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Beta Integrins ($\alpha 4\beta 1$, $\alpha 5\beta 1$) and Matrix Metalloproteinases Expression in Synovial Pannus Formation in Rheumatoid Arthritis

Hitoshi Ishikawa$^1$, Souichirou Hirata$^1$, Ryuichi Saura$^1$, Yoshihiro Andoh$^2$, Yasuhiko Imaizumi$^3$, and Kosaku Mizuno$^3$

To investigate the mechanism of synovial pannus formation in rheumatoid arthritis, immunohistochemical studies with monoclonal antibodies against the adhesion molecules, $\alpha 4\beta 1$, $\alpha 5\beta 1$ integrins and matrix metalloproteinases (MMP-1, MMP-3) and tissue inhibitor of metalloproteinase (TIMP-1), were carried out to determine the pattern of the distribution of these molecules at the rheumatoid synovial cartilage junction. Treatment with purified anti-human monoclonal antibodies, anti-$\alpha 4\beta 1$ and $\alpha 5\beta 1$, resulted in membrane staining of most of the cells infiltrating the synovial tissue and bordering the pannus cartilage junction. The $\alpha 4\beta 1$ and $\alpha 5\beta 1$ interaction appeared to be involved in the attachment of the pannus. Staining for MMP-1 and MMP-3 was particularly intense at the pannus-cartilage junction. These molecules were also strongly stained in the cartilage matrix bordering the tip of pannus invasion. TIMP-1 staining disclosed very weak cellular staining and weak extracellular staining at the pannus-cartilage junction. The present study indicates that a receptor-ligand interaction between $\beta 1$ integrin and cartilage matrix may occur at the early stage of pannus formation, and that this is followed by elevated proteolytic activity of MMPs and a decreased level of TIMP-1. And in this way, these changes may contribute to pannus invasion and cartilage destruction.

Key Words: Synovial-pannus formation, $\beta 1$ integrins, Matrix metalloproteinases (MMP-1, MMP-3), Tissue inhibitor of metalloproteinase (TIMP-1).

Introduction

In rheumatoid arthritis, proliferating synovial cells penetrate the cartilage in the form of a pannus, and cartilage destruction takes place in the zone of contact between the cells and the cartilage. In response to as yet unknown auto- coids, in addition to the presence of immune complexes in the superficial cartilage$^{1,2}$, the proliferating synovial tissue penetrates and degrades the cartilage. The mechanisms responsible for pannus formation are not fully understood, but there is general agreement as to the role of pannus, growing over the cartilage surface and

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invading the cartilage matrix, in the production of cartilage injury.\textsuperscript{3-6} In established disease, the pannus is commonly regarded as an extension of the synoviocytes of the joint lining layer, expanding under the influence of an immunologically mediated process,\textsuperscript{6} but little is unknown about when pannus first appears and its initial localization.

In a previous study, the authors demonstrated that recombinant human inteleukin-I (IL-I) stimulated monocyte and synovial cell attachment to rheumatoid cartilage in vitro.\textsuperscript{7} In this study, large numbers of monocytes from healthy individuals and cultured synovial cells were observed to attach to the rheumatoid articular surface in the presence of IL-1, suggesting that IL-I generated by adherent monocytes and synovial cells may increase the binding of these cells to cartilage matrix protein. Furthermore, the cells attached to the cartilage surface strongly expressed \( \alpha_4 \) and \( \alpha_5 \) integrins. In another previous study, using immunohistochemical and immunoelectron microscopic techniques, and monoclonal antibodies against the \( \beta_1 \) integrin adhesion molecules, we examined the pattern of distribution of these molecules at the rheumatoid synovial cartilage junction. In this study, we observed that \( \alpha_3 \), \( \alpha_4 \) and \( \alpha_5 \) integrins were the predominant \( \beta_1 \) integrins expressed by rheumatid synovial pannus.\textsuperscript{8} Since these three integrins all function as fibronectin receptors,\textsuperscript{9-11} it was suggested that the fibronectin-rich environment of the rheumatoid cartilage surface trapped pannus cells expressing high level of these molecules.

