeptide and mRNA for bFGF were detected in the synovial samples examined. 2.) However, increased bFGF staining was found in synovium-cartilage interface where joint destruction occurred and in hyperplastic synoviocytes of a subset of rheumatoid synovium. 3.) Strong cytoplasmic bFGF staining was localized in the majority of mast cells and vascular cells.

Conclusions: 1.) Synovial tissue from patients with RA, DJD and trauma express bFGF. 2.) Increased bFGF staining in the hyperplastic lining synoviocytes and at the pannus-cartilage interface suggests that bFGF may play a role in synovial hyperplasia and joint destruction. 3.) Strong cytoplasmic bFGF staining found in mast cells and vascular cells indicates that these cells are the major sources of tissue bFGF.

Introduction

Rheumatoid arthritis (RA) is an immunologically mediated disease of unknown etiology characterized by chronic articular inflammation that leads to the destruction of cartilage and bone in the afflicted joint. Although the devastating change in RA is the destruction of articular cartilage and bone that may lead to crippling, the earliest pathologic changes occur in the synovium. The synovial tissue in RA shows varying degrees of inflammatory changes with considerable hyperplasia and hypertrophy of synovial lining cells, increased vascularity, mononuclear cell infiltration, and focal aggregates of infiltrated lymphoid cells. Synovium with extensive growth and infiltration exhibits invasive behavior and ultimately causes erosion of articular cartilage and bone. The mechanisms underlying this disease are unknown. RA like other types of inflammation is an intricate disease process that may be mediated by a complex network of cytokines and polypeptide growth factors (3, 4). Several cytokines and growth factors such as interleukin (IL)-1α, IL-6, transforming growth factor beta (TGF-ß) (5), tumor necrosis factor alpha (TNF-a) (6, 7), platelet-derived growth factor (PDGF)-B chain and acidic fibroblast growth factor (8) have been localized in rheumatoid synovium. However, the profile of polypeptide growth factor expression in rheumatoid synovium is far from clear. Because synovial hyperplasia and neovascularization are often observed in active RA characterized by on-going joint destruction, we were intrigued to study possible involvement of growth factors with both potent mitogenic and angiogenic activities. A prominent member of such growth factors is basic fibroblast growth factor (bFGF).

Basic FGF is a potent mitogenic and angiogenic polypeptide that induces cell proliferation and neovascularization both in vitro and in vivo (for reviews see (9, 10)). Recent studies support the notion that bFGF may be involved in the RA disease process (11). We and others previously reported that cultured human synoviocytes synthesize

and bind bFGF, and also proliferate in response to it (1, 12). Recently, we observed that increased bFGF expression was closely correlated with bone destruction in rats with adjuvant arthritis and exhibited kinetics and a distribution pattern distinct from that of another polypeptide factor, platelet-derived growth factor A chain (2). Now we extend our study by detecting *in vivo* expression of bFGF in human synovial tissues from patients with RA, DJD and trauma. Using immunohistochemistry, Western blot, Northern blot, and RNase protection analyses, we demonstrate here that b-FGF is expressed by human synovial tissues and that increased staining for bFGF is observed in the interface of cartilage and invading synovium and in the hyperplastic lining synoviocytes of a subset of synovial samples from patients with RA.

Experimental Design

To detect local expression of bFGF, synovial samples were examined at both protein and mRNA levels. First, immunohistochemical staining was carried out to demonstrate localization of bFGF in the synovial tissues. In addition, 3 cell type-specific antibodies, two for macrophages (anti-CD68 and HAM56) and one for mast cells (anti-tryptase), were used to identify the cells stained for bFGF. Second, immunoblot analysis was performed to examine molecular weights of immunoreactive bFGF-like molecules from synovial samples to confirm the immunostaining specificity. To determine the origins of bFGF detected by immunostaining and immunoblot, Northern blot analyses were performed to examine mRNAs for these molecules. RNase protection assay was done to quantify bFGF mRNA levels. Synovial tissues from patients with DJD and trauma were also studied to contrast that from rheumatoid patients.

Results and Discussion

Distribution Patterns of bFGF in Synovial Samples

In all samples examined, immunostaining for bFGF was readily found in blood vessels, some scattered connective tissue cells and macrophage-like infiltrated cells. In vessel structures, staining was associated with the endothelial cells, basement membranes and pericytes of small capillaries, *tunica intima* and *tunica media* of non-muscular vessels (**Fig. 1 a - e**). In muscular arteries, only weak staining was observed in *tunica adventitia* while the smooth muscle cells in *tunica media* were not labeled by the anti-bFGF. Staining of the endothelial cells gradually disappeared as the size of the vessel increased. Of 32 rheumatoid synovial samples, 11 exhibited marked lining synoviocyte hyperplasia with variable changes in the sublining region in terms of cell infiltration and neovascularization. Increased immunostaining for bFGF was found in hyperplastic synoviocytes in 7 of these 11 cases. In contrast, of 9 rheumatoid synovial samples without apparent hyperplasia, 6 showed no bFGF staining and the remaining 3 exhibited only weak bFGF staining in lining synoviocytes. The results are summarized in Table 1. The bFGF staining appeared to be extracellular and on the surface of the hyperplastic lining synoviocytes (**Fig. 1a and c**). Strong bFGF staining was also found at the interface of cartilage and invading synovium in 3 out of 3 joint samples containing the areas of bone destruction (**Fig. 1d**). In contrast, only 4 of 35 synovia from patients with DJD showed moderate to strong staining for bFGF in lining synoviocytes. The staining of lining synoviocyte with anti-bFGF in the majority of samples from patients with DJD was minimal (**Fig. 1e**). The distribution pattern of bFGF staining in synovia from patients with accidental trauma was similar to that of synovia of DJD (not shown).

Two major types of cells in the sublining connective tissue were specifically labeled by anti-bFGF, one was scattered in the sublining region and exhibited strong cytoplasmic staining for bFGF (**Fig. 1c and e**), while another exhibited surface

labeling by anti-bFGF. The lymphocyte-like cells were not labeled by the anti-bFGF (**Fig. 1b**).

Double Immunolabeling with cell-type-specific antibodies

To identify the bFGF-positive cells in the sublining region, specific antibodies to macrophages (anti-CD68 and HAM56) and tryptase, a unique marker for mast cells, were used in conjunction with anti-bFGF for immunostaining of the synovial samples (**Table 2**). Dual immunostaining revealed that the majority of the scattered connective tissue cells with strong cytoplasmic staining for bFGF were also labeled by anti-tryptase (**Fig. 1e and f**) and that the majority of cells labeled by macrophage-specific antibodies were devoid of bFGF staining (**Fig. 1c**) in both RA and DJD. The distribution pattern of mast cells appeared to be altered in a subgroup of rheumatoid synovial samples. While mast cells were distributed throughout the sublining in synovium from patients with DJD, they were mainly found in deep sublining regions of the rheumatoid synovia with apparent hyperplasia.

Staining for bFGF was completely abolished after treatment with PBS containing 2 M NaCl. The high-salt treatment, however, did not affect staining for tryptase or macrophages (data not shown). Non-immune antibodies gave no staining in all samples examined. Pre-incubation of anti-bFGF with human recombinant bFGF resulted in a loss of the selective staining of blood vessels, mast cells and synoviocytes (not shown).

Detection of bFGF-like molecules by Western blot

To confirm the immunohistochemical staining results, we also used Western blot analysis to detect bFGF-like molecules in total protein extracts from RA and DJD synovial membranes. A single band with molecular weight of 17 kD was detected on the blot by anti-bFGF in all synovial samples examined. This band exhibited the same migration behavior as that of recombinant human bFGF (**Fig. 2**). The 17-kD bFGF-like molecule also showed high affinity to heparin and can only be dissociated from heparin

beads by washing in buffer with 2 M of NaCl. No consistent quantitative difference in the abundance of the detected bFGF-like molecules was found between RA and DJD samples. No band could be visualized on the blot when the anti-bFGF was replaced by non-immune IgG at the same concentration or when anti-bFGF was pre-incubated with bFGF (data not shown).

Analysis of bFGF mRNA by Northern Blot and RNase Protection Assays

In order to determine if bFGF was synthesized in the synovial tissue, Northern blot analysis of total RNA isolated from synovial membranes was carried out to detect mRNA for bFGF. The bFGF probe specifically hybridized with mRNA of the expected sizes, 7.0-kb and 3.7-kb, in all RA and DJD samples examined. Results from one such experiment are shown in **Fig. 3.** No consistent difference in the levels of these two message species was observed between RA and DJD samples. For more quantitative analysis, the relative amounts of bFGF mRNA in total RNA extracted from RA and DJD samples were compared by RNase protection assay. **Figure. 4.** displays the results for the same samples shown in **Fig. 3.** After normalization of densitometric measurements to cyclophilin levels, no apparent quantitative difference of bFGF transcript levels was observed between RA and DJD samples.

Since only small quantities of synovial membranes from patients with accidental trauma were available, immunoblot and RNA analyses were not performed in these cases.

Discussion

Basic FGF has been shown to play an important role during normal wound repair. However, its role in other types of inflammation, such as RA, remains unknown. In this report we demonstrate localization of bFGF in human synovial tissues. Regardless of clinical diagnosis and tissue morphology, immunoreactivity with anti-bFGF antibody was found to be closely associated with vascular structures in all cases

examined. This observation is consistent with previous reports that the endothelial cells and the adjacent extracellular matrix are a major source and reservoir of tissue bFGF (13, 14). A consistent distribution pattern of bFGF was observed in all but four DJD samples examined; it was in blood vessels and mast cells but absent from lining synoviocytes. The distribution pattern of bFGF was altered in synovial samples from patients with RA. Increased staining for bFGF was found in the hyperplastic synoviocytes of a subset of rheumatoid synovium. This observation, together with our previous findings that cultured synovial fibroblasts exhibit a proliferative response to bFGF (1) and that local proliferation of synovial fibroblasts contribute significantly to synovial hyperplasia (15), suggests that bFGF in the lining synoviocytes may be involved in synovial hyperplasia. Furthermore, staining for bFGF was minimal in regions with heavy lymphatic cell infiltration and lymph nodule formation and was absent from the majority of lymphocytes. Since hyperplasia and neovascularization are thought to be pathological changes usually found at early and active stages of RA while formation of lymph nodules and fibrosis characterize late and chronic stages, it seems that expression of bFGF is stage-dependent and may be related to the activity level of the disease. Interestingly, strong bFGF staining was also found at the leading edge of the invading pannus. These results are consistent with our recent observations made in a rat adjuvant model, suggesting that the presence of bFGF in arthritic joint correlates well with the activity of the joint inflammation and plays a role during joint destruction (2).

The distribution patterns of bFGF described here differ from that of acidic FGF, platelet-derived growth factor B chain and transforming growth factor beta that have been reported by others (8, 16, 17). First, the bFGF staining was less widely distributed in rheumatoid synovia. Second, not all rheumatoid samples exhibit increased staining for bFGF. Our findings indicate that the levels of bFGF in rheumatoid synovia were not in simple accord with the clinical diagnosis or the stage of RA. The variability in bFGF

staining reported here may also be attributed to the morphological and clinical heterogeneity typical of the disease and to medication administered to reduce disease activity.

The specificity of bFGF detection by immunostaining was confirmed by immunoblot and mRNA analyses. Both immunoblot and Northern blot analyses revealed protein and mRNA that bind to specific probes and exhibited migration identical to that of bFGF and bFGF mRNA respectively (1). These findings also indicate that bFGF is, at least in part, synthesized locally. However, neither immunoblot nor solution hybridization revealed a consistent quantitative difference of bFGF levels between rheumatoid synovia and synovial samples from patients with DJD. This may be attributed to the fact that when a gene product is measured at the tissue level, detection of differences among samples is difficult if the cells expressing the molecule do not constitute a major percentage of the cells in the tissue. This is especially a concern for RA synovial membranes where prominent infiltration of lymphocytes often occurs. On the other hand, it may also be possible to induce cellular responses by simply mobilizing and redistributing the growth factors locally without increasing their global synthesis. Increased expression of collagenases and metalloproteinases found in superficial regions of RA synovium (18-20), for example, provides a possible mechanism for release of bFGF from ECM pools. Localizing bFGF in mast cells also suggests that mast cell degranulation may be another mechanism of bFGF redistribution.

Although the anti-bFGF labeled a number of types of cells, the cytoplasmic bFGF staining was mainly found in vascular cells and mast cells, indicating that they are the major source of tissue bFGF. Double immunostaining revealed that the cytoplasmic staining for bFGF was found in majority of mast cells but was absent in most cells labeled by antibodies specific for macrophages. This is consistent with our recent report that mast cells serve as a major source of bFGF in several chronic proliferative diseases

including RA (manuscript submitted). We have also observed that cultured human mast cell line (HMC-1 and several murine mast cell lines express bFGF at both protein and mRNA levels (manuscript in preparation). Macrophages have been shown to be a major source of cytokines and growth factors that are suspected to mediate inflammation. In rheumatoid synovium, TNF-alpha, IL-1 and GM-CSF have been localized in macrophages (5, 6). Absence of cytoplasmic bFGF immunoreactivity in the majority of macrophages in the synovial samples examined is surprising. Although this may be a stage- or medication-related phenomenon, our findings suggest that the notion that macrophages are the major source of bFGF needs to be re-evaluated.

Mast cells (MCs) play an essential role during development of inflammation following chemical and immunological insults and have been implicated in fibrosis and angiogenesis (21). For example, mast cells are required for full expression of antigen-induced arthritis in mice (22) and inflammation in other experimental models (23, 24). Increased numbers of mast cells have also been noted in rheumatoid synovium (25-27). Since bFGF is a potent mitogenic and angiogenic factor and since synovial hyperplasia and neovascularization are often observed in active RA, our findings may help elucidate the contribution of mast cells to articular inflammation.

Materials and Methods

Reagents

Primary antibodies used in this study and their sources are listed in Table 2. Alkaline phosphatase conjugated goat anti-mouse IgG was obtained from (GIBCO BRL, Gaithersburg, MD). Horseradish peroxidase conjugated goat anti-rabbit IgG, biotinylated goat anti-rabbit IgG and horse anti-mouse IgG second antibodies as well as avidin-biotin complex (ABC) reagents were purchased from Vector Lab. (Burlingame, CA). Immobilan™ PVDF membrane was obtained from Millipore Corp. (Bedford,

MA). Fast Red substrate kit was from BioGenex Lab. (San Ramon, CA). The following reagents were purchased from Sigma Biochemicals (St. Louis, MO): non-immune mouse IgG (MOPC 21), diaminobenzidine tetrahydrochloride (DAB), heparin-acrylic beads and leupeptin.

Patients and Tissue Specimen Preparation

Tissue Processing

Synovial tissues were obtained during joint surgery from patients with RA (n = 32), degenerative joint disease (DJD) (n = 35) and trauma (= 7). Synovial samples were from knee, shoulder, hip and wrist joints. All patients with RA met the 1987 ACR criteria for that diagnosis (28). Information about all patients with RA was summarized in Table 1. No attempt was made to segregate RA or DJD patients on basis of duration and stage of the disease or treatment.

Dissection was performed to discard accessory muscle and fat tissues from the synovial samples. The synovial membrane was divided into parts and processed separately as the following: 1.) fixed in neutralized buffered formalin (NBF) for 1-2 days at 4 ^{0}C and subjected to routine processing until being embedded in paraffin. Paraffin-embedded tissues were cut into 3-μm sections and laid on poly-L-lysine coated slides. 2.) immediately snap frozen in liquid nitrogen and stored at -70 ^{0}C until use.

Immunohistochemical Staining for b-FGF

To localize bFGF in the synovial tissues, a two-step indirect immunostaining method was used. The tissue section were first treated with 0.2% hyaluronidase (Sigma H-6254) in 0.1 M acetate buffer at pH 5.2 for 30 minutes. The potential non-specific antibody binding sites were blocked by incubation in phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin (BSA) and 1% normal goat serum for 20 minutes. The sections were then incubated with the monoclonal anti-bFGF at 1:20,000

for 1 hour followed by three brief washes in PBS and incubation with alkaline phosphatase conjugated goat anti-mouse IgG (GIBCO BRL) at 1:100 for 30 minutes. The antibody-antigen complex was visualized by incubation for 30 minutes in Fast Red substrate (BioGenex) according to the supplier's instructions. Levamisole (2 mM) was added into the substrate solution to inhibit endogenous alkaline phosphatase.

Three control groups were used to confirm the staining specificity. In the first, the primary antibodies were replaced by an ascites fluid containing non-immune mouse IgG (MOPC21, Sigma) at the same concentration as that of the anti-bFGF. In the second, 1 ml of anti-bFGF at 1:20,000 was preincubated with 20 μg of human recombinant bFGF (UBI, Lake Placid, NY) for 1 hour at 37 ^{0}C before its incubation with sections. In the third, the sections were washed with PBS containing 2 M NaCl for 10 minutes after enzymatic treatment. The staining pattern obtained by the monoclonal antibody was further confirmed by using a rabbit anti-bFGF and control rabbit IgG as described elsewhere (2). For each subject, the staining was repeated at least once to ensure specificity and consistency. Staining was examined and scored by two researchers independently (one was masked to the diagnosis of the samples).

Dual Immunostaining for bFGF and cell-type-specific markers

To identify the cells labeled by anti-bFGF, dual immunostaining was performed using antibodies specific to mast cells (anti-tryptase) and macrophages (KP1 and HAM56) (Table. 2). For bFGF/CD68 or tryptase double labeling, the sections were first labeled with anti-bFGF, and then were stained with the cell type-specific antibodies using avidin-biotin complex (ABC) method (15). For HAM56/bFGF staining, labeling with HAM56 was carried out first using DAB as substrate because the enzymatic treatment necessary for bFGF staining greatly reduces the intensity of macrophage labeling with HAM56.

Immunoblot Analysis of bFGF-like Molecules in Synovial Samples

Total protein was extracted from frozen synovial tissue from cases 1-22 (Table. 1) by homogenizing the samples in 1.5-3 ml of lysis buffer (50 mM Tris, 400 mM NaCl, 1% NP-40, 1 mM MgCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, pH 7.4). The supernatants were collected after centrifugation at 10,000 X g for 15 minutes and the total protein concentrations were measured by a BCA protein assay (Pirece, Rockford, IL). For immunoblot analysis, 250 μg of total protein from each sample were incubated overnight at 4 ^{0}C with 15 μl of heparin acrylic beads that had been washed with the lysis buffer containing 3 M NaCl at pH 7.4 and equilibrated with lysis buffer. After incubation, the heparin beads were washed twice with the lysis buffer containing 0.6 M NaCl and equilibrated with 10 mM HEPES at pH 7.4. The beads were collected by brief centrifugation, resuspended in 30 μl of gel electrophoresis sample buffer (0.125 M Tris, 4% SDS, 30% glycerol and 2% 2-mercaptoethanol) and subjected to 15% polyacrylamide gel electrophoresis. The separated proteins were electroblotted onto PVDF membranes with a modified buffer containing 50 mM Tris base, 48 mM glycine, 10% methanol and 0.02% SDS, pH 9 at 10 ^{0}C. The transferring efficiency of bFGF to the membrane was monitored by silver staining of the polyacrylamide gel after blotting according to Wray et al. (29) and by probing a second layer of PVDF membrane. For a negative control, beads incubated with samples were washed with a 2 M NaCl buffer to remove the bound bFGF before adding SDS-PAGE sample buffer. Human recombinant bFGF was used as a positive control (Upstate Biotechnology Inc., Lake Placid NY).

Nonspecific binding to blots was blocked with PBS containing 5% normal horse serum, 2% non-fat milk and 2% BSA for 1 hour at room temperature. The blot was incubated with the monoclonal anti-bFGF at 1:10,000 for 90 minutes at room temperature

followed by incubation with HRP-conjugated horse anti-mouse IgG at 1:7,000 for 60 minutes. There were four 5-minute washes with PBS containing 0.5% Tween-20 following each step. The blot was treated with chemiluminescence reagent (NEL-102, DuPont NEN, Boston, MA) according to the vendor and exposed against Kodak film (X-OMAT™ AR, Kodak, Rochester, New York) for 1-2 minutes. The intensity of the specific bands were quantified by scanning densitometry using a GS-300-scanner (Hoefer Scientific Instruments, San Francisco, CA).

Northern Blot Analysis of mRNA for bFGF

Total RNA was extracted from the synovial samples from patients with RA and DJD with a modified LiCl/urea isolation procedure described by Auffray and Rougeon (30). 20 μg of total RNA from each sample were separated by electrophoresis on 1% agarose gel containing formaldehyde and transferred to Nytran filters.

The bFGF probe was a 270-bp EcoR I-Hind III insert that was from the human bFGF coding region and cloned into a PGM4Z vector (J.A.Abraham, California Biotechnology Inc. San Francisco, CA). Single-stranded cRNA probes were synthesized and labeled with ^{32}P dCTP (800 Ci/mMol) using T7 polymerase. Northern blot analysis was carried out as described elsewhere (31).

Solution Hybridization/RNase Protection Assay

To quantify bFGF mRNA from synovial samples, solution hybridization/RNase protection assay was performed as described (32). Briefly, hybridization incubations were performed with 4 μg of total RNA in a buffer containing 2.5 X 10^6 dpm each of ^{32}P-labeled bFGF and rat cyclophilin cRNA probes, 80% formamide, 40 mM PIPES (pH 6.4), 400 mM NaCl and 1 mM EDTA in plastic tubes at 45 ^{0}C for 16 hours . After hybridization, RNA samples were digested with RNase A (0.04 μg/μl) and T1 (0.002 μg/μl) for 1 hour at 37 ^{0}C. Protected hybrids were extracted by phenol/chloroform,

ethanol-precipitation, and electrophoresed on a 5% polyacrylamide/7.1 M urea denaturing gel. Multiple autoradiographic exposures from each gel were quantified by scanning densitometry using a GS-300-scanner (Hoefer Scientific Instruments, San Fransisco, CA). Labeled probes were also incubated with yeast tRNA at the hybridization step as a background control.

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Figure Legend

Fig 1. Immunohistochemical staining of tissues from patients with RA (a - d) and DJD (e - f). 1 a. shows increased staining for bFGF (pink) in the hyperplastic synoviocytes and blood vessels of a synovial sample (MW). Staining for bFGF appears to be on the surface of the lining synoviocytes. 1 b. shows bFGF staining in a rheumatoid synovial sample (S91-GT) with heavy lymphocyte infiltration but no hyperplasia. Note the absence of bFGF staining in lymphocytes. 1 c. demonstrates double staining for bFGF (pink) and HAM56 (orange), a macrophage marker, in a rheumatoid synovium (KEK). While increased staining for bFGF is found in the hyperplastic synoviocytes, the majority of macrophages (orange) are devoid of bFGF staining. Scattered mast cells exhibit strong cytoplasmic bFGF staining (pink) (see inset for an example). Intense bFGF staining was also found in the edge of synovial tissue invading bone cortex (Fig. 1 d, arrowheads). 1 e and f are from section double-labeled of a synovial sample from DJD by anti-bFGF (pink in 1 e) and anti-tryptase (green fluorescence in 1 f). Note the co-localization of bFGF and tryptase. Magnification: 400 X for 1 a - c; 200 X for 1 d - f.

Fig. 2. Immunoblot analysis of synovial samples. Synovial samples from RA (lanes 2 - 8) and DJD (lanes 9 -13) were probed with anti-bFGF. Recombinant human bFGF is used as a positive control (lane 1). All synovial samples exhibited a predominant band with an apparent molecular weight of 17 kD that shows migration identical to the recombinant bFGF. Note that no apparent quantitative difference is found between samples from RA and DJD.

Fig. 3. Northern blot analysis of synovial samples. 20 μg of total RNA from each sample were electrophoresed, blotted, and hybridized with the ^{32}P-cRNA probe for

bFGF. Two predominant bands of 7.0 and 3.7 kb, as expected for bFGF mRNA, were observed in both RA (lanes 2 - 6) and DJD (lanes 7 - 10) samples.

Fig. 4. Solution hybridization /RNase protection assay. Results from the same set of synovial samples in Fig. 3 are shown here. All mRNA samples examined protected a single fragment of 250 bp from the bFGF probe (top panel). The rat cyclophilin probe protected 3 different size regions of the human mRNA (bottom panel). No protected band was found when tRNA was used as the sample (lane 9). No apparent quantitative difference between samples from RA (lanes 1 - 5) and DJD (lanes 6 - 8) judging by densitometry. Lane 10 shows undigested bFGF probe..

Table 1. List of Patients with rheumatoid arthritis[¶] in the study

	Patient *	Age[†]	Sex	Duration[†]	Hyperplasia[§]	bFGF Staining[¥]
1.	BG	47	F	8	++	++
2.	BJF	59	F	27	+++	+++
3.	CED	71	M	NA	--	--
4.	DS	29	F	22	++	+
5.	EEK	62	F	52	++	--
6.	EMH	72	F	NA*	--	+
7.	ES	75	F	23	++	--
8.	FEA	75	F	14	+++	++
9.	GSH	76	F	NA	++	++
10.	GTS	49	M	19	+++	+++
11.	JAC	51	F	25	--	--
12.	JAC	70	F	39	++	--
13.	JAN	59	M	15	--	+
14.	JET	58	F	26	++	+
15.	KEK	19	F	NA	+++	+++
16.	LLF	65	F	22	++	++
17.	MW	60	F	30	+++	+++
18.	NLD	53	F	10	+++	+++
19.	PAB	66	F	NA	+	++
20.	RFU	59	M	NA	--	--
21.	RJL	67	M	3	++	--
22.	VCT	60	F	6	++	--

(Continue on next page)

Table 1. (Continue)

	Patient *	Age†	Sex	Duration†	Hyperplasia§	bFGF Staining¥
23.	S93-PDG	81	F	NA	+++	+++
24.	S91-DKM	25	F	NA	+++	+++
25.	S92-SDS	46	F	NA	--	--
26.	S91-SMS	55	F	NA	++	+
27.	S92-TLW	39	F	NA	+++	+
28.	S92-MRW	68	F	NA	+++	+
29.	S91-GT	53	F	NA	--	+
30.	S91-LDL	37	M	NA	--	--
31.	S92-DJH	55	M	NA	+++	+
32.	S93-PDO	81	F	NA	--	--

¶ All cases met the 1987 ACR criteria for RA diagnosis (28).

* Immunoblot and RNA analysis were not done for cases 23 -32.

† Age and duration are in years. Abbreviation: F-female, M-male, NA-not available.

§ +, ++ and +++ are defined as 3, 4, and ≥ 5 cell layers of lining synoviocytes respectively.

¥ +, ++ and +++ are assigned to weak, moderate and strong staining for bFGF on synoviocytes of the lining layers.

Table 2. List of Primary Antibodies used in this Study

Antibody	Species	Dilut./Concent.*	Treatment	Source
Anti-bFGF	Mouse§	1: 20,000 (< 3 μg/ml)	Hyaluronidase	ZymoGenetics. Inc., Seattle, WA
Anti-bFGF	Rabbit IgG	1: 500 (2 μg/ml)	Hyaluronidase	Biomedical Technologies Inc., Stoughton, MA.
Anti-tryptase	Mouse IgG	1: 100 (0.5 μg/ml)	Hyaluronidase	Dako Corp., Carpinteria CA
Anti-CD68 (KP1)	Mouse IgG	1: 100 (3.7 μg/ml)	Hyaluronidase	Dako Corp., Carpinteria CA
HAM56	Mouse IgM	1: 500 (1.4 μg/ml)		Dako Corp., Carpinteria CA

* Dilut./Concent.= Dilution/Concentration.

§ This antibody is raised against full length human recombinant bFGF and does not cross-react with acidic FGF upon immunoblot analysis (33). Clone number of this antibody is 148.6.1.1.1. The listed dilution is for 2-step indirect method. For ABC method, the dilution is 1: 10^6 (< 0.1 μg/ml).

Fig. 1 Immunohistochemical staining of synovial tissue

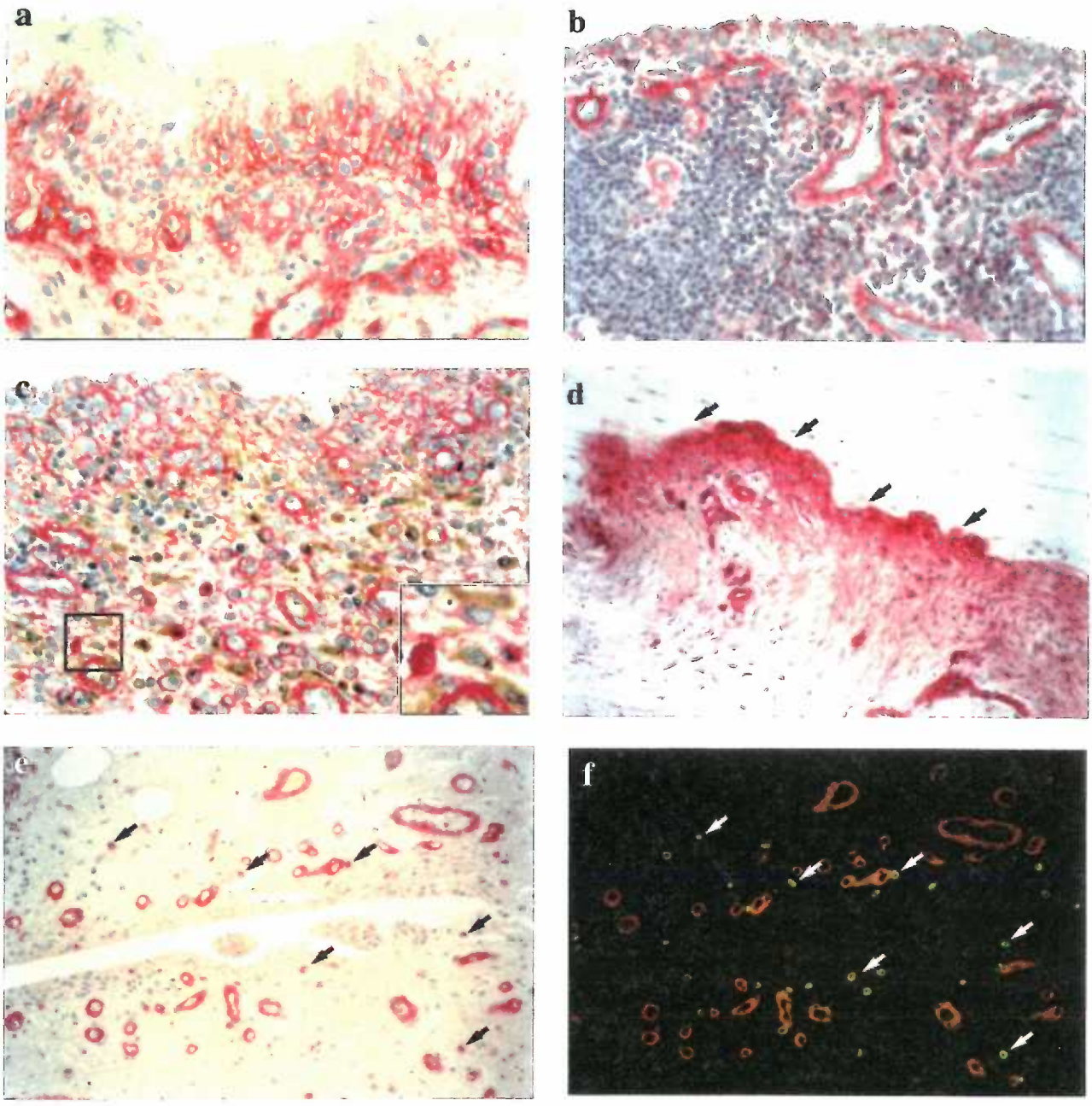


Fig. 2. Immunoblot analysis of synovial samples.

