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The Modulation of Integrin Expression by the Extracellular Matrix in Articular Chondrocytes

Sung Jae Kim[1], Eun Jung Kim[1], Yun Hee Kim[2], Soo Bong Hahn[2], and Jin Woo Lee[2]

[1]BK21 Project for Medical Science, [2]Department of Orthopaedic Surgery, Yonsei University College of Medicine, Seoul, Korea.

Normal articular cartilage is composed of chondrocytes embedded within an extracellular matrix (ECM). The patterns of integrin expression determine the adhesive properties of cells by modulating interactions with specific ECMs. Our hypothesis is that chondrocyte integrin expression changes in response to changes in their microenvironment. Porcine articular chondrocytes were encapsulated in alginate beads with several ECMs (collagen type I, collagen type II and fibronectin) for 7 days, subjected to RT-PCR, western blot analysis and immunofluorescence staining. It was found that chondrocytes in different ECMs showed different patterns of integrin expression. Integrin $\alpha 5$ and $\beta 1$ were strongly expressed in all groups, but integrin $\alpha 1$ was strongly expressed only in collagen type I and fibronectin conjugated alginate beads, and integrin $\alpha 2$ was strongly expressed only in collagen type II conjugated alginate beads. These findings suggest that the addition of different ECMs to chondrocytes can modulate the patterns and levels of integrin expression possibly through a feedback mechanism. These finding suggest that the modulation of ECM interactions may play a critical role in the pathogenesis of osteoarthritis.

Key Words: Articular chondrocyte, integrin, extracellular matrix, alginate culture, monolayer culture

INTRODUCTION

Normal articular cartilage is composed of chondrocytes, which are embedded within an extracellular matrix that consists primarily of collagen type II, aggrecan, and hyaluronic acid. In addition, the following proteins are present to a much lesser extent; collagen types VI, IX and XI, cartilage oligomeric matrix protein (COMP), thrombospondin, fibromodulin, decorin, and biglycan.[1,2] The unique expressions and organization of these macromolecules within the articular cartilage endows the tissue with exceptional compressive strength, resilience, and mechanical durability. The stability of the chondrocyte phenotype is determined by cell shape and cytoskeletal architecture, and the extracellular matrix (ECM), in turn, plays a key role in controlling these cellular characteristics. Culture conditions that allow chondrocytes to form a peri-cellular matrix promote the expression of cartilage-specific gene products, and thereby support the cartilage phenotype. However, culture conditions that induce cell spreading and the formation of focal contacts and stress fibers result in the loss of peri-cellular matrix, rearrangement of the actin cytoskeleton, and alteration of the collagen expression pattern. The mechanism by which the ECM controls chondrocyte phenotypic expression is unclear. Specific studies that have attempted to define the regulatory role of the interaction between chondrocyte and the matrix are few. Ramdi, et al.[3] examined the influence of collagen type I, collagen type IV and fibronectin on alginate-embedded rabbit chondrocytes. When cultivated in beads, entrapped cells maintained their differentiated phenotype over time; and the rates of proteoglycan quantity were similar to those of primary chondrocytes. Beekman, et al.[4] found that via an integrin-mediated mechanism, extracellular collagen down-regulated collagen synthesis in a negative-feedback manner. The integrin family of cell surface receptors appears to play a major role in the mediation of the cell-ECM interactions asso-

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Reprint address: requests to Dr. Jin Woo Lee, Department of Orthopaedic Surgery, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, Korea. Tel: 82-2-361-5640, Fax: 82-2-363-1139, E-mail: ljwos@yumc.yonsei.ac.kr

