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ARTICLE

An *in vitro* Model for Studying Mechanisms Underlying Synoviocyte-Mediated Cartilage Invasion in Rheumatoid Arthritis*

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Rheumatoid arthritis (RA) is a chronic inflammatory disease of joints involving the pathological development of an invasive and destructive pannus tissue which contributes to the loss of cartilage and bone. To further analyze the process of cartilage degradation and invasion, we have developed an *in vitro* model composed of cartilage matrix and synoviocytes (isolated from RA pannus tissue, as well as normal synovial membrane). The matrix is derived from pig articular cartilage and contains collagen type II and proteoglycans and is similar in composition to human cartilage. Data generated from this model reveal that synoviocytes isolated from RA pannus tissue invaded cartilage matrix in a manner which directly correlated with the severity of the disease. Analysis of mechanisms associated with the invasive process demonstrate that highly invasive RA synoviocytes maintain a round morphology during attachment and spreading on cartilage matrix, compared with their normal counterparts. Furthermore, the level of secretion of matrix metalloproteinase (MMP) activity was shown to correlate with the RA phenotype, which could be modulated with a novel MMP inhibitor. Normal synoviocytes could be "converted" to an RA phenotype by specific inflammatory cytokines, such that invasion of cartilage matrix was augmented by culturing these cells in the presence of 5 U/ml IL-1β or 18 U/ml TGFβ. Invasion was inhibited by 150 U/ml TNFα, and unaffected by 100 ng/ml PDGF. In addition, synovial fluid from RA patients induced invasion of normal synoviocytes, in a concentration dependent manner, from 150% to 460%; however, synovial fluid from another inflammatory arthritidy (Crohn's) did not augment invasion to the same degree. Moreover, this "conversion effect" appears to be specific for synoviocytes, since similar effects could not be achieved with human skin fibroblasts. This *in vitro* model of synoviocyte-mediated cartilage invasion allows for further molecular characterization of the invasive properties of the synoviocyte which contribute to RA. (Pathology Oncology Research Vol 2, No3, 157–166, 1996)

Key words: invasion, cartilage, pannus, matrix metalloproteinases, cytokines

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of joints leading to the loss of joint function by erosion of subchondral bone, invasion of cartilage by pannus, and stretching of ligaments around the involved joints.[1,2,5] While it is unclear if the disease is caused by a bacterial infection or is an autoimmune disorder,[30] the pathology of RA involves a slow progressive destruction of joint components resulting in increasing disability.[1,2,5]

Cartilage-pannus junctions have been described as erosive regions with pannus tissue invading into cartilage.[18] *In vitro* co-cultures of synoviocytes (isolated from RA pannus tissue, normal synovial membrane, and a patient with Reiters syndrome) and articular cartilage have demonstrated an RA specific ability to deplete cartilage of matrix components.[10] Further investigations into the destructive ability of pannus tissue indicate that a specific cellular component, the RA synoviocyte, is present at the leading front of the invasive tissue.[11] To further analyze synoviocyte invasion into cartilage, we have developed a clinically applicable *in vitro* invasion assay, which incor-

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Abbreviations: RA: rheumatoid arthritis; TNFα: tumor necrosis factor-alpha; TGFβ: transforming growth factor-beta; PDGF: platelet derived growth factor; IL-1β: interleukin-1-beta; MMP: matrix metalloproteinase; PBS: phosphate buffered saline

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porates normal and RA synoviocytes, cartilage matrix, and the Membrane Invasion Culture System (MICS).[14]
Essentially, the *in vitro* invasion model presented here is a modification of two previous systems using co-cultures with cartilage pieces.[10,15] In these prior studies, Hamerman and colleagues.[10] The observed RA synoviocyte-mediated cartilage depletion, while Janusz and Hare[15] made similar observations with transformed cell lines. The *in vitro* joint model reported here advances previous observations of cartilage destruction by challenging both normal and RA synoviocytes with a homogeneous cartilage matrix. We have used this invasion model to analyze mechanisms associated with RA synoviocyte cartilage matrix degradation and invasion, as well as factors involved in the conversion of normal synoviocytes to the RA phenotype.

Other key participants in RA joint destruction are matrix metalloproteinases (MMPs), which are capable of degrading cartilage matrix, and have been identified in RA synovial fluid.[35] Moreover , the levels of stromelysin and interstitial collagenase from plasma have been shown to correlate with RA progression and joint destruction.[22,24] While these observations suggest that MMPs are involved in cartilage destruction in RA, direct evidence for the role of these enzymes in advancing the pannus front into cartilage has not been demonstrated.

It has been proposed that inflammatory mediators associated with the active inflammatory immune response in RA induce the normal quiescent synovial membrane to proliferate into invasive pannus tissue.[11] In fact, inflammatory cytokines have been shown to affect RA synoviocyte growth characteristics,[21] including MMP gene expression,[4] and may be important in the conversion of normal synoviocytes to the invasive RA phenotype. This hypothesis was tested in our *in vitro* invasion model by culturing normal synoviocytes in either RA synovial fluid or in cytokines present in RA synovial fluid – namely, interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNFα), transforming growth factor-beta (TGFβ) or platelet derived growth factor (PDGF), and measuring subsequent modulation of invasiveness through cartilage matrix.

Herein is described an *in vitro* invasion assay for analysis of synoviocyte invasion of cartilage, with special focus on some of the mechanisms associated with the destruction of joint matrix.