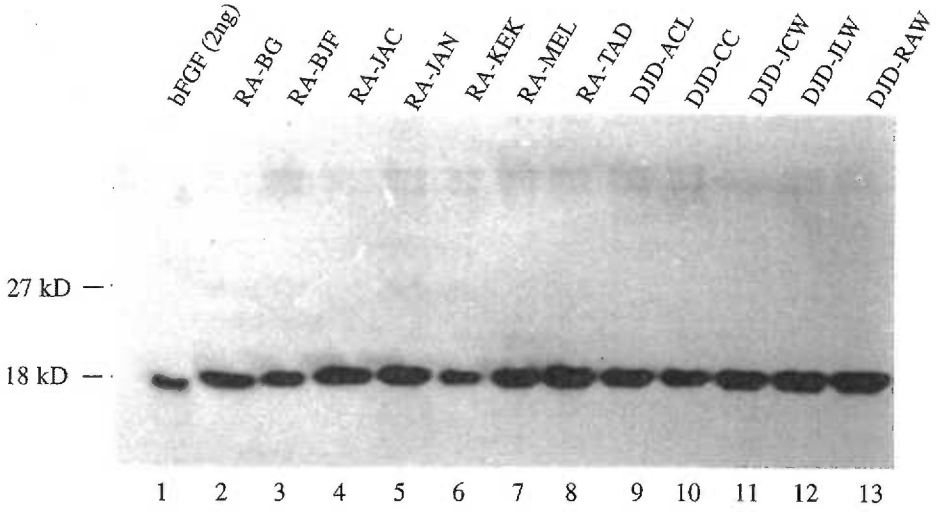


Fig. 3. Northern blot analysis of synovial samples.

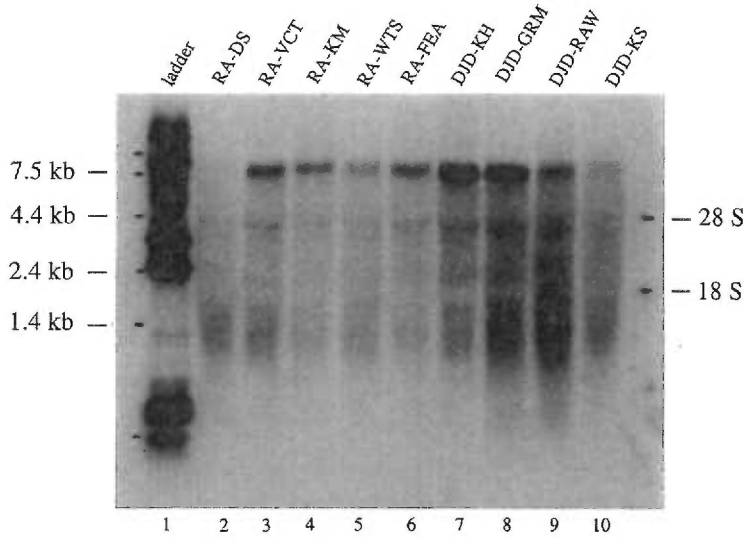
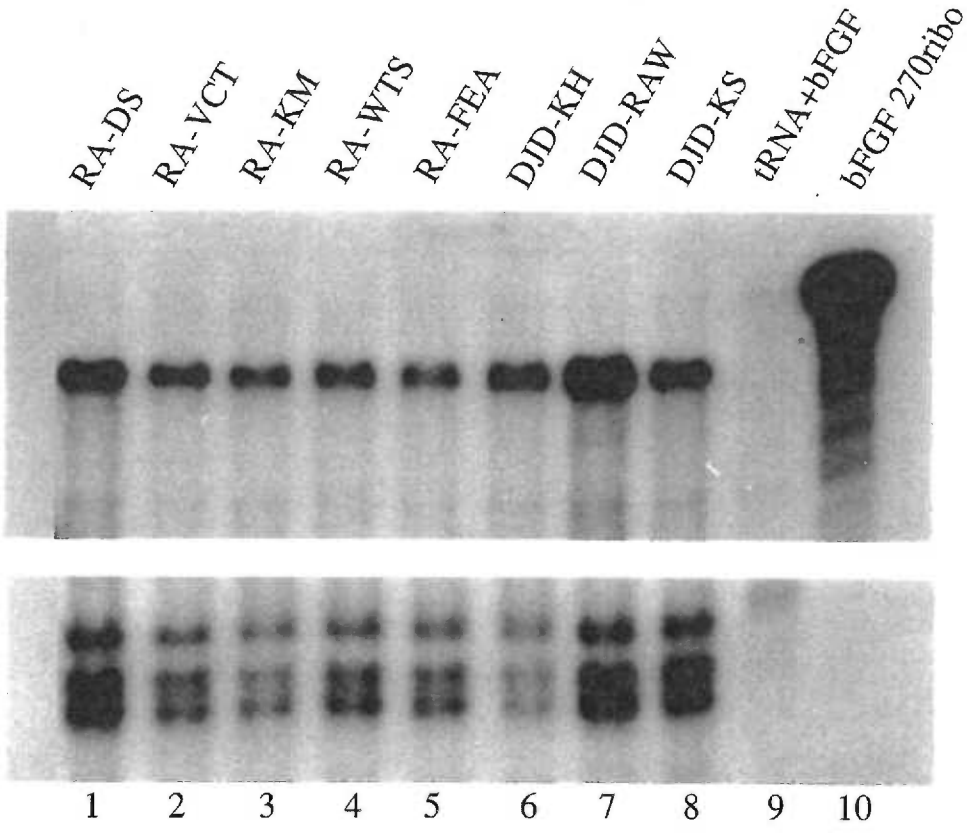


Fig. 4. Solution hybridization/RNase protection assay.



Immunolocalization of Basic Fibroblast Growth Factor and Platelet-derived Growth Factor-A during Adjuvant Arthritis in the Lewis Rat†

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Abstract

A prerequisite in defining the role of a growth factor in a disease is knowledge of its expression kinetics during the natural course of the disease. We, therefore, used immunohistochemical and immunoblot analyses to examine tissue distribution of basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF-A) during the development of destructive arthropathy in the rat adjuvant arthritis model.

In normal joints, bFGF was mainly localized in endothelial cells. In inflamed joints, increased staining for bFGF was found in the invading panni, hyperplastic synovium and thickened periosteum where bFGF was also co-localized with two cell proliferation markers. Staining for bFGF began to increase at the onset of arthritis (day 11-13), reached peak level on day 17-24 and gradually declined afterwards. In contrast, PDGF-A staining did not change until day 17 and the increased staining was restricted to areas of newly-formed bone. The distinct temporal and spatial distribution pattern of these two GFs during the destructive arthropathy strongly suggests that they play different roles during arthritis. While PDGF-A seems to be exclusively related to osteogenesis, bFGF may have a more extensive impact on synovial proliferation and bone destruction as well as bone formation.

Keywords: inflammation, osteogenesis, immunohistochemistry, synovium, joint destruction.

Abbreviations used in this manuscript

AA, adjuvant arthritis; ABC, avidin-biotin-complex; bFGF, basic fibroblast growth factor; BrdU, bromodeoxyuridine; GF(s), growth factor(s); HRP, horseradish peroxidase; PCNA, proliferating cell nuclear antigen; PDGF-A, platelet-derived growth factor; RA, rheumatoid arthritis; SCJs, synovium-cartilage junction(s).

Introduction

Rheumatoid arthritis (RA) is an immunologically-mediated chronic disorder characterized by invasive growth of inflamed synovial tissue that leads to joint destruction. The mechanism underlying this disease is largely unknown. Increasing evidence indicates that growth factors (GFs) and cytokines are involved in rheumatoid arthritis (RA) (for review see [1, 2]). Of these GFs, basic fibroblast growth factor (bFGF) is of particular interest. It is a potent mitogenic, angiogenic and chemotactic factor and has been implicated in the wound-healing processes [3-5] (for reviews see [6, 7]) which exhibit resemblance to aspects of RA. Recent studies seem to support the hypothesis that bFGF is involved in RA. Basic FGF is a potent inducer of a variety of metalloproteinases [8, 9] and plasminogen activator [10]. It upregulates interleukin-1 (IL-1) receptor expression in chondrocytes [11] and thereby potentiates IL-1-induced metalloproteinase production [8]. It also synergizes with tumor necrosis factor (TNF)-alpha in inducing the synthesis of matrix proteinases and the release of proteinases from synovial fibroblasts [12]. BFGF alone or synergizing with IL-1 induces synthesis and release of prostaglandin E by articular chondrocytes and rheumatoid synovial cells [8, 13-15]. The findings that repeated intraarticular injection of IL-1 results in a chronic synovitis without cartilage and bone destruction [16], while co-injection of IL-1 and bFGF induces degradation of articular cartilage [17], suggest an important role of bFGF in cartilage and bone destruction. Unlike other GFs or cytokines detected in arthritic joint tissue such as IL-1 or TNF α, bFGF seems to be involved not only in the destructive process but also in wound repair. It also has been implicated in bone formation *in vitro* as well as *in vivo* [18-21]. We and others reported that cultured synoviocytes also synthesize, bind and proliferate in response to bFGF [22, 23]. Two recent studies show that inhibition of nitric oxide synthesis results in suppression of arthritis in Lewis rats and that bFGF markedly inhibits interferon γ- /endotoxin-induced nitric oxide synthase [24]

[25]. Thus, bFGF may have dual effects on articular inflammation. It may play a role in destructive changes; it may also be involved in the wound-repair process and have beneficial effects in protecting joint tissue from inflammatory attack. In view of the simultaneous presence of both tissue destruction and repair in RA, we were intrigued to examine bFGF expression in relation to disease processes and pathological changes.

In order to define better the role of a growth factor in a disease process, it is very important to examine the temporal expression and localization of the growth factor during the initiation and development of the disease. However, efforts made to characterize the pathophysiological roles of GFs during rheumatoid changes *in vivo* are often hampered by three major obstacles. First, the detection of a growth factor can not be equated with proof that it plays a causal role. Second, synovial samples used for immunolocalization of growth factors are largely obtained from patients with long-established disease. It is, therefore, unclear whether the expression of a growth factor in the tissue samples is a primary (i.e. causal) or secondary (i.e. resultant) event. Third, medication has the potential to modulate expression of growth factors. We therefore used a well-characterized arthritis animal model [26, 27] to investigate the expression kinetics of bFGF in joint tissues in relation to pathological changes during the development of adjuvant arthritis. We focused on bone destruction in synovium-cartilage junction (SCJ) and osteogenesis. Since upregulated expression of multiple GFs appears to be a common occurrence during articular inflammation, we used PDGF-A, a GF with considerable functional overlap with bFGF for comparison. Our results show that, in contrast to immunostaining for PDGF-A, increased bFGF staining coincides with active cell proliferation, invasive growth of panni, and bone formation suggesting that bFGF may play an important role during articular inflammation.

Materials and Method

Induction of Adjuvant Polyarthritis

Pathogen-free female Lewis rats (Harlan Sprague Dawley, Indianapolis, IN) at 170-200 gram were used for the present study. The animals were kept for at least 2 days under constant environmental conditions with food and water *ad libitum* before experimentation. All the experiments were performed in accordance with the guideline approved by the Committee for Care and Use of Laboratory Animals at the Oregon Health Sciences University.

Adjuvant arthritis was induced by intradermal injection of 1-1.5 mg of mycobacterium butyricum (Difco, Detroit, MI) suspended in mineral oil (10 mg/ml) (Sigma) into the tail base. The extent of arthritis was quantitated by volumetric measurement of each of the 2 hind feet as determined by immersion of each in fluid up to a tattoo mark 5 mm above each ankle. The amount of water displaced served as a quantitation of limb swelling. The severity of arthritis in each hind limb was scored as the percentage increase in volume:

$$\text{Arthritic Score} = (\frac{V_2}{V_1} - 1) \times 100$$

where V1 represents the volume of the limb upon the initial measurement on day 0, while V2 represents the measurement upon sacrifice. Results from repeated measurements over 84 ankle joints in our pilot study showed that the error of measurement was ca. 4.5 $\pm$ 2.4% . The maximal increase of the score in normal rat as a result of natural growth in a 40-day period is less than 15%.

Rats were sacrificed by CO_2 inhalation on day-9 and at 5 - 7 day intervals starting at day 11 - 13 (onset) after tail injection (Table 1.). Untreated rats (n = 4) were used as control. Intraperitoneal injection of bromodeoxyuridine (BrdU) at 50 mg/kg and fluorodeoxyuridine (FldU) at 5 mg/ml was given 2-3 hours prior to sacrifice for pulse labeling of proliferating cells. Afflicted hind limbs with typical arthritic scores were selected at each time point and used for this study. Hind limbs with arthritic scores $\leq$

15% (n = 7) from five adjuvant-treated rats were considered as arthritis-free and were also examined.

Processing of the Tissues

For immunohistochemical study, afflicted hind limbs were amputated at the malleolus and the middle of the metatarsals after the skin was peeled off. The samples were fixed in Bouin's solution for 1-2 days at 4 °C and then decalcified in 0.1 M Tris buffer (pH 7.2) containing 10% EDTA with daily changes for 20-25 days. After extensive washing in Tris buffer, the tissues underwent routine processing until being embedded in paraffin. Immunohistochemical localization of bFGF and PDGF-A was carried out using the paraffin sections. To identify the actively proliferating cells, the tissue sections were also stained for proliferating cell nuclear antigen (PCNA) and BrdU.

For Western blot analysis of bFGF-like molecules, the hind limbs were dissected skin-free and immediately snap frozen and stored in liquid nitrogen. To extract total protein from the joint tissues, the samples were ground into powder in liquid nitrogen and dissolved in a lysis buffer (pH 7.3) containing 25 mM HEPES, 0.4 M NaCl, 2 mM MgCl, 1% NP-40, 2 mM phenylmethyl sulfonylfluoride (PMSF), 1 mM leupeptin and 1 mM aprotinin. The samples were homogenized with a Brinkmann PT3000 Polytron (Dispergir-und Mischtechnik, Luzern, Switzerland) at maximal speed for four 30-second pulses. After centrifugation at 1000 X g for 5 minutes, the supernatant was collected. To ensure maximal yield of soluble proteins, the pellets were dissolved in fresh lysis buffer and the extraction was repeated twice. The supernatants from repeated extractions were collected, mixed well, and the total protein was measured by Pierce BCA Protein Assay following the vendor's instruction (No. 23209, Pierce, Rockford, IL). The samples were then stored at - 70 °C until use.

Immunohistochemical staining for bFGF, PDGF and PCNA

Immunohistochemical staining utilized for this study was as previously described [28]. Briefly, paraffin-embedded tissues were cut into 4-6 micrometer sections and laid on poly-L-lysine coated slides. To improve adherence, the slides were heated at 60 °C for 30 minutes in a humid chamber. The sections were deparaffinized in xylene and rehydrated through graded series of alcohol and distilled water. A brief enzymatic treatment was carried out, when necessary, to retrieve the antigenicity (see Table 2). To block non-specific antibody binding, the sections were incubated in phosphate buffered saline (PBS) containing 0.3% bovine serum albumin (BSA) and 1% normal goat and horse sera for 20 minutes. A three-step avidin-biotin complex (ABC) protocol was used for staining unless otherwise specified. After washing and blocking steps, the slides were incubated with a primary antibody at an appropriate dilution (Table 2) overnight at 4 °C. Then, a biotinylated goat anti-rabbit or horse anti-mouse secondary antibody (adsorbed with rat serum) was applied at 1:200 for 40 minutes followed by incubation with alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated avidin-biotin-complex for 30 minutes according to the vendor's instructions (Vector Lab., Burlingame, CA). The antibody-antigen complexes were visualized by incubation for 30 minutes in Fast Red substrate containing 2 mM levamisole (BioGenex Lab., San Ramon, CA) according to the supplier's instructions or by incubation for 10 minutes with a HRP substrate solution containing 0.5 mg/ml 3,3'-diaminobenzidine, 0.1 M imdazole and 0.03% H_2O_2 in PBS. The sections were counterstained with Gill III hematoxylin and mounted in Crystal Mount (Biømeda Corp., Foster City, CA).

For bFGF staining with the mouse monoclonal antibody, a two-step indirect staining method was also used. After incubation with the monoclonal antibody to bFGF at 1:20,000 for 1 hour and then two brief washes in PBS, the sections were incubated with an alkaline phosphatase conjugated goat anti-mouse IgG (GIBCO BRL, Grand

Island, NY) at 1:100 in the diluting buffer containing 5% normal rat serum for 30 minutes. The antigen-antibody complexes were visualized by Fast Red as described above.

The control groups included 1) replacing the specific primary antibodies with nonimmune IgG at the same concentration from the same species; 2) preincubation of a primary antibody with the specific antigen before staining for bFGF and PDGF-A; 3) for bFGF staining, washing the sections with PBS containing 2 M NaCl following enzymatic treatment; and 4) for BrdU staining, tissue samples from rat not injected with BrdU.

Double or triple labeling of bFGF, PDGF and PCNA/BrdU

For double labeling of PCNA and bFGF, tissue sections were stained first for PCNA using the ABC-HRP method with DAB as substrate. A 2-step indirect method was used for bFGF staining as described above with Fast Red as substrate.

To seek the temporal correlation of bFGF and PDGF-A, double immunolabeling was performed in which the sections were stained first for bFGF using the ABC-AP method and Fast Red as substrate. Staining of PDGF-A was then carried out using the ABC-HRP method with DAB as substrate as described above.

For triple labeling, the sections, after bFGF/PDGF-A double staining, were treated with 2 N HCl for 15 minutes at room temperature and incubated with phosphate buffer saline (PBS) containing 0.3% bovine serum albumin (BSA) and 1% normal goat and horse serum for 20 minutes. The samples were then incubated with anti-BrdU at 1: 20 for 1 hour followed by incubation with FITC conjugated goat anti-mouse IgG at 1: 50 containing 5% of normal rat serum for 40 minutes.

Assessment of bFGF staining in relation to joint destruction and cell proliferation

To seek a correlation between bFGF staining and joint destruction, the number of synovium-cartilage junctions (SCJs) with bFGF staining was counted by two

investigators independently (one was blinded to the tissue samples). The frequencies of bFGF and PDGF-A staining at both intact and damaged SCJs of all joints in a limb were then compared with control. The number of periosteal cells stained positive for PCNA/BrdU was also counted and expressed as a percentage. The data were analyzed with Students' t test and the frequency was expressed as mean ± standard deviation (SD).

Western Blot analysis of bFGF-like molecules

The joint tissue lysates were analyzed for bFGF-like proteins by Western blot analysis using the mouse monoclonal antibody (148.6.1.1.1) (Table 2). Briefly, 1 mg of total protein from each sample was pre-cleaned by incubation with 20 µl of protein G /protein A-Agarose (IP05, Oncogene Science, Uniondale, NY) for 3 hours with agitation at room temperature. After brief centrifugation, the sample supernatant was transferred into a new 1.75-ml tubes containing 15 µl of heparin acrylic beads (H-5263, Sigma) that had been washed with the lysis buffer containing 3 M NaCl at pH 7.4 and equilibrated with lysis buffer. The sample was incubated with the beads overnight at 4 ^{0}C. The heparin beads were washed twice with the lysis buffer containing 0.6 M NaCl and equilibrated with 10 mM HEPES at pH 7.4. The beads were collected by brief centrifugation, resuspended in 30 µl of SDS electrophoresis sample buffer (0.125 M Tris, 4% SDS, 30% glycerol and 2% 2-mercaptoethanol) and subjected to electrophoresis in 15% polyacrylamide gels. The separated proteins were transferred onto PVDF membranes at 95 volts for 60 minutes at 10-12 ^{0}C in a Hoefer electroblotting tank (Hoefer Scientific Instru. San Francisco, CA). A modified buffer containing 50 mM Tris base, 48 mM glycine, 10% methanol and 0.02% SDS, pH 9.3 at 10 ^{0}C, was used to optimize the transfer and binding efficiency of bFGF. The transferring efficiency of bFGF to the membrane was monitored by silver staining of the polyacrylamide gel after blotting according to the method of Wray et al.[29] and by immunostaining a second layer of PVDF membrane. For a negative control, beads incubated with samples were

washed with a 2 M NaCl buffer to remove the bound bFGF before adding electrophoresis sample buffer. Human recombinant bFGF was used as a positive control with or without incubation with heparin beads.

Nonspecific binding to the blot was blocked with PBS containing 5% normal horse serum, 2% non-fat milk and 2% BSA for 1 hour at RT. The blot was incubated with the monoclonal anti-bFGF at 1:10,000 for 90 minutes at RT followed by incubation with HRP-conjugated horse anti-mouse IgG at 1:7,000 for 60 minutes at RT. There were four 5-minute washes with PBS containing 0.5% Tween-20 following each step. The blot was treated with chemiluminescence reagent (NEL-102, DuPont NEN, Boston, MA) according to the vendor and exposed against Kodak film (X-OMAT™ AR, Kodak, Rochester, New York) for 1-2 minutes. The intensity of the specific bands were quantified by scanning densitometry using a GS-300-scanner (Hoefer Scientific Instruments, San Francisco, CA).

Results

Hind limbs from normal rats exhibited no joint swelling with typical arthritic scores $\leq$ 15% during the period of experiment. No apparent inflammatory changes were observed. Periosteum exhibited 1-2 layers of cells with fibrocyte morphology. Staining for bFGF was observed in blood vessels, proliferating chondrocytes in the growth plate, a small number of osteoblasts and some scattered cells in loose connective tissue. In SCJs and periosteum, bFGF staining was limited to blood vessels (Fig. 1a). PCNA immunoreactivity was found in < 5% of periosteal cells (inner layer of periosteum) (data not shown). Although the BrdU labeling regimen labeled only a quarter of the proliferating fraction that stained for PCNA, the distribution patterns as well as the staining kinetics of both BrdU- and PCNA-cells were identical. Weak staining for

PDGF-A was present in tendon and smooth muscle cells in blood vessels but essentially absent from periosteum, synovium, bone matrix and cartilage (data not shown). Intense PDGF-A staining was only found in the cytoplasm of megakaryocytes in bone marrow (Fig. 3c) and in nerve fibers (data not show). Joint tissue from arthritis-free rats 9 days after adjuvant inoculation is indistinguishable to that from control rats pathologically and immunohistologically (Table 1).

Three major pathological changes were readily observed during development of the articular inflammation: peri- and intra-articular infiltration with inflammatory cells; erosion of cartilage, bone and tendon; and osteogenesis. Neovascularization and chondrogenesis were also often seen. Of these changes, bone erosion and formation, in particular, exhibited a great extent of temporal overlapping with bone erosion dominating the early stage while bone formation the late stage (Table 1). While staining for bFGF seemed to be related to both destructive and osteogenic processes, PDGF-A immunostaining, in contrast, was predominantly associated with bone formation.

Upon disease onset, on day 11 - 13, the afflicted hind limbs showed apparent redness and swelling with typical arthritic scores of 30-60%. Histology indicated acute inflammation with extensive acellular exudation and mild to moderate cell infiltration in synovium and in loose connective tissue adjacent to joints and tendons in all afflicted limbs examined. No obvious formation of panni or synovial villi and erosion of joint or tendon were observed. The periosteum proximal to inflamed joints exhibited hypertrophy. Staining for bFGF, however, began to appear in 3 major areas in addition to blood vessels: periosteum (Fig. 2a), SCJs, and scattered cells in inflamed loose connective tissue (Fig. 1b). Although less than 10% of SCJs exhibited bone erosion, over 60% SCJs were stained for bFGF (Table 1 and Fig. 5). Western blot analysis also revealed a 2-fold increase in the bFGF level of the joint tissues (Fig. 4). Double staining of PCNA/bFGF showed that PCNA and bFGF immunoreactivities were colocalized in

lining synovium (Fig.1b), periosteum and some capillaries (Fig. 2a). Anti-PDGF-A exhibited the same staining pattern as that seen before onset.

The magnitude of swelling of an afflicted limb reached its peak between days 17 - 24 and remained unchanged for about a week. The typical arthritic score during day 17 - 20 is 60-80%. Increased inflammatory cell infiltration in synovium and progressive erosion of cartilage, subchondral bone and tendon characterized pathological changes occurring by day 17 - 24. Histology showed an acute to subacute inflammation characterized by extensive infiltration of inflammatory cells, outgrowth of synovia, and formation of panni that progressively eroded tendon, subchondral bone and cartilage. Periosteum proximal to afflicted joints exhibited dramatic hyperplasia and began to give rise to osteoid between the original bone cortex and hyperplastic periosteum (Fig. 2b). Intense staining for bFGF was found in newly-formed panni, thickened periosteum, and capillaries in adjacent soft tissue (Fig. 1c and d; 2b). While there was about 20% increase in the number of bFGF-positive SCJs compared with that on day 11-13, the increase in the number of SCJs exhibiting bone erosion was dramatic (more than 50%) (Fig. 5). Over 80% of total SCJs exhibited staining for bFGF (Table 1. and Fig. 5). An increased number of PCNA-/BrdU-positive cells was also observed in the panni and periosteum (Fig.2b). PCNA and bFGF staining were highly colocalized in these regions (Fig. 2b-c and Table 1). The staining pattern of PDGF-A remained mainly unchanged except that weak to moderate PDGF-A staining was noticed within area of the osteoid tissue.

Extensive subchondral bone destruction, active intramembranous bone formation and the presence of PDGF-A immunoreactivity in the osteogenic tissue characterized the pathological changes observed on day 24 - 27. The majority of SCJs (> 80%) demonstrated bone destruction and positive staining for bFGF (Fig. 5). The subchondral bone was gradually replaced by invading panni that evolved into granular tissue

containing inflammatory cells and multinucleated giant cells (Fig. 1d). As a pannus continued to extend and expand into the subchondral region, bFGF staining became diminished in the connective tissue core of the expanded pannus. However, cells at the leading edge of the pannus continuously showed strong staining for bFGF (Fig. 1e). Synovial tissue exhibited heavy infiltration, neovascularization and hyperplasia as well as hypertrophy of lining synoviocytes. Intense immunostaining for bFGF was found in lining synoviocytes with dendritic morphology, small blood vessels and extracellular matrix in inflamed synovium (Fig. 3a). A great percentage of lining synoviocytes showed nuclear staining for PCNA ($\geq$ 50%) and BrdU ($\geq$ 15%) (Fig. 3a). Meanwhile active periosteal bone formation along adjacent bone shaft was observed. The newly-formed bone grew from the bone shaft and formed dactylate (finger-like) projections. Periosteum surrounding the newly-formed bone consisted of highly cellular soft tissue with fibroblast-like cells and blood vessels that exhibited strong bFGF staining (Fig. 2e). A great percentage of BrdU/PCNA-positive cells was found in the thickened periosteum and remained colocalized with bFGF immunoreactivity (Fig. 2f). Increased staining for PDGF-A was apparent in the newly-formed bone tissue, while the original bone matrix remained largely unstained (Fig. 2c and 2d).

Pathological changes seen on days 33-40 were marked by extensive osteogenesis and a gradual reduction in inflammatory infiltration along with a dramatic decrease in bFGF and PCNA/BrdU immunoreactivity. Histologically, subchondral bone of afflicted joints was largely destroyed and replaced by granular tissue, while the periosteal new bone expanded to form a large mass that stained strongly for PDGF-A. BFGF immunoreactivity in pannus and periosteum was greatly decreased, although weak to moderate staining for bFGF was still present in some of the blood vessels (Fig. 2g). Western blot analysis also showed a decrease in bFGF in the inflamed limb to levels substantially below normal controls (Fig. 4). A simultaneous decrease in the number of

total cells as well as in the percentage of PCNA/BrdU-positive cells in the periosteum accompanied the reduction of bFGF (Fig. 2e). In contrast, intense PDGF-A staining remained in the periosteal bone (data not shown). At the end of this period, granular tissue that had replaced the original subchondral bone gradually made a transition into granulation tissue with a greatly reduced number of infiltrated inflammatory cells and multinucleated giant cells. A transient local increase in bFGF but not PDGF-A staining in these areas was noticed. The granulation tissue then developed into woven bone which was stained for PDGF-A (data not shown).

In spite of the high incidence of polyarthritis in this model, about 10% of the adjuvant-treated rats remained arthritis-free with joint scores $\leq$ 15% and showed no sign of inflammation (joint swelling, redness, cell infiltration and bone erosion) until sacrifice ($\geq$ day 17). Moreover, in some adjuvant-treated rats only one hind limb was affected. In these arthritis-free hind limbs (n = 7) from adjuvant-treated rats, about 70% of SCJs exhibited increased staining for bFGF without apparent cell infiltration, pannus formation, bone destruction or osteogenesis (data not shown). However, they exhibited an identical staining PDGF-A pattern as the limbs form non-treated rats.

