Differential effects of IGF-binding proteins, IGFBP-3 and IGFBP-5, on IGF-I action and binding to cell membranes of immortalized human chondrocytes

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Abstract

Insulin-like growth factor-I (IGF-I) is an important anabolic factor for cartilage tissue and its action is, in part, regulated by IGF-binding proteins (IGFBPs). The object of this study was to investigate the effects of IGFBPs on IGF-I action and on binding of IGF-I to cells using a reproducible immortalized human chondrocyte culture model. Treatment of the C-28/I2 cells with IGF-I or des(1-3)IGF-I in serum-free medium stimulated cell proliferation in a dose-dependent manner. However, the effect of des(1-3)IGF-I was more potent, thereby suggesting that endogenously produced IGFBPs inhibited IGF action. The stimulatory effect of IGF-I was inhibited significantly by addition of IGFBP-3 but enhanced slightly by IGFBP-5. However, neither IGFBP-3 nor IGFBP-

5 had an effect on basal cell growth. Binding of ¹²⁵I-labeled IGF-I to the cells was displaced by both IGFBP-3 and IGFBP-5, although higher concentrations of unlabeled IGFBP-5 were required to displace IGF-I to the same extent as IGFBP-3. Treatment of the cells with IGF-I increased the levels of IGFBP-5 protein measured by Western ligand blotting, and stimulated a corresponding increase in IGFBP-5 mRNA while increasing type II collagen mRNA. Our findings indicate that the balance between IGFBP-3 and IGFBP-5 influences IGF receptor binding and its action on chondrocyte proliferation, and may thereby modulate cartilage metabolism.

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Introduction

The insulin-like growth factor (IGF)-I is a major growth and differentiation factor for cartilage tissue (McQuillan *et al.* 1986, Ohlsson *et al.* 1992, Seong *et al.* 1994) and its influence on chondrocyte functions is regulated, in part, by IGF-binding proteins (IGFBPs) (Lamson *et al.* 1991, Jones & Clemmons 1995). At present, six IGFBPs (IGFBPs-1 to -6) have been isolated and their cDNAs have been cloned (Shimasaki & Ling 1991). IGFBPs have been detected in culture media of cultured chondrocytes from various species (Froger-Gaillard *et al.* 1989, Olney *et al.* 1993, Sunic *et al.* 1995) and in intact bovine cartilage (Morales 1997).

We reported previously that the predominant IGFBPs produced by rat articular chondrocytes were IGFBPs-2, -3, -4 and -5, and that the concentration of IGFBP-5 in conditioned medium was increased by IGF-I in a dose-dependent manner by a transcriptional mechanism via the

type 1 IGF receptor (Matsumoto *et al.* 1996a). We also detected the presence of an IGFBP-5 protease (Matsumoto *et al.* 1996b), suggesting post-translational modification as an additional mechanism of IGFBP regulation. Although the physiological roles of these IGFBPs in cartilage metabolism remain to be clarified, various studies have begun to address how IGFBPs may exert differential effects on the systemic and local activities of IGF-I. In osteoarthritic cartilage, the normal anabolic function of IGF-I may be disrupted. Chondrocytes from animals with experimental arthritis and from patients with osteoarthritis are nonresponsive to IGF-I, although they express increased levels of IGF-I, IGF-I receptor, and IGFBPs-2, -3 and -5 (Middleton & Tyler 1992, Dore *et al.* 1994, Olney *et al.* 1996). Since IGFBP-3 and IGFBP-5 have been shown to have both similar and opposing effects in different experimental models, we investigated the interaction between IGF-I and these IGFBPs in human chondrocytes.

Materials and Methods

Materials

Recombinant human (rh) IGF-I and des(1–3)IGF-I were obtained from Gropep (Adelaide, Australia). Recombinant human IGFBP-3 and polyclonal antibody to IGFBP-2 and IGFBP-3 were generous gifts from Dr Y Oh (Oregon Health Sciences University, Portland, OR, USA). IGFBP-5 was purchased from Austral Biologicals (San Ramon, CA, USA). A polyclonal antibody to IGFBP-5 was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Dulbecco's modified Eagle's medium (DMEM) and heat-inactivated fetal calf serum (FCS) were purchased from Gibco BRL (Grand Island, NY, USA).