Materials and Methods

Human synoviocytes

Synoviocytes were isolated from RA pannus tissue and normal synovial membrane. Pannus tissue was obtained from RA patients undergoing joint replacement surgery. Normal synovial membrane was obtained from patients undergoing joint arthroscopy, primarily for sports-related injuries, who did not have clinical evidence of RA or other inflammatory arthropathies. Synoviocytes were isolated and cultured as previously described.[40] Cells were collected with trypsin (Gibco BRL, Grand Island, NY) and used after passage 2 and prior to passage 6, for RA synoviocytes, and prior to passage 10 for normal synoviocytes. Malme-3 normal human skin fibroblasts (ATCC, Rockville, MD) were used between passage 13 and 15. All cultures were randomly demonstrated to be *Mycoplasma*-free by testing with the Gen-Probe Rapid Detection System (Fisher, Pittsburgh, PA). In addition, cultures were shown to be negative for immune cells by immunofluorescence staining of HLA-DR and CD 11b.

Cartilage matrix

Articular cartilage was isolated from the joints of the front and rear legs (from the hip to the hoof) of 12-16 week-old conventional Yorkshire cross pigs, obtained from the Wilcox Hog Farm (Wilcox, AZ). Cartilage extract was prepared as previously described[7,33] by repeatedly extracting cartilage shavings in a 4M guanidine hydrochloride solution. The pooled extractions were cleared by centrifugation, dialyzed against 10mM ammonium bicarbonate, lyophilized and stored at -20°C until use. Cartilage matrix was resuspended in phosphate buffered saline (PBS) for further analysis and use in experiments. Morphological analysis of the matrix with light microscopy was performed using cartilage extract-coated culture dishes, stained with Coomassie blue. Cartilage extract was spread on a glass coverslip for scanning electron microscopy, fixed in cacodylate-buffered Karnovsky's fixative[16] followed by dehydration, using a series of ascending grades of ethanol. The matrix was coated with gold palladium prior to observation in the Etec Autoscan electron microscope, operating at an accelerating voltage of 20Kv. Cartilage extract proteins were electrophoretically separated in a standard 12.5% SDS polyacrylamide gel[20] and stained with Coomassie blue (0.25% Coomassie blue R235, 25% isopropanol, 10% glacial acetic acid). Proteoglycans in the extract were fractionated by first adding CsCl to 5.2 M followed by centrifugation. Subsequently, the gradient was divided into 4 equal fractions, dialyzed and electrophoretically separated in a 1.2% polyacrylamide-0.6% agarose gel and stained with Toluidine blue.[2,5,24]

Invasion assay

The Membrane Invasion Culture System (MICS) assay was performed as previously described[14] with slight modifications. A polycarbonate filter containing 10 μm pores (Poretics, Inc., Livermore, CA) was coated with a cartilage matrix spread to a uniform thickness and placed in the MICS chamber. Synoviocytes were seeded at a concentration of 1 X 10^5 cells/upper well of the MICS chamber in Dulbecco's Modified Eagles Medium (DMEM; GIBCO, BRL) containing Mito+ serum-free

(NuSerum, Collaborative Research, Bedford, MA), then incubated at 37°C with 5% CO_2 for 48 hr. Each culture of synoviocytes was assayed in triplicate and each experiment repeated at least twice. Invasion is expressed as the percentage of total cells which successfully invaded through the matrix compared to the initial number of cells seeded onto the upper wells and corrected for cell proliferation. [illegible] and statistics was performed using the [illegible].

The ability of a novel inhibitor of gelatinase activity, COL-3, a chemically modified tetracycline, along with a serine protease inhibitor α-1 antitrypsin to affect the invasive ability of RA synoviocytes was also examined. [illegible] the cartilage matrix was preincubated in the presence of 250 µg/ml α-1 antitrypsin for 4 hr prior to cell seeding followed by the addition of 50 µg/ml of COL-3 to the cells at 2 hr and 24 hr post-seeding.

Clinical evaluations

Patients were scored blindly for the severity of their disease activity. This was based upon the primary rheumatologist's evaluation of each patient's clinical history as derived from patient medical charts including the assessment of radiological testing.

Spreading assay

Culture dishes (35mm) were coated with the cartilage matrix, and $5x10^2$ synoviocytes were inoculated into each dish. Representative fields were photographed using inverted light microscopy, beginning with 30 min and ending at 24 hr post-seeding. The percentage of round cells was calculated by counting the number of cells demonstrating a round morphology, divided by the total number of cells in each field. A minimum of 17 cells per field was analyzed from triplicate fields per dish.

SDS-substrate polyacrylamide gel electrophoresis

Cell-free-culture supernatants were obtained from 48 hr cultures of $2x10^5$ synoviocytes in 500 µl DMEM containing 10% NuSerum (Collaborative Research) in a 24 well dish pre-coated with cartilage matrix. As a control, 500 µl of DMEM containing 10% NuSerum and no cells were incubated with the cartilage matrix. Cell-free culture supernatants were stored at -80°C for further analysis of MMP activity, as previously described.[38] Briefly, gelatinase A, gelatinase B and associated degradation products were detected in 10% SDS polyacrylamide gels containing 1 mg/ml gelatin (BioRad, Hercules, CA), and activity associated with stromelysin was detected using 10% SDS polyacrylamide gels containing 0.5 mg/ml α-casein (Sigma, St. Louis, MO). Confirmation of the zymographic analyses of MMPs was performed by Western blot analysis as previously described.