Immunostaining was abolished in control sections using nonimmune antibodies (Fig. 1f) or preabsorbed anti-bFGF and anti-PDGF-A. While washing sections with buffer containing 2 M NaCl completely eliminated staining for bFGF, it did not affect staining for BrdU or PCNA. Possible cross-reaction between the primary and secondary antibodies for double labeling was excluded by using a medullary bone sample as a positive control in which staining for bFGF and PDGF-A was found in osteoclasts and megakaryocytes respectively (Fig. 3c). The specificity of anti-bFGF used for immunohistochemical staining was further confirmed by immunoblot analysis. Upon the blot analysis, all samples exhibited a specific band of about 17 kD with migration identical to that of human recombinant bFGF (Fig. 4). Consistent with the

immunohistochemical result, an increase in bFGF level was observed at the time of onset (day-13) and the level showed apparent decrease on day-33 compared to that of normal (Fig. 4).

Discussion

Localization of bFGF at the SCJ and panni in inflamed arthritic joints but not at the SCJ of joints from control rats, suggests that this multifunctional factor may play a role in the process of bone destruction. The presence of bFGF at SCJ and panni of the inflamed joint may be responsible for initiation and progression of joint destruction. Alternatively, it may be a secondary event as a result of local response to the joint damage. Our data seem to support the former hypothesis. First, bFGF staining was present early upon the onset of arthritis preceding bone erosion. Second, bFGF levels, as shown by immunoblot analysis, were increased at the time of onset (day 11-13) of arthritis which was 5-7 days prior to microscopic bone destruction. Increased staining persisted through the phase of progressive bone destruction (day 17-24), although it was not reflected in the immunoblot. The much greater increase in the number of lymphocytes that did not express bFGF may have diluted the specific immunoreactivity of bFGF in tissue extracts during this period. In contrast, PDGF-A staining was not observed to increase in afflicted joints until active osteogenesis took place 7-10 days after onset, when extensive joint destruction had already occurred. Third, bFGF immunoreactivity was also present in periosteum proximal to SCJ where no bone erosion was apparent. In fact, active osteogenesis was observed in these areas, suggesting increased bFGF expression is not a secondary event resulting from bone destruction. It has been shown that bFGF is a potent inducer of a variety of metalloproteinases [8, 9] and plasminogen activator [10]. Increased expression of metalloproteinases in rheumatoid synovium has also been shown

by both immunohistochemistry and *in situ* hybridization [30-33]. Furthermore, the invasive growth of synovial panni requires active cell proliferation; colocalization of bFGF and BrdU/PCNA in cells at SCJ indicate that its mitogenic activity may be a major contributor to pannus formation. This speculation is consistent with a recent study which shows increased expression of tyrosine-phosphorylated proteins by synovial tissue from rats with AA [34], indicating GF-related cell activation in arthritic synovium. It seems that bFGF's role in bone destruction is at least two-fold. It may directly or indirectly initiate or upregulate expression of metalloproteinases which in turn degrade proteoglycan and collagens ultimately leading to joint destruction. In addition, it may stimulate cell proliferation leading to the formation of panni that invade and replace bone tissue.

Studies have also shown that bFGF stimulates bone formation *in vitro* as well as *in vivo* [18-21]. Increased bFGF expression throughout the healing process of bone fracture has also been reported [35]. Our observations are consistent with these findings and strongly suggest that bFGF may also function as an osteotrophic factor in periosteal bone formation during adjuvant arthritis. First, strong bFGF staining was always found in the periosteum adjacent to newly-formed bone tissue during the phase of active osteogenesis (day 17-27). Second, bFGF was colocalized with BrdU/PCNA at the thickened periosteum suggesting its involvement in active cell proliferation in this region. Furthermore, both cell number and staining for bFGF and BrdU/PCNA exhibited a drastic decrease as the disease process progressed into remission (day 33-40). Such tightly coupled staining patterns strongly implicated bFGF in the periosteal osteogenesis observed in this arthritic animal model. In contrast, PDGF-A staining showed a quite different temporal and spatial pattern in this region. Its presence lagged about a week behind that of bFGF and was restricted to the osteoid where no cell proliferation was detected. Such temporal and spatial differences may reflect a functional coordination between these GFs. The simplest scenario is that during osteogenesis, increased bFGF

with its mitogenic and angiogenic effects may have led to active periosteal cell proliferation for the formation and expansion of the osteogenic tissue. The subsequent expression of PDGF-A may be responsible for continuation of the osteogenic process i.e. differentiation, maturation and calcification. The immunostaining for both BrdU and PCNA in the nuclei of hyperplastic synovium observed in this study is consistent with our pervious observation made in human rheumatoid synovium that local cell proliferation contributes significantly to synovial hyperplasia [28].

Our observations are consistent with other studies suggesting that bFGF may have dual effects during articular inflammation (see Introduction). One important question is what dictates bFGF's effects on the arthritic disease process, i.e. what decides whether bone destruction or osteogenesis is elicited by locally increased bFGF expression. Two major factors may underlie such distinct tissue responses: the intrinsic difference of different types of target cells and the difference in microenviroments at SCJ and periosteum along the bone shaft. SCJ differs from the proximal periosteum in both of these two aspects. First, there are two major types of cells in SCJs that are absent in proximal periosteum: chondrocytes and synoviocytes. Both of them are important sources of metalloproteinases and are major targets of bFGF [22, 36, 37]. Second, dramatic infiltration of inflammatory cells occurs in synovium at SCJs and around tendon, while cell infiltration along bone shaft is usually mild, if present. The inflammatory cells are rich sources of a variety of cytokines such as IL-1 and TNF-alpha that are known to collaborate functionally with bFGF. For example, bFGF upregulates IL-1 receptor expression in chondrocytes [11] and thereby potentiates IL-1-induced metalloproteinase production [8]. Moreover, IL-1 and bFGF lead to a synergistic increase in prostanoid production by articular chondrocytes [8, 13]. bFGF also synergizes with TNF-alpha in inducing the synthesis of matrix proteinases and the release of proteinases from synovial fibroblasts [12]. It appears that increased bFGF at SCJs not

only stimulates cell proliferation to form pannus but also, with collaboration of IL-1 and TNF-alpha at the region, upregulates expression of matrix proteinases by chondrocytes and synoviocytes ultimately resulting in bone destruction. Increased bFGF at proximal periosteum, on the other hand, may target periosteal cells and, with collaboration of a different set of GFs such as PDGF-A, induce bone formation. In support of this are the findings that intraarticular infusion of bFGF alone promotes cartilage repair while co-injection of bFGF and IL-1 induces cartilage degradation in rabbits [17, 37]. Taken together, our observations and those of others suggest that the microenvironment is critical in programming cellular responses to bFGF and that collaboration of a number of growth factors may be important during the disease process.

The concomitant presence of increased bFGF expression and onset of the articular inflammation does not mean that bFGF initiated the disease process. In fact, absence of increased bFGF staining prior to the onset on day 9 and of articular inflammation in arthritis-free limbs from arthritic rats, despite increased bFGF staining, suggests that bFGF is not an initiating factor of the inflammation. Other GFs such as IL-1 and TNF-alpha are more likely candidate(s).

Functional overlap is known to exist among certain multifunctional GFs. Both PDGF and bFGF, for example, are mitogenic for mesenchymal cells; both potentiate IL-1-induced synthesis of metollaproteinase by chondrocytes [8, 38, 39], both accelerate the wound-healing process and the repair of bone fractures [35]; and both stimulate osteoblast proliferation and induce bone formation *in vitro* as well as *in vivo* [40-42]. It also seems a common occurrence that some GFs exhibit tissue colocalization. For example, acidic FGF, PDGF-B and TGF-beta are all reportedly expressed by synovial cells from the synovial membrane from patients with rheumatoid arthritis and Lewis rats with experimental arthritis [43-45]. Using high concentration of primary antibodies (20-50 μg/ml) and the ABC method, the investigators also localized these GFs in the synovial

samples [43-45]. Localization of other cytokines in rheumatoid synovial membrane has also been documented [1]. More recently, co-localization of aFGF and PDGF-B with phosphotyrosine-containing protein is reportedly present in arthritic joint tissue from human and rats [34]. In contrast, our studies indicate a striking disparity in the temporal and spatial expression patterns of two GFs with similar functions *in vitro*. These differences can, of course, be attributed to the distinct growth factors that were detected in each report. The reciprocal nature of the staining that we detect argues strongly for unique roles for each growth factor. More importantly, our study related the growth factors' expression with pathological changes in the course of the articular inflammation and suggested that bFGF may play an important role in joint destruction and new bone formation. Although functional collaboration of different GFs exists in articular inflammation, specific GFs may be responsible for a specific aspects of the disease process. Modulation of the expression or activity of a specific growth factor at an appropriate stage may effectively modulate the pertinent disease process. Experiments are currently in progress to test this hypothesis by *in vivo* modulation of bFGF activity.

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Figure Legend

Figure 1. Immunostaining of SCJs: staining for bFGF (pink) in normal SCJ is minimal and is mainly associated with blood vessels (arrows) (a) while increased bFGF staining is found at SCJ at early stage of inflammation (day-12) (b). Early bone erosion by a newly-formed pannus was accompanied by strong bFGF staining (pink) of the pannus (day-17) (arrowheads) (c). Strong bFGF staining (pink) is seen throughout the pannus at the peak of erosion (day-23) (d). As the erosion progresses, the pannus invades into and replaces subchondral bone. Strong bFGF immunoreactivity remains at the pannus/bone junction (arrowhead) (e). Note the periosteal bone formation (stars) (e). Replacing the anti-bFGF and anti-PCNA with nonimmune antibodies for the first and second labeling abolishes the specific staining (f). Magnification: a - c and f = 200X; e = 400X.

Figure 2. Immunostaining of periosteum and osteogenic tissue: double staining shows the colocalization of bFGF (pink) and the cell proliferation marker, BrdU (black), in thickened periosteum (a). Increased staining for bFGF (pink) and PCNA (black) is found as periosteum undergoes hyperplasia (b) and begins to develop into osteoid (arrowhead in b). Periosteal hyperplasia was followed by formation of osteoid (arrowheads) as shown in (c) and (d). Note the colocalization of bFGF (pink) and PCNA (black) in the hyperplastic periosteum and staining for PDGF-A (brown) in osteoid (d). Fig. 2e and f show triple-labeled osteogenic tissue on day-24. The newly-formed osteoid growing from original bone shaft is stained for PDGF-A (golden brown) and surrounded by thickened periosteum with bFGF staining (pink) (e). The same tissue field under fluorescence demonstrates localization of BrdU-positive cells (bright green) in the thickened periosteum (2f). Note the reciprocal staining pattern of PDGF-A and bFGF (e), and the absence of both bFGF and PCNA/BrdU staining in the osteogenic tissue.

Fig. 2g shows decreased bFGF (pink)/PCNA (dark brown, arrows) staining and cell number of periosteum (arrowheads) at late stage (day-40). Magnification: a and b = 400X; c-g = 200X.

Figure 3. Immunostaining of inflamed limbs with synovitis and tendinitis: (a) shows double-labeling of bFGF (pink) and PCNA (brown) in arthritic synovium. Increased bFGF staining is found both intra- and extracellularly in the inflamed synovium. Note that the majority of lining synoviocytes exhibit nuclear staining for PCNA. (b) demonstrates increased bFGF (pink) staining and PCNA (black) in an inflamed synovial sheath invading a tendon. (c) shows medullary bone sample as a positive control. Note that the positive staining for PDGF-A (brown) associated with megakaryocyte (arrows) and bFGF staining (pink) with blood vessel and osteoblasts (arrowheads) were mutually exclusive.. Magnification: a and c = 400X; b = 200X

Figure 4. Western blot analysis of total joint tissue for bFGF: a specific band with an apparent molecular weight of 16 - 17 kD that exhibits identical migration as human recombinant bFGF (lane 1) is found in all joint samples (lane 2 - 14). The high molecular weight bands (≥ 25 kD) result from cross-reaction of contaminating endogenous rat immunoglobulin with the (HRP-conjugated horse anti-mouse) secondary antibody as shown in lane 15.

Figure 5. Histogram shows the percentage of SCJs that exhibit bone erosion (closed box) and positive staining for bFGF (open box) during the course of AA. Determination of bFGF staining data on day 30-40 was not applicable due to massive destruction by invasive panni that completely replaced SCJs.

Table 1. Summary of Pathological Changes during the Arthritic Inflammation

Day	0	9	11 - 13	17 - 20	22 - 27	33-40
n =	4	5	6	24	11	5
Score range	0	0-10	30-60	60-80	60-80	50-80
Major pathologic changes						
Periosteum (cell layer)	1-2	1-21	hypertrophy	hyperplasia	hyperplasia	2-4
Infiltration*	---	---	+	++	+++	++
Neovascularization†	---	---	++	+++	+++	+
Pannus formation	---	---	---	+	+++	NA
Bone destruction§	---	---	+	++	+++	NA
Osteogenesis ¶	---	---	---	+	++	+++
bFGF/PDGF-A Immunostaining #						
Synovium	--/--	--/--	+/--	++/--	++/--	+/--
Periosteum	--/--	--/--	++/--	+++/--	+++/--	+/--
Pannus	NA	NA	NA	+++/--	+++/--	+/--
Osteogenic tissue	NA	NA	NA	++/+	++/++	+/+++

(Continue on the next page)

Table 1. (Continue)

Day	0	9	11 - 13	17 - 20	22 - 27	33-40
bFGF staining at SCJs (Mean + SD%)						
Intact SCJs	13.2±6.3	17.1± 10.3	56.1± 14.9	78.1± 18.4	80.6± 10.1	NA
Eroded SCJs	NA	NA	87.5± 9.8	87.2± 11.3	88.1± 9.2	NA
Total SCJs	13.2±6.3	17.1 ± 10.3	63.7± 9.1	81.8± 9.2	86.5± 10.5	NA

* Infiltration: infiltrated cells per field at 400X 20-50 = +, 50-200 = ++, > 200 = +++.

† Neovascularization: small vessels per field at 400X 3-5 = +, 5-8 = ++, > 8 = +++.

§ Bone destruction: ratio of thickness of replaced and original cortex < 1/2 = +, 1 = ++, > 1 = +++.

¶ Osteogenesis: ratio of thickness of newly-formed bone/original cortex ≤ 1/2 = +, 1 = ++, > 1 = +++.

Immunostaining: weak +, moderate ++, strong +++.

NA: not applicable.

Table 2. List of Primary Antibodies used in this Study

Antibody	Species	Dilut./Concent.*	Treatment	Source
Anti-bFGF	Mouse§	1: 20,000 (< 3 μg/ml)	Hyaluronidase	ZymoGenetics. Inc., Seattle, WA
Anti-bFGF	Rabbit IgG	1: 500 (2 μg/ml)	Hyaluronidase	Biomedical Technologies Inc., Stoughton, MA.
Anti-BrdU	Mouse IgG	1: 100 (0.5 μg/ml)	Pepsin/HCl	Becton Dickenson, San Jose, CA
Anti-PCNA	Mouse IgG	1: 100 (3.7 μg/ml)	None	Dako Corp., Carpinteria CA
Anti-PDGF-A#	Rabbit IgG	1: 500 (1.4 μg/ml)	Pepsin	Zymogenetics

* Dilut./Concent.= Dilution/Concentration.

§ This antibody is raised against full molecules of human recombinant bFGF and does not cross-react with acidic FGF upon immunoblot analysis [46]. Clone number of this antibody is 148.6.1.1.1. The listed dilution is for 2-step indirect method. For ABC method, the dilution is 1: 10^6 (< 0.1 μg/ml).

\# The antibody is rased against human recombinant PDGF-A and is cross-adsorbed with PDGF-B. No cross-rection with PDGF-B is observed [47].

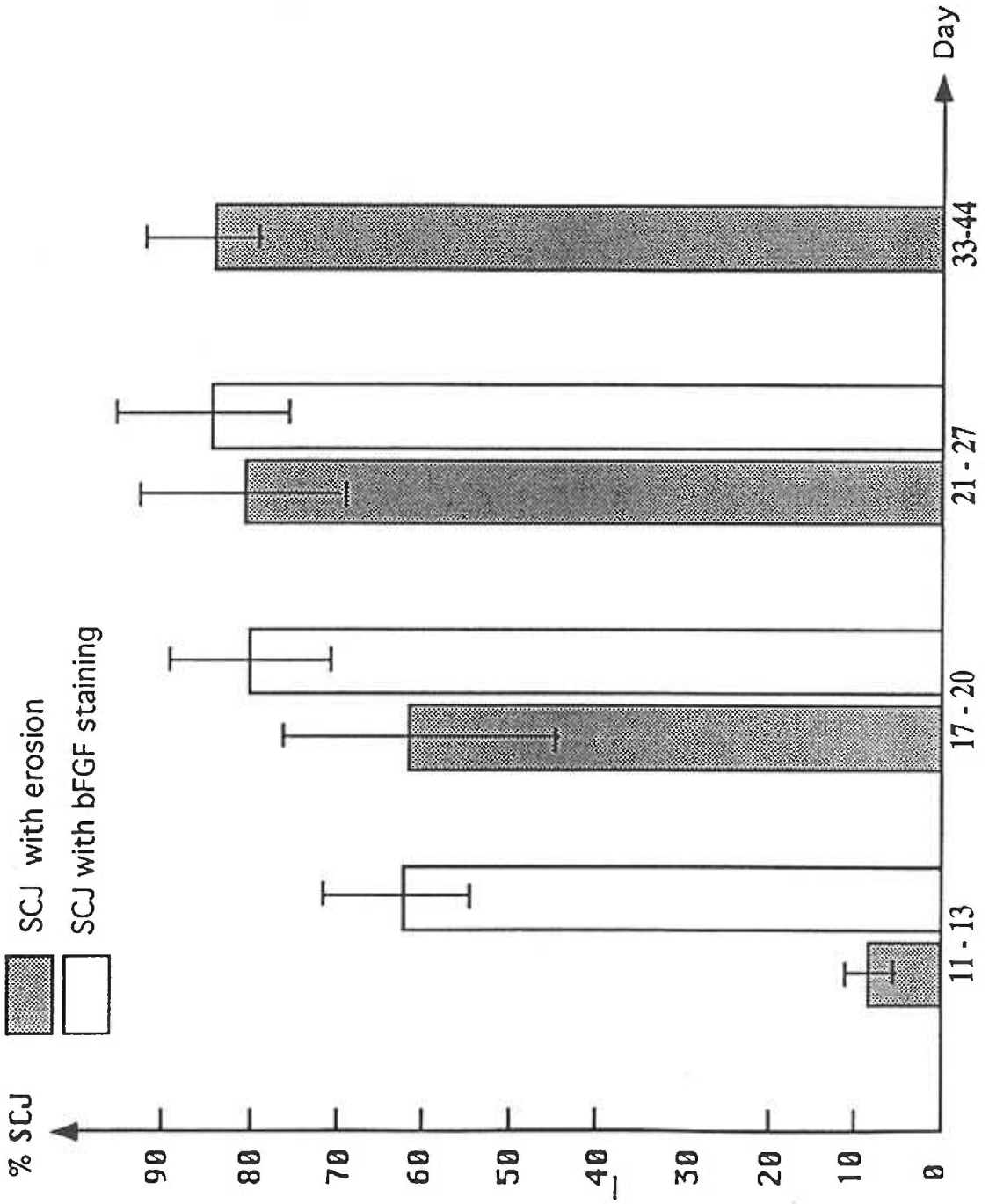

% SCJ
90
80
70
60
50
40
30
20
10
0
SCJ with erosion
SCJ with bFGF staining
11 - 13
17 - 20
21 - 27
33-44
Day

Fig. 5

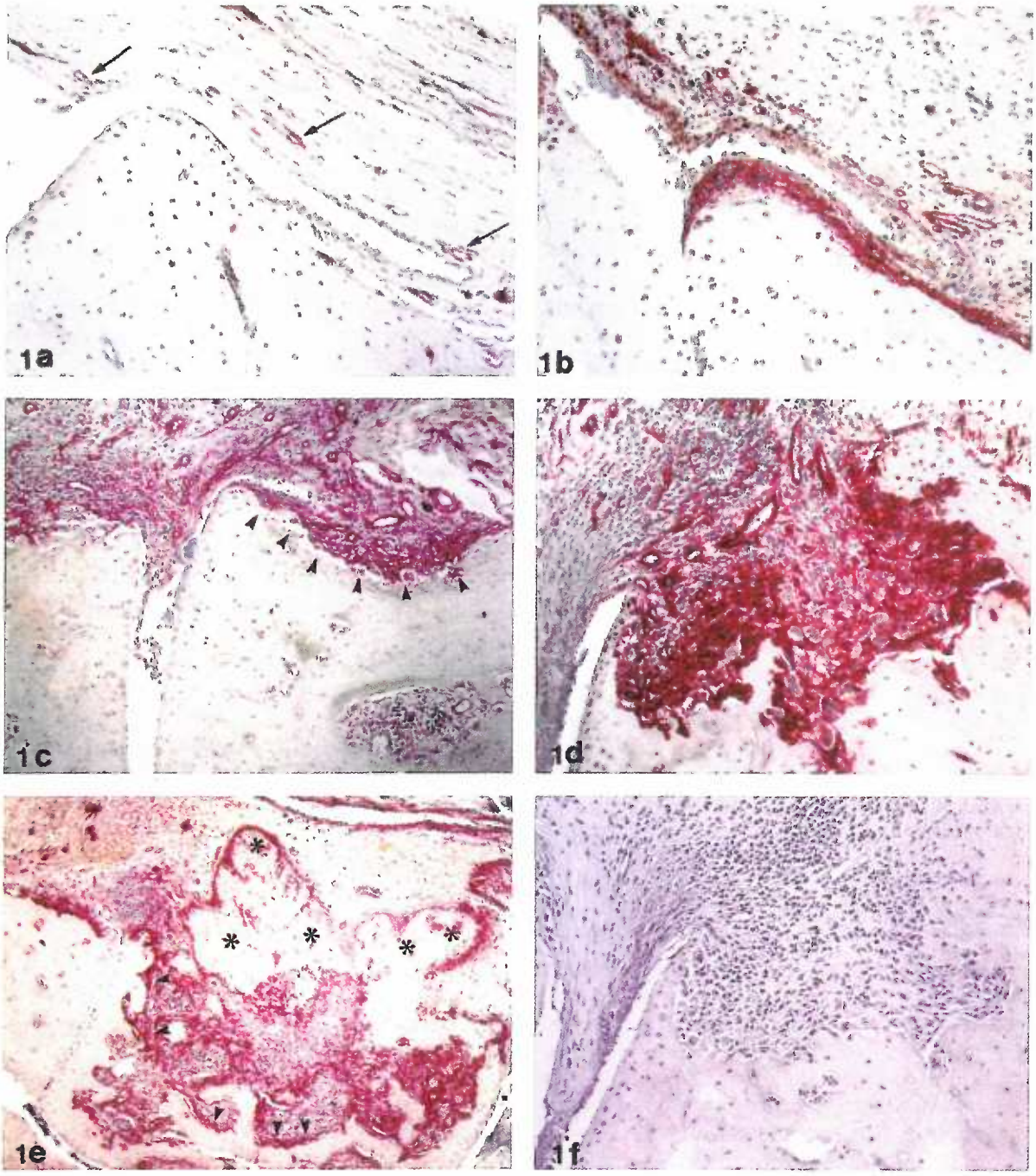
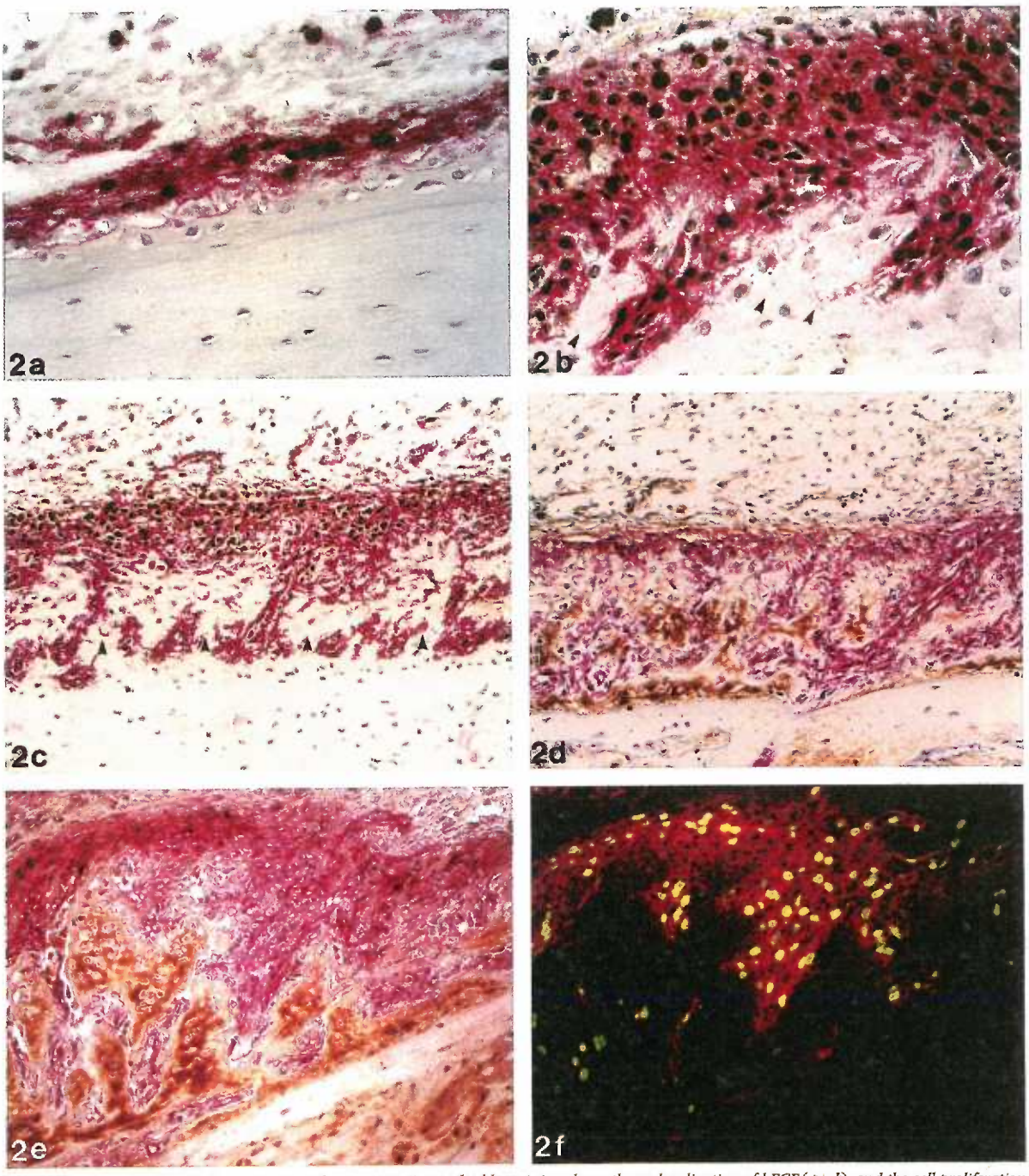


Figure 1. *Immunostaining of SCJs: staining for bFGF (pink) in normal SCJ is minimal and is mainly associated with blood vessels (arrows)* (a), *whereas increased bFGF staining is found at SCJ at early stage of inflammation (day 12)* (b). *Early bone erosion by a newly formed pannus was accompanied by strong bFGF staining (pink) of the pannus (day 17) (arrowheads)* (c) *Strong bFGF staining (pink) is seen throughout the pannus at the peak of erosion (day 23)* (d). *As the erosion progresses, the pannus invades into and replaces subchondral bone. Strong bFGF immunoreactivity remains at the pannus/bone junction (arrowhead)* (e). *Note the periosteal bone formation (stars)* (e). *Replacing the anti-bFGF and anti-PCNA with nonimmune antibodies for the first and second labeling abolishes the specific staining* (f). *Magnification:* a–c *and* f ×*200;* e ×*400.*



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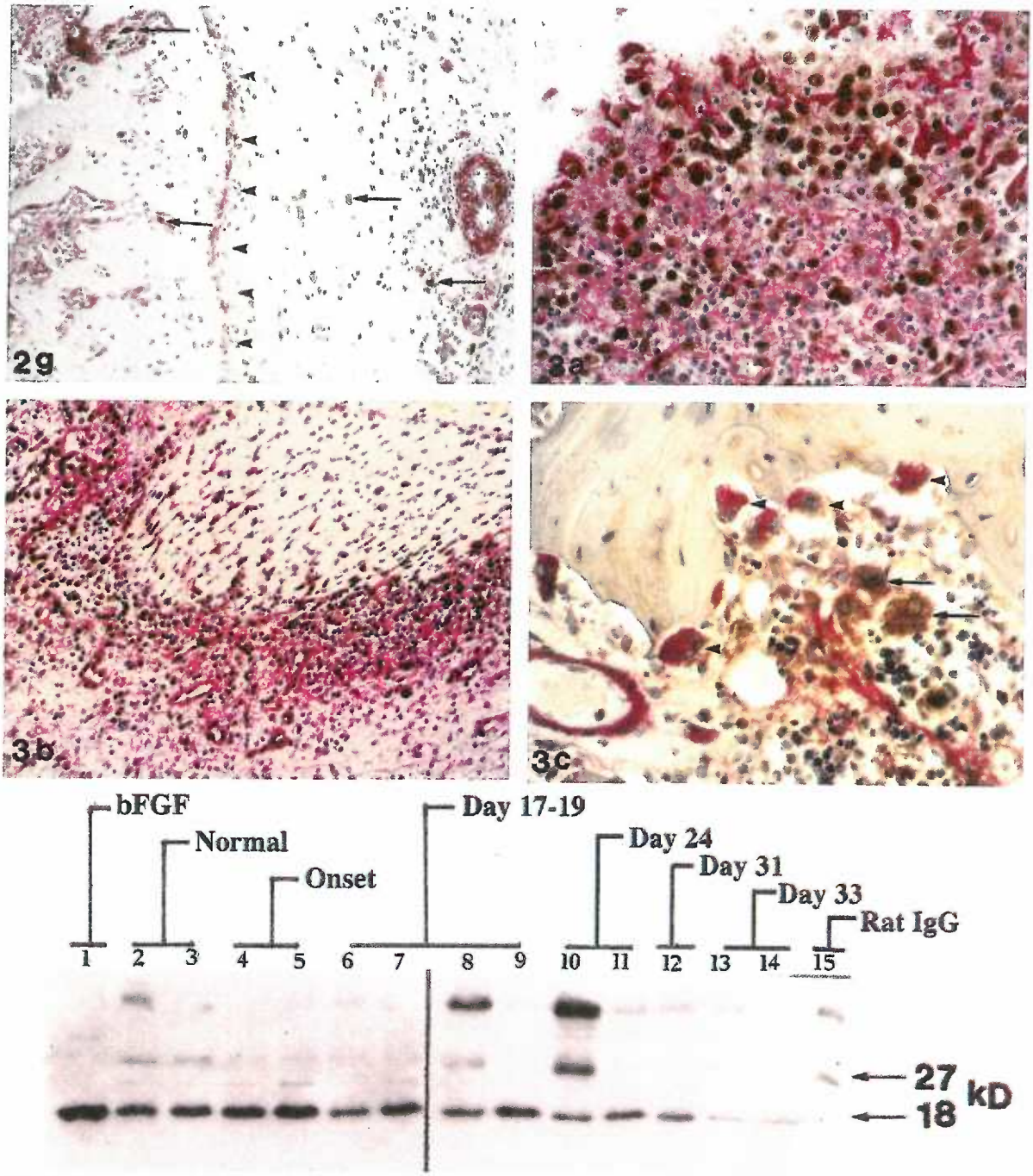

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Basic Fibroblast Growth Factor Attenuates Adjuvant Arthritis in Lewis Rats , While An Antagonist Exacerbates the Disease†

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Abstract

A large body of evidence suggests that polypeptide growth factors/cytokines are involved in articular inflammation. Increased expression of a variety of growth factors is found in synovial tissue from patients with rheumatoid arthritis and from experimental animals with polyarthritis. A commonly-held hypothesis is that synovial hyperplasia and joint destruction results from an increased expression of growth factors in response to the unknown triggering causes. Based upon this hypothesis, antagonists of such growth factors would be predicted to inhibit or abolish the development of articular inflammation. We recently observed striking temporal and spatial correlation between local basic fibroblast growth factor (FGF-2) expression and joint destruction during the development of adjuvant arthritis in Lewis rats. In an attempt to define further the role of FGF-2 during arthritis, we examined the arthritic changes following adjuvant inoculation in rats treated with FGF-2 or suramin. The latter is known to inhibit cellular responses to FGF-2 *in vitro* as well as *in vivo* by blocking ligand-receptor binding. Much to our surprise, continuous intravenous infusion of FGF-2 from day-7 to day-14 following adjuvant inoculation significantly ameliorated the articular inflammation while intravenous injection of suramin on day 7 and day 17 aggravated and prolonged adjuvant arthritis. These results, though paradoxical, together with our previous observations directly implicate FGF-2 in the experimental arthritis and suggest that FGF-2 plays an important role in articular inflammation. However, it would be overly simplistic to conclude that FGF-2 is exclusively pro- or anti-inflammatory. Elucidation of the underlying mechanism awaits further investigation.