Synovial cell invasion of cartilage is thought to be a multi-step process involving the adhesion of synovial cells to extracellular cartilage matrix components, proteolytic degradation of the matrix, and invasion through the digested matrix. It has been concluded that, although integrins play an important role in normal cell growth and differentiation, increased expression of these agents on tumor cells has been correlated with aggressive invasive behavior of these cells.\textsuperscript{12-14} These observations indicate that both \( \beta_1 \) integrin-mediated cell migration and protease activity are required for cellular invasion. The expression of metalloproteinases is controlled partly by direct signalling via \( \beta_1 \) integrins.\textsuperscript{15}

Matrix metalloproteinases (MMPs) are a family of enzymes that are secreted in zymogen form by connective tissue cells, inflammatory phagocytes, and a number of varieties of transformed cells.\textsuperscript{15} MMPs play an important role in various physiological changes such as tissue remodeling, morphogenesis and cellular invasion of extracellular tissue.\textsuperscript{2,16} Immunostaining experiments have shown intense staining of collagenase (MMP-1) and stromelysin (MMP-3) at the pannus cartilage junction.\textsuperscript{17,18} These studies have indicated that components of the ternary MMP-1/TIMP-1 (tissue inhibitor of metalloproteinase) /MMP-2 (gelatinase) complex are coexpressed in the extension of synovial lining cells to the hyaline articular cartilage where it leads to tissue destruction.\textsuperscript{18} A recent study has shown that there is a striking decrease in the amount of TIMP-1 secreted by rheumatoid synovial membrane compared with normal synovium.\textsuperscript{19} This has important implications for the pathogenesis of cartilage destruction and erosion in RA and has raised the possibility that the TIMP sys-
tem is overwhelmed by massive amounts of metalloproteinase production in RA.\textsuperscript{20} In the present study, an immunohistochemical investigation using immunoperoxidase staining methods has been carried out to determine more precisely whether $\beta 1$ integrin adhesion molecules expressed on pannus and cartilage, play a role in the proteolytic activation on MMP to enhance cellular invasion into cartilage matrix.

**Materials and Methods**

Thirty-one samples of rheumatoid articular cartilage covered with pannus from 31 patients were obtained during synovectomy or joint replacement surgery. All patients were considered to have moderate to severe active synovitis at the time of surgery. All patients were rheumatoid factor positive and fulfilled the ACR diagnostic criteria for rheumatoid arthritis. Several samples of pannus-cartilage junction from each patient studied were selected to contain active pannus on the basis of naked eye examination, and this was confirmed by light microscopy using haematoxylin and eosin staining. Fibrous pannus was discarded on the basis of absence of cellularity. Each specimen was stained immediately after collection.

Purified anti-human monoclonal antibodies denoted as CDw49d ($\alpha 4\beta 1$, VLA4) and CDw49e ($\alpha 5\beta 1$, VLA-5) were obtained from Immunotech (Marseilles, Cedex, France). These monoclonal antibodies had similar specific avidities for their antigen. Purified anti-human MMP-1,\textsuperscript{21} anti-human MMP3\textsuperscript{22} and anti-human TIMP-1\textsuperscript{23} were purchased from Toyama Pharmaceutical Co (Toyama, Japan). Purified mouse IgG was obtained from Cappel Laboratories (Cochranville, Pa). Avidin biotinylated peroxidase (ABC-kit) was obtained from Vector Laboratories (Burlingame, Calif), and 3-3'-diaminobenzidine was purchased from Sigma Chemical Co (St. Louis, Mo). Frozen sections, about 4-6-µm thick, were cut on a cryostat (Bright, Huntingdon, England) at $-20 \, ^\circ \text{C}$, and mounted on gelatin and egg albumin coated slides. After drying at room temperature, the sections were washed with phosphate-buffered saline (PBS). Normal goat serum, diluted 1:200, was applied to the sections for 20 min. After washing, they were incubated with 100-200 µl of diluted monoclonal antibody for 60 min. After washing with PBS, biotinylated peroxidase conjugated goat anti-mouse IgG antibody (Becton-Dickinson Monoclonal Center, Mountainview, Calif.) was added. The tissue was then incubated with 3mg of 3-3'-diaminobenzidine in 10 ml of Tris HCl buffer, pH 7.5, for 10 min. The specimens were then washed in PBS and dried at room temperature. Sections were stained with haematoxylin for background and nuclear staining of the cells. In each specimen, identifiable immunoreactive cells were scored by an immunohistochemical technique.

**Results**

A variety of cell types stained positively for $\alpha 4\beta 1$ and $\alpha 5\beta 1$ antibodies, including the synovial lining cells, mononuclear cells and endothelial cells of the post-capillary venules (PCV). The percentage of cells stained positively for different molecules at the pannus cartilage junction varied. The reasons for
such variation in the total cells counted were due to difference in specimen size and a wide variation in cellularity. The overall staining patterns of the cells distributed at the pannus cartilage junction for β1 integrins (α4, α5), metalloproteinases (MMP-1, MMP-3) and TIMP-1 is shown in Table 1. Some degree of hyperplasia of the synovial lining cells was observed.