Zymographic analysis was also performed on the conditioned media from the MICS assay used to measure the affects of COL-3 and α-1 antitrypsin on the RA invasive potential. Briefly, samples were split into two aliquots with one aliquot treated with 1 mM aminophenylmercuric acetate (APMA, Aldrich Chem. Corp., Milwaukee, WI) for 2 hr at 37°C, and the other aliquot incubated at 4°C. Two [illegible] of conditioned medium were added to one part Laemmli sample buffer (minus reductant) and loaded onto 10% SDS polyacrylamide gels containing 0.1% gelatin without prior boiling. After electrophoresis, the gel was incubated in 50 mM Tris-Cl (pH 7.5)/2.5% Triton X-100 plus 50µg/ml COL-3 for 30 min, followed by incubation in 50 mM Tris-Cl (pH 7.5)/10 mM $CaCl_2$/0.02% NaN_3 plus 50µg/ml COL-3 for 20 hr at 37°C. The gel was then stained and destained as previously described[illegible].

Cytokine or synovial fluid treatment of normal cells

Normal synoviocytes were inoculated into T-75 tissue culture flasks, allowed to grow to 95% confluence and then removed with trypsin. The cells were washed and inoculated equally among four T-75 tissue culture flasks and cultured with specific concentrations of selected cytokines, synovial fluid from patients with RA or Crohn's disease or complete DMEM as a control. TNFα (Genzyme, Cambridge, MA) was cultured with normal synoviocytes at 10 U/ml and 150 U/ml; TGFβ1 (Genzyme) was used at 0.5 U/ml, 3 U/ml and 18 U/ml; IL-1β (Genzyme) was used at 5 U/ml and 25 U/ml; PDGF was a kind gift from Amgen (Thousand Oaks, CA) and was used at 30 ng/ml and 100 ng/ml; and patient-derived synovial fluid was diluted in medium to final concentrations ranging from 0 to 50%. The medium was replaced every 3 days until the cells reached 80-90% confluence. Subsequently, the cells were harvested with trypsin for further analysis in the *in vitro* invasion model.

Results

Characterization of Cartilage Matrix

To analyze synoviocyte-mediated cartilage destruction, we adapted a cartilage matrix, isolated from porcine articular cartilage, that could be used as a barrier in an *in vitro* invasion assay. Biochemical characterization of the extract by polyacrylamide gel electrophoresis, as shown in *Fig.1A*, demonstrates the presence of multiple proteins in the extract, including collagen type II which is characteristic of cartilage. This cartilage matrix also contains a significant amount of proteoglycans which were fractionated by CsCl gradient centrifugation and visualized by polyacrylamide/agarose gels, as shown in *Fig.1B*. The uronic

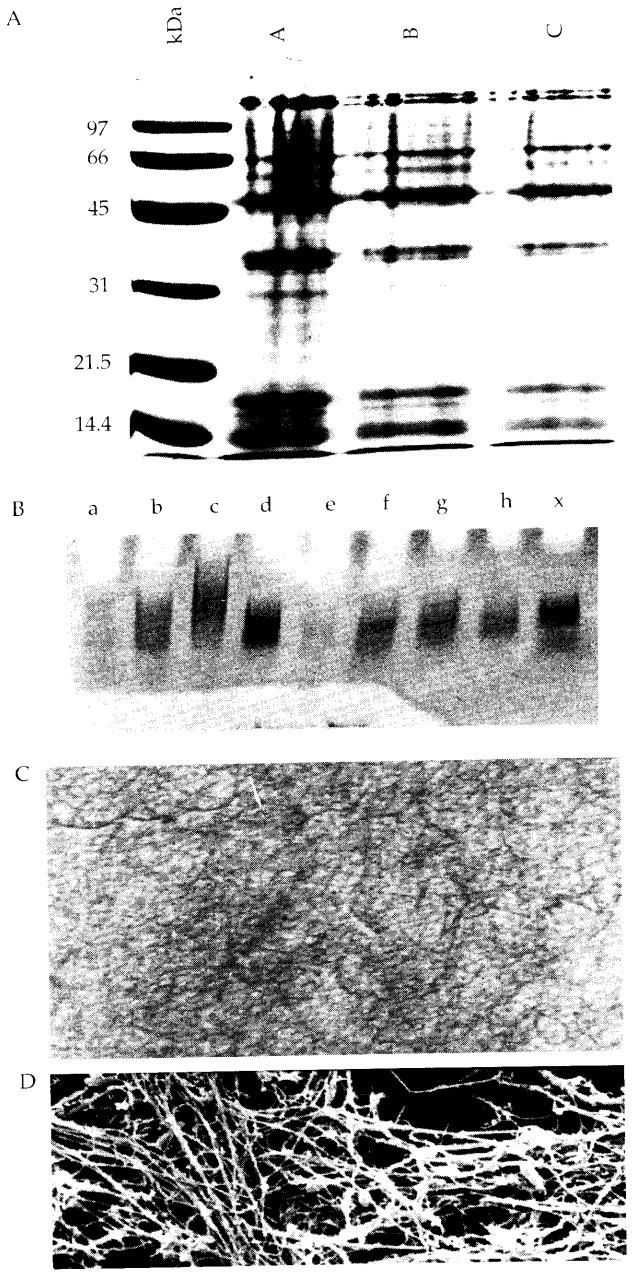

Figure 1. *Biochemical and morphological analyses of articular cartilage extract. A: SDS-polyacrylamide electrophoretic analysis of proteins in the extract as stained by Coomassie blue. Arrow indicates the α chain doublet of type II collagen. Internal lanes A, B and C represent serial dilutions of the cartilage extract to loading buffer (1 mg/ml) of: A,1:1; B,1:2; and C,1:3. B: CsCl fractionation and gel electrophoretic analysis of proteoglycans in the cartilage extract from CsCl fractions were obtained: a) least dense CsCl fraction collected from the top of the gradient; b) directly below fraction a; c) directly above fraction d; and d) most dense CsCl fraction collected from the bottom of the gradient. C,D: Morphological analysis of cartilage matrix; C: Inverted light microscopy of cartilage matrix coated onto a tissue culture dish and stained with Coomassie blue (original magnification 125X). D: Scanning electron microscopy of a cartilage-coated glass coverslip (original magnification 3000X).*