Cell cultures

The immortalized human chondrocytes used in this study, the C-28/12 cell line, were established using a retrovirus expressing simian virus 40 large tumor antigen (SV40-TAg) and the neomycin-resistance selection marker (neoR), as described by Goldring *et al.* (1994). These cells, which were derived from juvenile costal cartilage, express the differentiated chondrocyte phenotypic markers of type II collagen and aggrecan, but not the hypertrophic chondrocyte marker, type X collagen. Cultures were maintained in a 5% CO₂ incubator at 37 °C in DMEM supplemented with 10% FCS and were continuously passaged at subconfluency. Prior to experimental incubations, the chondrocytes were resuspended in DMEM supplemented with 10% FCS and seeded at a density of 200 000 cells/cm² in 96-well plates for cell proliferation studies, in 24-well plates for receptor binding studies, in 12-well plates for affinity cross-linking studies, or in 10-cm plates for total RNA extraction. The medium was then changed to serum-free medium containing 0.3% bovine serum albumin.

Cell proliferation

In order to determine the effects of IGF-I and IGFBPs on cell proliferation, the C-28/12 cells were plated in 96-well plates. The cells were grown to subconfluence for 3 days, following which the cultures were changed to serum-free DMEM and incubated for 24 h. The medium was then replaced with DMEM containing various concentrations of IGF-I or IGFBPs, and incubations were continued for a further 48 h. Cell proliferation was then determined by a nonisotopic method based on measurement of acid phosphatase levels using a cell counting kit (Abacus Cell Proliferation Kit, Clontech, Palo Alto, CA, USA). Six replicates were used for each treatment.

Effect of IGFBPs on IGF-I binding to cells

In order to determine the effects of IGFBPs on IGF-I binding to chondrocytes, cells were plated in 24-well

plates. After 24 h incubation without serum, various concentrations of IGFBPs were added and then cells were incubated with ¹²⁵I-labeled IGF-I (40 000 c.p.m.) for 3 h at room temperature. ¹²⁵I-labeled IGF-I bound to cells was extracted with 0.5 M NaOH and counted using a γ -counter.

Affinity cross-linking

Confluent chondrocytes in 12-well plates were washed with PBS and incubated with ¹²⁵I-labeled IGF-I or ¹²⁵I-labeled IGF-II (4 × 10⁵ c.p.m./well) with or without unlabeled IGFs in 50 mM HEPES binding buffer containing 1% BSA, overnight at 4 °C. The buffer was aspirated, and cross-linking was performed with disuccinimidyl substrate for 15 min at 4 °C. The reaction was quenched with 0.1 M Tris–10 mM EDTA. Cells were solubilized and the lysates were separated by 10% SDS-polyacrylamide gel electrophoresis. Gels were dried and exposed to X-ray film for 3 days.

Western ligand blotting and immunoblotting

Western ligand blots (WLB) were carried out according to the method of Hossenlopp *et al.* (1986). Briefly, conditioned medium samples (100 μ l) from chondrocytes treated with or without IGF-I for 48 h were separated by 12.5% SDS-polyacrylamide gel electrophoresis. Proteins were then electrotransferred onto nitrocellulose filters. The filters were washed in 3% NP40 in Tris-buffered saline (TBS) for 30 min, and then incubated with 1% BSA in TBS for 2 h. The treated nitrocellulose filters were probed with 1 × 10⁶ c.p.m. radiolabeled IGF-I and IGF-II overnight. The filters were visualized by autoradiography.

For immunoblotting, filters were blocked with 5% BSA overnight at 4 °C, incubated for 2 h with antiserum (1:500 dilution), and then incubated for 2 h with goat anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase conjugate. Antigen-antibody reactions were visualized using amplified alkaline phosphatase immunoblotting reagents following the manufacturer's instructions (Bio-Rad, Richmond, CA, USA).