**Beta 1 integrins**

When specimens were treated with anti-α4β1 and anti-α5β1, most of the cells located at the cartilage border showed strong membrane staining with both antibodies (Figure 1). The α5β1 positive cells usually outnumbered the α4β1 positive cells. The percentage of total cells that stained with α5β1 in sections remained fairly constant at 50-75%, regardless of the numbers of various cell types present. There was strong anti-α5β1 staining on chondrocytes at or close to the pannus-cartilage junction. In addition, α4β1 and α5β1 integrin molecules were present in pericellular and also in interterritorial cartilage ma-

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The mean percentage of cells stained are represented as follows: non stained (−); ~25% (+), 26~50% (++); 51~75% (+++)

**Figure 1.** α4β1 and α5β1 staining of the rheumatoid synovial pannus-cartilage junction. Diffuse and weakly positive α4β1(a) and strongly positive α5β1 fibroblastic cells and macrophages are observed at the pannus-cartilage border (b). Original magnification X200 (CA cartilage, PA pannus)
Matrix metalloproteinase distribution in pannus cartilage junction

Staining for MMP-1 and MMP-3 was particularly intense at the pannus-cartilage junction. Similarly, at the synovial-pannus junction, the pannocytes were intensively positive for MMP-1 and MMP-3. These molecules were also strongly stained in the interterritorial matrix of cartilage of the tip of pannus invasion (Figure 3a, b).

Tissue inhibitor-1 of matrix metalloproteinase (TIMP-1)

Although TIMP-1 staining was detectable in lining cells, there was an absence of TIMP-1 staining in the perivascular areas. There was very weak cellular staining and weak staining of extracellular TIMP-1 at the pannus cartilage junction (Figure 3c). In contrast to TIMP-1, MMP-1 and MMP-3 were strongly expressed in the cells of the invasive pannus tissue. The cells of the pannus and the interface of the synovial-cartilage junction were weakly positive or negative.

Matrix MMPs and TIMP-1 distribution in chondrocytes and extracellular cartilage matrix

Chondrocytes beneath the pannus...
were intensely stained for MMP-1 (Figure 4a) and MMP-3. Furthermore, these molecules were found diffusely around chondrocyte lacunae in the form of a “halo”, and chondrocytes surrounded by these “halos” appeared to be necrotic as indicated by the presence of empty lacuna (Figure 4b). These changes were observed to a depth of 200 mm beneath the pannus cartilage border. TIMP-1 staining of chondrocytes beneath the pannus was either very weak or negative (Figure 4c).

**Discussion**

Although the rheumatoid pannus is characterized by increased fibroblast proliferation, the initial triggering factors contributing to pannus formation are still unclear. Synovial cell invasion of articular cartilage is a multi-step process, involving the adhesion of synovial cell to extracellular matrix (ECM) components, proteolytic degradation of the matrix, and invasion through the digested matrix. In a previous study, we have reported that binding of monocytes and synovial cells to cartilage matrix was increased in the presence of IL-1. Synovial cell attachment to cartilage may be the initial step in pannus formation. In another study, we showed that α4β1 and α5β1 integrins are the predominant β1 integrins expressed by rheumatoid synovial pannus. In this study, we suggested that receptor-ligand interaction between α5β1 and cartilage matrix may occur in the early stages of pannus formation. Furthermore, we emphasized that an increase in β1 integrin may be necessary for the growth of the pannus and also for the upregulation of the VLA molecules, leading secondarily to increased attachment.

Most of the ECM integrins include a b1 subunit, which can be combined with any one of the α1-α7 chains. Recently, it has been shown that treatment of glioma cells with an antibody to α5 increased their invasiveness significantly. This suggests that when the synovial tissue comes in contact with an appropriate ECM molecule, there may be an increased expression of integrins on the cell surface that would facilitate the invasion process. In the present study, we have therefore, investigated the morphological character and distribution of cells expressing α4β1, α5β1 molecules as well as MMP-1, MMP-3 and TIMP-1 at the synovial cartilage junction.