ciated with structural and functional changes in surrounding tissues.[5] The integrins are heterodimeric glycoproteins that are composed of an α and a β subunit, each of which has extracellular and cytoplasmic domains. These extracellular domains bind to a number of ECM proteins, including collagen types II and VI, fibronectin, and matrix Gla protein. Patterns of integrin expression determine the adhesive properties of cells by modulating their interactions with specific ECM proteins. In studies of the integrin-mediated attachment of chondrocytes to ECM proteins, it has been observed that the adhesiveness of chondrocytes increases when the cells are maintained in monolayer culture prior to use in attachment assays.[6] These results suggest that chondrocyte-ECM adhesive interactions can be regulated by changes in integrin expression or function. Several recent studies have provided evidence that articular chondrocytes express integrins.[7-12] Salter, et al.[11] used immunohistochemical staining in normal adult human articular cartilage, and noted that the integrin $\alpha5\beta1$ was the most prominently expressed chondrocyte integrin. In contrast, the expressions of the integrin $\alpha1$ and $\beta3$ subunits were weak and variable. A more recent study demonstrated that the chondrocyte expressions of integrin $\alpha1\beta1$, $\alpha5\beta1$, and $\alpha v\beta5$ were accompanied by the weak expressions of integrin $\alpha3\beta1$ and $\alpha v\beta3$.[10] Although the amount of information regarding the role of the integrins as mediators of chondrocyte regulation has increased, little is known about the nature of the communication between chondrocytes and the extracellular matrix. In this study, we hypothesized that chondrocyte integrin expression changes in response to their microenvironment. To test this hypothesis, we examined integrin expressions, known to involve chondrocyte-ECM interactions under different culture conditions with or without several ECM.

MATERIALS AND METHODS

Materials

Integrin $\alpha1$, $\alpha2$, $\alpha5$ and $\beta1$ antibodies were purchased from Chemicon (Chemicon, Temecula, CA, USA). Tissue culture reagents, including Dulbecco's modified Eagle medium (DMEM), penicillin-streptomycin, and HEPES, were obtained from GIBCO (GIBCO BRL, Grand Island, NY, USA). Collagenase type II and hyaluronidase were purchased from Worthington (Worthington Biochemical Corporation, Lakewood, NJ, USA). Collagen type II was purchased from Chondrex (Chondrex, Seattle, WA, USA) and fibronectin from GIBCO. Low viscosity alginate, collagen type I and other general chemical reagents were purchased from Sigma (Sigma, St. Louis, MO, USA).

Cell culture

Articular cartilage was obtained from the knee joints of pigs slaughtered at a local abattoir. Cartilages were shaved from the articular surface under sterile conditions, and finely minced and washed several times in phosphate-buffered saline (PBS). The tissue was then incubated at 37℃ for 4 h in DMEM-high glucose (DMEM-HG) containing 0.1% collagenase and 0.065% hyaluronidase. The digested material was filtered through nylon filters (100μm pore size), and the resulting cell suspension centrifuged at 13000 rpm for 10 min. The pellet obtained was washed several times by re-suspending it in DMEM-HG containing 10% fetal bovine serum (FBS) and centrifugation. Chondrocytes were encapsulated in alginate beads immediately after isolation, according to the method of Hauselmann, et al.[13] Briefly, 2×10^6 cells/ml were suspended in three 4 mg/ml ECMs (collagen type I, collagen type II and fibronectin) and encapsulated in 1.2% (w/v) alginate. The cell suspension was passed dropwise through a 19-gauge needle into a 6-well plate containing 102 mM $CaCl_2$ solution (80 beads per flask, with each bead containing an average number of 2.5×10^4 cells). After being polymerized for 5 min, the beads were washed three times with normal saline, once with DMEM-HG and finally they were cultured in DMEM-HG containing 10% FBS for 7 days.

Semi-quantitative reverse transcription-polymerase chain reaction

To evaluate changes in integrin expression,

semi-quantitative reverse-transcription polymerase chain reactions (RT-PCR) were performed, as previously described.[14] Total RNA was isolated from chondrocytes using a RNeasy kit (Qiagen, Valencia, CA, USA), and quantitated by spectrophotometry. The primer sets used for the integrins and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were derived from sequences from GeneBank, and are detailed in Table 1. Total RNA was reverse-transcribed using an Omniscript kit (Qiagen). Aliquots (2 μl) of the resulting cDNA were amplified in a total volume of 50 μl containing PCR buffer, 0.2 M d-nucleoside triphosphate mixture, 1.5 mM $MgCl_2$, 0.5 M of each primer, and 1 U of Taq DNA polymerase (Qiagen). Each of the PCR products was analyzed by separating the amplicon in a 1.5% agarose gel at 60 V/cm in Tris-Borate-EDTA buffer, and this was followed by staining with ethidium bromide. The photographs obtained were quantitated using a densitometer.