Extraction and analysis of mRNAs

The cells were grown to subconfluence in 10-cm dishes for 3 days, following which the medium was changed to serum-free medium containing 0.3% bovine serum albumin and incubation continued for 16–24 h. IGF-I (100 ng/ml) was then added to test cultures without medium change and incubations continued for a further 48 h. Total RNA was extracted from cell layers using guanidine thiocyanate with high volume LiCl precipitation, followed by phenol/chloroform/isoamyl alcohol extraction and EtOH precipitation as described (Cathala *et al.* 1983, Goldring 1996). Semi-quantitative reverse

Figure 1 Effects of IGF-I and des(1-3)IGF-I on cell proliferation. Chondrocytes were incubated for 48 h with or without (control) IGF-I or the IGF analog, des(1-3)IGF-I. Cell numbers were examined as described in Materials and Methods. Values are expressed as a percentage of the untreated control. Error bars give the standard deviation. * $P < 0.001$ compared with control.

transcriptase-polymerase chain reaction (RT-PCR) for rapid screening of mRNAs was carried out using the GeneAmp ThermoStable rTth RT-PCR Kit (Perkin Elmer, Foster City, CA, USA). Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and collagen II were as described by Lum *et al.* (1996), and for IGFBPs-2, -3, -4 and -5 were as described by Olney *et al.* (1995).

Statistical analysis

The results are presented as means $\pm$ s.d. Statistical analysis was undertaken using one-way analysis of variance (ANOVA) with Scheffé's F test.

Results

Effects of IGF-I and des(1-3)IGF-I on cell proliferation

IGF-I at 10, 100 and 1000 ng/ml stimulated proliferation of chondrocytes to 106 ± 8 , 125 ± 10 and $143 \pm 17\%$ of control respectively. However, des(1-3)IGF-I, which is an analog of IGF-I with >100 -fold reduced affinity for IGFBPs but normal affinity for the type 1 IGF receptor, was more potent than native IGF-I in stimulating cell growth at 10 ng/ml ($142 \pm 15\%$ of control) and 100 ng/ml ($147 \pm 21\%$ of control) (Fig. 1). These findings suggest that interaction of native IGF-I with its receptor may be attenuated by bound IGFBP(s) which do not interfere with receptor binding of the analog.

Inhibition of IGF-I-stimulated cell proliferation by IGFBP-3 but not by IGFBP-5

Since IGFBP-3 and IGFBP-5 have been shown to have both stimulatory and inhibitory actions on IGF-I action

and are expressed differentially in normal and arthritic chondrocytes, we examined the effect of IGFBP-3 or IGFBP-5 on IGF-I-stimulated cell proliferation when co-incubated with IGF-I. Neither IGFBP-3 nor IGFBP-5 (100–1000 ng/ml) had any significant effect on proliferation of chondrocytes in the absence of IGF-I (Fig. 2). At 100 ng/ml, IGF-I stimulated cell proliferation to $139 \pm 7\%$ of control, and this stimulatory effect of IGF-I was inhibited significantly to $123 \pm 7\%$ and $111 \pm 3\%$ in the presence of 500 and 1000 ng/ml IGFBP-3 respectively ($P < 0.001$ versus IGF-I-treated without IGFBP). In contrast, IGFBP-5 did not show any inhibitory effect but rather a slightly stimulatory effect on the IGF-I-induced cell proliferation, to $152 \pm 5\%$ at 500 ng/ml ($P < 0.001$ versus IGF-I-treated without IGFBP).

Displacement of binding of 125 I-labeled IGF-I to C-28/I2 cells by IGFBP-3 and IGFBP-5

We examined the effects of IGFBPs on binding of IGF-I to the cell membrane by incubating cells with 125 I-labeled IGF-I with or without unlabeled IGFBP-3 or IGFBP-5 (1–500 ng/ml). The binding of 125 I-labeled IGF-I to the cell membrane was displaced by either IGFBP-3 or IGFBP-5 in a dose-dependent manner. However, the reduction in the binding of IGF-I tracer produced by IGFBP-3 was more potent than that produced by IGFBP-5. At 250 ng/ml unlabeled IGFBP-3 or IGFBP-5, the binding of 125 I-labeled IGF-I to chondrocytes (B/B_0) was decreased to 18% or 77% respectively (Fig. 3).

