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Differential Effect of ECM Molecules on Re-Expression of Cartilaginous Markers in Near Quiescent Human Chondrocytes

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The limited source of healthy primary chondrocytes restricts the clinical application of tissue engineering for cartilage repair. Therefore, method to maintain or restore the chondrocyte phenotype during in vitro expansion is essential. The objective of this study is to establish the beneficial effect of ECM molecules on restoring the re-expression of cartilaginous markers in primary human chondrocytes after extensive monolayer expansion. During the course of chondrocyte serial expansion, COL2A1, SOX9, and AGN mRNA expression levels, and GAG accumulation level were reduced significantly in serially passaged cells. Exogenous type II collagen dose-dependently elevated GAG level and induced the re-expression of cartilaginous marker mRNAs in P7 chondrocytes. Chondroitin sulfate did not show significant effect on P7 chondrocytes, while hyaluronic acid inhibited the expression of SOX9 and AGN mRNAs. Upon treatment with type II collagen, FAK, ERK1/2, and JNK were activated via phosphorylation in P7 chondrocytes within 15 min. Furthermore, GFOGER integrin blocking peptide, MEK inhibitor and JNK inhibitor, not p38 inhibitor, significantly reduced the type II collagen-induced GAG deposition level. Finally, in the presence of TGF-β1 and IGF-I, P7 chondrocytes cultured in 3D type II collagen matrix exhibited better cartilaginous features than those cells cultured in the type I collagen matrix. In conclusion, type II collagen alone can effectively restore cartilaginous features of expanded P7 human chondrocytes. It is probably mediated via the activation of FAK-ERK1/2 and FAK-JNK signaling pathways. The potential application of type II collagen in expanding a scarcity of healthy chondrocytes in vitro for further tissue engineering is implicated.

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Articular cartilage is an avascular tissue with limited capacity of self-repair. In the adult articular cartilage, chondrocytes are the only cell type dispersed among the dense cartilage-specific matrix, which is abundant of type II collagen and glycosaminoglycans. This histological feature makes that the articular cartilage bears only limited spontaneous healing ability (Hunter, 1995). One of the surgical attempts to restore normal cartilage surface of the joint is made by the subchondral drilling technique. Bleeding from the subchondral bone promotes wound healing and results in a hyaline-like but more cellular tissue at the defect site of the cartilage (Mainil-Varlet et al., 2003). During recent years, intensive efforts have been made to develop cell therapies for cartilage repair, such as the autologous transplantation of chondrocytes (Brittberg et al., 1994; Convery et al., 1996; Ghazavi et al., 1997). Large quantities of healthy cells from limited source are required in such cell-based therapies. Consequently, in vitro expansion of chondrocytes for cell implantation cannot be avoided.

Abbreviations: COL2A1, type II collagen alpha 1 chain gene; COL1A1, type I collagen alpha 1 chain gene; AGN, aggrecan gene; SOX9, SRY-box 9 gene; ITGA2, integrin alpha 2 gene; ITGB1, integrin beta 1 gene; CS, chondroitin sulfate; HA, hyaluronic acid; COL II, type II collagen; COL I, type I collagen; ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinase.

Li-Hsuan Chiu and Shih-Ching Chen contributed equally to this work.

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Problem emerges when primary chondrocytes are extensively expanded in the monolayer culture system. The cells eventually lose their phenotypic features, including the attenuation of type II collagen production and the reduction in proteoglycan accumulation (Benya et al., 1978; Benya and Shaffer, 1982). The rapid loss of cartilaginous features of the cells during gross expansion poses a key limitation to the application of autologous chondrocyte transplantation. To counter this problem, chondrocytes have been suspended in three-dimensional environments such as agarose, alginate, pellet culture, or three-dimensional scaffolds (Benya and Shaffer, 1982; Fortier et al., 1999; Martin and Buckwalter, 2000; Qi and Scully, 2003). However, only cells up to passage 3 has been able to re-express native chondrocyte phenotype, and it takes at least 2 weeks to restore the phenotype of the chondrocytes (Yaeger et al., 1997; Lemare et al., 1998). Moreover, the chondrocytes must be isolated from alginate beads for further applications. All the above disadvantages make the three-dimensional culture system ineffective. Therefore, a better culture system to maintain or restore the phenotypic features of chondrocytes after monolayer expansion is prerequisite to the success in clinical application of chondrocyte autologous transplantation.