![Figure 4. MMP-1, MMP-3 and TIMP-1 staining on chondrocytes. Chondrocytes beneath the pannus was intensely stained for MMP-1 (a), and MMP-3 was diffusely stained in the extracellular cartilage matrix around the chondrocytes like “halo” (c).](image-url)
Adhesion molecules such as the \( \beta_1 \) integrins are critically important for the binding of inflammatory cells to ECM components, such as fibronectin and collagen.\(^9,^{10}\) The present study describes the in situ staining patterns of the \( \alpha_4 \) and \( \alpha_5 \) members of the \( \beta_1 \) integrin family of the cells bordering the interface between the pannus and the cartilage surface. \( \alpha_4\beta_1 \) and \( \alpha_5\beta_1 \) positive cells were present in large numbers in the pannus, the \( \alpha_5\beta_1 \) positive cells usually outnumbering the \( \alpha_4\beta_1 \) positive cells. The increased cartilage-pannus junction staining for \( \alpha_4\beta_1 \) and \( \alpha_5\beta_1 \), associated with binding to cartilage matrix, may result in the activation of the pannus cells. Since these integrins function as fibronectin receptors,\(^59\) it seems likely that the fibronectin-rich environment of the rheumatoid cartilage surface\(^30,^{31}\) may effectively bind pannus cells expressing high levels of these molecules. It has also shown that fibronectin present in the superficial region of cartilage potentiates pannus extension and proteoglycans and immune complexes inhibits pannus extension.\(^{32}\)

The strong expression of \( \alpha_5\beta_1 \) on chondrocytes, as observed in the present study, would suggest that interactions between chondrocytes and fibronectin may occur in the course of pannus formation through the activation of chondrocytes by various cytokines.\(^7,^{33}\) Many of the known ligands for integrins, including collagen, fibronectin, laminin and thrombospondin, are present in articular cartilage. The role of integrins in collagen-chondrocyte interaction is, as yet unclear.\(^{34,35}\) Recent studies suggest that certain chondrocyte-ECM interactions may be mediated by integrins.\(^{29,33,36,37}\)

It is unknown whether the invasive behavior of inflammatory cells may be dependent on the action of proteases, as has been observed in the metastasis of solid tumors.\(^{16}\) Recently, it has been shown that the expression of the fibronectin-degrading protease is down-regulated by crosslinking of \( \alpha_4\beta_1 \) integrin receptors on T lymphocytes.\(^{15}\) It was concluded that the expression of these enzymes is controlled partly by lymphocyte activation signals and by direct signalling via \( \beta_1 \) integrins. The cells that infiltrate the pannus are made up mainly of fibroblasts, macrophages and lymphocytes. All these cells produce and secrete matrix metalloproteinases with cell-specific patterns of the individual MMPs. These appear to be prerequisites for the induction and control of expression of MMPs and their functional roles in the mediation of immunity and inflammation.\(^{38}\)

The molecular mechanisms responsible for the invasiveness and collagenolytic properties of the pannus are not clear. Fibroblast-type collagenase or MMP-1 has long been considered as the key enzyme in the destruction of collagen at the pannus-cartilage junction.\(^{39,40}\)

In the present study, MMP-1 and MMP-3 are strongly expressed on synovial lining cells and cells penetrating cartilage. Furthermore, these components of the pannus were strongly stained in chondrocytes beneath the pannus and in pericellular lacunae. These observations strongly suggest that matrix metalloproteinases such as MMP-1 and MMP-3 digest the cartilage matrix. MMP-1 cleaves interstitial types I, II and III collagen, and MMP-3 exhibits a broader substrate specificity.
hydrolyzing collagens found in basement membrane, as well as glycoproteins (fibronectin and laminin) and proteoglycans.\textsuperscript{38} It has recently been shown that in contrast to MMP-1, MMP-3 is very active against cartilage type II collagen and aggrecans.\textsuperscript{41,42} These investigation concluded that two enzymes, an interstitial collagenase and gelatinase, are required for the complete dissolution of stromal collagen during cellular invasion.

In the present study, TIMP-1 was weakly stained at the cartilage-pannus junction. Synovial lining cells also showed very weak staining. The regulation of MMP activity is complex and closely controlled by TIMPs. Specific tissue inhibitors of TIMP provide fine control by binding to this component in a 1:1 complex with MMPs and inhibiting their action.\textsuperscript{43} The reason for the low levels of TIMP-1 secretion by pannus tissue or RA synovium is unclear. As suggested by Jackson et al.\textsuperscript{44} it is possible that reduced TIMP-1 secretion by rheumatoid synovium is an acquired functional abnormality of infiltrated cells. Also it has been suggested that excessive MMP activity is a major causative factor in joint destruction in RA.\textsuperscript{45,46} It appears likely to us that the increased MMP activity of the RA synovium also contributes to joint destruction by promoting angiogenesis. Normal articular cartilage is avascular and resistant to invasion by new vessel growth. In RA, this barrier is broken and blood vessels grow into the articular cartilage as a result of \( \beta1 \) integrin expression, elevated proteolytic activity of the MMPs and decreased levels of TIMP-1, and in this way contributing to cartilage destruction.

References

β1 integrins and MMPs in RA pannus