Affinity cross-linking of 125 I-labeled IGF-I and 125 I-labeled IGF-II to chondrocytes

The affinity cross-linking of 125 I-labeled IGF-I and 125 I-labeled IGF-II to the cell surface was then examined (Fig. 4). After separation of 125 I-labeled IGF-I cross-linked proteins by SDS-polyacrylamide gel electrophoresis under reducing conditions, a band of 137 kilodaltons (kDa), consistent with the size of the IGF-I receptor α -subunit, was observed (Fig. 4, lanes 1 and 2). This band was attenuated in the presence of unlabeled 200 ng/ml IGF-I (lanes 3 and 4) and to a lesser extent by 200 ng/ml IGF-II (lanes 5 and 6). Two bands of more than 200 kDa may represent cross-linked α -subunits of the IGF-I receptor.

After separation of 125 I-labeled IGF-II cross-linked proteins by SDS-polyacrylamide gel electrophoresis, a band of more than 200 kDa, consistent with the IGF-II/mannose 6-P receptor, was seen (Fig. 4, lanes 7 and 8). This band was not abolished by addition of 200 ng/ml IGF-I, but it was attenuated by 200 ng/ml IGF-II. 125 I-labeled IGF-II also bound to a doublet protein at approximately 50 kDa, and the binding was decreased by unlabeled IGF-II (Fig. 4, lanes 7 and 8). After subtraction of the molecular weight of the ligand (IGF-II), these bands were found to correspond to a cell-associated IGFBP-3

Figure 2 Effects of IGFBPs on basal and IGF-I-induced cell proliferation. Panel A shows the effects of increasing concentrations of IGFBP-3 on cell proliferation in the presence (solid squares) or absence (open squares) of 100 ng/ml IGF-I. Panel B shows the effects of increasing concentrations of IGFBP-5 on cell proliferation in the presence (solid circles) or absence (open circles) of 100 ng/ml IGF-I. Values are expressed as a percentage of the untreated control. Error bars give the standard deviation. * $P < 0.001$ compared with the IGF-I control (treated with 100 ng/ml IGF-I without IGFBP).

doublet of 40 kDa and 38 kDa. In addition, a faint band at 37 kDa was detected, which was diminished by 200 ng/ml IGF-I and IGF-II, thus suggesting the presence of cell-associated IGFBP-5. However, in order to determine that the binding was to specific membrane-associated proteins, rather than to the proteins secreted during the incubation period, further studies using membrane preparations will be needed.

Figure 3 Effect of IGF-I and IGFBPs on the binding of ^{125}I -labeled IGF-I to cells. Competition by IGF-I (open squares), IGFBP-3 (solid circles), and IGFBP-5 (solid squares) incubated simultaneously with labeled IGF-I. Results are expressed as a percentage of binding ^{125}I -labeled IGF-I in the absence (B_0) and presence (B) of cold ligand. Error bars give the standard deviation.

Stimulation of IGFBP-5 by IGF-I at the protein and mRNA levels

To identify the IGFBPs produced by the C-28/I2 cells and to determine IGF-mediated changes, the cells were treated with IGF-I for 48 h and the serum-free conditioned medium was subjected to WLB analysis. In the absence of IGF-I, binding to radiolabeled IGF-I was detected as a 39/43 kDa doublet band, a 33 kDa band and a 24 kDa band. IGF-I induced the appearance of a band at 29 kDa, with a maximal effect at 100 ng/ml, but also slightly increased the other IGFBPs (Fig. 5). Immunoblotting with anti-IGFBPs-2, -3, -4, and -5 antibodies showed that the 33 kDa band was IGFBP-2, the 39/43 kDa doublet was IGFBP-3, the 24 kDa band was IGFBP-4, and the 29 kDa band was IGFBP-5 (Fig. 6). Treatment of the C-28/I2 cells with IGF-I at 100 ng/ml also stimulated a corresponding increase in IGFBP-5 and type II collagen mRNAs, but not IGFBPs-2, -3, and -4 mRNAs (Fig. 7).

Discussion

In the present study, we found that IGFBP-3 and IGFBP-5 differentially modulated the action of IGF-I on cell proliferation in immortalized human chondrocytes. Although neither IGFBP affected cell proliferation in the absence of IGF-I, IGFBP-3 but not IGFBP-5 inhibited IGF-induced cell proliferation. A role for endogenous

Figure 4 Affinity cross-linking of ^{125}I -labeled IGF-I (lanes 1 to 6) and of ^{125}I -labeled IGF-II (lanes 7 to 12) to chondrocyte monolayers. Samples were analyzed by SDS-PAGE on 10% polyacrylamide gels under reducing conditions. Lanes 1 and 2, no unlabeled IGF-I; lanes 3 and 4, 200 ng/ml IGF-I; lanes 5 and 6, 200 ng/ml IGF-II; lanes 7 and 8, no unlabeled IGF-II; lanes 9 and 10, 200 ng/ml IGF-I; lanes 11 and 12, 200 ng/ml IGF-II.