acid content of the cartilage extract fractions indicated that proteoglycans comprised 13.6% of the extract and were of a composition typical of cartilage. For our application, it was important that the extract also form a matrix that could be incorporated as a barrier in an *in vitro* invasion assay. To demonstrate the architectural "matrix" forming properties of the cartilage extract, a culture dish and glass coverslip were coated with the extract, and the morphology observed with light and high resolution scanning electron microscopy, respectively *(Fig.1C,1D)*. These morphological analyses demonstrate that the extract forms a complex matrix containing interlocking and interweaving fibrils.

Correlation of in vitro Synoviocyte Invasion of Cartilage Matrix with RA Clinical Progression

To measure the ability of synoviocytes to invade the cartilage matrix, the MICS chamber[14] was adapted to incorporate both the cartilage matrix and synoviocytes. In this model, normal and RA synoviocytes were able to invade through cartilage matrix-coated filters in a 48 hr assay. Normal synoviocyte invasion ranged from 0.2% to 3.4% *(Fig.2A)*. By comparison, RA synoviocyte invasion of cartilage matrix ranged from 0.3% to 7.3%. The variability in the invasive nature of RA synoviocytes isolated from separate patients prompted a comparison of in *vitro* invasiveness vs. progression of disease. The disease activity was determined from medical histories and X-ray analysis of bone erosions. In *Fig.2B*, the clinical progression of RA vs. the invasiveness of RA synoviocytes were statistically analyzed, and a correlation is shown between RA synoviocyte invasion in the MICS assay and disease progression. (Spearman's rank, r=0.78)

Synoviocyte Attachment and Spreading on Cartilage Matrix

Attachment and spreading are key steps in the process of matrix remodeling and invasion[23]. To determine if synoviocyte association with cartilage matrix differed between highly invasive RA synoviocytes (RA-02) and normal synoviocytes (N-O1), we observed the ability of these cell phenotypes to attach and spread. As shown in *Fig.3*, at 30 min post-plating, both normal and RA synoviocytes have attached to the cartilage matrix in a similar manner with the majority of the cells maintaining a rounded morphology. At 2 hr post-plating, normal synoviocytes were well into the spreading process, as indicated by the presence of extended, dendritic-like processes extended by the cells and a lack of reflected light around the flattened cell bodies. Conversely, the highly invasive RA synoviocytes (RA-02) at 2 hr post-plating were less spread, demonstrated by increased reflected light around the cell bodies, and retained the more rounded morphology. Hence, these

data indicate that a differential spreading ability exists between the poorly invasive and highly invasive synoviocytes on cartilage matrix.

Metalloproteinase Enzyme Activity

Rheumatoid synoviocytes have been shown to secrete a variety of MMPs[36]; however, there have been no comparative studies performed with normal human synoviocytes. To determine if the extracellular levels of specific MMPs produced by synoviocytes correlated with the RA phenotype, enzyme activity was measured by substrate-incorporated SDS-polyacrylamide gel electrophoresis (zymography). Supernatants were collected from normal synoviocytes (N-02 and N-03), and RA synoviocytes (RA-02, RA-03, RA-04), after 48 hr incubation with cartilage matrix. As shown in *Fig.4A*, using a gelatin substrate, all cell types produced gelatinases A and B; however, only the RA samples produced the *active* form of gelatinase B, as well as additional breakdown products of gelatinase A. All cell types showed stromelysin activity using a casein substrate (data not shown). Further determination of MMP gene expression by Northern blot analysis corroborated these data (results not shown).

In an attempt to inhibit the degradative, and hence, invasive activity of RA synoviocytes, we tested the effect(s) of COL-3 (an inhibitor of gelatinolytic activity[9]) in addition to α-1-antitrypsin (an inhibitor of elastase activity)[32] in the *in vitro* invasion assay. The data shown in *Fig.4B* demonstrate a marked decrease in the invasive ability of RA synoviocytes, when treated during the invasion assay, and a synergistic effect with α-1-antitrypsin plus COL-3, resulting in a greater diminution in invasive activity.

Modulation of Normal Synoviocyte Invasion by Cytokines and Synovial Fluid

A compelling argument has been made for cytokine mediated joint destruction observed in RA. Thus, with our model, we tested the hypothesis that specific cytokines, found in synovial fluid, have the ability to modulate the invasive capability of normal synoviocytes *in vitro*. *Table 1* shows the maximum dose effect tested of specific inflammatory cytokines: IL-1β, TGFβ, TNFα and PDGF, on several normal synoviocyte cell strains. After 8 days of treatment with each respective cytokine, the cells were washed and seeded onto cartilage matrix in the invasion model. Clearly, IL-1β and TGFβ increased the invasive ability of these cells, while TNFα reduced their invasiveness through cartilage matrix, and PDGF had little effect. Depletion of TNFα from the media, using neutralizing antibodies, resulted in the restoration of invasive activity similar to the levels achieved by the respective IgG control.

