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Parallel changes in fibronectin and $\alpha_5\beta_1$ integrin in articular cartilage in type II collagen-induced arthritis

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Interactions of cells with extracellular matrix (ECM) are mediated through specific cell surface receptors, belonging to the integrin family of transmembrane proteins. Integrins have been shown to be involved in chondrocyte-matrix interactions in the cartilage. In this study, the status of a matrix glycoprotein fibronectin (FN) and its receptor $\alpha_5\beta_1$ integrin in the articular cartilage in collagen type II-induced experimental arthritis in rats, as well as in synovial fluid from osteoarthritic patients was investigated. Experimental arthritis was induced by intradermal injection of type-II collagen (300 μg/100 g body wt) and Freund's complete adjuvant. Saline-treated animals served as control. Clinical severity was indicated by increase in paw volume. Significant increase in the activities of lysosomal enzymes β-glucuronidase and β-hexosaminidase was observed in synovial effusate, serum and cartilage of arthritic animals, when compared to untreated control, indicating dysfunction of cartilage. Changes in FN and $\alpha_5\beta_1$ integrin were studied by ELISA. A progressive increase was observed in the FN level in synovial effusate and cartilage of arthritic animals, when compared to untreated controls. FN levels were also significantly high in synovial fluid of osteoarthritic patients. A significant increase in the levels of $\alpha_5\beta_1$ integrin was found in cartilage of arthritic rats. Parallel changes in FN and $\alpha_5\beta_1$ integrin indicated that alterations in FN and $\alpha_5\beta_1$ integrin in chondrocytes constituted one of the molecular mechanisms during progression of arthritis.

Keywords: Fibronectin, Integrins, Arthritis, Synovial fluid, Cell-matrix interaction

Chondrocyte-matrix interaction is a critical factor in regulation of various biological processes, such as cell adhesion, differentiation, growth and survival, which are important to cartilage homeostasis and repair. These interactions are mediated by cell surface receptors, particularly the heterodimeric transmembrane integrin family of receptors[1]. Several integrin receptors have been found in cartilage cell. Chondrocytes express $\alpha_5\beta_1$, $\alpha_2\beta_1$ and $\alpha_1\beta_1$ integrins, which serve as receptors for collagen type II, IV and VI. Other receptors expressed on chondrocytes include $\alpha_5\beta_3$ integrin, a common receptor for vitronectin and osteopontin, laminin receptor $\alpha_6\beta_1$ integrin and fibronectin (FN) receptor $\alpha_5\beta_1$ integrin[2-4].

Integrins transduce signals that modulate cellular activities and gene expression[5]. Interaction of the cells with extracellular matrix (ECM) ligands like FN mediated by integrins lead to activation of major signaling pathways, such as the jun kinase pathway[6] and ECM-regulated kinase pathway, which is specific for cytokines-like platelet-derived growth factor (PDGF) and epidermal growth factor (EGF)[7,8]. Expression of integrins is regulated by insulin-like growth factor 1 (IGF 1) and is enhanced by transforming growth factor β (TGF β)[9]. Cross-talk between integrin signaling pathways play a role in cartilage homeostasis. Changes in α_2 integrin, the major collagen receptor, in response to cyclic strain in articular chondrocytes from osteoarthritic knee joints suggest a critical role for the integrins in transduction of mechanical stimuli[10-12].

Role of integrins in chondrocyte function has been reported in fetal cartilage and chondrocyte-matrix interaction mediated by β_1 integrin is found to regulate chondrocyte differentiation. Studies have shown that β_1 integrin expression is linked with the severity of lesions in osteoarthritic cartilage and relative distribution of α subunits is altered in cultured chondrocytes[13-15]. Induction of cyclic strain in human

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Abbreviations: ECM, extracellular matrix; EGF, epidermal growth factor; FN, fibronectin; HRP, horseradish peroxidase; IGF 1, insulin-like growth factor1; MMPs, matrix metalloproteinases; PDGF, platelet derived growth factor; TGF, transforming growth factor; TNF-α, tumor necrosis factor-α.

chondrocytes causes alteration in the expression of type-II collagen[16].

FN, a major glycoprotein in the ECM promotes cell adhesion, cell migration and influences a number of cellular activities, which are mediated through its interaction with the integrin receptor $\alpha_5\beta_1$. In other systems, it increases expression of matrix metalloproteinases (MMPs), which play a key role in matrix degradation in osteoarthritis[17-19]. FN fragments have been found in osteoarthritic cartilage and influence the progression of osteoarthritis (OA)[20]. In this study, the status of a matrix glycoprotein FN and its receptor $\alpha_5\beta_1$ integrin has been investigated in the articular cartilage in collagen type II-induced experimental arthritis in rats, as well as in synovial fluid from osteoarthritic patients.