Western blot analysis

Integrin expressions under different ECM conditions were confirmed at the protein level by Western blot. Cell extracts were prepared by adding lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 10% glycerol and a cocktail of protease inhibitors (Sigma). Lysates were clarified by centrifugation at 12,000 g for 10 min at 4℃ and the protein contents of supernatants were determined using a modified Bradford assay. Diluted 50 μg protein samples were loaded onto a 4% stacking gel and a 6% polyacrylamide mini-gel and electrophoresed in SDS running buffer at 60-80 V for approximately 2-3 h. The proteins were electrophoretically transferred onto a Hybond-P membrane (Amersham Pharmacia, Piscataway, NJ, USA) in a 1 × Transfer buffer at 50 mA overnight. Membranes were blocked with 5% skimmed milk and TBS (50 mM Tris HCl, 150 mM NaCl) containing 0.1% Tween (TBST) at room temperature for 1 h. Primary antibodies (integrin α1, α2, α5, and β1) were diluted 1/3000 in TBST and incubated with the membranes overnight. After washing three times with TBST, the membranes were incubated for 1 h with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Pharmacia). After washing the membranes three times in TBST, signals were visualized using ECL detection reagents (Amersham Pharmacia) for 5 minutes and

Table 1. List of Integrin Primers

Primer	Sequences	Length	Size (bp)	cycle
Bovine	5′ CAC TCA AAT CCA GCC ACA GCA GC 3′	23	464	26
Integrin β1	3′ CAA CCA CCT TAC ACT GTG CCG AC 5′	23		
Human	5′ GTT CCC AGT GAG TGA GGC CCG AGT A 3′	25	419	34
Integrin β3	3′ GCG ATT GAC TGG TCC ACT GGG CGA A 5′	25		
Human	5′ GCT TAT TGG TTC GTT AGT TGG C 3′	22	461	30
Integrin α1	3′ TTT TGC ACT GGG TAC TCA AGT TGG A 5′	25		
Bovine	5′ TCA GAA GTC TGT TAC CTG CAA TGT G 3′	25	361	34
Integrin α2	3′ TAG GTG TAG GGA GTT ATG TGG TTT C 5′	25		
Bovine	5′ ACC GGC TGC AAA GAC GGA TGT TCC T 3′	25	294	23
Integrin α5	3′ TCA CCT AGT TCC GTC TTC CGT CGG T 5′	25		
Human	5′ TTG GAG CAT CTG TGA GGT CGA AAC 3′	24	395	30
Integrin αV	3′ CAC CGA CAG CCT CTA AAG TTA CCA 5′	24		
Human	5′ ACC ACA GTC CAT GCC ATC AC 3′	20	450	24
GAPDH	3′ ATG TCG TTG TCC CAC CAC CT 5′	20		

exposed to radiographic film for 1 to 10 minutes.

Immunofluorescence staining

Cultured beads were fixated in p-formaldehyde and embedded in paraffin. After deparaffinization, the sections (5μm) were fixed in cold methanol for 15 min, and rinsed in PBS. Sections were then incubated with the primary antibodies (integrin α1, α2, α5, and β1), diluted 1/100 for 5 h at room temperature. Sections were incubated with the goat-anti-rabbit immunoglobulin conjugated with FITC (Santa Cruz, Santa Cruz, CA, USA) (diluted 1 : 80) and PI (Santa Cruz) (diluted 1 : 500) for 1 h at room temperature. Finally, the sections were rinsed, air-dried and examined under a confocal microscope.

Statistical analysis

Sample quantities are expressed as means and standard deviations for three experiments, each performed in triplicate. Statistical significance was determined by multiple comparison tests, and accepted when $p < 0.05$.