Type II collagen has known to contribute to the chondrocyte proliferation and phenotype regulation (Loeser, 2000; Tarone et al., 2000). Several reports also suggested that hyaluronic acid may stimulate proteoglycan and chondroitin sulfate syntheses (Bansal et al., 1986; Frean et al., 1999; Kawasaki et al., 1999). Thus, it is hypothesized that these ECM molecules can be applied as better culture systems to restore the phenotypic features of chondrocytes after extensively monolayer expansion. In this study, the biological significances of various exogenous ECM molecules, including chondroitin sulfate, hyaluronic acid, and type II collagen in serially passaged P7 articular chondrocytes were extensively investigated. The effects of growth factors, TGF-β1 and IGF-I, in combination with type I collagen or type II collagen on directing chondrocyte redifferentiation in 3D gel culture system were analyzed as well. The results demonstrate the potential of using type II collagen to effectively restore the phenotype of in vitro expanded chondrocytes from a scarcity of healthy cartilage for future tissue engineering or regeneration application.

Materials and Methods

Reagent

Dulbecco's Modified Eagle Medium-low glucose (DMEM-LG) medium, fetal bovine serum (FBS) and other cell culture medium-related supplies were from Invitrogen (Carlsbad, CA). Antibodies against pFAK397, pFAK576/577, pFAK925, FAK, pERK1/2, ERK1/2, pJNK, JNK, pp38, p38 and β-actin were obtained from Cell Signaling Technology (Danvers, MA). Hyaluronic acid (H5388) and chondroitin sulfate (C4384) were obtained from Sigma–Aldrich (St. Louis, MO). RT-PCR related reagents include TRIzol® and SuperScript® III RT systems were purchased from Invitrogen (Carlsbad, CA). SYBR Green I qPCR system was obtained from Roche applied science (Indianapolis, IN).

Isolation of type I and type II collagen

Type I collagen and type II collagen were isolated from rat tail tendon and porcine sternum, respectively, as previously described (Lai and Chen, 2003; Lai et al., 2003; Chen et al., 2005). Briefly, the rat tail tendon or porcine sternum were minced to pieces and pretreated with 4M guanidine HCl overnight, followed by 4.5 M NaCl–50 mM Tris, pH 7.5 for another 24 h. Then, the washed residual tissue was extracted with 3 mg/ml pepsin in 0.5 M acetic acid followed by salting-out type II collagen in 0.9 M NaCl–0.5 M acetic acid. The type II collagen pellet was washed three times with 70% ethanol in PBS to remove excessive salts and acid, and also to sterilize. Finally, the type II collagen pellet was dissolved in 5 mM acetic acid, quantified and stored at 4°C until use.

Chondrocyte isolation

The cartilages were placed in chilled Hank's solution containing antibiotics including penicillin (100 unit/ml), streptomycin (100 μg/ml), and fungizone (250 ng/ml) (P/S/F). After several washings, the tissue was aseptically sliced into little pieces and subsequently digested with collagenase (1 mg/ml), hyaluronidase (1 mg/ml) and trypsin (0.25%) (Sigma) solution at 37°C with shaking for 1 h. This step was repeated 3–4 times. Finally, the enzyme-released cells were combined, spun down and resuspended in Dulbecco's Modified Eagle's medium/F12 (DMEM/F12, Hyclone, Logan, UT) containing 10% fetal bovine serum (FBS, Gibco, Grand island, NY), 40 mM L-proline, and 1× P/S/F.

Chondrocyte culture

Human articular chondrocytes were seeded onto 10-cm tissue culture dishes at a density of 5×10^5 cells/dish. Cells were cultured in DMEM/F12 medium containing 10% fetal bovine serum, 40 mM L-proline, and 1× P/S/F at 37°C, in 5% CO_2, and 85% humidity. Cell culture medium was changed every 4 days. As soon as reaching 80% confluence, cells were trypsinized, pooled together and counted using a hemocytometer. Then, the cell suspension was divided into four aliquots, spun down and the residual medium removed. The cell pellets were resuspended in DMEM/F12 medium containing various concentrations of type II collagen ranged from 0, 50, 100, to 200 μg/ml, or containing either hyaluronic acid or chondroitin sulfate at designated concentrations, and 50 μg/ml L-ascorbic acid. Then, chondrocytes were cultured in 6-well tissue culture dishes at a density of 4×10^4 cells/well as described above.