Materials and Methods

Gelatin, Freund's complete adjuvant, type II-collagen, anti-human FN, anti-rabbit IgG conjugated horseradish peroxidase (HRP), *p*-nitrophenyl-N-acetyl-β-D-glucosaminide and *p*-nitrophenyl-β-D-glucuronide were from Sigma Chemical Co., USA. Antibody against $\alpha_5\beta_1$ integrin was from GIBCO BRL Products, USA. Anti-rat FN was raised in rabbit.

Induction of arthritis

The 8-10 weeks old male wistar rats weighing 175-200 g were divided into two groups of 6 animals each. Arthritis was induced in one group by intradermal injection of an emulsion containing type II collagen (300 μg/100 g body wt.) and Freund's complete adjuvant, and the other group of animals treated similarly with normal saline served as control[18]. After 3 weeks, one more injection was given and 1 week after, the animals were sacrificed and serum and cartilage were collected for analysis. The study was approved by the Institutional Ethical Committee.

Collection of synovial effusate and synovial fluid samples

To assess the progress of disease, synovial effusate was collected and analyzed every week. To collect synovial effusate, 200 μl of sterile saline was injected into the femur joints of animals and effusate was aspirated immediately, centrifuged at 13,000 *g* at 4°C for 5 min and the clear supernatant was used for analysis. Synovial fluid from osteoarthritic patients and accident victims were collected from Medical College Hospital and SP Fort Hospital, Thiruvananthapuram respectively. It was centrifuged at 13,000 *g* at 4°C for 5 min and the clear supernatant was used for analysis. Human samples were collected after obtaining informed consent.

Assay of lysosomal enzymes

To assess the progression and severity of disease, lysosomal enzymes such as β-glucuronidase[22] and β-hexosaminidase[23] in synovial effusate, cartilage and serum were assayed. β-Glucuronidase and β hexosaminidase were assayed using *p*-nitrophenyl β-D-glucuronide and *p*-nitrophenyl-N-acetyl-β-D-glucosaminide as substrates respectively and activity was expressed as micromoles of *p*-nitrophenol liberated/min.

Quantitation of FN and integrin by ELISA

Articular cartilage was collected, homogenized in detergent buffer and clear supernatant was used for assay of FN by ELISA. Different concentrations of antigens in PBS (50 μl) were coated in the wells of multi-well plates by incubating for 3 h at room temperature. The wells were washed with PBS and free-binding sites were blocked using 0.2% gelatin in 0.05% PBS-Tween. Primary antibody (1:500) in PBS-Tween was added to the wells and incubated for 2 h at room temperature. After extensive washing with PBS-Tween, the wells were treated with HRP-conjugated secondary antibody for 1 h at room temperature. *o*-Dianisidine reagent was added and absorbance was measured at 400 nm[24].

Statistical analysis

Statistical analyses were done using one-way ANOVA, followed by Duncan's post-hoc test to identify the differences and Levenes '*t*' test using SPSS 10. Differences of $p<0.05$ were considered to be significant.

Results

Experimental induction of arthritis

Development of arthritic condition in experimental animals was assessed both clinically and biochemically. Inflammation of joints was visible within a week after treatment with type II collagen. Clinical severity was assessed by measuring paw volume changes at regular intervals. A significant increase in paw volume was observed after injection in experimental animals, compared to control. This was further evaluated biochemically by determination of the activities of lysosomal hydrolases viz., β-glucuronidase and β-hexosaminidase. A significant

increase in the activities of both the enzymes was observed in synovial effusate, serum and cartilage of arthritic animals, when compared to untreated controls (Fig. 1).

Changes in FN in synovial effusate and cartilage

In order to study whether the development of arthritis was associated with changes in FN, amount of FN was determined in synovial effusate and cartilage using ELISA. Analysis of synovial effusate collected at regular intervals from both arthritic and normal controls showed a progressive increase in the level of FN (increased to about 10-fold over the basal level) in arthritic animals during a period of 1 month after treatment with type II collagen; whereas in normal control, FN level was very low and remained unaffected (Fig. 2A). Results of changes in FN in cartilage during the development of arthritis in experimental animals are shown in Fig. 2B. A significant increase in FN level was observed in cartilage of arthritic animals, when compared to untreated animals.

Changes in FN in synovial fluid of osteoarthritic patients

To study whether similar changes in FN also occurred in osteoarthritis, FN was determined in synovial fluid from osteoarthritic patients using ELISA and the results are shown in Fig. 3. A significant increase in FN level in synovial fluid of osteoarthritic patients was observed, when compared to normal subjects. This was further confirmed by immunoblot analysis using specific antibody against FN (data not shown).