RESULTS

Expression of the integrin α1, α2, α5, αv, β1 and β3 subunits by RT-PCR

It was found that the addition of ECM to chondrocytes changed their pattern of integrin expression. By RT-PCR, all samples were founded to express the integrin α1, α5, αv and β1 subunit mRNAs, but not to express the integrin α2 and β3 subunits. In fresh culture, the integrins were more strongly expressed than integrin expression in monolayer culture. When chondrocytes were cultured in alginate beads, the pattern of integrin expression was maintained with time. However, the magnitudes of the integrin expressions were altered, as the integrin α5, αv and β1 subunits were more strongly expressed, but the α1 subunit was found to be expressed very weakly under all conditions. Integrin α1 was shown in alginate bead culture at day 4, but disappeared at day 7 (Fig. 1). Experiments were repeated three times and results were expressed as mean values ± standard deviation.

Western blot analysis of the integrin α1, α2, α5 and β1 subunits in porcine articular chondrocytes

By western blot analysis, the integrin α5 and α1 subunits expressed strongly and showed minimal change with time under all culture conditions. Integrin α1 was expressed to a lesser extent in fresh and monolayer cultures compared to alginate bead culture regardless of time. Integrin α1 was strongly expressed in alginate bead culture compared with monolayer culture, regardless of the presence of ECM, and integrin α2 expression increased with time in collagen type II conjugated alginate beads, while the levels of control proteins for GAPDH and other integrins did not. Integrin α2 expression decreased with time in collagen type I and fibronectin conjugated alginate beads and in monolayer culture (Fig. 2). Experiments were performed in triplicate and results are expressed as mean values ± standard deviation.

Immunofluorescence localization of integrin α1, α2, α5 and β1 subunits in porcine articular chondrocytes

To confirm the presence of integrins, paraffin sectioned alginate beads were stained with integrin α1, α2, α5 and β1 antibodies. Sections were visualized under the confocal microscope; FITC-labeled cytoplasm appeared green and PI-labeled nuclei as red images (Fig. 3). Integrin α5 and β1 antibodies stained strongly in all alginate bead sections, regardless of time. No sample stained positively for integrin α1, except collagen type I and fibronectin conjugated alginate bead sections. Integrin α2 was also absent in alginate bead sections, except collagen type II conjugated alginate bead sections. FITC-labeled integrin α2 expression was detected with time in collagen type II conjugated alginate bead sections.

DISCUSSION

The coupling of chondrocytes with their microenvironment is necessary to give the cells ability