Cell proliferation assay

Chondrocytes were cultured in 10-cm tissue culture dishes at a density of 5×10^5 cells/dish in DMEM/F12 medium containing 10% fetal bovine serum, 40 mM L-proline, and 1× P/S/F at 37°C. Medium was changed every 4 days. Chondrocytes were grown to confluence, trypsinized and subcultured in new dishes repetitively until the cells reached passage 7. Cell morphology was observed throughout the entire culture interval using inverted light microscope (Olympus, Tokyo, Japan). Cell numbers of each group of triplicate wells were counted with a hemocytometer (Burker, Marienfeld, Germany) under light microscope at a magnification of 400×. To accurately assess total live cell number, trypan-blue exclusion method was performed prior to cell counting with hemocytometer. Triplicate aliquots from each well of the group were counted to minimize variation.

Glycosaminoglycan (GAG) staining

The accumulated GAG level was measured via Alcian blue staining. Briefly, the cells were fixed with 10% formaldehyde for at least 30 min, rinsed with distilled water followed by incubated in 0.0018 M H_2SO_4 for 30 min. Then, the acid solution was removed completely before adding Alcian blue solution (1% Alcian blue 8GX in 0.0018 M H_2SO_4). The staining step took 3 h, followed immediately by washing with 0.018 M H_2SO_4 for another 3 h to remove redundant dye. Finally, the bound dye was eluted with dissociation buffer (4 M guanidine hydrochloride in 33% 1-propanol with 0.25% Triton X-100). The absorbance of each sample was then measured at 600 nm using spectrophotometer.

RNA extraction and RT-PCR

Total RNA of chondrocytes was extracted with TRIzol® reagent as described by the manufacturer (Life Technologies Ltd., Paisley, UK)

and stored at −80°C until use. RNA (500 ng) was reverse-transcribed in a 50 μl reaction mixture containing 1xBca 1st buffer, 5 mM $MgSO_4$, 0.5 mM dNTPs, 2.5 mM oligo(dT) primer, 2.5 μM random 9mers, 20 units of RNase inhibitor, and 22 units of BcaBEST polymerase (Takara Shuzo Co. Ltd., Tokyo, Japan). In realtime PCR analysis, aliquots of cDNA were amplified by Real Time PCR (Roche, LightCycler® FastStart DNA Master SYBR Green I) using specific primer sets. The relative quantification of gene expression level of each marker was performed with LightCycler® software by using Pffafl's method (Pfaffl, 2001). All primer sequences were as listed in Table 1.

Western blotting

Whole cell lysates were prepared by using a protein extracting buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% SDS and supplemented with protease inhibitors. The proteins were then size-fractionized on SDS–PAGE and transferred to PVDF membrane. The membrane was blocked with 5% non-fat dry milk prior to incubating with each primary antibody (1:1,000), followed by respective HRP-conjugated secondary antibody (1:2,000). The membrane was then washed with Tris-buffered saline after each incubation and further visualized with HRP substrates (Thermo Scientific, Rockford, IL). The activation of FAK, ERK and JNK were evaluated by using antibodies specific to the activated, tyrosine- and threonine-phosphorylated FAK, ERK and JNK (Cell Signaling Technology, Danvers, MA), respectively.

3D culture of chondrocyte-collagen constructs

In 3D culture experiment, 4×10^5 chondrocytes were suspended in 2 ml of 2× DMEM/F12 medium containing 20% fetal bovine serum, 80 mM L-proline, 2× P/S/F and mixed with an equal volume of 2 mg/ml type I or type II collagen solution in 10 mM acetic acid. After polymerization of collagen matrix at 37°C for 1 day, the cell-collagen constructs were cultured with fresh DMEM/F12 medium containing 10% fetal bovine serum, 40 mM L-proline, 1× P/S/F. The chondrocyte-collagen constructs were cultured for 14 or 28 days with medium change every 3–4 days. Then, all samples were fixed in 4% formalin for at least 24 h, then embedded in paraffin, sectioned at 5 μm, and stained with H&E stain and Safranin O for histological and proteoglycan level evaluation.

Statistical analysis

Each datum point was derived from three independent experiments or an experiment of triplicate assay and was presented as mean ± SD. The statistical analysis was performed by one-way ANOVA and Duncan's multiple range test.