Introduction

Growth factors (GFs) and cytokines by the nature of their mitogenic, angiogenic and chemoattractant properties are believed to be involved in destructive articular inflammation such as rheumatoid arthritis (RA) in humans and experimental arthritis in animals. A variety of GFs and cytokines have been detected in rheumatoid synovium from humans and joint tissues from animals with experimental arthritis. (for review see (1, 2)). Of the GFs and cytokines involved in joint inflammation, basic fibroblast growth factor (FGF-2) is of particular interest due to its broad spectrum of target cells and potency of biological activities. The finding that repeated intraarticular injection of (interleukin-1) IL-1 results in a chronic synovitis without cartilage and bone destruction (3), while co-injection IL-1 and FGF-2 induces degradation of articular cartilage in experimental animals (4), suggests an important role of FGF-2 in cartilage and bone destruction. Several lines of evidence support this notion. First, FGF-2 is a potent inducer of a variety of metalloproteinases (5, 6) and plasminogen activator (7). Second, it synergizes with both IL-1 and tumor necrosis factor (TNF)-alpha in inducing the synthesis of matrix proteinases and the release of proteinases from synovial fibroblasts (6, 8). Third, FGF-2 alone or in a synergistic manner with IL-1 induces the synthesis and release of prostaglandin E2 by articular chondrocytes and rheumatoid synovial cells (6, 9-11). In addition, we and others previously demonstrated that cultured synovial fibroblasts proliferate in response to FGF-2 (12). Recently, we reported that increased FGF-2 is localized at the synovium-cartilage junction and in the invading pannus of joint tissue from rats with adjuvant arthritis (AA); such an increase in FGF-2 precedes joint destruction and overlaps the active stage of the disease (13). Similar findings have also been made in human rheumatoid synovium (14). These observations suggest that FGF-2 may play an important role in the joint destruction.

A current hypothesis concerning joint destruction during RA and experimental arthritis is that the invasive growth of inflamed synovium results from sustained increases in GF expression in response to persistent inflammatory stimuli. The hypothesis implies that selective inhibition of the bioactivities of the increased GF(s) may attenuate the increased synovial growth and thereby prevent joint destruction. Conversely, supplement of exogenous growth factor(s) may have the opposite effect. Following this rationale, we attempted to modulate articular inflammation in Lewis rats with adjuvant arthritis using exogenous FGF-2 and a growth factor antagonist, suramin.

Suramin is a symmetrical urea compound originally used for treatment of African trypanosomiasis (15). It also functions as an antagonist to growth factors such as FGF-2, platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-beta). For example, it inhibits cellular responses to FGF-2 and PDGF *in vitro* by blocking ligand-receptor binding (16). Its inhibitory effect on cell proliferation induced by growth factors in different tumor cell lines promotes its clinical application as an anti-tumor treatment (for review see (17)). Its relative low specificity and broad spectrum of inhibitory effects on cell growth induced by different GF(s) make this compound a good candidate for blocking disease processes attributed to multiple GFs. We expected that suramin, by the virtue of its antagonism against FGF-2, would inhibit the experimental arthritis in rats. Much to our surpprise, suramin aggravated and prolonged the articular inflammation whereas systemic FGF-2 ameliorated the disease process.

Materials and Methods

Induction of Adjuvant Polyarthritis

Pathogen-free female Lewis rats (Harlan Sprague Dawley, Indianapolis, IN) at 160-180 grams were kept for at least 2 days under constant environmental conditions with

food and water *ad libitum* before experimentation. All the experiments were performed in accordance with the guidelines approved by the Committee for Care and Use of Laboratory Animals at the Oregon Health Sciences University. Adjuvant arthritis was induced by intradermal injection of 1-1.5 mg of mycobacterium butyricum (Difco, Detroit, MI) suspended in mineral oil (10 mg/ml) (Sigma) into the tail base as previously described (13).

Administration of Exogenous Human Recombinant FGF-2

The animals were anesthetized with 0.15 - 0.2 ml of a cocktail containing 50 mg/ml Ketaset (Ketamine HCl) (Aveco Co., Inc.), 1 mg/ml PromAce® (Acepromazine Maleate) (Aveco) and 5 mg/ml Gemini™ (Xylazine) (Rugby Lab. Inc.). Both intraarticular injection and the vascular cannulation surgery were performed under sterile conditions.

Human recombinant FGF-2 (147 aa) was carrier protein-free with total endotoxin content > 0.1 U according to the vendor (R &.D System, Minneapolis, MN). For intraarticular injection (n =4), 0.5 μg of FGF-2 dissolved in 10 μl saline was injected into ankle joint using a 27 gauge needle on days-8, -12, and -14. As a control, the contralateral ankle was given only the vehicle solution.

For continuous intravenous infusion, an Alzet@ osmotic mini pump (model 2ML1, flow rate = 10 μl/hr, loading volume = 2 ml) (ALZA Corporation, Palo Alto, CA) containing 50 μg of FGF-2 in 2 ml of a vehicle solution (1.6% glycerol, 0.02% sodium azide and 100-200 U/ml heparin) was embedded subcutaneously and connected to a Silastic® Translucent Tubing (VWR Scientific, Seattle WA) that was inserted into vena cava through the right jugular vein. The continuous infusion was carried out for 8 days at 10 μl per hour. For the control rats, the osmotic pump contained only the vehicle solution. Upon the completion of the experiment, the osmotic pump was examined for signs of malfunction such as excess retention of loaded solution or disconnection from the catheter.

Administration of Suramin

Suramin obtained through Centers for Disease Control from Germany was given intravenously at 20 - 25 mg/per rat in 0.5 ml of saline on days-7 and -17 following adjuvant inoculation.

Assessment of Joint Inflammation

The extent of arthritis was quantitated by volumetric measurement of each of the 2 hind feet as determined by immersion of each in fluid up to a tattoo mark 5 mm above each ankle. The amount of water displaced served as a quantitation of limb swelling. The severity of arthritis in each hind limb was scored as the percentage increase in volume as described elsewhere (13). Isoflurane (Ohmeda Caribe Inc., Liberty Corner NJ) in oxygen was used as an inhaled anesthetic during the measurement of foot volumes.

To determine the extent of bone destruction, radiographs of hind limbs were taken on day-17 and -24 and -52 at 25 KV, 4 OmAs with 9.2 compression using a Lorad M III mammograph system (Lorad M III, Danbury, CT). The degree of joint destruction as determined by bony decalcification was scored by an investigator who was masked to the experimental design. To score the bone destruction, one point was assigned to each site of apparent bone lose in the hind feet. Thus bone loss at calcaneum was give 1 point, talus was given 2, distal tarsal bones was given 5, proximal end of metatarsal bones 5, distal end of metatarsal bones (5). Two extra points were assigned for general severity of the bone loss. The maximal total scores in one limb would be 20.

Results

Continuous Intravenous Infusion of FGF-2 Had Time-dependent Effects

To avoid joint trauma while testing the effect of FGF-2 on the course of articular inflammation, FGF-2 was given intravenously through continuous infusion. When the

treatment started at or shortly after onset of arthritis, no apparent beneficial effect on afflicted joints was observed. The degree of inflammation and severity of bone destruction appeared comparable to that of control rats (data not shown). No change in the disease course was noted. However, when the infusion started 4 to 5 days prior to the onset of arthritis, FGF-2 significantly attenuated the articular inflammation and ameliorated severity of joint destruction (Table and Fig. 2c and 2d). The treated rats exhibited reduced paw swelling and redness, high mobility, and a steady increase in body weight. Radiographs revealed greatly reduced decalcification of subchondral bone in the afflicted joints compared to the control rats (Fig. 2c and d).

Suramin Exacerbated the Joint Inflammation

To test if inhibition of FGF-2 activity would affect the articular inflammation, suramin, a urea compound that is known to block FGF-2-induced cell proliferation (18-20), was given to the rats inoculated with adjuvant. As shown in Table I, treatment with suramin significantly increased the incidence of polyarthritis, exacerbated the joint inflammation, worsened the severity of joint destruction, altered the disease course, and prolonged the destructive phase of the polyarthritis. Administration of suramin markedly increased foot swelling (Table I., and Fig. 3) and severity of bone destruction (Fig. 2b and 2c). Suramin-treated rats also showed severe weight loss and lethargy.

Intraarticular Injection of FGF-2 had no Significant Effect on the Disease

To test if FGF-2 was directly involved in joint inflammation, FGF-2 was given intraarticularly to rats inoculated with adjuvant. Administration of 0.5 μg FGF-2 on days 8, 12, and 14 had no significant effect on the inflammation in all treated limbs. Paw volume and severity of bone destruction were comparable to the contralateral control limb (data not shown). The arthritic changes followed the characteristic course of this model. Joint swelling started at onset (day-11, -13) and reached its peak on day 20, and severe bone destruction as demonstrated by bony decalcification on X-ray was observed in day

20-24. No apparent intraarticular bleeding nor bacterial infection due to injection was observed.

Discussion

Although our previous study suggests that increased FGF-2 may be a driving force behind the joint destruction during articular inflammation, intraarticular administration of exogenous FGF-2 failed to have significant impact on the disease. Dosage, timing or limited delivery to a critical microenvironment could affect these results. Alternatively, an increased expression of endogenous FGF-2 as a result of articular inflammation might lead to saturation of receptors for FGF-2. We previously demonstrated increased immunohistochemical staining in both rheumatoid synovium from patients with RA and joint tissues from rats with adjuvant arthritis suggesting a locally increased level of FGF-2 during the articular inflammation (13). It is conceivable that increased endogenous FGF-2 may saturate its available receptors, and thereby prohibit further stimulation by exogenous FGF-2.

FGF-2 is one of the most potent angiogenic factors known. It has been shown that treatment with AGM-1470, an angiogenesis inhibitor, significantly suppresses collagen-induced polyarthritis in rats (21). That intravenous infusion of FGF-2 initiated 5 days prior to, but not after, the onset of inflammation significantly ameliorated the articular inflammation and joint destruction appears to be paradoxical. There are at least three possible explanations for this observation. First, the pre-onset infusion of FGF-2 may lead to downregulation of its receptor expression, which may subsequently attenuate the cellular response to endogenous FGF-2 when the disease occurs. Second, high levels of systemic exogenous FGF-2 may operate on immune system and change the immune response to adjuvant. Third, FGF-2 at the therapeutic dose may induce tissue responses different from those elicited by physiologic local concentrations of FGF-2. The time-dependent effects of exogenous growth factor on a disease process has also been

observed in other experimental disease models (22, 23). The potential effects of FGF-2 on immune response are incompletely studied. Nevertheless, our study clearly demonstrates that the disease process of articular inflammation can be significantly attenuated by exogenous FGF-2 and indicates that FGF-2 does play an important role during joint inflammation.

Our finding that the therapeutic effect of exogenous FGF-2 depended on the route of administration has several analogies. TGF-beta, for instance, induces rapid onset of articular inflammation when given intraarticularly (24, 25), but it protects the animals from experimental arthritis when given systemically (26, 27). We have recently observed that locally injected IL-10 dramatically inhibits endotoxin-induced eye inflammation while intraperitoneally injected IL-10 appears to worsen endotoxin-induced eye inflammation (Rosenbaum and Angell, submitted for publication). However, in contrast with TGF-beta and IL-10, FGF-2 has not been shown to have immune inhibitory effects.

Although suramin inhibits bioactivity of many polypeptide growth factors by binding to them and causing growth factor aggregation (16), it has been shown that treatment of cells with suramin also leads to an increased expression of receptors for FGFs *in vitro* as well as *in vivo* (28, 29). Thus, the net effect of suramin on cell response to FGF-2 may be determined by the magnitude of the increase in the receptor number and the level of local FGF-2. The marked increase in FGF-2 in inflamed joint tissue as we reported elsewhere (13, 14) may favor an exaggerated cellular response to FGF-2. Although the low specificity of suramin as a growth factor antagonist and its opposing effects on growth factor activity and FGF-2 receptor number make definitive interpretation of this finding difficult, the result is consistent with those from FGF-2 infusion experiments: FGF-2 attenuates while an antagonist exacerbates the articular inflammation.

In summary, our data indicate that properly timed infusion of FGF-2 can ameliorate adjuvant arthritis whereas a growth factor antagonist, suramin, worsens the disease. Although these observations are contrary to what we initially expected, they do not necessarily refute our previous findings because changes in the levels of receptors for FGF-2 might reverse the direct effect of FGF-2. An alternative tantalizing explanation for these observations is that synovial inflammation represents "a wound that does not heal". As in wound healing, growth factor expression in inflammation plays a critical role and exogenous growth factor can accelerate the healing process. The cytokine/growth factor network represents a complex interaction with positive and negative feedback loops at many junctures. Perturbing this balance alters the course of adjuvant arthritis. It would be overly simplistic to conclude that FGF-2 is either exclusively pro-inflammatory or anti-inflammatory. A more sophisticated understanding of the role of GFs in inflammation is needed to be able to control this network successfully for therapeutic benefit.

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Figure Legend

Figure 1. Schematic demonstration of joint scoring. a) Lateral view of hind limb indicating (with dots) 8 sites where bone loss was scored. b) Dorsal view of hind limb indicating an additional 9 sites where bone loss was scored.

Figure 2. X-ray radiography of hind feet: a) Represents a normal foot. It exhibits clear joint space and distinct shape of tarsal bones. No apparent soft tissue swelling or bone loss is observed. b) shows an arthritic foot from a suramin-treated rat on day 24 . Severe joint destruction and bone loss are easily seen in tarsal and metatarsal bones characterized by patchy low density areas and disappearance of joint spaces. c) demonstrates an arthritic foot on day 23 from a rat treated with vehicle solution only as the control for FGF-2-treatment. Moderate bone destruction is found at peripheral ends of the tibia bone, central tarsal and the proximal end of several metatarsals. Although the afflicted bones lost their distinct shapes, the joint spaces are generally distinguishable. The severity of joint inflammation in this foot is also typical of that from arthritic rats without any treatment. d) shows an arthritic foot on day 25 from a rat treated with continuous infusion of FGF-2 for 8 days starting on day 7. The treatment greatly attenuated the joint inflammation. Although several tarsal bones exhibit some patchy bone destruction, changes in their shape and general structure are minimal. The destructive changes are mild.

Figure 3. Photography of hind limbs from normal (A) and arthritic rats (B and C). Hind limbs from the arthritic rats exhibit apparent swelling. Treatment with suramin exacerbated the joint inflammation reflected by the increased foot swelling (C) compared to nontreated arthritic control (B). Vasodilatation caused by isoflurane anesthesia leads to the redness of limbs and masked the original difference in the color between the normal and arthritic rats.

Table I. Effects of FGF-2 and Suramin on Articular Inflammation

	Suramin Treatment			FGF-2 Treatment †		
	Control (n = 62)		Suramin-treated (n = 22)	Control (n = 9)		FGF-2 treated (n = 5)
Incidence	87.2%		100%	88.8%		20%
Foot Volume *	58 ± 32%	(p ≤ 0.01)	110 ± 21%	76 ± 36%	(p ≤ 0.01)	5.6 ± 3.9%
Joint Involvement	Mainly hind-feet		Both hind- and fore-feet	Mainly hind-feet		Hind-feet
Radiograph	Moderate		Severe	Moderate		Mild
Remission	Day 35-40		Later than day 40			

† FGF-2 was administrated by continuous infusion starting on day 7 as described in Materials and Methods.

* Foot volume was obtained at the peak of foot swelling (day 18-20) and expressed as percentage increase relative to day 0.

Figure 1. Schematic demonstration of joint scoring.

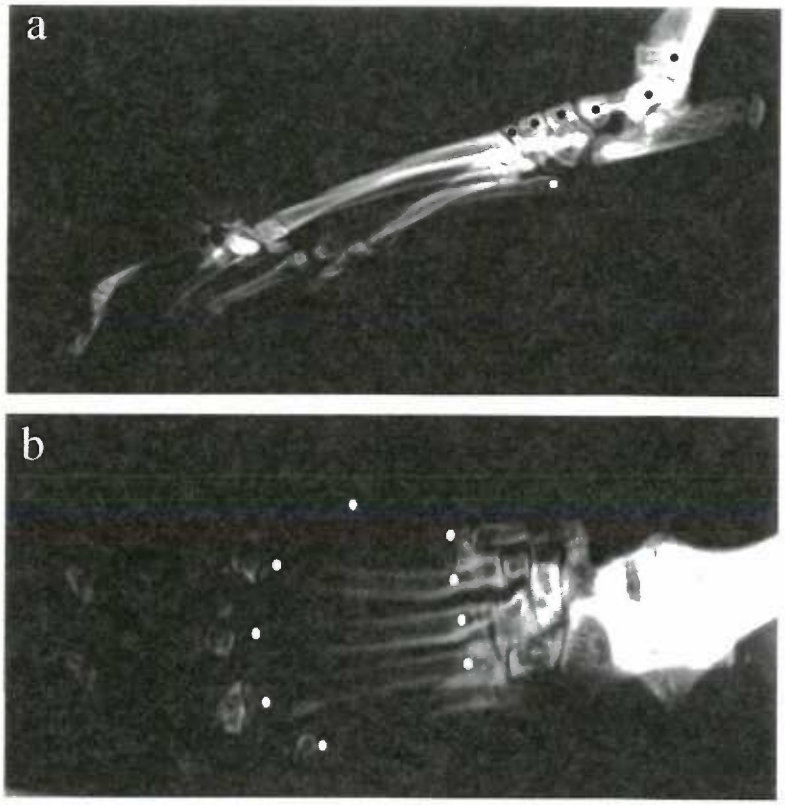

Figure 2. **X-ra radiography of hind Limbs of normal and arthritic rats.**

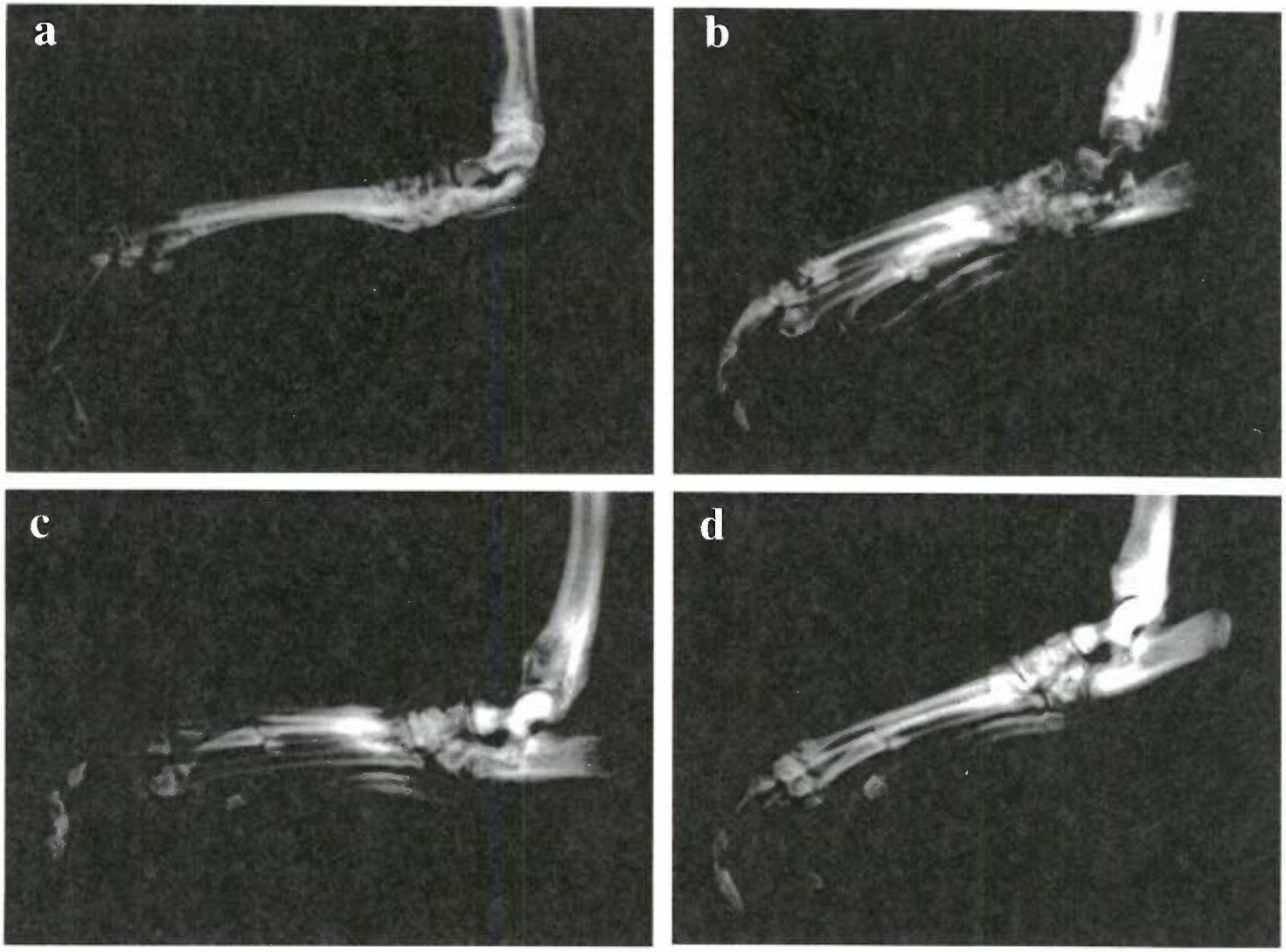

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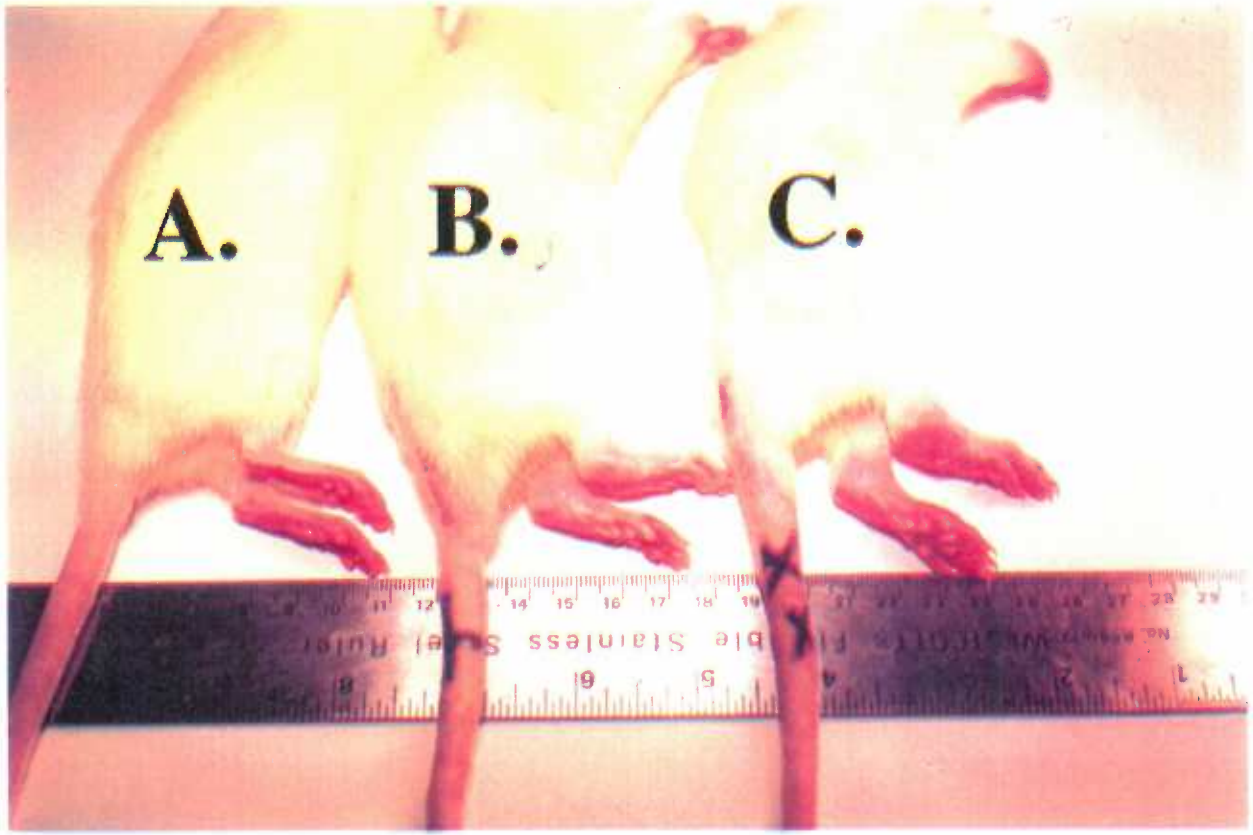
Figure 3. Photogaphy of hind limbs of normal and arthritis rats



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Local Proliferation of Fibroblast-like Synoviocytes Contributes to Synovial Hyperplasia

----- Results of Proliferating Cell Nuclear Antigen/Cyclin, c-myc, and Nucleolar Organizer Region Staining†

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Brief Index:

In rheumatoid synovium, hyperplasia of lining synoviocytes was previously attributed solely to migration of monocyte/macrophage from blood stream. This report shows that active proliferation of fibroblast-like synoviocytes contributes to synovial hyperplasia

Abstract

Objective To test the hypothesis that local proliferation contributes significantly to the hyperplasia of rheumatoid synovium..

Methods Immunohistologic and chemical staining was used to identify 3 markers 0f cell proliferation: proliferating cell nuclear antigen (PCNA), c-myc proto-oncogene and nucleolar organizer regions (NOR). Synovium from 21 patients with rheumatoid arthritis, 34 with degenerative joint disease, and 7 with joint trauma was examined.

Results All 3 markers indicated substantial, active proliferation of synovial lining cells in synovium with hyperplasia. Proliferating cells showed type I procollagen immunoreactivity but were negative for CD68, a monocyte/macrophage marker. Proliferation was greater in rheumatoid arthritis than in the other other conditions evaluated.

Conclusion *In situ* proliferation of fibroblast-like synoviocytes in the synovium lining contributes considerably to the increase in cell numbers in rheumatoid synovium.

Introduction

Hyperplasia of the synovial lining cell layer is a morphologic feature of rheumatoid arthritis (RA). However, the exact origin of the hyperplastic synovial lining in chronic inflammatory joint diseases is still a subject of controversy. Some evidence suggests that new hyperplastic lining synoviocytes are derived from monocytes which have migrated from the blood rather than from direct division of resident cells. First, mitotic figures of synovial lining cells are rarely observed. Second, immunohistochemical staining for Ki-67, a cell proliferation marker, is reportedly absent from rheumatoid synovium [1]. Third, after lethal irradiation of mice and heterologous bone marrow transplantation, the synovial macrophages are gradually replaced by cells with genetic markers of the bone marrow donor strain [2]. Fourth, synovial lining cells express a wide range of macrophage antigens which favors a bone marrow origin [3].

On the other hand, morphologic changes in synovial lining cells and ^{3}H-thymidine incorporation experiments are compatible with some degree of *in situ* proliferation of the synovial lining cells in RA. Rheumatoid synovium with hyperplasia of the lining synoviocytes but without inflammatory cell infiltration in the underlying region is occasionally observed suggesting that there might be *in situ* cell proliferation of lining synoviocytes. Furthermore, *in vivo* or explant cell labeling with ^{3}H-thymidine has shown that both normal and inflamed synovial lining cells incorporate ^{3}H-thymidine into DNA, although the frequency is less than 1% [4, 5]. In the present study we used proliferating cell nuclear antigen (PCNA), *c-myc*, and nucleolar organizer regions (NOR) staining to identify the proliferating activity of synovial tissues from patients with RA.