The next set of experiments tested the ability of synovial fluid, derived from two patients with progressive RA and one patient with Crohn's disease (an inflammatory bowel disorder), to augment the invasive phenotype of normal synoviocytes (*Fig.5*). Following a similar 8 day pretreatment protocol, *Fig.5A* demonstrates a concentration dependent increase in the invasive ability of the normal cells to respond to synovial fluid (diluted to a final

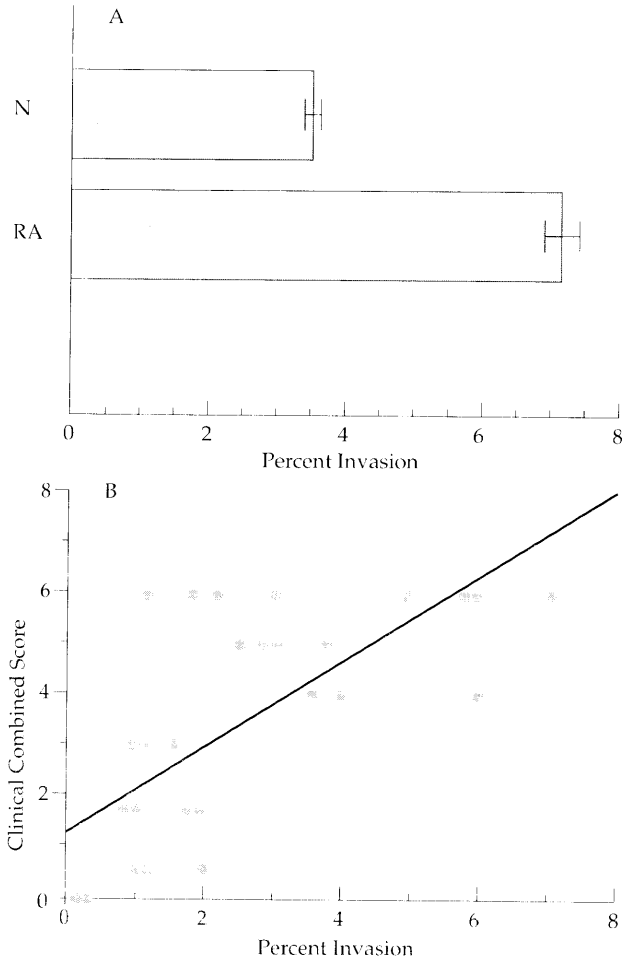

Figure 2. *A. Bar graphs showing actual percent invasion of normal synoviocytes (N) versus RA synoviocytes (RA) through cartilage matrix in vitro. B. Scatter graph of RA synoviocytes invasion rates and combined score of clinical and bone evaluations (statistical analysis of Spearman's rank r=0.78). Invasion through cartilage matrix was measured using the MICS assay in which RA and normal synoviocytes were analyzed for their ability to penetrate a cartilage extract-coated polycarbonate filter, containing 10μm pores. Error is expressed as standard error of the mean of n=3 wells minimum from a representative experiment; invasion rates are expressed as the percentage of cells seeded onto the cartilage-coated filter compared with the post-invasive cells collected from the undersurface of the filter. Scores were derived from patient evaluation(s) for clinical progression of disease and bone involvement at the time of surgery. Scores range from 0 (mild disease activity and little bone involvement) to +4 (aggressive disease with extensive bone involvement). Statistical analysis was performed by comparing the RA invasiveness to paired normal synoviocyte invasion.*

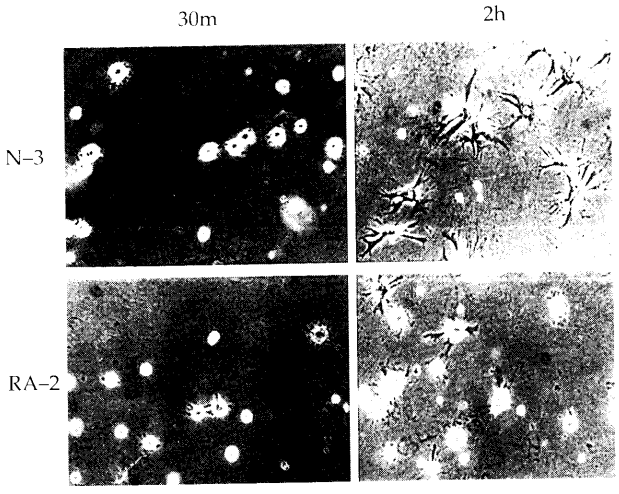

Figure 3. Phase contrast micrographs of normal (N-3) and rheumatoid arthritis (RA-2) synoviocyte on cartilage matrix. At 30 min, all samples are composed of 100% rounded cells. At 2 hr, 15% of N-3 are rounded; while 39% of RA-2 remain rounded (original magnification 125X).

concentration of 10%, 25% and 50% in media) by augmenting invasion up to 460% compared with control. We also tested whether this "conversion" effect was specific for synoviocytes by treating Malme human skin fibroblasts with synovial fluid from the same RA patient, and found little to no effect on invasiveness. Synovial fluid from another patient with RA (*Fig.5B*), diluted as described above,10%, 25% and 50%, showed an augmentation of invasive potential of synoviocytes through cartilage matrix, similar to that demonstrated in *Fig.5A*. We then used gelatin zymography to analyze the conditioned medium from normal synoviocytes treated with another RA synovial fluid (50% dilution), to determine if increased invasive activity correlated with potential changes in gelatinolytic activity (inset). Consequently, this treatment resulted in a 4-fold increase in the amount of extracellular active gelatinase A (lane 1 compared to lane 3) and corresponding increase in the amount of APMA activatable gelatinase A (lane 2 compared to lane 4). (We also verified the presence of specific cytokines in these synovial fluid samples, and found IL-1β IL-2, IL-2R, TNF-α, IL-6, TGF-β and PDGF, similar to what was shown in *Table 1.*) Lastly, we tested the ability of Crohn's synovial fluid to induce the invasive phenotype of normal synoviocytes, and found this induction was not concentration dependent and did not significantly alter invasiveness above two-fold (*Fig.5D*).