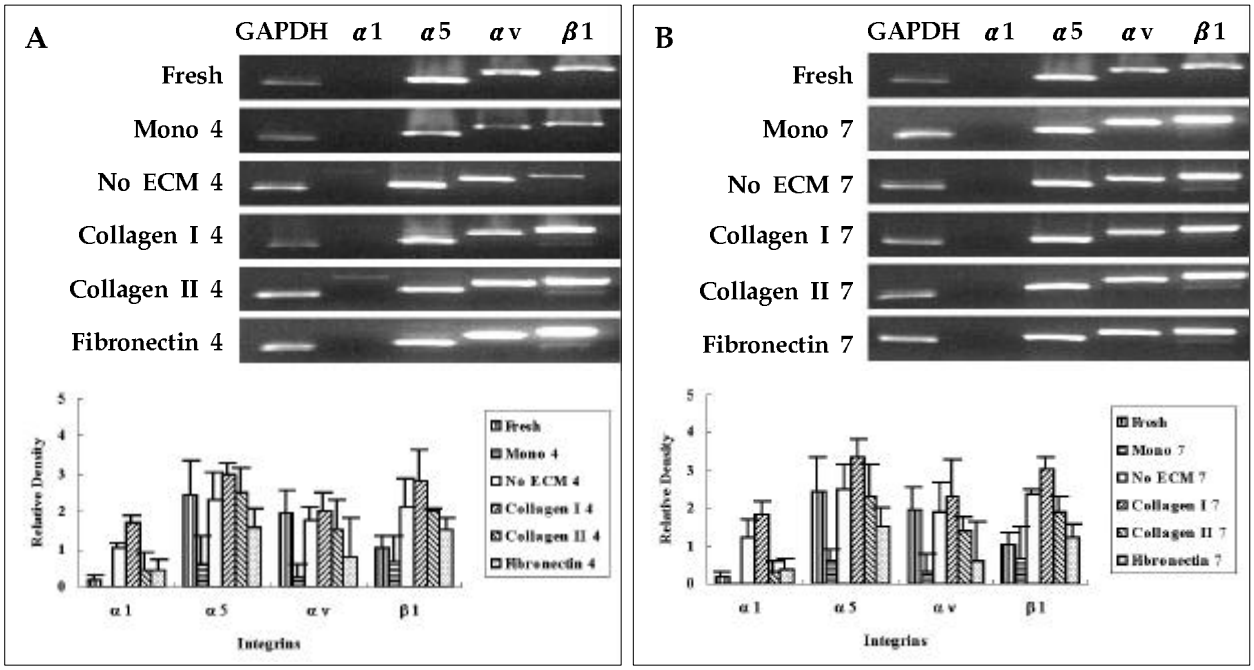

Fig. 1. Results of semi-quantitative RT-PCR of integrin expression in porcine articular chondrocytes. (A) With use of the primers identified in Table 1, RT-PCR analysis of RNA from freshly isolated chondrocytes and chondrocytes cultured in different conditions was performed. Culture conditions were: freshly isolated chondrocytes (Fresh), monolayer cultured chondrocytes for 4 days (Mono 4), chondrocytes cultured in alginate beads without extracellular matrices for 4 days (No ECM 4), and chondrocytes cultured in alginate beads in the presence of type-I collagen (Collagen I 4), type II collagen (Collagen II 4) and fibronectin (Fibronectin 4) for 4 days. Densitometric analysis of integrin expression in triplicate. Results are mean ± standard deviation. (B) With use of the primers identified in Table 1, RT-PCR analysis of RNA from freshly isolated chondrocytes and chondrocytes cultured in different conditions was performed. Culture conditions were: freshly isolated chondrocytes (Fresh), Monolayer cultured chondrocytes for 7 days (Mono 7), chondrocytes cultured in alginate beads without extracellular matrices for 7 days (No ECM 7), and chondrocytes cultured in alginate beads in the presence of type-I collagen (Collagen I 7), type II collagen (Collagen II 7) and fibronectin (Fibronectin 7) for 7 days. Densitometric analysis of integrin expression in triplicate. Results are mean ± standard deviation.

to respond to external changes and to maintain the tissue composition and the mechanical properties of the articular cartilage. Chondrocytes receive information from the external environment via three potential sources: mechanical signals, soluble mediators, and interactions with the ECM.

Chondrocyte attachment to the ECM appears to be mediated by integrins, and the binding of ECM ligands to integrins can induce cellular responses. After binding to the ECM, integrins bind to cytoskeletal elements and promote cytoskeletal reorganization. Moreover, cytoskeletal alterations in response to a specific integrin-ligand combination may result in increased or decreased gene expression.[7] To elucidate the nature of the interaction between the ECM and the integrins, we investigated the integrin expressions of chondrocytes after they had been cultured under the following conditions: freshly isolated, and monolayer, alginate gel including collagen type I, collagen type II or fibronectin cultured. By RT-PCR, porcine articular chondrocytes were found to express integrin $\alpha 1$, $\alpha 5$, αv and $\beta 1$ subunits but integrin $\alpha 2$ was not detected. There are two explanations for this. One is that the sequence of porcine integrin $\alpha 2$ differs from the bovine or human sequence, because we used sequence of bovine or human integrin. The other is that porcine chondrocytes do not express integrin $\alpha 2$. Since we could detect integrin $\alpha 2$ expression by western blot analysis, we could reason that integrin $\alpha 2$ was not detected due to difference of species-specific sequence. By western blot analysis and immunofluorescence staining, integrin $\alpha 1$ was stained strongly in collagen type I and fibronectin conjugated alginate