PCNA (or cyclin) is a 36-kd nuclear protein whose expression is associated with the S-phase of the cell cycle (for review see [6]). It is an auxiliary protein of DNA polymerase ∂ and plays a critical role in the initiation of DNA synthesis [7-10]. The presence of PCNA has been found to correlate with other cell proliferation indices such as

^{3}H-thymidine incorporation [11], and Ki-67 immunoreactivity [12, 13]. Immunohistochemical staining of routine paraffin-embedded tissue for PCNA has been accepted as a simple, reproducible, and reliable technique for identifying proliferating cells in normal and tumor tissues [13-15].

The *c-myc* proto-oncogene is the cellular homolog of the transforming gene of the avian myelocytic leukemia virus [16]. It is expressed in a wide range of cell types and is involved in DNA replication, transcription, and cell differentiation (for review, see [17, 18]). The *c-myc* gene encodes a nuclear protein that is expressed in proliferating cells but is absent from quiescent cells [19, 20]. Induced expression of *c-myc* has been found in different types of cells after mitogenic stimulation. Antisense oligonucleotides of *c-myc* inhibit entry of T lymphocytes into S phase and cell proliferation [21, 22]. Induction of the expression of c-myc under the control of a dexamethasone-inducible promoter results in increased cell proliferation of BALB/c 3T3 cells [23]. This evidence implicates *c-myc* in cell proliferation and validates it as a cell proliferation marker. Although *c-myc* protein is expressed by proliferating cells throughout the cell cycle, its half-life is estimated to be < 30 minutes [24]. This makes the immunohistologic detection of the protein very difficult but specific for the proliferating cell.

Nucleolar organizer regions (NOR) represent nucleic acid binding proteins and loops of DNA which are located on the short arms of the acrocentric chromosomes and possess genes for ribosomal RNA (for review, see [25]). NORs can be readily visualized in cultured cells and paraffin-embedded tissue sections by a single-step silver colloid staining method (AgNOR). They appear as black dots within the nucleus by virtue of the argyrophilia of the associated proteins [26, 27]. A clear correlation has been found between NOR counts per cell and cell proliferative activity measured by ^{3}H-thymidine incorporation [28], Ki-67 immunostaining [29-31] and by flow cytometry [32]

in tissues as well as in cultured cells. Thus, cell kinetic, immunologic and cytogenetic studies have shown that the number of NORs is correlated with cell proliferation.

Using PCNA, *c-myc* and NOR staining, we show that proliferation of fibroblast-like synoviocytes occurs in the hyperplastic lining layers. In contrast, macrophage-like cells are not involved in active proliferation. Our data provide direct evidence of *in situ* proliferation of synovial lining cells *in vivo* and suggest that the active proliferation of fibroblast-like cells may contribute to the hyperplasia observed in lining synovia in RA.

Patients and Methods

Tissue Processing

Synovial tissues were obtained during joint surgery from patients with RA (n = 21), degenerative joint disease (DJD) (n = 34) and trauma (= 7). The mean + SD ages in year for the patient groups are 60.3 (SD $\pm$ 14) for RA, 68.7 (SD $\pm$ 7.2) for DJD and 35.2 (SD $\pm$ 16.6) for trauma. There were 17 female and 4 male patients with RA, 2 female and 30 male patients with DJD (unknown in 2), and 1 female and 6 male patients with accidental trauma. Synovial samples were from knee, shoulder, hip and wrist joints. Clinical information about all patients with RA or trauma, and six patients with DJD examined with NOR staining is summarized in Table I. All RA patients met the 1987 ACR criteria for that diagnosis [33]. Tissue samples were divided into two parts: one fixed in 10% neutralized buffered formalin (NBF) and the other in B_5 fixative (0.22 M HgCl, 0.15 M sodium acetate and 4% formalin) for 1 - 2 days at 4 ^{0}C. After routine paraffin-embedding, tissues were cut into 4-micrometer sections and laid on poly-L-lysine coated slides. The sections were deparaffinized in xylene and rehydrated through graded series of alcohol and distilled water. For each subject, the staining was repeated at

least once to ensure specificity and consistency. Human tonsil tissue was used as a positive control and was processed the same as synovial tissue samples.

PCNA and *c-myc* immunohistochemical staining

For labeling of PCNA and c-myc, tissues fixed in NBF and B5 were used respectively. The avidin-biotin-complex (ABC) method was used according to Hsu et al. [34]. Briefly, after rehydration the sections were covered with blocking solution (phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA), 0.05% NaN_3, and 1.5% normal horse serum) to inhibit non-specific binding. The sections were incubated with the primary antibodies at 4 ^{0}C overnight. Mouse monoclonal antibodies specific for PCNA (clone PC10, Signet Inc., New York, NY) and for c-myc (Oncogene Sci. Inc., Manhasset, NY) were used at 0.08 μg/ml and 1 μg/ml respectively. After washing, the slides were incubated with biotinylated horse anti-mouse IgG antibody (Vector Lab., Burlingame, CA) at 1: 200 for 30 minutes at room temperature followed by treatment with 3% H_2O_2 in PBS to quench endogenous peroxidase. The slides were extensively washed in PBS and then incubated with ABC solution prepared as the vendor recommended (Vector Lab.) for 30 minutes at room temperature. The antigen-antibody complexes were visualized by incubation for ten minutes with substrate solution containing 0.5 mg/ml 3,3'-diaminobenzidine, 0.1 M imidazole and 0.03% H_2O_2 in PBS. The slides were then counterstained with methyl blue or Mayer's hematoxylin (Sigma Chemical, St. Louis, MD) and mounted in Crystal Mount (Biømeda Corp., Foster City, CA).

Double staining of cell proliferation markers and cell type specific markers

To identify PCNA/c-myc positive cells, samples were also labeled with antibodies to CD68, a monocyte/macrophage marker [35, 36], and type I procollagen, a fibroblast

marker [37]. For double labeling of PCNA or c-myc and macrophage markers, the alkaline phosphatase anti-alkaline phosphatase (APAAP) method was used for the second labeling. After staining of PCNA or c-myc, the slides were incubated in 1.5% normal goat serum in PBS and then in a monoclonal antibody specific for CD68 (Dako, Carpinteria, CA) at 1:1000 for 30 minutes followed by incubation with a goat anti-mouse link antibody at 1:100 (Dako). A mouse APAAP (Dako) at 1:100 was then applied to the sections for 20 minutes. Fast Red was prepared and used as the substrate according to the vendor (BioGenex Lab., San Ramon, CA). The sections were mounted as described above. Brief enzymatic digestion of the sections with protease type XXIV (Sigma) at 0.01% in PBS (pH 7.4) at 37 ^{0}C was carried out before second labeling.

Double labeling of PCNA and type I procollagen was performed using the ABC method. After PCNA staining, the sections were incubated in PBS containing 1% normal rabbit serum and biotin to block non-specific binding. Then a rat monoclonal antibody specific to human type I procollagen was applied at 1:1000 dilution (0.99 μg/ml) (Chemicon, Temecula, CA) followed by a biotinylated rabbit anti-rat IgG secondary antibody and alkaline phosphatase ABC (Vector) following the vendors' instruction. Fast Red was used as the substrate.

Non-immune immunoglobulin from the same species as the primary antibodies was used to replace the primary antibodies in first and second labeling as negative controls for each specimen. Double labeling of c-myc/CD68 and PCNA/CD68 or type I procollagen was performed in all samples used in this study. The slides were examined and graded on a scale of 0 - 3 + (see Table) by two researchers (one was blinded).

NOR staining

Six hyperplastic synovia from patients with RA and six non-hyperplastic samples from patients with DJD were stained for NOR according to the method of Crocker and Nar [27] using formalin-fixed samples. The staining solution was prepared by mixing

50% aqueous silver nitrate with 2% gelatin in 1% aqueous formic acid at a proportion of 2:1. The slides were incubated in the solution at room temperature for 45 minutes in darkness, and then washed extensively with double distilled water. Then the slides were dehydrated graded alcohol to xylene and mounted in PRO-TEXX mounting medium (Lerner Lab., New Haven, CT).

Each section was examined separately by 3 of us (ZQ, CHG, and LMO), two were blinded as to the tissue source). 1000 synovial lining cells of each sample were studied under an oil immersion lens at magnification of 1000 X. The average number of NOR per nucleus and percentage of cell populations containing different numbers of NOR numbers were compared between RA and DJD. The data were evaluated with both Student's t test and Mann-Whitney U test. All results were expressed as the mean ± SD.

Results

Immunohistochemical staining for PCNA

Of 21 synovia from patients with RA examined for PCNA immunoreactivity, 6 exhibited extensive hyperplasia of lining synoviocytes characterized by an increase to 5 to 8 cell layers and stratification of the synoviocytes with heterogeneous histological changes in terms of inflammatory infiltration, increased vascularity and fibrosis in the sublining region. 15 showed no or limited hyperplasia with 1 to 4 layers of lining synoviocytes (see Table). The lining synoviocytes were separated from the sublining region by underlying loose connective tissue or infiltrated lymphocytes. All rheumatoid synovia with marked hyperplasia exhibited extensive and strong PCNA immunoreactivity in the superficial synoviocytes. The frequency of positive cells ranging 30 - 70% varied from region to region within the same sample and from case to case. Staining for PCNA was found in the nuclei of the synoviocytes throughout the lining layers (Fig. 1a) and most PCNA-positive synoviocytes showed round nuclei with scarce cytoplasm or typical

fibroblast-like morphology with spindle-shaped or ovoid nuclei. In contrast, no staining for PCNA in macrophage-like cells with abundant cytoplasm and large round nuclei or multinucleated cells in the lining or sublining regions was observed (Fig 2a). In all of the RA cases examined, approximately 10 cells with mitotic figures were encountered and all of these cells were PCNA positive (not shown). In synovia from patients with RA with no or only limited hyperplasia, the frequency of PCNA-positive cells ranged from 5 - 20% of the lining synoviocytes (see Table). In control tonsil tissue, nuclear staining for PCNA was found in germinal centers and in scattered cells in the paracortex of lymphoid tissue (not shown).

In 18 of 32 DJD cases, staining for PCNA was absent in most lining synoviocytes. Single cells or 2 to 3- cell clusters with strong nuclear staining for PCNA were occasionally encountered in the sublining region. These PCNA positive cells were usually located in the basal layer of lining synoviocytes and made up about 1-3% of the lining synoviocytes (Fig. 1c). At increased concentration of primary antibody and increased incubation time with substrate, these synovial samples also exhibited PCNA immunoreactivity in up to 50% of superficial synovial cells. However, the staining was diffuse and much weaker than that seen in RA. In synovia from patients with trauma, positive staining for PCNA was rarely (< 1% in 6 of 7 samples) encountered.

The non-immune immunoglobulin control gave no staining in all specimens examined (Fig. 1b).

Immunostaining of c-myc

Staining for c-myc in synovia from patients with RA, DJD and trauma exhibited similar patterns as that for PCNA. Figure 2c, for example, illustrates the distribution of c-myc staining in synovium from a patient with RA. However, the staining was weaker and more diffuse than that of PCNA. Weak cytoplasmic staining was often noted in cells

with positive nuclear staining. In control tonsil tissue, staining for c-myc was also found in cells in the germinal center (data not shown).

Labeling proliferating cells with cell type specific markers

To characterize PCNA- and c-myc-positive cells, we also labeled the synovial samples with antibodies specific for CD68, a monocyte/macrophage marker [35, 36]. CD68 has been found to be restricted to cells of monocyte/macrophage lineage including macrophages of all different lymph node compartments and multinucleated giant cells, but not in granulocytes or dendritic reticulum cells [35, 36]. As expected, the anti-CD68 antibody selectively labeled the cytoplasm of perivascular monocyte/macrophage-like cells, multinucleated cells and a proportion of lining synoviocytes (Fig. 2a). No staining for CD68 was observed in lymphocytes and endothelial cells. Most of CD68-positive lining synoviocytes were devoid of nuclear staining for PCNA (Fig. 2a) or c-myc (Fig. 2c) suggesting that the proliferating cells may be "fibroblast-like" cells.

To confirm our speculation, samples were also labeled with an antibody specific to human type I procollagen, a fibroblast marker. In DJD, staining for type I procollagen was rarely encountered (<1%) in synovial lining layers (data not shown). A dramatic increase in number of the cells stained for the procollagen in synovial lining layers was observed in RA where hyperplasia and PCNA immunoreactivity were present, and the majority of PCNA-positive cells were also labeled by the anti-procollagen antibody (Fig. 2b). CD68 and type I procollagen staining seemed to be mutually exclusive in lining synoviocytes (Qu et al., in preparation).

The negative controls for both first and second labeling showed no staining.

NOR staining

Six synovial samples with extensive hyperplasia from RA patients and six non-hyperplastic samples from patients with DJD were stained for NOR (see Table). The

mean NOR number in lining synovial cells was 2.4 (SD ± 0.3) per nucleus in these RA and 1.5 (SD ± 0.2) in DJD. The difference in the RA and DJD means was significant ($p < 0.001$). Cells containing 4 and ≥ 5 NOR made up 2.9 (SD ± 1.2)% and 1.1 (SD ± 0.9)% of the total cells counted in DJD, while they made up 12.0 (SD ± 3.0)% and 9.1 (SD ± 5.4)% respectively in RA ($P < 0.001$) (Fig. 4). In DJD, the majority of lining synoviocytes contained 0 - 3 NORs per nucleus and the NORs were usually large (≥ 0.5 μm), dark brown and located in the center or peripheral region (Fig. 3b). In contrast, NORs in hyperplastic lining synoviocytes of RA usually numbered 3-5 per nucleus and tended to be granule-like black dots (≤ 0.3 μm) diffusely distributed (Fig. 3a). NOR number and pattern in RA tissue without synovial lining hyperplasia were similar to those in DJD. Endothelial cells and lymphocytes contained 0 - 1 and 0 - 3 large NORs respectively. In multinucleated cells NOR count was less than 1 per nucleus. In control tonsil tissue, higher NOR numbers were found in the germinal centers than in the perigerminal regions (data not shown). Cells in germinal centers were also strongly labeled by PCNA antibody (data not shown). In synovia from patients with accidental trauma, NORs were morphologically identical to those in DJD. Due to limited availability of the traumatic synovial membranes no statistical data could be obtained.

Discussion

The present study was designed to determine whether the hyperplasia of lining synoviocytes occurs solely as a result of cell migration or whether local proliferation of synoviocytes also contributes significantly to the hyperplasia. Using specific staining for PCNA, c-myc, and NOR to detect proliferating cells in synovium, we report here that lining synoviocytes do proliferate *in situ* . The lining synoviocytes labeled by anti-PCNA and -c-myc antibodies also react with type I procollagen antibody but usually exhibit no immunoreactivity with anti-CD68 suggesting that they are fibroblast-like cells instead of cells of hemopoietic lineage such as the infiltrated monocytes/macrophages. Hyperplastic

synovial samples from patients with rheumatoid arthritis show significantly higher NOR scores and greater number of PCNA-, c-myc-positive cells than that from patients with DJD or trauma. Taken together our data indicate that active proliferation of non-macrophage-like lining synoviocytes occurs in rheumatoid synovium and contributes considerably to hyperplasia.

The specificity of our PCNA immunostaining and its validity as a proliferating cell marker are supported by several lines of evidence. First, anti-PCNA from hybridoma clone PC10 has been reported to stain selectively the nuclei of proliferating cells in a wide range of histological materials routinely processed [13, 38]. Second, in control tonsil tissue, only cells in lymphoid germinal center where active cell division is known to occur were stained for PCNA. Third, all cells with mitotic figures encountered in this study were also labeled by the PCNA antibody. Since PCNA antibodies can be found in serum of patients with autoimmune disorders [39, 40], one may suspect that the PCNA staining is a disease-related marker. However, staining for PCNA in synovial lining cells was found in almost all synovial samples with hyperplasia regardless of the clinical diagnosis and was minimal when no hyperplasia was observed. This supports the notion that PCNA is a cell proliferation- but not disease-specific marker.

To confirm the PCNA staining results, the synovial tissues were also stained with c-myc antibody. C-myc is a growth related gene expressed throughout cell cycle in proliferating cells but absent in quiescent cells [19, 20]. One would expect that c-myc and PCNA antibodies would label the same cell population in our synovial samples if the labeling is proliferation-specific. As we predicted, the c-myc antibody gave similar staining patterns as PCNA.

As a third assay for assessing cell proliferation, we compared NOR scores of lining synoviocytes in samples from hyperplastic RA with those from non-hyperplastic DJD . The mean value of NOR scores in RA (n = 6) is significantly greater than in DJD

($n = 6$) ($P < 0.001$). Also, a greater percentage of superficial synoviocytes contains ≥ 4 NOR per nucleus in RA than in DJD. We also observed in these RA cases altered morphology and localization of NOR within nuclei that have been documented in studies of a variety of proliferative abnormalities *in vitro* and *in vivo* [28, 41, 42]. These findings reinforce our conclusion that direct proliferation of the lining synoviocytes contributes to the increase in cell number during this chronic inflammatory joint disease.

It is commonly believed that the hyperplastic lining cells are derived from monocytes which have migrated from blood circulation (see introduction). Our observation of expression of the markers associated with cell proliferation by lining synoviocytes, however, does not exclude blood-originated macrophage-like synoviocytes as a source of synovial hyperplasia, since the migrated monocyte/macrophage might also proliferate extensively in synovium. To exclude such a possibility, the synovial samples were also stained with an antibody specific for CD68, a monocyte/macrophage marker. Double-labeling showed that most PCNA- and c-myc-positive cells were not labeled by anti-CD68, while conversely, CD68-positive cells usually did not exhibit PCNA or c-myc immunoreactivity. This result indicated that the proliferating cells are, at least predominantly, fibroblast-like synoviocytes. To confirm such a claim, samples were also labeled with an antibody to human type I procollagen. The results clearly demonstrated that the majority of PCNA-positive cells, but not the multinucleated cells, were also stained for type I procollagen. The observation of markers associated with cell proliferation in lining synoviocytes in hyperplastic synovium together with the presence of PCNA staining in procollagen- but not CD68-positive lining cells strongly argues that blood-originated macrophage-like synoviocytes are not the only source of hyperplasia and that the *in situ* proliferation of fibroblast-like synoviocytes is significant. This is different from the result presented in a study using Ki-67 immunostaining by Lalor et al. [1] which suggests that the hyperplastic synovial lining cells are predominantly composed

of macrophages and do not arise by cell division but by cell migration from the underlying synovial blood vessels. Our results are consistent with the notion that as monocytes infiltrate into inflamed tissue and undergo maturation toward macrophages, they lose their proliferating potential. In synovial tissue culture, less than 1% of synoviocytes that survive three passages have macrophage markers. This has been attributed to the loss of proliferating potential of macrophage and macrophage-like synoviocytes [43]. Our results are also consistent with ^{3}H-thymidine incorporation experiments [4, 5].

We also noticed that both PCNA and c-myc immunoreactivities are present in synovium from patients with DJD, although the magnitude is lower than that in hyperplastic RA synovium. This finding is consistent with the notion that DJD synovium may also exhibit proliferative change.

We and others have observed considerable histologic variability in terms of hyperplasia, lymphoid infiltration, fibrosis, and neovascularization in rheumatoid synovium. Such variability may be affected by multiple factors which include age of the patients, medications and/or disease duration. The observation that extensive and strong staining for PCNA and c-myc was not found in all RA synovial samples or in all samples from younger patients appears to indicate that these cell proliferation markers are not disease-specific or age-related (Table. 1). However, we do not think any definite conclusion as to whether age and other clinical factors may affect the staining can be made based on the limited number of cases examined. Additional study is needed to test the hypothesis that proliferative status can be used as a diagnostic or prognostic criterion. We and others have also shown that growth factors such as basic fibroblast growth factor and platelet-derived growth factor are also detected in the lining synoviocyte in RA (Qu, et al., in preparation). Active synoviocyte proliferation may play an important role in rheumatoid arthritis.

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Figure Legend

Fig. 1. Staining of PCNA in synovial tissue. Positive immunoreactivity is located in the nuclei. Extensive staining for PCNA (black) is found in lining synoviocytes of RA (BJF) (a), while negative control (non-immune IgG) gives no staining (b). In DJD (CFW) positive staining (arrows) is minimal (c). Magnification. 200X for (a) and (b); 400X for (c).

Fig. 2. Double labeling of cell proliferation- and cell type-specific markers in RA synovium (MW): (a) shows double staining for PCNA (black) and CD68 (pink); (b) shows staining for PCNA (black) and type I procollagen (pink). Note the PCNA-positive cells (arrows) are devoid of staining for CD68 (2a) but are also labeled by anti-type I procollagen (2b) whereas the CD68-positive cells (arrowheads) are not labeled by either anti-PCNA (2a) or by anti-type I procollagen (2b). Fig. 2c shows double staining for c-myc (arrows) and CD68 (arrowheads). Note, the staining of cells for c-myc in the nucleus and CD68 in the cytoplasm is mutually exclusive. Magnification 400X.

Fig. 3. NOR staining in RA (BJF) (a) and DJD (AH) (b) synovium. Note increased layers of lining synoviocytes (arrowheads mark the synovial surface) and higher NOR number per cell in RA (a) than in DJD (b). Synoviocytes with ≥ 4 NORs per cell are labeled with arrows (a). Magnification 1000X.

Fig. 4. Percentage distribution of lining synoviocytes containing 0 to ≥ 5 NORs in RA and DJD. P values are obtained by both Student's t and Mann-Whitney U tests.

Table I. List of Patients Examined in This Study

Patient	Age	Sex	Diagnosis	Duration	Hyperplasia≠	PCNA§
BG	47	F*	RA	8	++	++
BJF†	59	F	RA	27	+++	+++
DS	29	F	RA	22	++	+
EEK	62	F	RA	52	++	--
EMH	72	F	RA	NA*	--	+
ES	75	F	RA	23	++	--
FEA†	75	F	RA	14	+++	++
GSH	76	F	RA	NA	++	+
GTS†	49	M	RA	19	+++	+++
JAC	51	F	RA	25	--	+++
JAC	70	F	RA	39	++	+++
JAN	59	M	RA	15	--	--
JET	58	F	RA	26	++	+
KEK	19	F	RA	NA	++	++
LLF†	65	F	RA	22	++	++
MW†	60	F	RA	30	+++	+++
NLD†	53	F	RA	10	+++	+++
PAB	66	F	RA	NA	+	++
RFU	59	M	RA	NA	--	--
RJL	67	M	RA	3	++	--
VCT	60	F	RA	6	++	--

(Continue on the next page)

Table I. (Continue)

Patient	Age	Sex	Diagnosis	Duration	Hyperplasia≠	PCNA§
ACL†	65	M	DJD	NA	+	--
AH†	62	M	DJD	NA	--	+
CCG†	73	M	DJD	5	--	--
DLT†	66	M	DJD	NA	--	--
VDC†	79	M	DJD	NA	--	--
VHG†	78	M	DJD	40	+	+
CW	14	F	Trauma		+	+
JHD	33	M	Trauma		--	--
LJB	56	M	Trauma		--	--
MLO	50	M	Trauma		--	--
MMR	39	M	Trauma		--	--
RLL	19	M	Trauma		--	--
RWK	NA	M	Trauma		--	--

* Age and duration are in year. Abbreviation: F-female, M-male, NA-not available.

† Samples used for NOR staining.

≠ --, +, ++ and +++ are defined as < 3, 3, 4, and ≥ 5 cell layers of lining synoviocytes respectively.

§ +, ++ and +++ are assigned to 10-20, 20-30 and ≥ 30% of cells with moderate to strong staining respectively. -- indicates negative or minimal (< 10%) staining for PCNA.

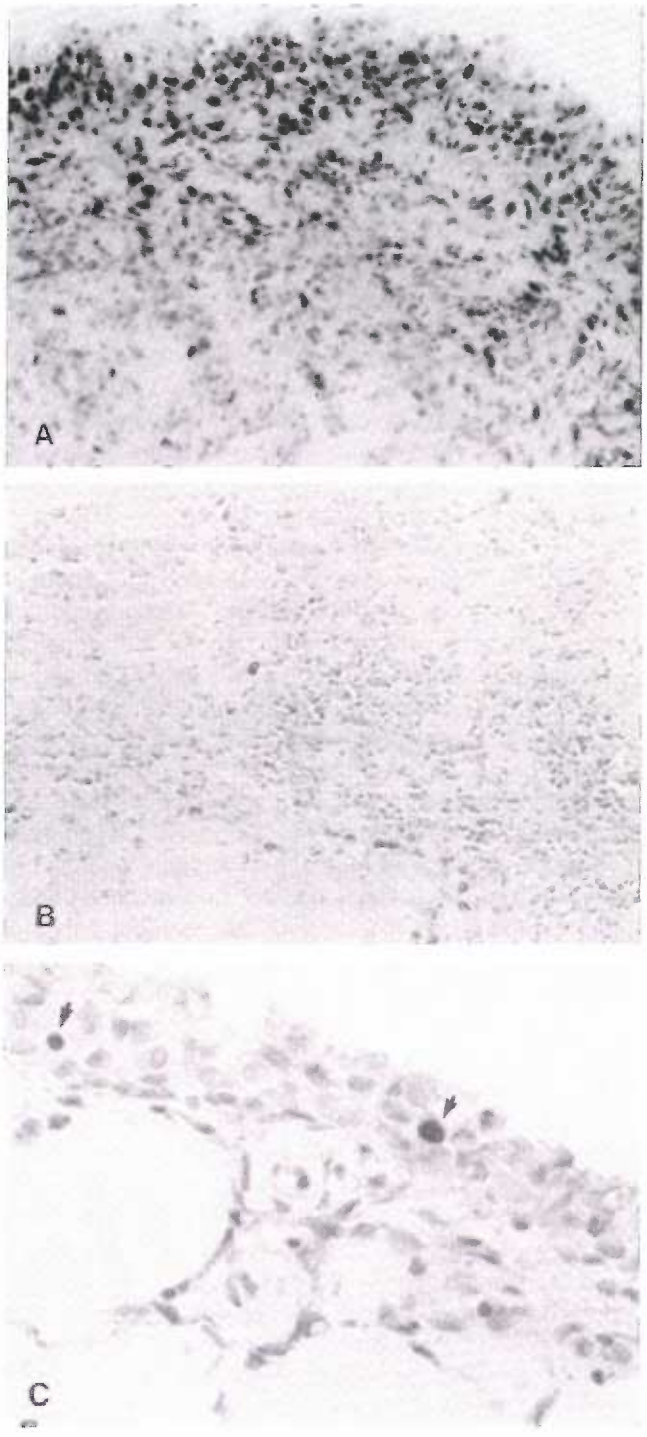

Figure 1. Proliferating cell nuclear antigen (PCNA) staining in synovial tissues of patients with rheumatoid arthritis (RA) and osteoarthritis (OA). Positive immunoreactivity is located in the nuclei. **A,** There is extensive staining in the lining synoviocytes of RA tissues (patient BJF). **B,** A negative control (nonimmune IgG) shows no PCNA staining. **C,** There is minimal staining (**arrows**) in the lining synoviocytes of OA tissues (patient CFW). (Original magnification × 200 in **A** and **B**, and × 400 in **C**.)

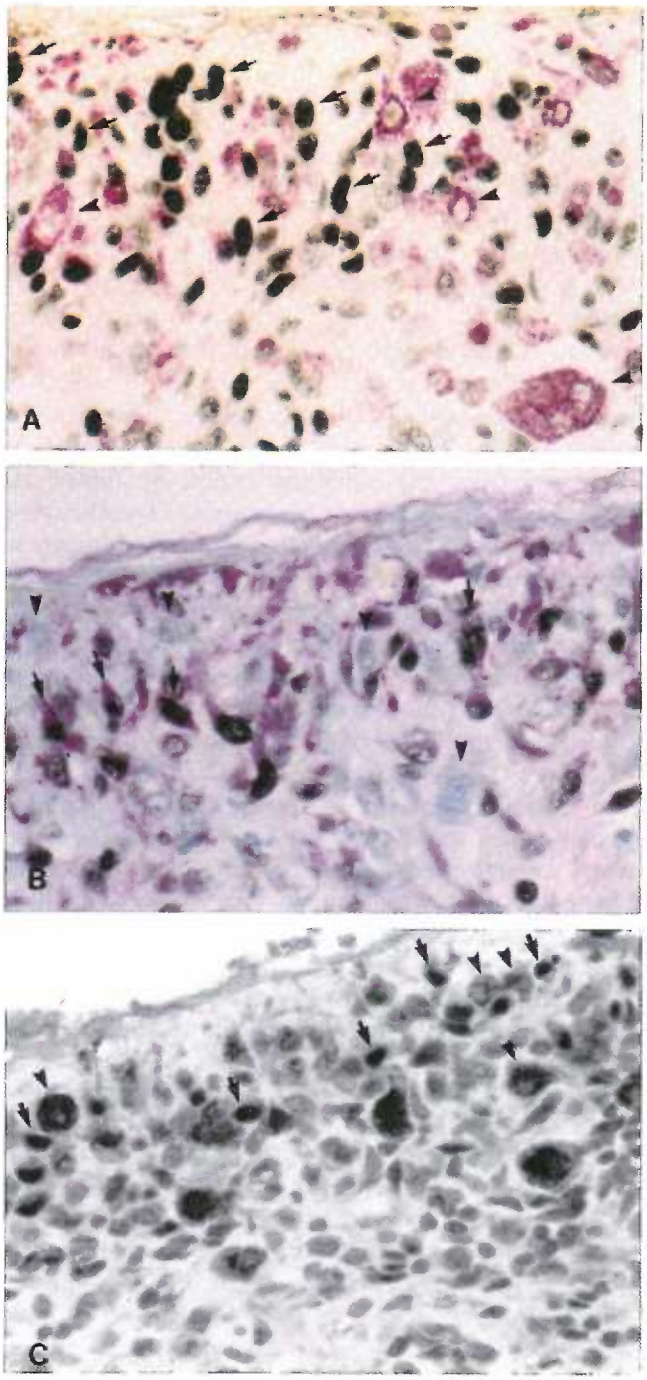

Figure 2. Double labeling of cell proliferation–specific and cell type–specific markers in RA synovium (patient MW). **A,** There is double staining for PCNA (black) and CD68 (pink), and **B,** for PCNA (black) and type I procollagen (pink). The PCNA-positive cells (**arrows**) are devoid of staining for CD68 but are positive for type I procollagen, whereas the CD68-positive cells (**arrowheads**) are not labeled by either PCNA or type I procollagen. **C,** There is double staining for c-*myc* (**arrows**) and CD68 (**arrowheads**). The staining of cells for c-*myc* in the nucleus and for CD68 in the cytoplasm is mutually exclusive. See Figure 1 for definitions. (Original magnification × 400.)