Discussion

The pathology of RA is mediated by the conversion of the normal quiescent synovial membrane into the highly metabolic pannus tissue.[11,26] The invasive and destructive properties of RA pannus tissue have been observed *in vitro* in both human patients and in animal models, as well as *in vitro* using co-cultures of pannus tissue and joint components.[11] To further analyze the ability of pannus tissue to invade cartilage, we have developed an *in vitro* model of synoviocyte invasion which incorporates an articular cartilage matrix and essentially simulates a joint.

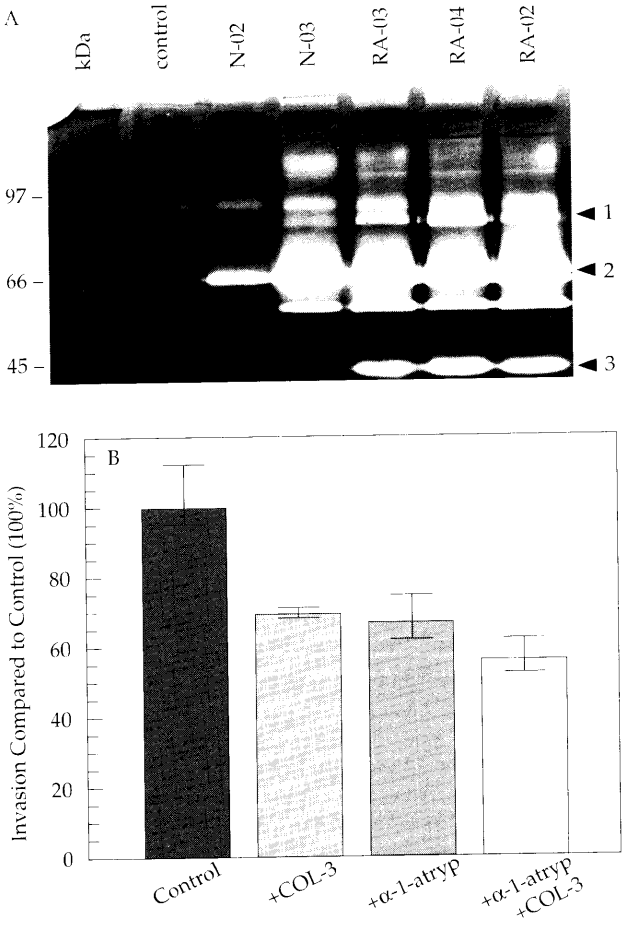

Figure 4. A. Zymographic analysis of gelatinolytic activity from 48 hr supernatants of normal and RA synoviocytes cultured on cartilage matrix. Control(s) consists of media supernatant from cartilage matrix without cells. Enzyme activity is denoted by clear zones where the substrate contained in the gel has been degraded and is not stained by Coomassie blue. Arrow 1 points to gelatinase B (MMP-9), with an underlying zone of associated activity, primarily in the RA samples. Arrow 2 points to gelatinase A (MMP-2), with an underlying zone of associated activity in N-03, RA-03, RA-04 and RA-02. Arrow 3 points to additional breakdown (or proteolytic) forms of gelatinase A, only in RA samples. B. Analysis of the effect(s) of COL-3, α-1-antitrypsin, or α-1-antitrypsin with COL-3 on the ability of RA synoviocytes to degrade and invade cartilage matrix, compared with untreated control cells, over 48 hr in vitro. Invasive potential of control was normalized to 100% invasion, and the invasiveness of treated cells was determined as a percentage of control. Error bars are based on n=8, and the SE was determined relative to control cells.

Using this model, we have investigated mechanisms associated with invasion, including cell-matrix interactions and MMP activity production as well as the ability of inflammatory cytokines and RA synovial fluid to induce an invasive phenotype in normal synoviocytes. We have also demonstrated the utility of testing inhibitor(s) of MMP activity for their efficacy in dimishing joint matrix degradation.

The unique component of the *in vitro* model is the cartilage matrix barrier generated from a porcine articular cartilage extract which contains collagen type II and proteoglycans – the major constituents of articular cartilage.[13] Quantification of uronic acid, which is a measure of proteoglycans, indicates that the extract contains 13.6% proteoglycans, thus mimicking intact human cartilage.[12] In addition, the extract can be spread over a surface creating a microfibrillar array of interweaving fibers, similar to that found in joint matrix.