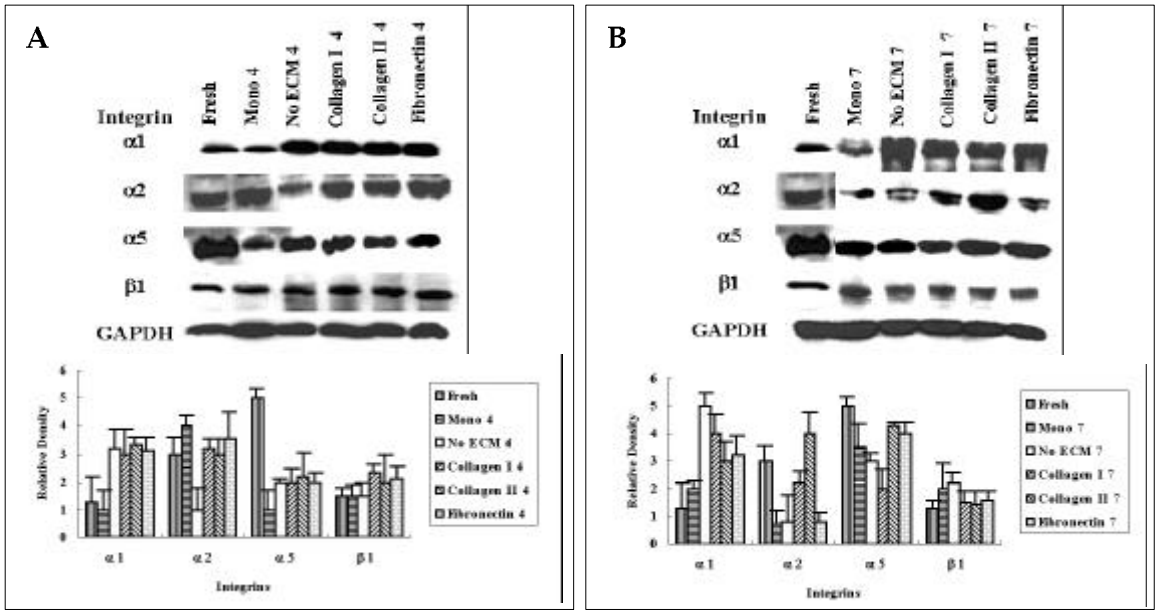

Fig. 2. Western blot analysis of the integrin $\alpha 1$, $\alpha 2$, $\alpha 5$ and $\beta 1$ subunits in porcine chondrocytes cultured under different conditions. (A) Western blot analysis of protein from freshly isolated chondrocytes and chondrocytes cultured in different conditions was performed. Culture conditions were: freshly isolated chondrocytes (Fresh), monolayer cultured chondrocytes for 4 days (Mono 4), chondrocytes cultured in alginate beads without extracellular matrices for 4 days (No ECM 4), and chondrocytes cultured in alginate beads in the presence of type-I collagen (Collagen I 4), type II collagen (Collagen II 4) and fibronectin (Fibronectin 4) for 4 days. Densitometric analysis of porcine chondrocytes integrin subunits cultured in different conditions. Results are mean ± standard deviation. (B) Western blot analysis of protein from freshly isolated chondrocytes and chondrocytes cultured in different conditions was performed. Culture conditions were: freshly isolated chondrocytes (Fresh), monolayer cultured chondrocytes for 7 days (Mono 7), chondrocytes cultured in alginate beads without extracellular matrices for 7 days (No ECM 7), and chondrocytes cultured in alginate beads in the presence of type-I collagen (Collagen I 7), type II collagen (Collagen II 7) and fibronectin (Fibronectin 7) for 7 days. Densitometric analysis of porcine chondrocytes integrin subunits cultured in different conditions. Results are mean ± standard deviation.

bead sections, and integrin $\alpha 2$ was stained in collagen type II conjugated alginate bead sections. Scully, et al.[15] noted that the presence of collagen type II in the alginate bead cultures diminishing integrin $\alpha 2$ mRNA expression using RT-PCR in bovine articular cartilage, and altered the cellular distribution pattern of the integrin $\beta 1$ receptors. In our study, we could not detect the $\alpha 2$ mRNA expression, but it was increased in the protein level in collagen type II conjugated alginate bead. Loeser, et al.[16] found that adult human articular chondrocytes expressed integrin $\alpha 1\beta 1$ but not integrin $\alpha 2\beta 1$, while human fetal articular chondrocytes, human chondrosarcoma cell lines, and bovine calf chondrocytes expressed integrin $\alpha 2\beta 1$ and little or no integrin $\alpha 1\beta 1$. So, this discrepancy may depend on differences of species, antibody specificities, or the detection techniques used.