IGFBPs in regulating IGF-I action was also suggested by the fact that des(1–3)IGF-I, which exhibits unaltered affinity for the IGF-I receptor, but weak or almost no binding to IGFBPs, was more potent in increasing cell proliferation than native IGF-I. The mechanism of the inhibitory effect might be explained by the ability of soluble IGFBPs to prevent IGF-I from binding to the type 1 IGF receptor. As expected, IGFBP-3 clearly inhibited the binding of IGF-I to cell membranes in this study. The inhibitory effect of IGFBP-3 on IGF-induced cell proliferation has also been shown in fibroblasts (DeMellow & Baxter 1988, Okajima *et al.* 1993) and osteoblasts (Schmid *et al.* 1991, Andress & Birnbaum 1992); however, some conflicting observations were reported (Cornell *et al.* 1987, Conover *et al.* 1990). Different experimental conditions, such as preincubation rather than coinubation with the IGFBP, or alteration in the molar ratio of IGF to IGFBP, may affect these results. How changes in the endogenous levels of IGF-I and IGFBPs may influence the responses in these cells remains to be clarified.

On the other hand, IGFBP-5 did not inhibit, but rather slightly enhanced IGF-I-induced cell proliferation. Although the inhibitory effect of IGFBP-5 on IGF-I binding to cell membranes was much less than that of IGFBP-3, high concentrations of IGFBP-5 prevented

IGF-I binding to the cells in this study. These findings give no appropriate explanation for why IGFBP-5 did not inhibit IGF-I-induced cell proliferation. Although molar excess of IGFBP-5 was reported to inhibit IGF-I action in osteosarcoma cells (Kiefer *et al.* 1992), the usual observation is that IGFBP-5 enhances IGF-I activity on mitogenesis in osteoblasts or fibroblasts via binding to the extracellular matrix (Andress & Birnbaum 1992, Jones *et al.* 1993). Whether a given IGFBP potentiates or inhibits IGF-I action appears to depend upon the relative levels of cell-associated and soluble extracellular IGFBP, as well as on the ratio of IGFBP to IGF-I (Jones & Clemmons 1995, Kelley *et al.* 1996). Although it is more complicated to understand the potentiation mechanism of IGFBPs, it is assumed that cell-associated IGFBPs, having a lower affinity for IGFs than soluble IGFBPs (McCusker *et al.* 1990, McCusker & Clemmons 1997) may play some part in enhancing IGF activity. Furthermore, there is the other possibility that IGFBP-5 proteolysis has effects on IGF action; however, we have not yet examined the IGFBP-5 protease in this model.

The immortalized cell line, C-28/12, used in this study exhibited a pattern of IGFBP expression similar to that which we reported previously in rat chondrocytes (Matsumoto *et al.* 1996a). In a preliminary study, the

Figure 5 Western ligand blot of IGF-BPs in the conditioned medium of C-28/I2 cells treated with IGF-I for 24 h. The samples were subjected to SDS-PAGE on a 12.5% acrylamide gel as described in Materials and Methods. Lanes 1 and 2 represent cells under basal conditions, while lanes 3 and 4 are cells treated with 10 ng/ml IGF-I, lanes 5 and 6 with 100 ng/ml IGF-I, and lanes 7 and 8 with 1000 ng/ml IGF-I. The molecular mass markers of the IGF-BPs are indicated on the ordinate.

Immunoblotting

Figure 6 Immunoblotting of IGF-BPs. Conditioned medium of C-28/I2 cells was subjected to 12.5% SDS-PAGE, then transferred to filters. The filters were immunoblotted with IGF-BPs-2, -3, -4 and -5 antisera as described in Materials and Methods.