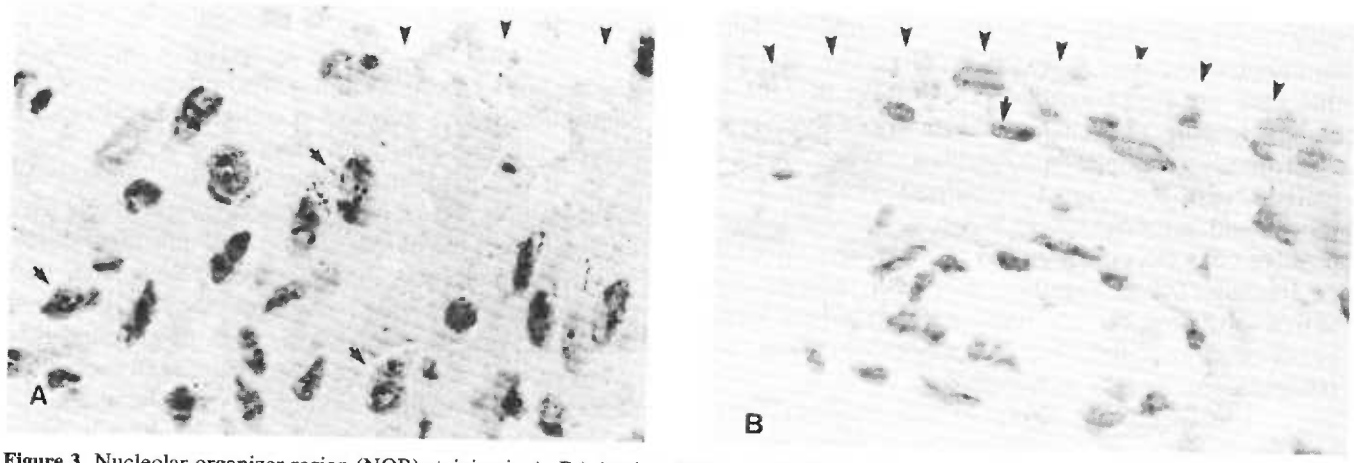

Figure 3. Nucleolar organizer region (NOR) staining in **A**, RA (patient BJF) and (**B**) OA (patient AH) synovium. Note the increased layers of lining synoviocytes and higher numbers of NOR per cell in RA than in OA. **Arrows** show synoviocytes with ≥4 NORs per cell; **arrowheads** show the synovial surface. See Figure 1 for other definitions. (Original magnification × 1,000.)

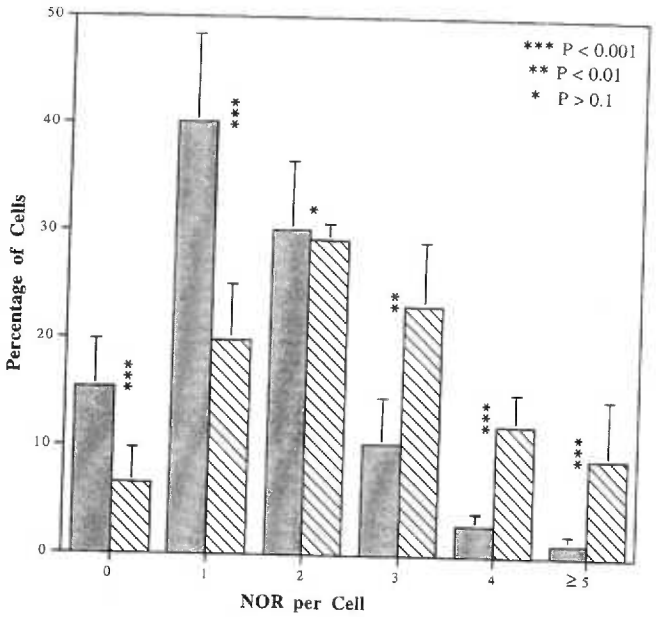

Figure 4. Percentage distribution of lining synoviocytes containing 0 to ≥5 nucleolar organizer regions (NOR) in RA (hatched bars) and OA (shaded bars) synovium. *P* values refer to the difference between RA synovium and OA synovium, determined by both Student's *t*-test and Mann-Whitney U test. Values are the mean and SD. See Figure 1 for other definitions.

Immunohistochemical Evidence for an Important Role of the Fibroblast in Joint Destruction during Rheumatoid Arthritis†

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Keywords: type I procollagen, rheumatoid arthritis, synovitis, fibroblast, cell proliferation, joint destruction.

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Abstract

Objective. To determine if sublining synovial fibroblasts are directly involved in synovial hyperplasia and joint destruction.

Methods. Immunohistochemistry with specific antibodies to type I procollagen and cell proliferation markers was used to identify proliferative activity and distribution patterns of synovial fibroblasts in joint tissue from patients with trauma (n = 5), rheumatoid arthritis (RA) (n = 17), and Degenerative joint disease (DJD) (n = 15).

Results. Active proliferation and a disordered distribution pattern of synovial fibroblasts with increased type I procollagen expression were found in those rheumatoid synovia that exhibited marked hyperplasia. These activated fibroblasts were localized not only in the sublining region but also at the leading edge of invading panni and the synovial surface layers. In contrast, their distribution is highly restricted in synovium from patients with trauma or DJD.

Conclusion. Sublining synovial fibroblasts are activated during arthritis and are directly involved in synovial hyperplasia and joint destruction. The fibroblast needs to be considered as an active participant in the rheumatoid synovium.

Introduction

Although the devastating outcome of rheumatoid arthritis (RA) is due to the destruction of articular cartilage and bone that ultimately leads to disability, the initial pathologic changes occur in the synovium. The synovial tissue in RA shows varying degrees of inflammatory changes with considerable hyperplasia and hypertrophy of synovial lining cells, increased vascularity, mononuclear cell infiltration and focal aggregates of lymphoid cell infiltration. Active rheumatoid synovium exhibits invasive behavior and may cause erosion of articular cartilage and bone. The exact destructive process remains largely unknown. It is believed that monocytes/macrophages are responsible for the joint destruction while fibroblasts play a secondary role (1, 2). Despite the fact that RA is considered to be a chronic proliferative disease, much investigative attention has been given to infiltrating inflammatory cells and macrophage-like synoviocytes that are thought to be derived from blood-born monocytes/macrophages. This can be attributed to several factors. First, lymphocytes and macrophages are key mediators of the immune response, an underlying mechanism of RA. Second, infiltrated inflammatory cells such macrophages and lymphocytes are not seen in a normal synovium, so their presence during RA is much more readily apparent compared to the increased number of fibroblasts that are already present in normal synovium. Third, the availability of specific antibodies to cell markers for lymphocytes and monocyte/macrophage allows infiltrating inflammatory cells to be the focus of research. In contrast, synovial fibroblasts are conceptually considered to be a ubiquitous component of soft connective tissue that only play a passive supporting role Antibodies specific to fibroblasts are limited. Consequently, the sublining synovial fibroblast eludes most investigation.

In the last few years, several lines of evidence have emerged suggesting that synovial fibroblasts may play a vital role during synovial hyperplasia and joint

destruction. Synovial fibroblasts have been identified as a major source of metalloproteinases that degrade a wide range of connective tissue components (3O, 4-6). In culture, large amount of collagenase, as well as prostaglandin E2, are produced spontaneously by rheumatoid synovial fibroblasts (7, 8). Production of metalloproteinases by synovial fibroblasts *in vivo* has also been documented (9-11). Moreover, it has been shown that synovial fibroblasts from both patients with RA and Lewis rats with streptococcal cell wall-induced arthritis exhibit anchorage-independent growth in culture (12). In experimental arthritic animal models, depletion of macrophage-like synoviocytes abolishes inflammatory cell influx (infiltration) but not cartilage/bone destruction (13, 14). Recently we also demonstrated that active proliferation of fibroblast-like cells occurs in rheumatoid synovium and contributes significantly to hyperplasia (15). These findings prompted us to examine further the role of the synovial fibroblast during RA. Using immunohistologic techniques, we examined proliferation and distribution patterns of the synovial fibroblast cell in synovium from patients with trauma, Degenerative joint disease (DJD) and RA. We found striking differences between synovial samples from a subset of patients with RA compared to samples from patients with joint trauma or DJD in terms of synovial fibroblast distribution and proliferation. The increased cell proliferation and disordered growth patterns of sublining synovial fibroblasts with increased type I procollagen expression observed in hyperplastic rheumatoid synovium indicate activation of these cells and suggest their direct involvement in joint destruction.

Materials and Methods

Tissue Processing

Synovial tissues from knee, shoulder, hip and wrist joints were obtained during joint surgery from patients with RA (n = 17), Degenerative joint disease (DJD) (n = 15) and trauma (= 5) (Table). Specimens containing synovium-cartilage junction from 5 patients

with RA were also available. All RA patients met the 1987 ACR criteria for this diagnosis (16). Tissue samples were divided in to two parts and fixed in neutralized buffered formalin (NBF) and Bouin's solution respectively for 1 day at 4 ^{0}C. Samples containing synovium-cartilage junction were decalcified in 0.1 M Tris buffer containing 10% EDTA with daily change for 20-30 days following fixation. All samples were routinely processed and embedded in paraffin.

Tissue sections cut at 4-micrometer were laid on poly-L-lysine coated slides. The sections were deparaffinized in xylene and rehydrated through a graded series of alcohol and distilled water. For each subject, the staining was repeated at least once to ensure specificity and consistency.

Immunohistochemical staining

For immunohistochemical staining, a three-step avidin-biotin complex (ABC) protocol was used as previously described (15). Briefly, tissue sections were incubated in phosphate buffer saline (PBS) containing 0.3% bovine serum albumin (BSA) and 1% normal serum from the same species as the secondary antibody for 20 minutes to block non-specific antibody binding. The tissue sections were incubated overnight at 4 ^{0}C with a primary antibody at appropriate dilution (see below). Then, a biotinylated secondary antibody specific for the primary antibody was applied at 1:200 for 30 minutes followed by incubation with alkaline phosphatase conjugated avidin-biotin-complex (ABC-AP) for 30 minutes at room temperature (RT) according to the vendor's instructions (Vector Lab., Burligame, CA). The antibody-antigen complex was visualized by incubation for 30 minutes in Fast Red substrate (BioGenex Lab., San Ramon, CA) following the supplier's instructions. 2 mM levamisole was added into the substrate solution to inhibit endogenous alkaline phosphatase. Following the primary antibody, brief rinse and washes with PBS containing 0.05% Tween-20 were performed after each step. Antibodies used in this study include a rat monoclonal antibody specific to the

carboxyterminal domain of human type I procollagen used at 1: 2,000 (Chemicon International, Inc., Temecula, CA), mouse monoclonal anti-CD68 at 1:1,000 (Dako Corporation, Carpinteria, CA), mouse monoclonal antibody (PC10) at 1:100 (Dako Corporation) against proliferating cell nuclear antigen (PCNA), and mouse monoclonal anti-human alkaline phosphatase at 1:3,000 (Sigma Chemical, St Louis, MO). CD68, Type I procollagen, PCNA and alkaline phosphatase were used as markers for monocyte/macrophage, activated fibroblast, proliferating cells, and osteoblasts, respectively.

Double immunostaining

For double labeling (procollagen/CD68 and Procollagen/PCNA), the sections were first labeled using ABC-HRP (horseradish peroxidase) method with 3'3-diaminobezidine (DAB) as the chromogen and then the second labeling were carried out using alkaline phosphatase and mouse anti-alkaline phosphatase (APAAP) method (Dako). Briefly, the sections were incubated again in PBS containing BSA and normal goat serum for 20 minutes at room temperature. A primary antibody specific for a second antigen to be labeled was applied for 1 hour at room temperature. After rinse and washes, the sections were incubated with a goat anti-mouse link antibody at 1:100 (Dako) for 40 minutes by APAAP complex at 1:100 for 40 minutes at room temperature. Fast Red was used as chromogen for the second labeling as described above.

The sections were counterstained with Mayer's hematoxylin and mounted in Crystal Mount (Biømeda Corp., Foster City, CA).

Results

Histological changes during RA lack disease-specific features and are similar to those occurring in the normal wound healing process. During normal wound healing, active wound repair is characterized by an increased number of fibroblasts with an

augmented expression of type I procollagen and proliferative activity in the wound area (17-19). We, therefore, used antibodies to type I procollagen and PCNA to identify activated fibroblasts in synovium. Immunohistochemical labeling of synovia from patients with trauma or DJD or RA with antibody to type I procollagen revealed two strikingly different staining patterns. First, staining for type I procollagen was weak in 14/15 samples from patients with DJD, while very strong staining was found in 7/9 samples from patients with RA whose tissue exhibited marked hyperplasia and infiltration of non-lymphocytic cells. Moderate staining was present in 1/6 tissues from rheumatoid synovium that exhibited extensive lymph nodule formation but minimal hyperplasia. Second, an increased number of the procollagen-positive cells were found in the hyperplastic synovia (Table I and Fig. 1a and e). Third, in synovial samples from patients with trauma or DJD cells stained positive for type I procollagen exhibited relatively homogeneous fibroblast morphology with a fusiform and an oval or cigar-shaped nucleus and were mainly found right beneath lining synoviocytes (Fig. 1d). In the RA synovium with marked hyperplasia and strong immunostaining for type I procollagen, a completely different distribution pattern was observed. The procollagen-positive cells exhibited heterogeneous morphology and were found throughout the synovium. They exhibited increased numbers in the sublining region adjacent to the surface and frequently invaded the lining layers (Fig. 1e). In the superficial regions in RA synovium where the procollagen-positive cells were abundant, the boundary between lining synoviocytes and sublining connective tissue was often obscured due to the invasion of the procollagen-positive cells into the lining layers of synovium (Fig. 1e). In rheumatoid synovial tissue that exhibited lymph follicle formation but no hyperplasia, the numbers of synoviocytes or fibroblasts stained for PCNA were minimal in both lining layers and sublining region. However, weak staining for type I procollagen was seen in lining layers and sublining regions (Fig. 1c). Although staining for type I procollagen

and PCNA was readily found in rheumatoid synovium with marked hyperplasia, it did not seem to be associated exclusively with clinic diagnosis of RA. Moreover, the staining patterns of both type I procollagen and PCNA in rheumatoid synovium with heterogenous morphology varied from site to site. Different staining patterns could be seen in tissue samples from the same patient.

Careful examination of synovial pannus that invaded bone or cartilage revealed that the leading edge of over 60% of the pannus is composed predominantly of fibroblast-like cells stained for type I procollagen (Fig. 1f). These fibroblast-like cells were not labeled by anti-alkaline phosphatase, suggesting they are osteoblasts that are involved in bone formation. An increased number of adjacent fibroblast-like cells with strong immunoreactivity to anti-procollagen was also observed. CD68-positive cells could be found at the leading edge of invading pannus that already invaded into subchondral medullary bone (Fig. 1f).

To determine the proliferative activity of synovial fibroblasts, synovial samples were also labeled with antibody specific to proliferating cell nuclear antigen (PCNA). As shown in Table I, increased number of PCNA-positive cells were seen in rheumatoid synovia with marked hyperplasia and increased cellularity. The distribution patterns of these PCNA-positive cells resembled that of fibroblast-like cells stained for type I procollagen. An increased number of the PCNA-positive cells were found not only in the lining layers but also in the sublining connective tissue. Double immunohistochemical staining showed that most of the PCNA-positive cells were also labeled by anti-procollagen antibody (Fig. 1e). The number of CD68-positive cells (macrophages) stained for PCNA is negligible (data not shown).

No apparent cross-reaction of the anti-procollagen antibody with macrophage or macrophage-like synoviocytes was observed as shown by double labeling for type I procollagen and CD68 indicating that the two antibodies distinguished two different cell

populations in synovium. Double staining also showed that the majority of the PCNA-positive cells were also labeled by anti-procollagen (Fig. 1e). Replacing the primary antibodies in either first and/or second labeling abolished the staining in all samples.

Discussion

In this study we used antibodies to PCNA and type I procollagen to identify proliferation activity and distribution patterns of synovial fibroblasts in synovial tissue from patients with trauma, DJD and RA. We observed a striking difference between synovial tissue from trauma/DJD and tissue with marked hyperplasia from a subset of rheumatoid synovium. Our results are consistent with those reported by Konttinen et al. (20). However, our study shows that staining for type I procollagen is not only associated with synovial fibroblasts in the sublining region but also in the hyperplastic lining layers of rheumatoid synovium. This observation is consistent with our previous report that local proliferation of fibroblast-like synoviocytes contribute to synovial hyperplasia (15). We have also observed that staining for type I procollagen and PCNA are closely associated with local pathological changes rather than clinic diagnosis of RA, indicating that this may be a disease stage-dependent or activity-dependent feature.

Although RA is considered as a proliferative disease, few studies have been devoted to the assessment of cell proliferation *in vivo*. It is believed that the excessive expression of GFs is responsible for the invasive growth of rheumatoid synovium. Increased expression of a variety of GFs such as TGF-beta and PDGF has been found in rheumatoid synovium (21, 22). However, cellular responses to the elevated levels of local GFs during RA remains largely undefined. Expression of type I procollagen by fibroblasts in culture is regulated by GFs such as TGF-beta (23-25), FGF (26) and IL-1 (27). We previously showed that cultured synovial fibroblasts proliferate in response to basic fibroblast growth factor (28). Recently, we demonstrated increased immunostaining for basic fibroblast growth factor in hyperplastic rheumatoid synovium

(29). These observations suggest that the increased cell proliferation and disordered growth pattern of synovial fibroblasts shown in the present report may reflect local cellular response to increased expression of growth factor such as bFGF in RA.

The inflamed synovium differs from the normal healing wound in that it exhibits unlimited growth and invasive behavior. The outgrowing synovium often invade adjacent joint tissue such as tendon, articular cartilage and subchondral bone ultimately resulting in destruction of the afflicted joint. The process of cartilage/bone erosion and the exact destructive element(s) are largely unknown. A current consensus is that monocytes/macrophages are responsible for this destructive process while fibroblasts play a secondary role. Such a consensus largely comes from immunohistochemical studies of monocyte/macrophage markers in arthritic joint. It has been reported that there is a significant increase in numbers of macrophages in RA synovial lining and in synovial pannus (1, 30). These activated macrophages are the most abundant cell adjacent to the cartilage (2). We observed, however, that the leading edge of over 60% invading panni is composed predominantly of fibroblast-like cells as determined by increased expression of type I procollagen. Furthermore, we found that these fibroblast-like cells were not labeled by antibodies specific to the monocyte/macrophage. Our observations are consistent with findings by Fassbender et al. (31) who reported that fibroblastic pannus occurs in 70% of cartilage-pannus junctions in RA. Similar findings were also reported by Barrie (32). The active cell proliferation, disordered growth pattern and increased cellular activity of sublining fibroblasts at both hyperplastic synovium and synovium-cartilage junction shown here strongly suggest possible direct involvement of the sublining synovial fibroblasts in synovial hyperplasia, pannus growth and joint destruction. Additional evidence can be obtained from studies by other research groups. Synovial fibroblasts have been identified as a major source of proteinases *in vitro*. (3O, 4-6). Expression of several metalloproteinases by fibroblast-like synoviocytes in

rheumatoid synovium has also been documented (9-11). In contrast, macrophages derived from human peripheral blood monocytes produce low levels of collagenase even when activated. It has been shown that synoviocytes from both patients with RA and Lewis rat with streptococcal cell wall-induced arthritis exhibit anchorage-independent growth in culture (12). Together, these observation are strongly indicative of the functional importance of synovial fibroblasts during joint destruction. However, data from our study do not refute the role of macrophages in joint destruction since macrophages were also present in the leading edge of some invading pannus (see Results). In vitro studies have shown that macrophages are required for the production of metalloproteinases by synovial fibroblasts. Whether localization of macrophages adjacent to synovial fibroblasts at the leading edge of pannus such functional interaction or it is associated with post erosion events such as cleaning and disposing of degraded tissue components following fibroblast-mediated destruction remains to be tested.

Histological evaluation of the activity of RA has been based upon the intensity of exudation and infiltration of inflammatory cells, which usually do not show a close correlation with the degree of joint destruction. There is no reliable pathological marker that can be used to predict the invasiveness of rheumatoid synovial pannus. RA is considered as an impaired wound healing process. During normal wound healing, an increased number of fibroblasts exhibits active cell proliferation and expression of type I procollagen (17-19). Expression of type I procollagen by fibroblasts in wound area can also be greatly enhanced by exogenous GFs that accelerate wound healing (33, 34). Production of type I procollagen fibroblasts has also been found in several inflammatory conditions such as chronic skin ulcers (35), systemic sclerosis (36), and streptococcal cell wall-induced granulomatous inflammation and liver fibrosis (37). In almost all these studies, type I procollagen is undetectable or minimal in normal or non-inflammatory tissue controls. Our findings are consistent with the results from these studies and

suggest that an increased number of synovial fibroblasts that exhibit expression of type I procollagen, the altered distribution pattern and the increased PCNA-positive fraction may have potential prognostic value in RA.

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Figure Legend

Figure 1. Immunohistochemical localization of type I procollagen, CD68, and PCNA in synovium: a) shows a rheumatoid synovium with marked hyperplasia labeled with anti-pCol.I (brown). Strong cytoplasmic staining for pCol.I was found in fibroblast-like cells that were localized in both sublining and surface of the synovial membrane. b) demonstrates a negative control section from the same patient as a). c) is from a rheumatoid sample with distinct lymph follicles but no apparent hyperplasia. Selective labeling of cells by anti-CD68 (pink) is easily seen. However, staining for type I procollagen (orange) is weak. d) shows staining for pCol.I. (brown) and CD68 (pink) of a sample from patient with OR. The restricted distribution of fibroblasts with weak staining for pCol.I is in contrast with that shown in a). Note the absence of pCol.I-positive cells on synovial surface and the clear boundary between the lining cells and sublining connective tissue. e) demonstrates a sublining region of a rheumatoid synovium with marked hyperplasia. Double immunostaining revealed localization of PCNA (black) in the nuclei of fibroblast-like cells that were also stained for pCol.I. (pink). Note the obscured demarcation between surface lining synoviocytes and sublining connective tissues. F) reveals distribution of CD68 positive (black) and pCol.I-positive cells (pink) at the pannus-bone junction. The leading edge of pannus is composed mainly of pCol.I-positive cells. Magnification 200 X for a), b) and e); 400 X for c), d), and f).

Figure 1. Immunohistochemical localization of type I procollagen, CD68, and PCNA in synovium

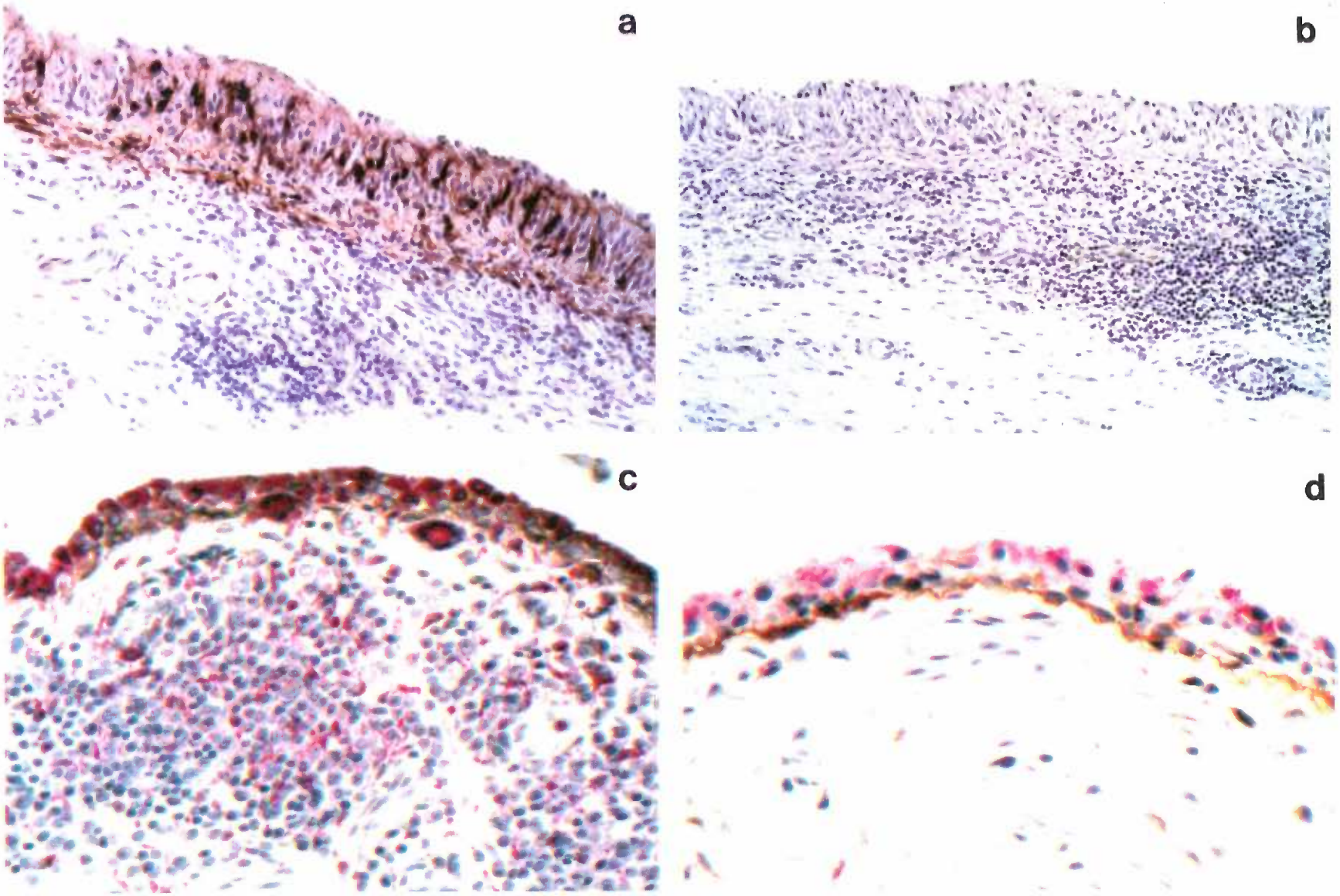

Figure 1. Immunohistochemical localization of type I procollagen, CD68, and PCNA in synovium

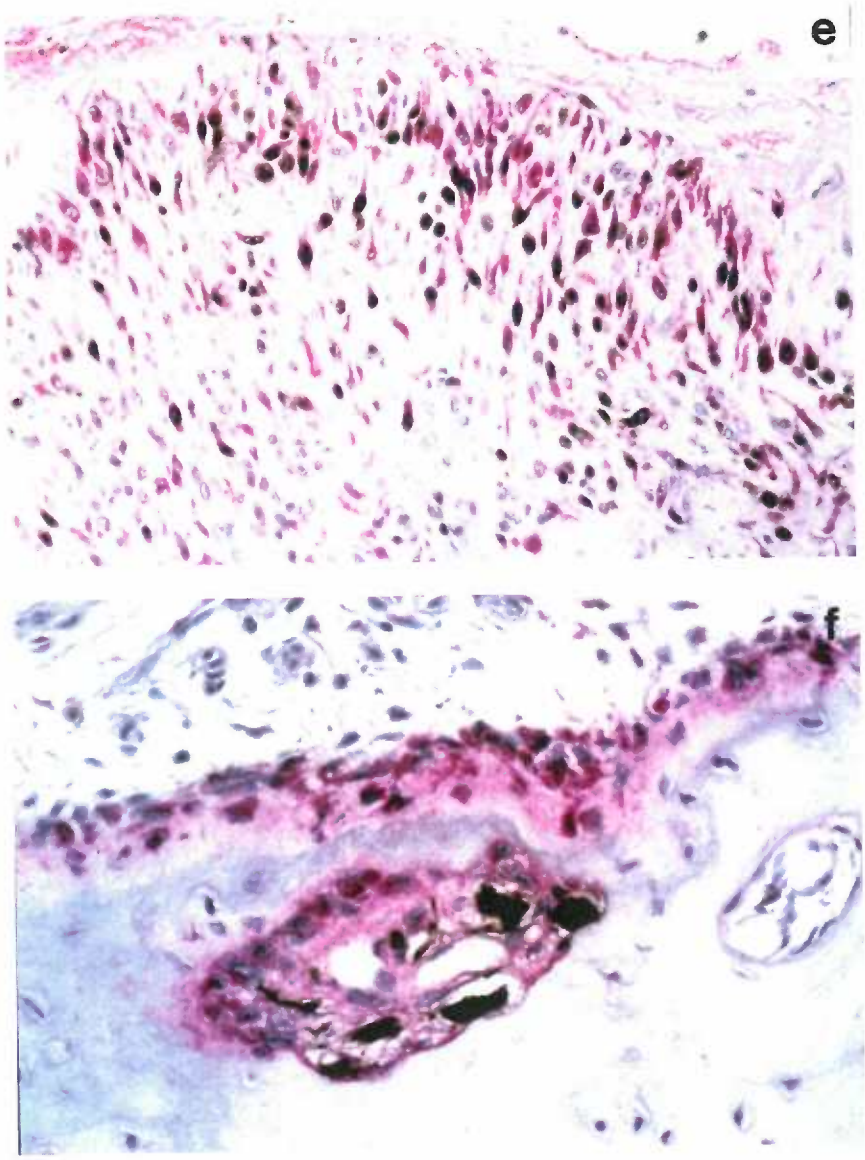

Table I. Morphological and Immunohistochemical Features of Synvoium Samples Examined

Diagnosis	n =	hyperplasia*	pCol.I staining§	pCol.I-positive cell/HPV†	PCNA/pCol.I-positive cells¶
RA	9	++ ~ +++	+++	75.1 ± 29.7	52.3 ± 16.2%
RA	8	-- ~ +	-- ~ ++	16.4 ± 3.0	7.7 ± 2.4%
DJD	15	--	-- ~ ++	10.3 ± 1.9	2.8 ± 1.1%
Trauma	5	--	-- ~ +	8.7 ± 1.3	< 1%

* Graded -- to +++, according to the number of lining cell layers: -- < 3, + = 3, ++ = 4, and +++ = ≥ 5.