It is likely that integrin $\alpha 2\beta 1$ has an important function in signal mediation between the chondrocytes and the cartilage matrix. Integrin $\alpha 2\beta 1$ is a more dynamic integrin, which is upregulated during changes in cell-matrix interactions, such as, during matrix turnover, remodeling, or mechanical stress.[17-21] It has also been shown that the integrin $\alpha 2\beta 1$ subunit is upregulated in fibroblasts in contracting collagen gels,[22] that is during the reorganization of the collagen matrix. This supports the idea that integrin $\alpha 2\beta 1$ can respond to changes in the extracellular matrix. The activation of integrin $\alpha 2\beta 1$ expression could be a repair response, and the newly expressed integrin $\alpha 2\beta 1$ could recognize the formation of a new peri-cellular matrix around isolated chondrocytes, rather than mediating chondrocyte-collagen interactions in the tissue.[23] In this particular study, integrin $\alpha 5$ and $\beta 1$ subunits were found to be strongly expressed and to show minimal change with time under all culture conditions. The presence of the integrin $\alpha 5$ and $\beta 1$ subunits of

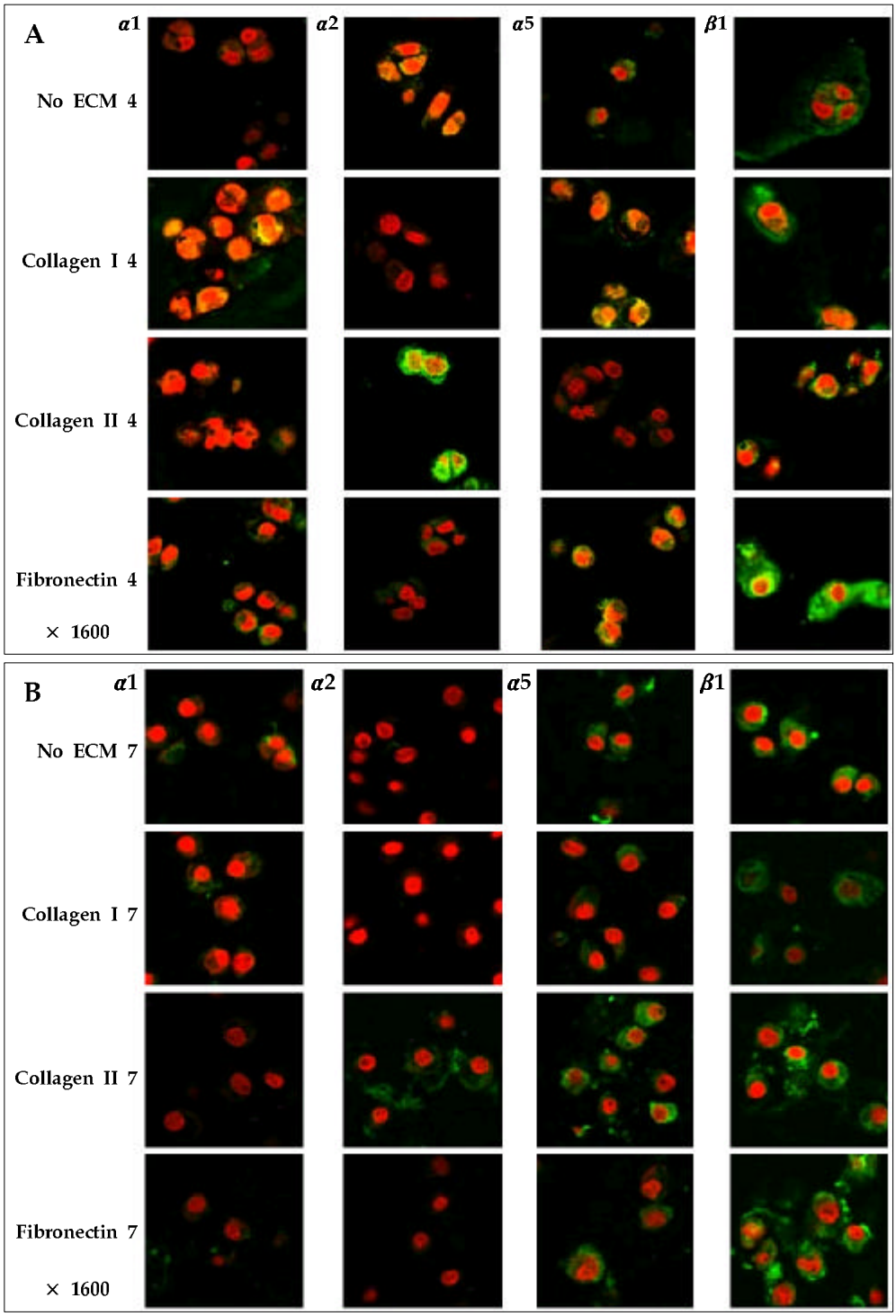

Fig. 3. Immunofluorescence-microscopic demonstration of the integrin $\alpha 1$, $\alpha 2$, $\alpha 5$ and $\beta 1$ subunits in alginate bead culture. These images are the result of the simultaneous acquisition of FITC-labeled cytoplasm (green) and the PI-labeled nuclei (red) acquired using a confocal microscope (× 1600). (A) Culture conditions were: chondrocytes cultured in alginate beads without extracellular matrices for 4 days (No ECM 4), and chondrocytes cultured in alginate beads in the presence of type-I collagen (Collagen I 4), type II collagen (Collagen II 4) and fibronectin (Fibronectin 4) for 4 days. (B) Culture conditions were: chondrocytes cultured in alginate beads without extracellular matrices for 7 days (No ECM 7), and chondrocytes cultured in alginate beads in the presence of type-I collagen (Collagen I 7), type II collagen (Collagen II 7) and fibronectin (Fibronectin 7) for 7 days.