Changes in $\alpha_5\beta_1$ integrin in experimental arthritis

In order to study whether changes in FN level could affect cell-matrix interactions, which are critical in the maintenance of structure and functions of cartilage,

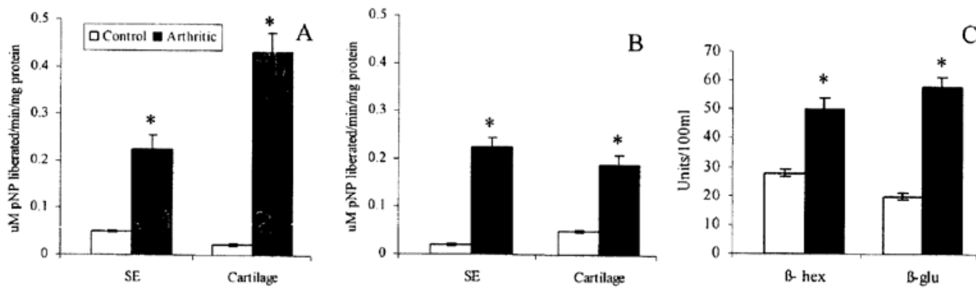

Fig. 1—Changes in the activity of lysosomal enzymes on induction of arthritis [Synovial effusate (SE) and cartilage extract were used for the assay of lysosomal enzymes ß-D-glucuronidase (A) and β-hexosaminidase (B) using *p*-nitrophenyl ß-D-glucuronide and *p*-nitrophenyl N-acetyl-ß-D-hexosaminide as substrates respectively. Activity of these enzymes were also determined in serum (C) and expressed as units/100 ml. One unit of enzyme was equivalent to micromoles of *p*-nitrophenol liberated/min. Saline-treated animals served as control. Values represented the average of five experiments ± SEM* (p<0.05)]

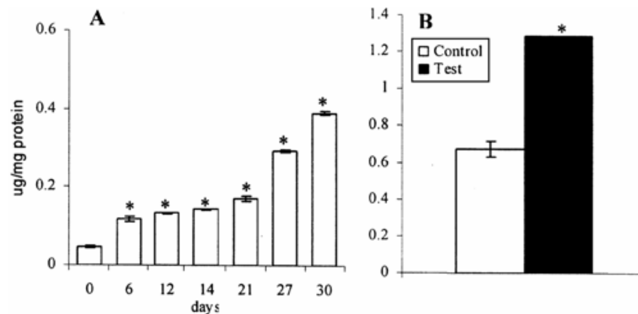

Fig. 2—Changes in FN content in synovial effusate and cartilage on induction of arthritis [Synovial effusate collected at regular intervals (A) and cartilage extract prepared after induction of arthritis (B) were coated on a multi-well ELISA plate as antigens, subjected to ELISA using specific antibody against FN and developed using *o*-dianisidine as substrate. Values given as µg/mg protein were average of five experiments ± SEM* (p<0.05)]

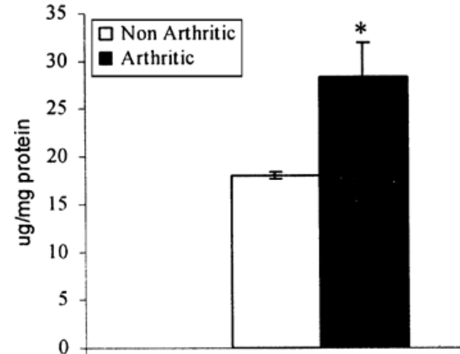

Fig. 3—Changes in FN content in synovial fluid [Synovial fluid of arthritic patients (n = 7) and non-arthritic subjects (n = 3) were coated on a multi-well ELISA plate as antigens, subjected to ELISA using specific antibody against FN and developed using *o*-dianisidine as substrate. Values given as µg/mg protein were mean ± SEM* (p<0.05)]