Incorporating the cartilage matrix barner into the MICS assay[14] led to the development of the *in vitro* invasion assay in which both normal and RA synoviocytes could be observed to actively invade. Normal synoviocyte invasion was not unexpected since fibroblasts as well as fibroblast-like cells have been shown to have matrix modifying characteristics that would enable these cells to degrade matrix components.[25]

Based on observations using the *in vitro* invasion assay, we began to investigate key steps in the invasive process, which have been described and partially characterized in relation to tumor cell metastasis. Liotta et al.[23] describes invasion as a series of steps beginning with attachment to the basement membrane, followed by dissolution of the membrane, and culminating in cellular movement through the degraded matrix. Applying these basic principles governing tumor cell invasion to the study of synoviocyte invasion, we first compared the ability of highly invasive and normal synoviocytes to spread on cartilage matrix, as others have demonstrated a correlation between cell spreading and invasiveness.[3] Highly invasive RA synoviocytes maintained a rounded morphology for a longer period of time than their normal counterparts when initially plated on cartilage matrix. Although there is no difference between the ability of normal and RA synoviocytes to attach to the matrix substrate, the less spread morphology

***Tabel 1.* Cytokine modulation of normal synoviocyte invasion of cartilage matrix**

	N-O1	*N-02*		*N-03*	*N-O5*
IL-1β (5U/ml)	123±5	669±59		167±27	ND
TGFβ (180U/ml)	280±11	ND		ND	387±27
TNFα (150U/ml)	20±5	ND		23±3	23±5
Anti-TNFα (6μg/ml)	—	—	110±12*	—	—
PDGF (100 ng/ml)	ND	137±16		116±12	ND

Invasion of synoviocytes (N) cultured in media alone was arbitrarily set to a value of 100%, and the cytokine cultured samples were compared to this control.
* TNFα activity was neutralized with a monoclonal antibody to TNFα, and invasion values compared with those associated with the IgG control arbitrarily set at 100%.

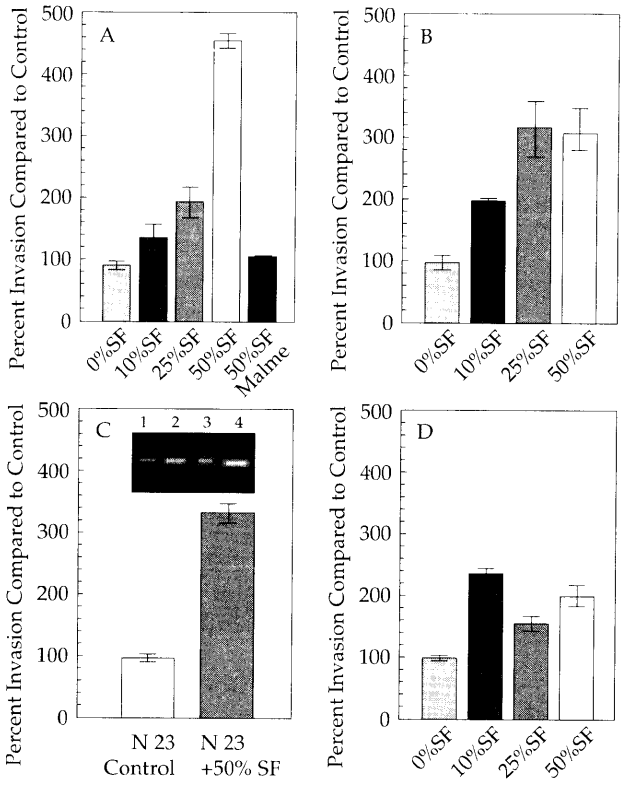

Figure 5. *Bar graphs representing percent invasion compared to control of normal synoviocytes treated for 8 days with synovial fluid (SF), 0%, diluted 10%, 25% or 50% with medium, from 3 patients with RA (A), and (B), (C) or Crohn's disease (D) prior to testing invasiveness through cartilage matrix in the MICS assay. In (A), the effect of 50% SF from an RA patient was also measured with respect to the invasive potential of Malme human skin fibroblasts. Standard error bars are expressed as standard error of the mean of n=3 wells minimum per sample, with each experiment performed in duplicate. Percent invasion of experimental wells receiving SF was compared with percent invasion generated from control without SF, in which the values were arbitrarily set at 100%. In (C), inset shows the zymographic analysis of supernatants from normal synoviocytes, either non-treated control or pretreated with 50% RA conditioned medium, prior to testing invasiveness in vitro. This treatment resulted in a 4-fold increase in the amount of extracellular active gelatinase A (MMP-2), shown in lane 1 compared with lane 3, and a corresponding increase in the amount of APMA activatable gelatinase A, shown in lane 2 compared with lane 4.*

displayed by the RA cells may be conducive to a more migratory phenotype, as has been demonstrated previously with tumor cells.[28] As an aside, we also tested whether this adhesion was mediated by integrins via treatment of both normal and RA cells with RGD peptide, as well as RGE control peptide. These data, although not presented in this study, showed a 70% inhibition of attachment in both cell types with RGD, thus implicating the involvement of integrins in the process of attachment.