C-28/I2 cells were found to produce IGF-BP-4 and IGF-BP-5 at approximately five fold higher levels than IGF-BP-3, and a barely detectable level of IGF-I (Goldring *et al.* 1996). Although our results showed direct effects of

IGFBP-3 and IGF-BP-5 on IGF action, the presence of other IGF-BPs, including IGF-BP-4, must also be considered (Schiltz *et al.* 1993). The importance of IGF-BP-5 in regulating IGF-I action in these cells was reflected in our

Figure 7 Comparison of IGFBP, type II collagen (COL2A1) and GAPDH mRNAs in monolayer cultures of C-28/I2 cells. The cells were passaged and cultured for 3 days in serum-containing medium. The medium was then changed to DMEM containing 0.3% BSA, IGF-I (100 ng/ml) was added the next day, as indicated, and incubation was continued for 48 h. The cell layers were then harvested for RNA extraction and analysis by RT-PCR, as described in the Materials and Methods. A 100 bp DNA ladder is shown in the left lane of each gel.

observation that IGF-I upregulated IGFBP-5 at both the mRNA and protein levels. While the other IGFBPs were also observed by WLB to increase dose dependently after addition of IGF-I, the small increases in expression of corresponding mRNAs suggested that the increased secretion of IGFBPs may have been due to post-translational modification.

Although the profiles of IGFBPs expressed by SV40-immortalized human fibroblasts and osteoblasts were shown to be similar to those expressed by their normal counterparts (Conover *et al.* 1993, Durham *et al.* 1995), IGFBP-5 has also been shown to be downregulated in SV40-transformed human fibroblasts (Reeve *et al.* 1995, Schenker & Trueb 1998). Nevertheless, our results indicate that there must be clonal variability in the responses of cells to stable expression of SV40-TAg. Since a limited number of other SV40-immortalized chondrocyte cell lines obtained by the same procedures did not express IGFBP-5 (Goldring *et al.* 1996), the presence of SV40 large T antigen did not appear to be a major consideration in our studies. Since the C-28/I2 cells do not form tumors in nude mice (Goldring *et al.* 1994), they are termed 'immortalized', rather than 'transformed', and thus may be expected to retain features of the chondrocyte phenotype. Although p53, which is inactivated by SV40-TAg, is known to induce IGFBP-3 with growth inhibitory properties in tumor cells carrying an inducible wild-type p53

transgene (Buckbinder *et al.* 1995), IGFBP-3 is present in the SV40-immortalized C-28/I2 chondrocytes at levels consistent with those reported in normal chondrocytes. Therefore, our studies indicate that the C-28/I2 cells constitute a representative model for clarifying the roles of IGFBPs in the modulation of IGF actions on chondrocytes.

In our previous studies and those of others, synovial fluid levels of IGF-I and IGFBP-3 in patients with osteoarthritis (OA) or rheumatoid arthritis (RA) were significantly increased as compared with the levels in normal subjects (Fernihough *et al.* 1996, Matsumoto *et al.* 1996c). Despite increases in IGF-I levels, destruction of cartilage tissue is found in these diseases, suggesting that OA or RA chondrocytes are hyporesponsive to IGF-I. This may be explained, in part, by the involvement of IGFBPs in the pathological process, as reported by others (Middleton & Tyler 1992, Dore *et al.* 1994, Olney *et al.* 1996). Furthermore, increased levels of IGFBP-3 in cell lysates of osteoarthritic chondrocytes were observed while expression of the type 1 IGF receptor was normal (Tardif *et al.* 1996). Hyporesponsiveness to IGF-I may also explain the ineffective repair associated with the decreased capacity of arthritic chondrocytes to synthesize cartilage-specific matrix macromolecules. Since IGF-I induction of IGFBP-5 mRNA was associated with increased type II collagen mRNA in our culture model, it is tempting to speculate that changes in the balance of IGFBP-3 to IGFBP-5 may contribute to abnormal cartilage repair in arthritic joints. Further studies are needed to determine the effects of IGFBPs not only on cell proliferation but also on cartilage matrix synthesis.

In this study, we have shown the different effects of IGFBP-3 and IGFBP-5 on IGF action and binding of IGF-I to the cell membrane of chondrocytes. We conclude that interactions among IGF, IGFBPs and IGF receptor may play important roles in regulating cartilage metabolism, and that alterations of the balance among them may result in abnormal maintenance of cartilage tissue.

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