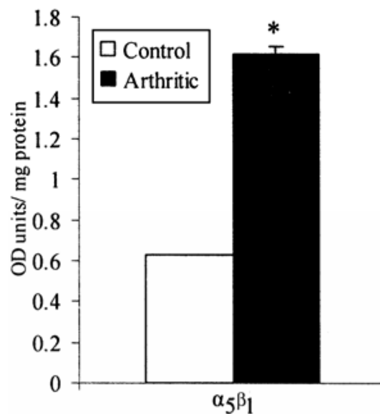

Fig. 4—Changes in $\alpha_5\beta_1$ integrin in cartilage on induction of arthritis [Cartilage extract equivalent to same amount of tissue was coated on a multi-well ELISA plate as antigens, subjected to ELISA using specific antibody against $\alpha_5\beta_1$integrin and developed using *o*-dianisidine as substrate. Values given as OD units/mg protein were average of five experiments ± SEM* ($p<0.05$)]

the level of $\alpha_5\beta_1$ integrin, the cell surface receptor for FN was investigated. $\alpha_5\beta_1$ integrin level in articular cartilage of experimentally-induced arthritic rats was determined by ELISA and the results are shown in Fig. 4. A significant increase in the level of $\alpha_5\beta_1$ integrin was observed in cartilage extract of arthritic animals, compared to untreated control.

Discussion

Interaction of chondrocytes with the matrix is important in maintenance of cartilage structure and function. Alterations in these interactions can be caused by changes in the activity of matrix-degrading enzymes, such as matrix metalloproteinases (MMPs), leading to alterations in cartilage structure and functions in pathological conditions like arthritis[25]. Besides, alteration in molecular mechanisms involved in mediating cell-matrix interactions can also affect interaction of cells with the ECM in cartilage. Results presented above on the changes in FN and its receptor $\alpha_5\beta_1$ integrin in the cartilage of experimentally-induced arthritis indicate that apart from matrix-degrading enzymes, alteration in cell surface receptors for matrix protein occurs in the pathogenesis of arthritis.

FN, a cell adhesion protein is also present as a soluble protein in body fluids. Interactions between chondrocytes and ECM help regulate biological processes, important to cartilage homeostasis and repair, including cell attachment, differentiation, growth and survival[26]. Integrin family of cell surface receptors plays an important role in mediating chondrocyte-matrix interaction[4]. Several integrin receptors for matrix proteins like collagen IV, laminin, vitronectin and FN including $\alpha_2\beta_1$, $\alpha_6\beta_1$, and $\alpha_5\beta_1$ have been reported to be expressed on the chondrocytes[1,27-30].

Our results show an increase in the amount of FN in arthritic group, compared to normal control. Changes in the FN level in arthritic cartilage indicate the possible alteration in cell-matrix interaction during progression of disease. FN fragments have been reported in the synovial fluid of osteoarthritic patients[31]. But, immuno blot analysis has not shown any significant amount of FN fragments in our samples. The present study also shows a parallel change in $\alpha_5\beta_1$ integrin and FN in arthritis. Parallel changes in collagen and its integrin receptor have been reported in conditions of cyclic strain in cartilage[12]. Our results also indicate a similar parallel change in FN and its receptor in articular cartilage during development of arthritis, suggesting that alterations in cell surface receptors for matrix protein, particularly integrin, is one of the key mechanisms involved in the alteration in cell-matrix interactions leading to arthritis.

Increase in FN level in type II collagen-induced experimental arthritis and its increase in synovial fluid of osteoarthritic patients suggest that change in FN is associated with cartilage dysfunction and may not be a general inflammatory response. Further evidence for cartilage dysfunction is provided by increase in the activity of lysosomal enzymes like β-glucuronidase and β-hexosaminidase in both synovial effusate and articular cartilage of arthritic animals. These hydrolytic enzymes are involved in the lysosomal degradation of glycosaminoglycans and other macromolecules of cartilage matrix[32]. Matrix-specific proteases such as MMPs whose activity is also increased in arthritic condition (data not shown) can contribute to degradation of cartilage matrix.

Alterations in FN and its receptor $\alpha_5\beta_1$ integrin in cartilage may have serious consequences to cellular activities in cartilage. FN-dependent upregulation of MMPs, particularly MMP-2 and MMP-9 have been reported in monocytes[33], hepatic stellate cells[34] and a number of other cells in culture[35]. MMPs appear to play a key role in the pathogenesis of arthritis and cause degradation of cell surface and ECM proteins[36]. Increase in their activity in synovial fluid of osteoarthritic patients and in experimental arthritis has been reported[37]. The increase in FN and its receptor

$\alpha_5\beta_1$ integrin in arthritis is particularly important, in the context of modulation of MMP production in different cell types by FN through $\alpha_5\beta_1$ integrin-dependent pathways[38]. It is likely that alteration in FN-chondrocyte interactions may be one of the factors contributing to the upregulation of MMPs, resulting in degradation of ECM during arthritis.

In conclusion, our results indicate a parallel change in expression of FN and $\alpha_5\beta_1$ integrin, suggesting that alterations in the cell surface receptor, particularly integrin receptor for FN constitute one of the mechanisms for alteration in cell-matrix interaction during progression of arthritis.

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