The second step in the invasive process involves the dissolution of matrix components. MMPs are a family of enzymes capable of degrading matrix components and have been associated with the invasive ability of tumor cells[23] and synoviocytes,[36] and have been identified in RA synovial fluid.[35] In the present study, a comparison was made between MMPs produced by normal synoviocytes vs. RA synoviocytes plated on cartilage matrix. These experiments revealed that gelatinase A (MMP-2), gelatinase B (MMP-9) and stromelysin I (MMP-3) were secreted by both normal and RA synoviocytes; however, the active form of gelatinase B was observed in the RA samples but not in the normals. Furthermore, additional breakdown products of gelatinase A were only observed in the RA samples. Indeed, one of the most interesting observations disclosed in this report is the inhibitory effect of COL-3 (tested at a physiological dosage of 50 μg/ml) on the invasive and degradative ability of RA synoviocytes *in vitro*. Current studies in our laboratory indicate a dose response to COL-3 is achievable, ranging from 3 μg/ml to 50 μg/ml, with the greatest inhibition of degradative activity being associated with the 50 μg/ml dosage. This compound belongs to a class of chemically modified tetracyclines with anti-gelatinolytic activity, which have been shown to be efficacious in the treatment of cancer, oral ulcerations, and osteoarthritis[9]. In this study, a synergistic effect of COL-3 with α-1-antitrypsin resulted in the greatest inhibition of invasive activity. Furthermore, with the recent finding that collagenases can also degrade serum α-1-antitrypsin (a major endogenous serpin inhibitor of serine proteinases), there is strong evidence to suggest that chemically modified tetracyclines may "protect" extracellular matrices from not only the collagenase and gelatinase MMPs, but from a wider spectrum of neutral proteases.[32] We next tested the hypothesis that inflammatory cytokines, found in RA synovial fluid, have the potential to convert normal synoviocytes to the invasive RA phenotype. We analyzed IL-1β, TNFα, TGFβ and PDGF, as these cytokines have been consistently identified in RA synovial fluid.[19] Normal synoviocyte invasion was augmented by 5 U/ml IL-1β or 18 U/ml TGFβ. Previous studies demonstrated IL-1β can induce collagenase production in human RA synoviocyte[4], which may contribute to the invasive nature of normal synoviocytes exposed to IL-1β. TGFβ contributes to the pathology of RA, as demonstrated by intra-articular injections of TGFβ promoting an inflammatory condition in the SCW rat model.[17] In this study, TGFβ promotion of the invasive phenotype of normal synoviocytes support the previous observations of Wahl and colleagues in the SCW rat model.[17] Normal synoviocytes cultured with 150 U/ml TNFα were much less invasive than the untreated control cells in our study, thus suggesting a negative regulatory role for this cytokine. In fact, the role of TNFα in suppressing tumor growth and metastasis has been established in a study where tumor cells were genetically altered to express TNFα, which formed only small tumors and no metastases,[37] and, thus, associates this cytokine with the suppression of metastatic behavior. On the other hand, TNFα has been associated with the pathology of RA, as antibodies to TNFα have been shown to significantly reduce the severity of collagen-induced arthritis in mice.[27] This apparent discrepancy may be explained by the role of TNFα in enhancing the immune response. For instance, T cells exposed to TNFα demonstrate enhanced proliferation in response to IL-1β,[39] and TNFα enhances the T cell response to antigenic challenge.[1] Therefore TNFα may contribute to the inflammation of RA, thus generating other factors involved in the development of the invasive pannus tissue. With respect to PDGF, up to 100 ng/ml showed no significant enhancement of the invasive phenotype of normal synoviocytes; yet, it has been shown to induce proliferation in fibroblasts.[6,29,34] However, it is important to keep in mind that proliferation and invasion are two distinct processes which are essentially mutually exclusive events in our invasion model. During the 48 hr window of observation for the invasion assay, proliferation of either normal or RA synoviocytes does not occur.

The most fascinating aspect of this study is the experimental manipulation of normal synoviocytes, by RA synovial fluid, resulting in up to 4.6-fold increase in invasion compared with control. The maximum effect achieved was with the 50% SF concentration from two different RA patients. As an additional control in the performance of these experiments, we addressed whether the "converting" activity could be partially attributed to the cartilage matrix. Accordingly, cells were treated with RA synovial fluid while growing on either cartilage or just plastic, followed by testing in the invasion assay. The results indicated that both conditions lead to a similar augmentation in invasive potential, thus suggesting that the "converting" activity of the synovial fluid is at a cellular level and not derived from inductive, extracellular *stimuli* (data not shown). Of special significance is the obseivation related to the "nonconversion" of the Malme human skin fibroblasts by 50% RA SF, indicating that the effect of cytokines on synoviocytes is quite specific, potentially via cytokine cell surface receptors. Just as intriguing is the observation of the Crohn's SF on normal synoviocytes, which does not demonstrate the same degree of

"conversion" potential as RA synovial fluid. Although Crohn's disease is considered an inflammatory connective tissue disorder, it differs from RA with its association with immune complex pathology and more obvious complement mediated vasculitis. Hence, the net effect of the normal synoviocyte conversion studies suggests that: 1) these cells can be converted to an RA phenotype by treatment with synovial fluid isolated from RA patients; 2) the upregulation in invasive ability through cartilage matrix is accompanied by a concomitant increase in MMP activity; and 3) this effect appears to be concentration dependent and specific to synoviocytes.

RA is a disease process that may culminate in joint destruction. This study describes the development and utilization of a clinically relevant invasion assay reflecting the *in situ* environment of cartilage-pannus junctions. An analysis of mechanisms associated with the process of invasion reveals that cell-matrix interactions involved in spreading of synoviocytes on cartilage matrix may contribute to the highly invasive RA phenotype. A further analysis of MMP production indicated both normal and RA synoviocytes secrete MMPs; however, more *active* gelatinolytic activity is associated with RA cells which may contribute to joint destruction. Furthermore, treatment of RA cells with inhibitors of gelatinolytic and collagenolytic activity resulted in a 50% decrease in invasive activity over 48 hr. In addition, the invasive ability of normal synoviocytes was modulated by specific cytokines known to be present in the synovial fluid of RA patients as well as RA synovial fluid itself. Hence, the model allows for further analysis of the invasive phenotype of synoviocytes, the mechanisms associated with cartilage destruction in this disease, and evaluation if additional MMP inhibitors for their efficacy in reversing the clinical progression of RA.

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