articular chondrocytes suggests that interactions between chondrocytes and fibronectin might be important *in vitro*. Fibronectin has a known number of effects on cultured chondrocytes, namely, they stimulate attachment and spreading, and alter the morphology and inhibit the synthesis of collagen types I and II. Durr, et al.[24] reported that bovine articular cartilage chondrocytes adhere to fibronectin-coated plastic with a higher avidity than they do to albumin-coated plates. In addition, this attachment was found to be inhibited by competition with an RGD-containing peptide, suggesting the need for an RGD sequence binding interaction, which characterizes the binding of integrin $\alpha5\beta1$ to fibronectin. These findings are corroborated by the report of Iwamoto, et al.[19] which found that chondrocytes initially attach to either collagen type I or collagen type II independently of integrin $\alpha5\beta1$, but after 2-3 days in monolayer culture, the attachment was found to be dependent on $\alpha5\beta1$. Accordingly, these findings speculated that initial adhesion occurred through annexin V or some other adhesion molecules, and with increasing time and dedifferentiation in culture became dependent on fibronectin-binding integrin. In other words, this type of adhesion may be mediated by a matrix-to-matrix attachment (collagen to fibronectin) by endogenously produced fibronectin rather than by an interaction between receptors and the matrix. Homandberg, et al.[25] claimed that fibronectin fragments (Fn-fs) enhance levels of catabolic cytokines as in osteoarthritis, and thus, potentially are earlier damage mediators than catabolic cytokines. Fn-fs up-regulate matrix metalloproteinase (MMP) expression, significantly enhance degradation and the loss of proteoglycan (PG) from cartilage and temporarily suppress PG synthesis, which are all events observed in osteoarthritis.

The current study represents a first attempt to examine the interaction between the ECM and the integrins of porcine chondrocytes. In this study, we found that integrins $\alpha1$ and $\alpha2$ were the most sensitively involved receptors in chondrocyte-ECM interactions *in vitro*. In addition, we reasoned that integrin expression could be influenced by the disease state, the age of cartilage, as well as by culture conditions. The further elucidation of these local regulatory mechanisms may contribute to a better understanding of the pathogenesis of articular cartilage diseases. Moreover, a comprehensive understanding of chondrocyte regulation may enable the manipulation of the chondrocyte reparative function, perhaps through both soluble and matrix components, to promote intrinsic repair of diseased articular cartilage.

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