§ Graded -- to +++, acording to staining intensity for pCol.I: -- = no staining, + = weak, ++ = moderate, and +++ = intense.

† Numbers of cells stained positive for type I procollagen under high power view (HPV) (= 400 X).

¶ Percentage of pCol.I-positive cells that are also labeled by anti-PCNA.

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Mast Cells are A Major Source of Basic Fibroblast Growth Factor in Chronic Inflammation and Cutaneous Hemangioma†

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Abstract

Mast cells (MCs) play an essential role during development of inflammation following chemical and immunological insults, and have been implicated in tissue fibrosis and angiogenesis. The exact contribution of mast cells to these conditions is largely unknown. In this report, we show that a potent angiogenic and mitogenic polypeptide, basic fibroblast growth factor (bFGF), is localized to the majority of mast cells in tissue samples characterized by fibrosis, hyperplasia and neovascularization. Using specific antibodies to mast cell tryptase, tissue macrophage and bFGF, we demonstrate that cytoplasmic bFGF immunoreactivity is localized to 96.8± 9.6% of tryptase-positive cells in human fibrotic lung tissue (n = 10), 82.3 ± 6.9% of tryptase-positive cells in rheumatoid synovia (n = 6), 97.6 ± 3.3% of tryptase-positive cells in nasal polyposis (n = 8) and 93.1 ± 4.8% of tryptase-positive cells in skin hemangioma (n = 5). Moreover, these tryptase-positive cells comprise a major portion (65 - 97%) of nonvascular cells exhibiting cytoplasmic bFGF staining in these tissues. In contrast, macrophage-like cells contribute less than 6.5% of the bFGF-positive cells in the same samples. The specificity of the immunostaining results was supported by the finding that cultured human MC (HMC-1) express both bFGF mRNA and protein upon activation. Our data indicate that MCs, a primary source of heparin, also serve as a significant source of a heparin-binding growth factor, bFGF, in these disease processes. These observations suggest that MCs may contribute to these pathological conditions by releasing this polypeptide.

Keywords: tryptase, HMC-1, pulmonary fibrosis, rheumatoid arthritis, immunohistochemistrty.

Introduction

Increasing evidence suggests that mast cells (MCs) may play an important role in inflammatory processes. For example, mast-cell-deficient mice have reduced inflammation in ozone- and silica-induced mouse lung injury models (1, 2). Replenishing the mast cell-deficient animals with MCs restored the inflammation to its full extent. MCs are also required for full expression of cutaneous acute inflammation induced by phorbol ester (3) and antigen-induced arthritis in mice (4). MCs have also been implicated in diverse fibrotic or proliferative diseases such scleroderma, neurofibromatosis, and psoriasis (for review see (5)). An increased number of MCs are found in synovium from patients with rheumatoid arthritis (6, 7) and in lung tissue from patients with idiopathic fibrosis (8). These studies indicate that MCs are important for tissue inflammatory responses to different types of injury. However, the exact contribution of MCs in inflammation remains largely unknown. Recent studies suggest that growth factors and cytokines, such as tumor necrosis growth factor-alpha and interleukins (IL) are important inflammatory mediators related to MCs (for review see (9)). Bradding et al.(10) recently immunolocalized IL- 4, -5, and -6 to MCs in the normal and inflamed nasal mucosa. However, the profile of MC-related growth factors *in vivo* is far from clear.

MCs also have been implicated in neovascularization. Increased numbers of MCs have been noted in diseases associated with neovascularization such as wound repair, rheumatoid arthritis (6, 7), diabetes mellitus (11), hemangioma (12) and other types of tumors (for a review see (13)). Kessler et al. (14) reported that a marked (40-fold) increase in the number of MC was found to precede angiogenesis induced by tumor implants on the chick chorioallantoic membrane. Recently, in a series of experiments, Norrby et al. (15-17) demonstrated that active MC secretion induced by repeated intraperitoneal injection of compound 48/80, a highly selective mast cell secretagogue,

resulted in marked mesenteric neovascularization in rats and mice as determined by vascularized area and the vascular density. More recently, Jakobsson (18) reported that the vascular changes in this experimental model included neovascularization and remodeling that lasted over a period of two months. Such marked organized angiogenic responses of long duration do not seem to be attributable to the structurally simple and short-lived mediators, such as heparin, released from MCs.

Basic FGF is a multifunctional polypeptide that affects growth and differentiation of a broad spectrum of cell types (for reviews see (19, 20)). It is a potent mitogenic and chemotactic factor, and has been implicated in the wound-healing process (21-23). Basic FGF is also one of the most potent angiogenic factors known and has been shown to induce angiogenesis *in vitro* as well as *in vivo*. In the course of characterizing the distribution of bFGF in inflamed tissues, we noted that a group of tissue cells that showed cytoplasmic staining for bFGF also exhibited a distribution pattern similar to that of MCs. We therefore used specific antibodies to identify these cells. We elected to study tissue from four diseases which are notable for a recognized contribution by mast cells and/or prominent neovascularization or fibrosis. Results from dual immunohistostaining of the tissues with anti-bFGF and cell-type-specific antibodies, and analysis of a cultured mast cell line indicate that MCs may function as a major source of bFGF. Our study suggests that bFGF may contribute to cell proliferation and angiogenesis associated with mast cells *in vivo*.

Materials and Methods

Immunohistochemistry

Human tissue samples were obtained from patients with idiopathic pulmonary fibrosis, rheumatoid arthritis, nasal polyposis (secondary to cystic fibrosis in 6 of 8 patients) and cutaneous hemangioma (Table 1). Hemangioma specimens were selected based on prominence of mast cell infiltration and dense vascularity. The samples were

fixed in neutralized buffered formalin and subsequently embedded in paraffin. A three-step avidin-biotin complex (ABC) (Vector Lab., Burligame, CA) method or alkaline phosphatase-anti-alkaline-phosphatase (APAAP) method (Dako Corporation, Carpinteria, CA) was used to localize bFGF and other cell markers as described elsewhere (24). Information about the antibodies used in this study is summarized in Table 2. The antibody-antigen complexes were visualized by incubation for 30 minutes in Fast Red substrate (BioGenex Lab., San Ramon, CA) or Vector Blue (Vector) containing 2 mM levamisole according to the suppliers' instructions. The sections were counterstained with Gill III hematoxylin when indicated and mounted in Crystal Mount (Biømeda Corp., Foster City, CA).

For double immunostaining of bFGF and tryptase or macrophages, the sections were first labeled for bFGF using ABC-AP method with Vector Blue as substrate. Potential non-specific binding resulting from the first labeling was blocked with Biotin/Avidin System following the vendor's instruction (Vector). The sections were then incubated with anti-tryptase at 1:200 (0.5 μg/ml) or HAM56 at 1:50 (2.5 μg/ml) overnight at 4 °C followed by incubation with biotinylated horse anti-mouse at 1:200 and then avidin-FITC at 1:100 for 1 hour at room temperature. Brief washes were performed after each step.

For CD68/tryptase double labeling, the samples were first labeled by antibodies to macrophages using ABC-AP method with Fast Red as the substrate. The second labeling was performed using APAAP method with Vector Blue as substrate.

Two control groups were used to confirm the staining specificity: 1) replacing the specific primary antibodies with nonimmune immunoglobulins of the same isotype at the same concentration from the same species as the antibodies and 2) washing the sections with phosphate-buffered saline (PBS) containing 2 M NaCl following enzymatic

treatment. Nonimmune immunoglobulin was also used to replace the primary antibody during the second labeling to determine the specificity of the second labeling.

At least 2 specimens from each case were examined for each antibody. Two approaches were used to determine the spatial relationship of cells labeled with antibodies to bFGF, macrophages and tryptase or Toluidine blue. In the first, the tissue sections were cut sequentially at 2-3 μm and stained separately with antibodies to bFGF, macrophages and tryptase or Toluidine blue. In the second, the sections were double-stained for bFGF/tryptase, bFGF/HAM56 or tryptase/CD68 using combined colorimetric and fluorescent methods. The spatial relationship of positive cells labeled by two different antibodies on each section was determined by bright field and fluorescent microscopic examination. The samples were examined by at least two independent researchers. 500 cells from three randomly selected areas in samples from fibrotic lung and cutaneous hemangioma were examined. In samples from rheumatoid synovium and nasal polyps, cells (ranging from 150 to 300) were counted in four randomly selected areas which included lining synovium or mucosa and underlying connective tissue regions.

Cell Culture:

The human mast cells line, HMC-1, was plated in Iscove's medium with 10% defined bovine calf serum (Hyclone, Logan Utah) at 1.5 X 10^5 cells/ml and maintained in a water - vapor saturated atmosphere with 5% CO_2 for 24 hours. Then, phorbol 12-myristate 13-acetate (TPA) and calcium ionophore A23187 dissolved in dimethyl sulfoxide (Sigma Chemicals, St. Louis, MO) were added to 80 nM and 1 μM, respectively. The control group was treated with vehicle only. Cells were harvested 24 hours after stimulation and analyzed by immunoblot and reverse transcription - polymerase chain reaction (RT-PCR) (see below).

Detection of bFGF mRNA by RT-PCR:

Total RNA was isolated from HMC-1 cells by an acid guanidium thiocyanate-phenol-chloroform extraction procedure (25). 150 ng of the RNA from each sample was reverse-transcribed as previously described (26).

4 µl of the reverse-transcribed material from each sample were amplified in 50 µl reaction volumes by PCR (26). Two different sets of primers were used. They contain the sequences unique to bFGF that are not found in other known genes. The sequence of the first set (sense 5'-TGT ACT GCA AAA ACG GGG GCT TCT CCT GCG CAT CC-3' and antisense 5'-CGT AAC ACA TTT AGA AGC CAG TAA TCT TCC ATC TTC C-3') corresponded to the region of nucleotides 561 to 760 of a human bFGF mRNA. The sequence of the second set (sense 5'-CAA GCA GAA GAG AGA GGA GTT GTG TC-3' and antisense 5'-CAG TTC GTT TCA GTG CCA CAT ACC-3') included the region from 659 to 857 of human bFGF mRNA. A restriction site for endonuclease Hinf I is located at position 789. Treatment of PCR product generated by the second pair of primers should result in two fragments of 130 bp and 68 bp respectively.

For the endonuclease digestion assay, PCR amplified products were treated with Hinf I (GibcoBRL, Grand Island, NY) for 1 hour at 37 °C. The reaction mixture (30 µl) contained 50 mM Tris (pH 8.0), 10 mM $MgCl_2$ and 50 mM NaCl and 10 U Hinf I. The digestion fragments were analyzed by gel electrophoresis as described above.

Western Blot analysis of HMC-1 for bFGF:

Approximately 10^7 HMC-1 cells were lysed in 10 mM of HEPES buffer (pH 7.3) containing 0.4 M NaCl, 1 mM $MgCl_2$, 5% NP-40, 10 mM EDTA, 2 mM PMSF, 2 µg/ml leupeptin and 2 µg/ml aprotinin. Four mg of total protein from each sample were incubated overnight at 4 °C with 25 µl of precleaned heparin acrylic beads (Sigma). The heparin beads were collected by centrifugation, washed twice with the lysis buffer

containing 0.6 M NaCl, equilibrated with 10 mM HEPES at pH 7.4 and then subjected to gel electrophoresis and immunoblot analysis as described elsewhere (27).

Results

Immunohistochemistry

When the tissue samples were labeled with antibodies specific to bFGF, staining for bFGF was associated with tunica media and tunica adventitia of small veins and venules, hyperplastic synoviocytes and epidermal cells (Fig. 1a and c). Intense vascular staining for bFGF was found in all rheumatoid synovia and nasal polyps examined. The intensity of bFGF staining associated with vascular cells exhibited great variability in fibrotic lung and cutaneous hemangioma. Two different bFGF-specific antibodies used in this study gave identical staining patterns. The anti-bFGF antibodies selectively gave cytoplasmic staining of a group of scattered connective tissue cells. The distribution pattern of these scattered bFGF-positive cells closely resembled that of mast cells. Cell-type-specific antibodies were therefore utilized in conjunction with Toluidine blue staining to identify the bFGF-positive cells in these samples. Human tryptase is a glycosylated serine proteinase that is essentially an exclusive product of MCs and is considered a specific marker for MCs (for review see(28, 29)). The anti-tryptase antibody specifically reacts with tissue MCs by immunohistochemistry (30-32). Since macrophages are commonly accepted as a major source for tissue cytokines/growth factors and are thought to play a major role during angiogenesis by releasing angiogenic factors including bFGF (33), tissue samples were also stained with two monoclonal antibodies (KP1 and HAM56) to human monocyte/macrophage (34, 35).

Careful examination of 2-3 μm sequential sections labeled by anti-bFGF and anti-tryptase separately revealed almost identical distribution patterns of bFGF- and tryptase-positive cells (Fig. 1a and 1b; 2a and 2b). Similar results were obtained by staining of the sequential sections with Toduidine blue and anti-bFGF (Fig. 2c and d). The anti-

tryptase did not label endothelial cells, smooth muscle cells, lymphocytes, macrophages, basement membrane or epidermal cells in any specimen examined. In contrast, the distribution pattern of cells labeled by anti-macrophages exhibited no resemblance to that of bFGF-positive cells or tryptase-positive cells (Fig. 2a and b).

To confirm the association of bFGF and MCs, double immunohistochemical staining was then performed. Double immunohistochemical/immunofluorescent staining for bFGF/tryptase revealed that the majority of tryptase-positive cells were also labeled by anti-bFGF in all samples examined (Fig. 1c and d). More importantly, these mast cells comprise a large fraction of cells with cytoplasmic staining for bFGF in these samples (Table 1). In contrast, the majority of cells labeled by the antibodies to macrophages were devoid of cytoplasmic bFGF staining (Fig. 2b) and the fraction of bFGF-positive cells reactive to macrophage antibodies was very small (Table 1). Anti-tryptase and anti-macrophage antibodies labeled two different cell populations with negligible overlap in all samples examined. In the specimens of fibrotic lung, for example, tryptase-positive cells were mainly localized in the interstitial tissue and fibrotic regions, while the majority of cells within alveoli were labeled with antibodies to macrophage (Fig.2a). The staining pattern of each antibody by double labeling was consistent with that obtained by single labeling. No nonspecific staining due to cross-reaction between antibodies and labeling reagents for the first and second labeling was observed following protocols described here (data not shown).

Since tissue mast cells have been reported to exhibit nonspecific staining due to their intrinsic affinity to avidin (36, 37) and to some types of immunoglobulins (38), several approaches were carried out to confirm the specificity of tryptase and bFGF staining. In addition to a standard control, an alternative method, alkaline phosphatase-anti-alkaline-phosphatase (APAAP) staining, was used to label the specimens and the result obtained was compared to that obtained by the avidin-biotin complex (ABC)

method. Moreover, a brief wash of tissue sections with a buffer containing 2 M NaCl prior to staining, a procedure known to abolish bFGF staining presumably due to dissociation of bFGF from heparin (27), was also performed. As we expected, replacing specific primary antibodies with a counterpart immunoglobulin from nonimmune animals completely abolished the staining. The APAAP method gave a staining identical to that obtained by the ABC method for each antibody used (data not shown). Washing with buffer containing 2 M NaCl selectively abolished bFGF staining but did not affect the tryptase staining (data not shown). These results indicated that staining of the mast cells by both anti-tryptase and anti-bFGF was not due to the intrinsic affinity of heparin to avidin or immunoglobulins.

Immunoblot Analysis and RT-PCR

To examine the association of bFGF and MCs further, expression of bFGF by MCs was examined *in vitro* using a human mast cell line, HMC-1 (39) by RT-PCR and immunoblot analyses. We failed to detect bFGF mRNA in cultured HMC-1 cells by RT-PCR (Fig. 3). However, following exposure to calcium ionophore A23187 and phorbol 12-myristate 13-acetate (TPA), marked induction of bFGF expression was observed at both mRNA and protein levels (Fig. 3 and 4). Amplification of mRNA from HMC-1 treated with TPA/A23187 by RT-PCR using two different pairs of primers gave rise to single bands with the predicted mobility (about 200 bp) upon gel electrophoresis. The specificity of the RT-PCR was confirmed by endonuclease mapping. Treatment of the amplified products with restriction endonuclease, Hinf I, generated a banding pattern as predicted according to the sequence of bFGF to be amplified (Fig. 3). Furthermore, immunoblot analysis of total protein from HMC-1 revealed a dominant band with an apparent molecular weight of 17 kD that exhibited migration behavior similar to that of human recombinant bFGF. Several bands with higher molecular weights also reacted

specifically with the anti-bFGF. Treatment of HMC-1 cells with TPA/A23187 resulted in a significant increase in the level of bFGF-like molecules (Fig. 4).

Discussion

In this report, we present evidence for the first time indicating that, in addition to vascular cells, mast cells may serve as a significant source of tissue bFGF in angiogenic and some chronic inflammatory conditions. Using specific antibodies, we demonstrate a marked spatial correlation between bFGF-positive and mast cells in all samples examined by immunohistochemistry. Double immunohistochemical staining revealed that the majority of tryptase-positive cells in fibrotic lung samples and cutaneous hemangiomas were also stained for bFGF and *vice versa* , indicating that most of the MCs were closely associated with bFGF. Although the fraction of tryptase-positive cells reactive with anti-bFGF in nasal polyps and rheumatoid synovia is somewhat lower than that in fibrotic lung tissue and hemangioma, it still constitutes a major portion of the nonvascular bFGF-positive cells in these samples (see Table 1). Such marked correlation indicates that MCs may function as a source of tissue bFGF during inflammation and neovascularization. Consistent with this hypothesis is the finding that cultured HMC-1 cells respond to TPA/A23187 treatment by markedly increasing expression of both bFGF mRNA and protein. The finding that bFGF immunoreactivity was found in a much larger number and fraction of MCs than that of monocytes/macrophages in all tissue specimens examined strongly argues against the hypothesis that monocyte/macrophages are the major source of tissue bFGF during inflammation in lung (40-42) and neovascularization (33). However, it is possible that expression of bFGF by monocytes/macrophages is disease stage-dependent and is more prominent in the early or acute stage of these diseases (43) than in the chronic condition as shown here. Studies in progress on animal models may help to clarify this.

Immunohistochemically detected bFGF in mast cells may come from two potential sources: it may be synthesized by activated mast cells or/and it may be synthesized by other cells and stored in MCs. Since it has been shown that bFGF has high affinity to heparin and that mast cells contain abundant heparin, it is conceivable that MCs may function only as a storage compartment of tissue bFGF. However, our *in vitro* study clearly demonstrated that cultured HMC-1 do express bFGF at both mRNA and protein levels and established that at least a subpopulation of MCs has the potential to express bFGF when activated. It is reasonable to predict that MCs may not only function as a storage site for bFGF but also produce bFGF *in vivo* when activated. Definite proof of this hypothesis requires further study employing other techniques such as *in situ* hybridization. The presence of protein but not mRNA for bFGF in non-stimulated HMC-1 cells could reflect: 1) insufficient sensitivity of the RT-PCR; 2) passive accumulation of bFGF from the culture medium; or 3) synthesis of protein from mRNA that was expressed during prior culture conditions.

Since bFGF is a potent mitogenic and angiogenic polypeptide, our findings may help to explain the close association of MCs with chronic inflammation and angiogenesis. First, MCs have been implicated in neovascularization, while bFGF is one of the most potent angiogenic factors known. Thus, neovascularization induced by mast cell degranulation is likely to be mediated by bFGF. Second, increased numbers of MCs have been found in tissues with chronic inflammation which by nature is also a wound healing process, while bFGF is known to accelerate normal wound healing. Neovascularization and proliferation of fibroblasts that occur during normal wound healing are also characteristic of rheumatoid synovium and nasal polyposis. Thus, increased numbers of mast cells in these disease conditions may provide bFGF required for the healing process.

In contrast to most other growth factors that function through a paracrine mechanism, bFGF lacks the signal peptide sequence for secretion. In spite of intensive studies, the mechanism(s) of bFGF release is still a subject of controversy. Basic FGF exhibits high affinity for heparin and formation of bFGF-heparin complex is known to stabilize bFGF structurally and preserve its bioactivity by protecting it from extracellular enzymatic degradation (44-46). Moreover, heparin and heparan sulfate are required for binding of bFGF to its receptors and, therefore, for induction of cellular responses to bFGF (47). The mast cells' potential to express bFGF upon appropriate stimulus, together with their two other unique characteristics -- high heparin content and degranulation, renders them an ideal candidate for regulation of local bFGF levels and bioactivity. First, increased bFGF expression may result from the tissue response to a specific insult(s). Second, the presence of abundant heparan sulfate and heparin with high binding affinity to bFGF implies a large capacity for bFGF storage. In addition, MCs may potentiate the bioactivity of bFGF through release of heparin. Third, mast cell degranulation may provide an efficient mechanism for bFGF release and another line of regulation of tissue bFGF levels. Preliminary data from our ongoing study of several murine mast cell lines suggests that cultured MCs release bFGF upon degranulation induced by substance P, compound 48/80 and A_{23187} (unpublished observations). These observations strongly implicate mast cell degranulation as a potential mechanism of bFGF release.

In summary, our data show that mast cells are a major source of bFGF. In contrast to mast cells, macrophages rarely expressed bFGF detectable by immunohistochemical staining in the samples examined. Our findings suggest that bFGF may contribute to cell proliferation and angiogenesis associated with MC-degranulation *in vivo*. The localization of bFGF to a cell which synthesizes heparin may help elucidate

the physiology of bFGF. This study helps clarify the contribution of the mast cells in angiogenesis, pulmonary fibrosis, and inflammatory joint disease.

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Footnote

List of abbreviations used in this paper:

ABC: avidin-biotin-complex.

APAAP: alkaline-phosphatase-anti-alkaline-phosphatase

bFGF: basic fibroblast growth factor.

MCs: mast cells.

RT-PCR: reverse transcription - polymerase chain reaction

TPA: phorbol 12-myristate 13-aceta.

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Figure Legend

Figure 1. Immunohistological localization of bFGF and mast cells in hemangioma and rheumatoid synovium : a.) and b.) show paried 2-μm sequential sections from hemangioma stained for bFGF (a) and tryptase (b). The sections were counterstained with hematoxylin. Arrows indicate some of the cells that react with both anti-tryptase and anti-bFGF antibodies. Note that only anti-bFGF labels epidermis (arrowheads). c.) and d.) are from a section from rheumatoid synoviuma double-labeled with anti-bFGF (pink) and anti-tryptase (green). d.) shows the same section visualized by fluorescent microscopy. Note the colocalization of bFGF with the mast cell marker. Original magnification: 400X.

Figure 2. Staining of fibrotic lung tissue for bFGF, mast cells and macrophages: a.) and b.) are sequential sections stained for CD68 (pink) and tryptase (dark blue) (a), and for bFGF (dark blue) (b). Arrows indicate some cells labeled by both anti-tryptase and anti-bFGF. Note the absence of bFGF staining in CD68-positive cells (arrowheads). c.) and d.) are also paired sequential sections stained with anti-bFGF (c) and Toluidin blue (d). Arrows indicate some of the cells labeled by both anti-bFGF and Toluidin blue. Original magnification: 400X (Nomarski).

Figure 3. Detection of bFGF mRNA from HMC-1 cells and rheumatoid synovium by RT-PCR. bFGF mRNA is not detectable in untreated HMC-1 cells (lane 2) whereas amplification of RNA samples from TPA/A23187-treated HMC-1 cells (lane 3) and rheumatoid synovium (lane 4) give rise to a single band of ~200 bp as predicted according to the sequence of human bFGF mRNA. Treatment of the amplified product with Hinf I generates two bands of 130 bp and 68 bp respectively as predicted (lane 5). mRNA quantitation and integrity were verified for all samples by RT-PCR with primers

for a constitutive gene, cyclophilin (26) (data not shown here). As a negative control (lane 6), RNA was omitted from the RT reaction.

Figure 4. Immunoblot analysis of HMC-1 cell lysates: samples from untreated (lane 1) and TPA/A23187-treated HMC-1 (lane 2) are probed with anti-bFGF. As positive controls, recombinant human bFGF is directly loaded (lane 3) or processed with heparin beads (lane 4) along with HMC-1 samples. A predominant band with an apparent molecular weight of 17 kD that exhibits identical migration as human recombinant bFGF is detected by anti-bFGF. The slower migrating bands (~32 kD) found here may be high molecular weight forms of bFGF reported by other researchers (49).

Table 2. List of Primary Antibodies used in this Study

Antibody	Species	Dilut./Concent.*	Treatment	Source
Anti-bFGF	Mouse‡	1: 20,000 (< 3 µg/ml)	Hyaluronidase	ZymoGenetics. Inc., Seattle, WA
Anti-bFGF	Rabbit IgG	1: 500 (2 µg/ml)	Hyaluronidase	Biomedical Technologies Inc., Stoughton, MA.
Anti-tryptase	Mouse IgG	1: 100 (0.5 µg/ml)	Hyaluronidase	Dako Corp., Carpinteria CA
Anti-CD68 (KP1)	Mouse IgG	1: 100 (3.7 µg/ml)	Hyaluronidase	Dako Corp., Carpinteria CA
HAM56	Mouse IgM	1: 500 (1.4 µg/ml)		Dako Corp., Carpinteria CA

* Dilut./Concent.= Dilution/Concentration.

‡ This antibody is raised against full length human recombinant bFGF and does not cross-react with acidic FGF upon immunoblot analysis (48). Clone number of this antibody is 148.6.1.1.1. The listed dilution is for 2-step indirect method. For ABC method, the dilution is 1: 10^6 (< 0.1 µg/ml).

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Table 1. Immunocolocalization of bFGF and Cell Type-Specific Antigens in Tissue Sections*

Source	n	bFGF/Tryptase†	Tryptase/bFGF‡	bFGF/HAM56§	HAM56/bFGF‖
Lung	10	96.8 ± 9.6%	95.7 ± 4.7%	< 2%	< 5%
Hemangioma	5	93.1 ± 4.8%	97.2 ± 3.6%	NA	NA
Synovium	6	82.3 ± 6.9%	86.2 ± 9.1%	< 5%	< 10%
Nasal polyp	8	97.6 ± 3.3%	65.5 ± 17.7%	19.4 ± 14.8%	6.5 ± 4.6%

* Data (mean ± standard deviation) are based on counting 150-500 cells per section. n = number of patients. NA = not applicable.

† percentage of tryptase-positive cells that co-express bFGF.

‡ percentage of bFGF-positive cells that are also stained for MC tryptase. bFGF-positive cells are the cells that exhibits cytoplasmic staining for bFGF.

§ percentage of HAM56-labeled cells that are stained for bFGF. Endothelial cells were excluded.

‖ percentage of bFGF-positive cells that are labeled by HAM56.

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Figure 1. Immunohistological localization of bFGF and mast cells in cutaneous hemangioma and rheumatoid synovium.

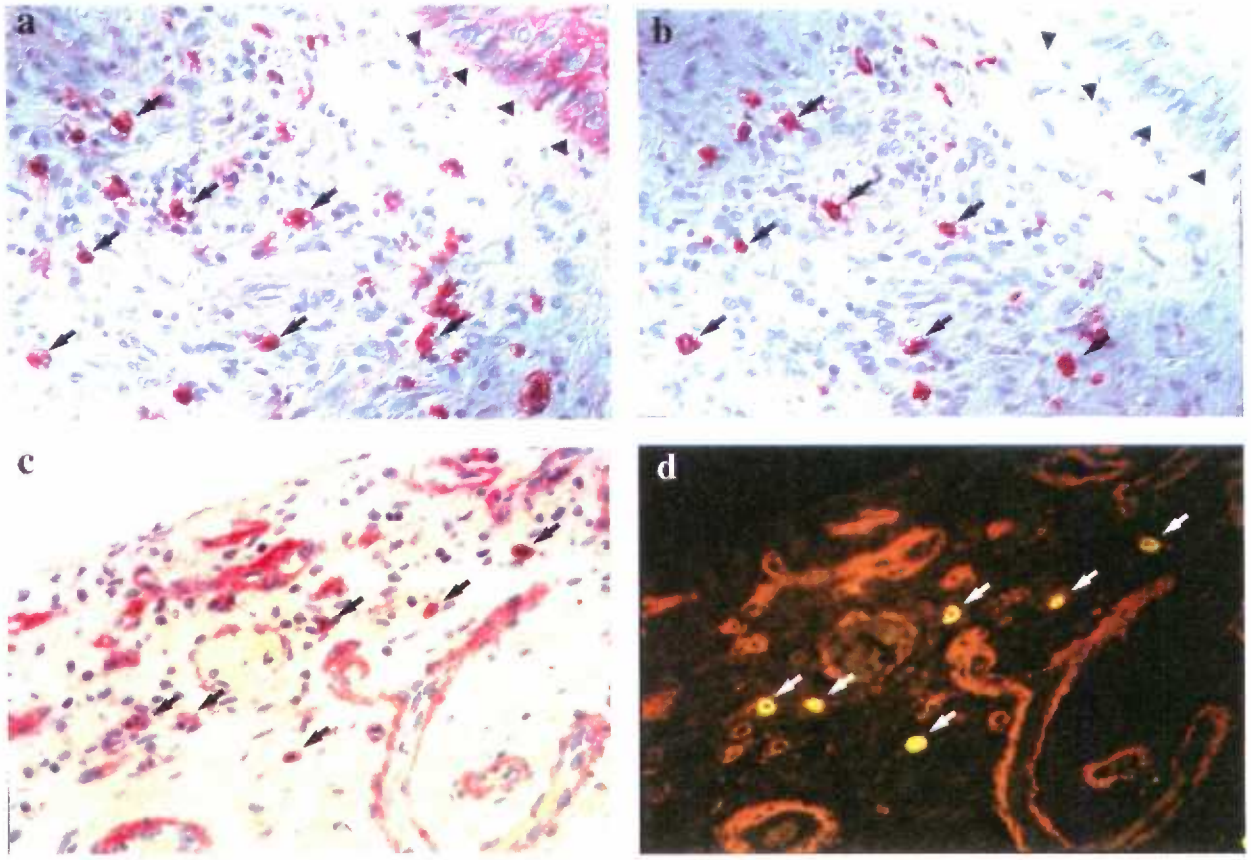

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Figure 2. Histochemical staining of bFGF, tryptase, and macrophages in lung tissue.

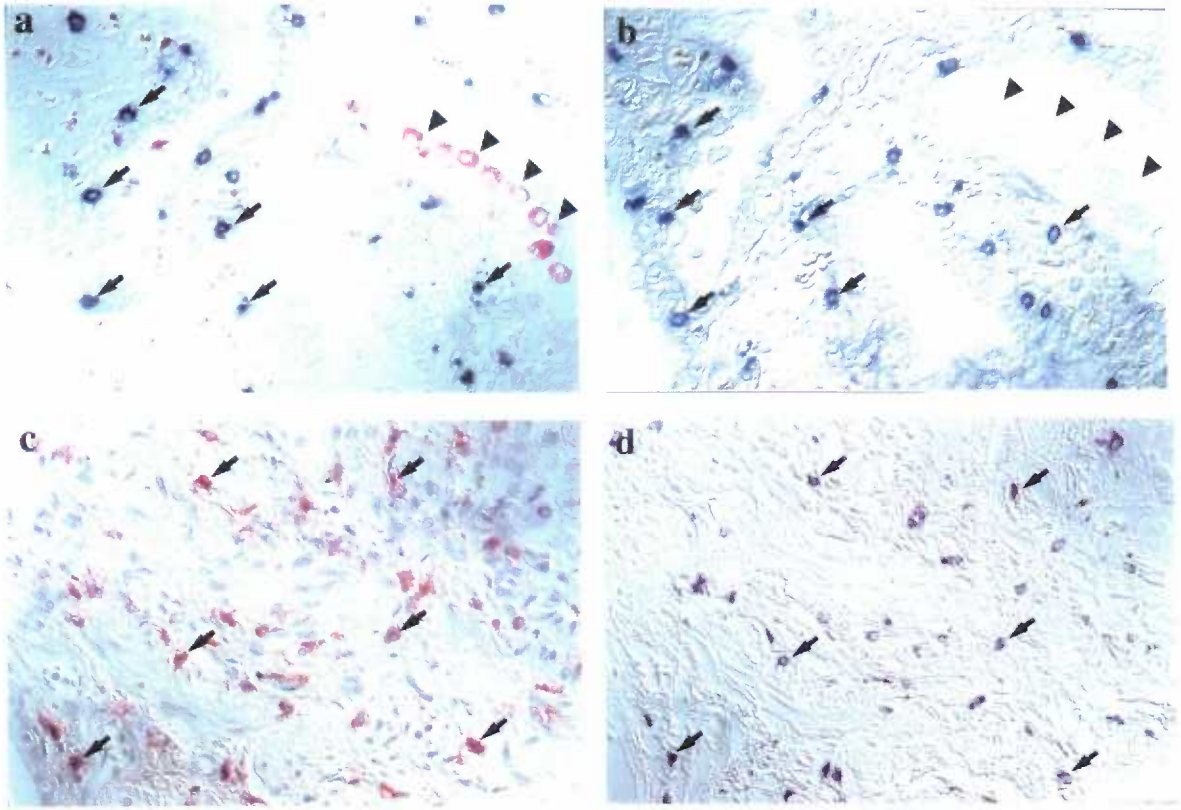

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Figure 3. Detection of bFGF mRNA by RT-PCR.

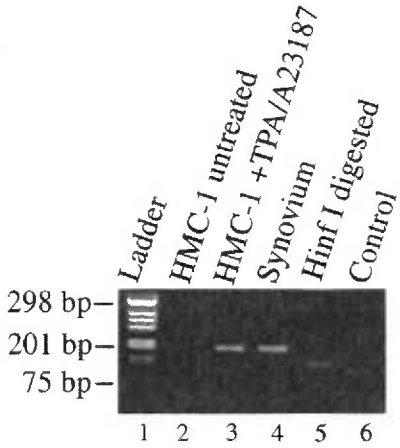
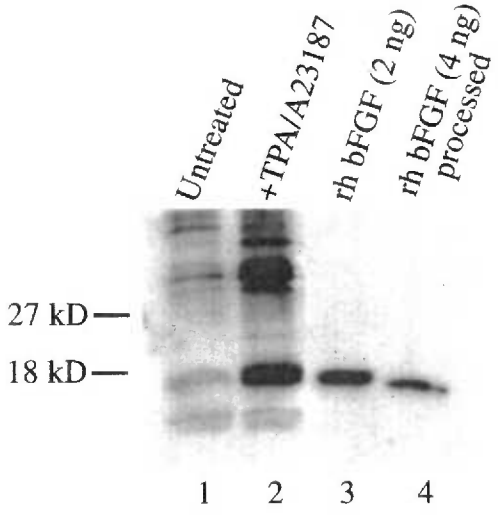


Figure 4. Immunoblot analysis of HMC-1 cell lysates.



MURINE MAST CELLS SYNTHESIZE AND RELEASE BASIC FIBROBLAST GROWTH FACTOR (FGF-2) †

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Abstract

Basic fibroblast growth factor (FGF-2) is a potent angiogenic and mitogenic polypeptide. Unlike most other growth factors that exert their biological effects through paracrine mechanism, FGF-2 lacks a conventional signal sequence for secretion. The mechanism(s) of its release is still a subject of controversy. We recently reported that human mast cells are a major source of FGF-2 in several diseases characterized by cell proliferation and neovascularization. The objective of this study was to test the ability of cultured murine mast cells to release FGF-2. Four murine mast cell lines (CFTL-12, CFTL-15, ABFTL-3 and P815) at 4 X 10^5 cells/ml were treated with 3 different stimulants: substance P (100 nM), compound 48/80 (2-5 μg/ml) and calcium ionophore A23187 (1 μM). The conditioned media were examined for FGF-2 by ELISA from 5 to 360 minutes following stimulation. The magnitude of cell death was determined by trypan blue exclusion and lactate dehydrogenase (LDH) assays. Of the 4 cell lines examined, three (CFTL-12, CFTL-15 and ABFTL-3) constitutively expressed FGF-2 as judged by Northern and Western blot analyses. Increases in FGF-2 levels (up to 16 pg/ml) were found in the culture media within 20 minutes following stimulation. Trypan blue exclusion and LDH assays indicated that cell death could not account for the release. Preliminary studies using a protein synthesis inhibitor (cycloheximide) and ER-Golgi route inhibitor (monensin and brefeldin A) and time course observations suggested that extracellular FGF-2 may be attributed to a pre-formed non-secreted product that is released during mast cell degranulation induced by the stimulants.

Introduction

FGF-2 is a multifunctional polypeptide with potent angiogenic and mitogenic activities. FGF-2 lacks a conventional signal sequence for secretion and the mechanism for its release from host cells is still a subject of controversy. Some authors attribute FGF-2 release to the compromised integrity of plasma membrane; others consider it to be an intracellular protein. As illustrated below, recent studies by us and others indicate that mast cells may serve as a major source of FGF-2 in several chronic proliferative diseases such as rheumatoid arthritis (Qu, Liebler et al. 1994; Reed, Albino et al. 1994). In the present study, we test the ability of cultured human and murine mast cells to express and release FGF-2.

Materials &. Methods

One human (HMC-1) and four murine mast cell lines (CFTL-12, CFTL-15, ABFTL-3 and P815) at 4 X 10^5 cells/ml were treated with 3 different stimulants: substance P (100 nM), compound 48/80 (2-5 μg/ml) and calcium ionophore A23187 (1 μM). The conditioned media were examined for FGF-2 by ELISA (R &. D System) 30 minutes following stimulation. FGF-2 levels were assayed by ELISA. The magnitude of cell death was determined by trypan blue exclusion and lactate dehydrogenase (LDH) assays. Total RNA and protein were isolated from the non-treated cells and analyzed by Western and Northern blot analysis.

Results

Of all cell lines examined, three (CFTL-12, CFTL-15 and ABFTL-3) constitutively expressed FGF-2 as judged by immunocytofluorescent staining, Northern and Western

blot analyses. CFTL-12 and ABFTL-3 also constitutively release detectable levels of FGF-2 into the medium. Increases in FGF-2 levels (up to 16 pg/ml) in the medium can be induced by treating the cells with compound 48/80 and A23187. Trypan blue exclusion and LDH assays indicated that cell death or plasma membrane disruption could not account for the release. Preliminary studies using a protein synthesis inhibitor (cycloheximide) and time course observations suggested that extracellular FGF-2 may be attributed to a pre-formed non-secreted product that is released during mast cell degranulation induced by the stimulants.

Conclusion &. Discussion

1. Murine mast cells can synthesize multiple forms of FGF-2.
2. FGF-2 can be constitutively released by two murine mast cell lines.
3. Release of FGF-2 can be induced or potentiated by degranulating agents, compound 48/80 and A23187.
4. The inducible release of FGF-2 seems to involve a mechanism unrelated to cell death or injury.

References

Qu, Z., J. M. Liebler, et al. (1994). "Mast cells are a significant source of heparin-binding basic fibroblast growth factor in chronic inflammation and cutaneous heangioma." (manuscript submitted for publication).

Reed, J. A., A. P. Albino, et al. (1994). "Human cutaneous mast cells express basic fibroblast growth factor. (Abstract)." Lab. Invest. **70**: 48a.

Figure Legend

Fig. 1. Immunoblot analysis of cell lysates (panel A) and conditioned medium (panel B) for FGF-2: Cell lysates from all cell lines but P815 exhibited a 17-kD band with migration similar to that of recombinant human FGF-2. Higher molecular weight bands are also detected by the anti-FGF-2 antibody. An increased level of FGF-2 was found in the conditioned medium after treatment with compound 48/80 (ABFTL-3/release in panel B).

Fig. 2. Detection of FGF-2 mRNA by Northern blot: Total RNA (20 μg) were extracted from non-stimulated cells and examined by Northern blot analysis. Ethidium bromide staining confirmed equal loading of the RNA. Hybridization of the blot with a cRNA probe specific to FGF-2 visualized a band of 7.0 kb in three of the five samples.

Fig. 3. Detection of FGF-2 and LDH in CFTL-12 cell culture media: Cells in fresh medium were treated with the degranulating agents for 30 minutes, and the levels of FGF-2 and LDH in the media were determined by ELISA and LDH assay. Increased levels of FGF-2 were found following the stimulation while no significant increase in LDH was observed.

Fig. 4. Detection of FGF-2 and LDH in ABFTL-3 cell culture media: Cells in 2-day (panel A) or fresh medium (panel B) were stimulated with degranulating agents for 30 minutes. Constitutive release of FGF-2 was observed. Stimulation with compound 48/80 and A23187 potentiated the release.

Figure 1. Immunoblot analysis of cell lysates (panel A) and conditioned medium (panel B) for FGF-2.

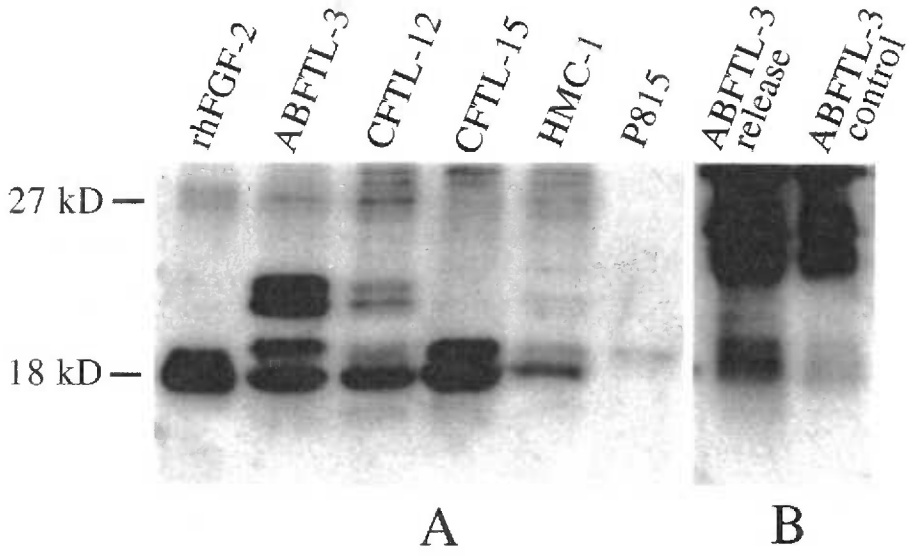


Figure 2. Detection of FGF-2 mRNA by Northern blot.

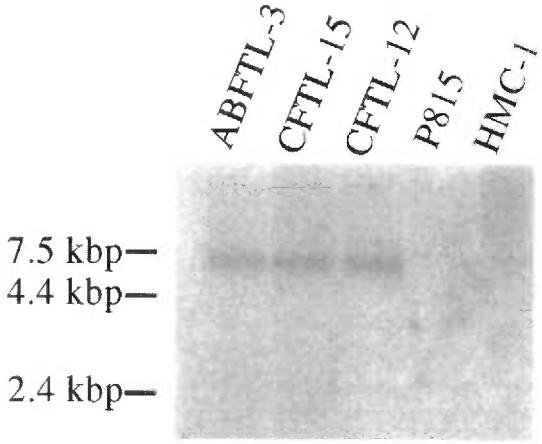
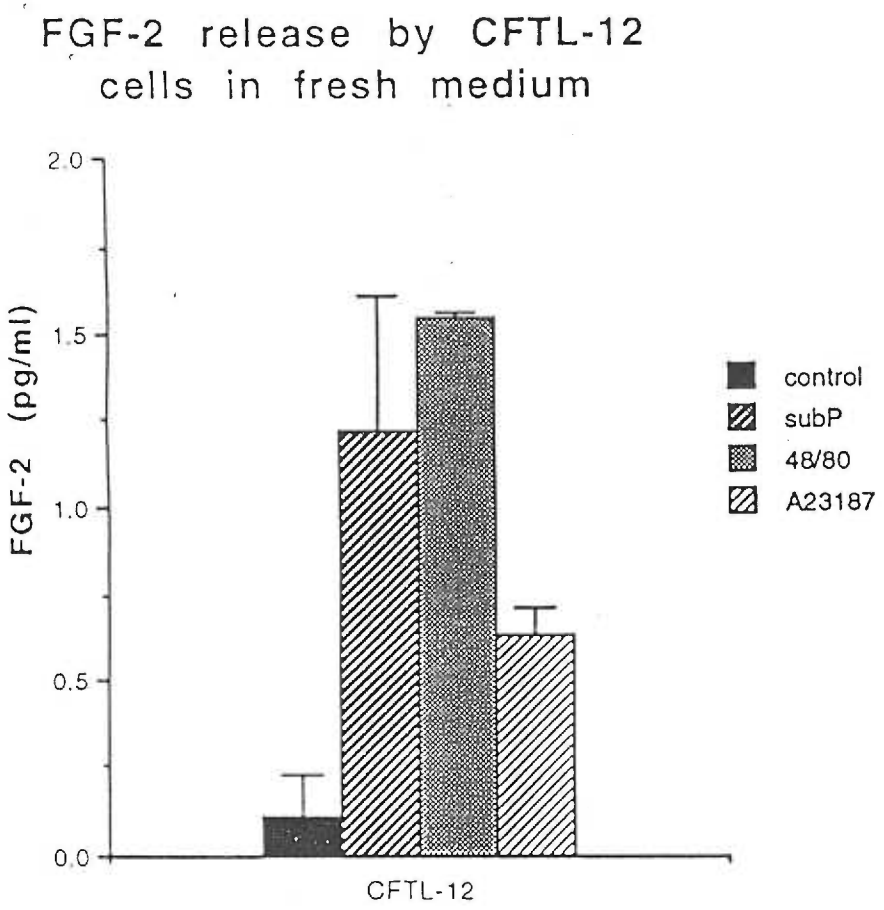


Fig. 3. Detection of FGF-2 and LDH in CFTL-12 cell culture media



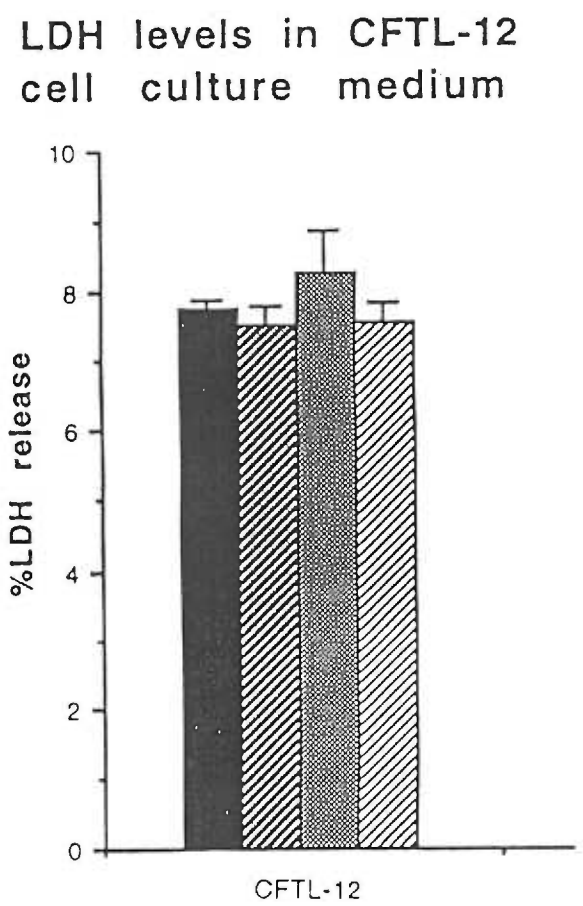


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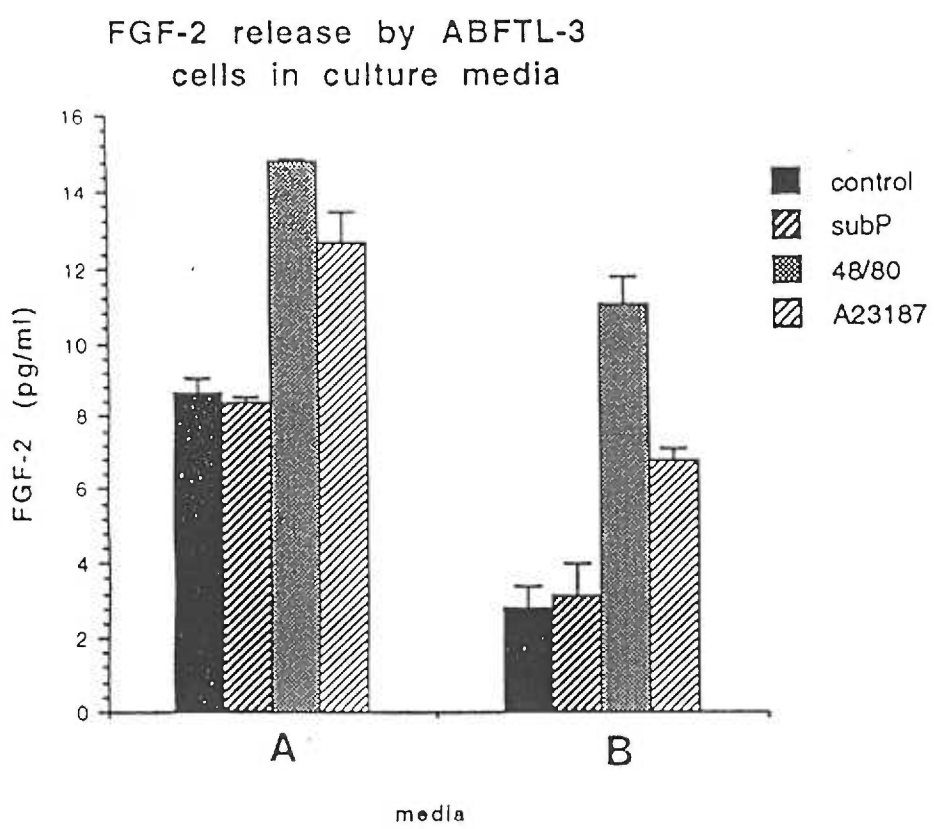
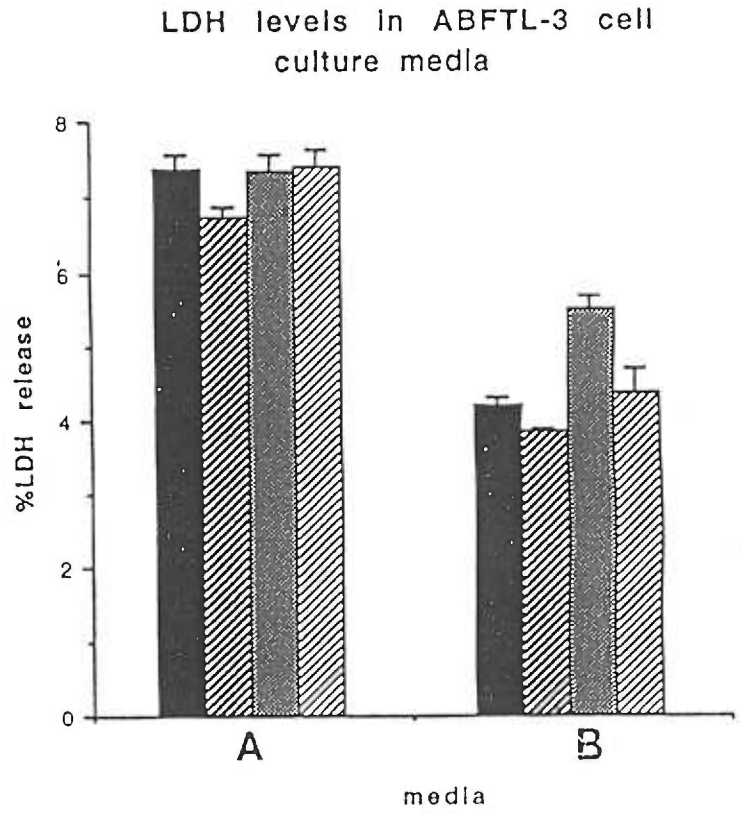
Fig. 4. Detection of FGF-2 and LDH in ABFTL-3 cell culture media





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IV. Discussion and Conclusions

Studies presented in this thesis fall into 3 related areas. First, it directly assesses the role of FGF-2 in arthritis by 1) localization of FGF-2 in inflamed joint tissue; 2) correlation of FGF-2 expression with pathological changes during the development of arthritis; and 3) modulation of experimental arthritis with exogenous FGF-2 and a GF antagonist. Second, it helps one better understand the cellular mechanism(s) of synovial hyperplasia and related proliferative changes in RA by identifying the synovial fibroblast as a potential cell target of GFs including FGF-2. Finally, it characterizes a major cellular source of local FGF-2 in RA and several other proliferative diseases by 1) immunolocalization of FGF-2 in mast cells and 2) detection of FGF-2 expression in human and several murine mast cell lines.

FGF-2 is Expressed by Synovial Tissue

Previous studies by us and others have implicated FGF-2 in RA. Based on these observations, we therefore hypothesized that FGF-2 may play a direct role in RA. As the first step to test this hypothesis, I attempted to determine if FGF-2 can be found in synovial tissue. The study has shown that FGF-2 is expressed by synovial tissue (III. Manuscript 1., In press in Lab. Invest. 1995). Increased staining for FGF-2 found in the hyperplastic synoviocytes of a subset of rheumatoid synovium suggests that bFGF in the lining synoviocytes may be involved in synovial hyperplasia. The distribution patterns of FGF-2 in rheumatoid synovium differ from that of acidic FGF, PDGF-B and TGF-beta that have reportedly been found throughout rheumatoid synovium by others (52, 90, 91). The variability in FGF-2 distribution patterns may reflect the morphological and clinical heterogeneity typical of the disease and the effect of medication administered to reduce disease activity. This is the first report on FGF-2 distribution in a large number of rheumatoid synovial samples. The observation of localization of FGF-2 in mast cells

ultimately led to the finding that mast cells are a major source of tissue FGF-2 in RA and several proliferative diseases (see section III. Manuscript 6).

Local FGF-2 Expression is Correlated with Disease Course in Experimental Arthritis

Although a variety of GFs/cytokines have been found in synovial tissue from patients with RA and from animals with experimental arthritis, only one other report on the expression kinetics has been published (92). Thus it is not clear whether presence of the majority of the GFs/cytokines in the inflamed joint tissues is the cause or the consequence of the disease. The finding of temporal and spatial correlation of FGF-2 expression with the disease process during the development of experimental arthritis in rats (III. Manuscript 2., published in Am. J. Pathol. 145: 1127-1139, 1994) suggests that the increased local FGF-2 expression may be a causal factor for the pathological changes during the articular inflammation. This study also provides strong evidence that FGF-2 may play a direct role in articular inflammation. Another common finding by other investigators is a similar distribution pattern or tissue colocalization of different GFs/cytokines (6, 90-93). In contrast, our study has revealed a striking disparity in the temporal and spatial expression patterns of FGF-2 and PDGF-A. The reciprocal nature of the staining that we detect argues strongly for unique roles for each growth factor. Finally, the kinetic and spatial patterns of FGF-2 expression observed in this study provide evidence for the first time that a GF may induce completely opposite tissue responses in the same disease, depending on the microenviroment.

Articular Inflammation can be Modulated by Exogenous FGF-2 and An Antagonist

To directly assess the role of FGF-2 in articular inflammation, an attempt has been made to modulate the disease process with exogenous FGF-2 and an antagonist, suramin. The findings that FGF-2 ameliorates while suramin worsens the experimental arthritis seem to be paradoxical, since the previous study of FGF-2 in this arthritis model suggested that FGF-2 may be responsible for the joint destruction. However, these

findings do not necessarily refute the possible destructive effect of FGF-2 during articular inflammation. First, down- or up-regulation of receptors for FGF-2 by FGF-2 and suramin respectively may result in such paradoxical effects opposite to the direct effects of FGF-2 and suramin. Second, possible effects of FGF-2 on immune response offer another explanation. Third, FGF-2 at the therapeutic dose may induce tissue responses different from those elicited by endogenous FGF-2. On the other hand, it is also possible that the increased expression of GFs including FGF-2 reflects the frustrated tissue response to a "wound that does not heal", or a defensive mechanism of the injured tissue. Adding exogenous GFs, therefore, may accelerate the healing process. Although the exact mechanism(s) underlying the tissue responses to exogenous FGF-2 and suramin await further investigation, results from this study clearly demonstrate that the experimental arthritis can be modulated by FGF-2 thus directly implicating FGF-2 in polyarthritis.

Excessive Cell Proliferation Contributes to Destructive Changes in RA

Although RA is considered as a proliferative disease characterized by hyperplasia and pannus/villus formation, previous studies by other investigators have attributed these changes mainly to the infiltration of inflammatory cells of bone marrow origin such as lymphocytes, plasma cells and macrophages (14, 17, 18). In two studies of this thesis work (see Section III. Manuscript 1, published in Arthritis Rheum. 37:212-220, 1994; and Manuscript 5, submitted to Arthritis Rheum. 1994), evidence for active local cell proliferation in rheumatoid synovium is presented. The implication of these studies is three fold. First, they reveal a cellular mechanism for the tissue response to increased GF expression during RA. Second, they identify synovial fibroblasts as a potential target of increased GFs in rheumatoid synovium. Third, they correct a long-standing discrepancy in this field -- synovial hyperplasia and joint destruction are mainly attributed to macrophages, but it is synovial fibroblasts, not macrophages, that respond to GFs by

proliferation and expression of proteinases *in vitro* (36, 48, 80). Results from these two studies in this thesis are consistent with these *in vitro* findings. These results are also supported by several recent *in vivo* studies showing that synovial fibroblasts express proteinases and are involved in bone destruction (8, 9, 40, 43).

Mast Cells are a Major Source of FGF-2 in Rheumatoid Synovium

The search for the origin of FGF-2 in rheumatoid synovium has led to the finding that mast cells are a major source of tissue FGF-2 in RA and several proliferative diseases (see Section III. Manuscript 6, submitted for publication). Mast cells have long been implicated in neovascularization and diverse fibrotic or proliferative diseases such as scleroderma, neurofibromatosis, pulmonary fibrosis, and psoriasis (for review see (94)). However, the exact contribution of mast cells to these conditions is largely unknown. Since FGF-2 is a potent angiogenic and mitogenic factor, the findings that mast cells express FGF-2 and function as a major source of FGF-2 in several of these chronic proliferative diseases strongly suggest that FGF-2 may be an important mediator of mast cells. These findings may lend insight into cell and molecular mechanisms underlying the diseases related to mast cells. On the other hand, FGF-2 is known to play a critical role during embryonic development, but its role during other physiologic and pathologic processes *in vivo* remains elusive. The direct link between FGF-2 and mast cells may provide important clues to its biologic function(s) during these processes. Finally, extracellular matrix components such as proteoglycans play an important role in regulating bioactivities of FGF-2. However, FGF-2 lacks a conventional secretory peptide in its structure and the mechanisms for its release is still a subject of controversy. Mast cells are characterized by high content of heparan sulfate and inducible release of their contents. Discovery of the association between mast cells and FGF-2 opens a new field for study of the mechanisms of regulation of expression, bioactivities and release of FGF-2.

Although studies presented here have vigorously tested the initial hypothesis and shown that FGF-2 plays an important role in articular inflammation, they, like other research work, also raise many new questions at the same time. Answers to these questions will help further understand the role of FGF-2 in destructive joint inflammation and the mechanism(s) of RA. Several important questions are currently being investigated. First, are the changes in the levels of FGF-2 receptors responsible for the paradoxical tissue responses to exogenous FGF-2 and suramin? Second, can the active proliferation of synovial fibroblasts in the inflamed synovium be attributed to increased local FGF-2 expression? Answers to these two questions may come from study of the expression of receptors for FGF-2. It is also important to determine how a specific antagonist to FGF-2 such as neutralizing antibody that does not change the level of FGF-2 receptors would affect the disease process in the experimental arthritis. Third, does FGF-2 also exert the protective role in other types of experimental arthritis? Answer to this question will help extrapolate information obtained from the animal models and apply it to RA in human. Fourth, how are the expression and release of FGF-2 by mast cells regulated? Elucidation of the mechanisms underlying these processes will helps us design effective therapeutic strategy to intervene in a variety of FGF-2-related proliferative diseases.

In summary, studies presented in this thesis have revealed that FGF-2 plays an important role in destructive articular inflammation. In addition, these studies also suggest that active cell proliferation and disordered growth patterns of synovial fibroblasts occur in rheumatoid synovium may reflect a mitogenic response of synovial fibroblasts to polypeptide growth factors and contribute to synovial hyperplasia and joint destruction. Finally, they have demonstrated that mast cells may serve as a major source of FGF-2 in rheumatoid synovium.