TABLE 1. Primers for human cartilaginous marker mRNA detection

Gene	Primer sequence	Reference[a]
COL2A1	5′-CTCCTGGAGCATCTGGAGAC-3′ 3′-ACCACGATCACCCTTGATCT-5′	NM_001844
COL1A1	5′-TTCCCCCAGCCACAAAGAGTC-3′ 3′-CGTCATCGCACAACACCT-5′	NM_000089
Aggrecan	5′-AGTATCATCAGTCCCAGAATCTAGCA-3′ 5′-AATGCAGAGGTGGTTTCACTCA-3′	NM_001135
SOX9	5′-ATCTGAAGAAGGAGAGCGAG-3′ 5′-TCAGAAGTCTCCAGAGCTTG-3′	NM_014587
ITGA2	5′-GTGCCTTTGGACAAGTGGTT-3′ 5′-GGGCAACTCTGTGCTTGATT-3′	NM_002203
ITGB1	5′-ATGAATGAAATGAGGAGGATTACTTCG-3′ 3′-AAAACACCAGCAGCCGTGTAAC-5′	NM_002211
β-Actin	5′-GCATCCCCCAAAGTTCACAA-3′ 3′-AGGACTGGGCCATTCTCCTT-5′	NM_001101

[a]Gene bank accession number.

Results

Phenotypic changes in chondrocytes during serial expansion

Primary chondrocytes were cultured and expanded in DMEM/F12 medium until passaged 7 (P7). The cell proliferation rates through the entire culture period were evaluated. The average slope of growth curve for each passage was decreased from passage 1 to passage 7 (Fig. 1), indicating that the cell proliferation rate of chondrocytes was gradually down regulated. The accumulated glycosaminoglycan (GAG) level at each passage was determined by alcian blue staining. A significant decrease in GAG level per cell was observed from passage 1 to passage 7 (Fig. 2a). The mRNA expression levels of several cartilaginous marker genes were also examined by RT-PCR analysis. As shown in Figure 2b,c, the mRNA levels of *COL2A1*, *AGN*, and *SOX9* genes were all down regulated from passage 1 to passage 7. However, the expression of *COL1A1* mRNA was gradually increased during the course of cell expansion. By passage 7, the mRNA levels of secretory proteins such as *COL2A1* and *AGN* were too low to be detected, while the expression level of *SOX9* was reduced to a barely detectable level under the experimental condition. A two-way ANOVA was performed to examine the gene expression levels during various passages of chondrocytes for COL1A1, COL2A1, SOX9, and AGN. A significant main effect was found between the gene expression level and passage number ($P < 0.001$), indicating that the passage number of the cells affects the expression levels of various genes. The expression of integrin subunit *ITGA2* and *ITGB1* mRNAs were also examined (Fig. 2b). The mRNA expression level of *ITGA2* appeared to increase slightly while that of *ITGB1* did not exhibit detectable changes along the passages, respectively. The data demonstrated that

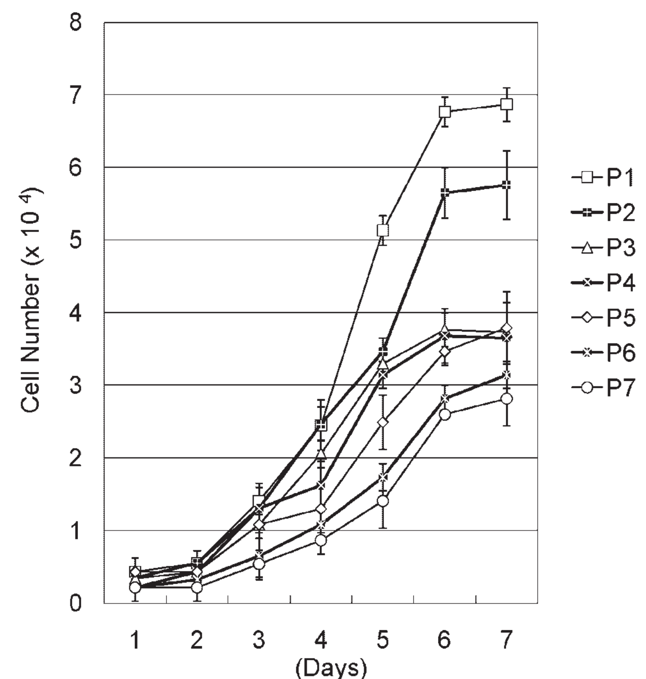

Fig. 1. The proliferation curves of serially expanded chondrocytes from P1 to P7: Chondrocytes from each passage were analyzed at 7 days after each subculture and the cell numbers of the serially passaged cells were plotted. The error bars on the graph indicate the standard deviations. The data represent means ± SD (n = 3).

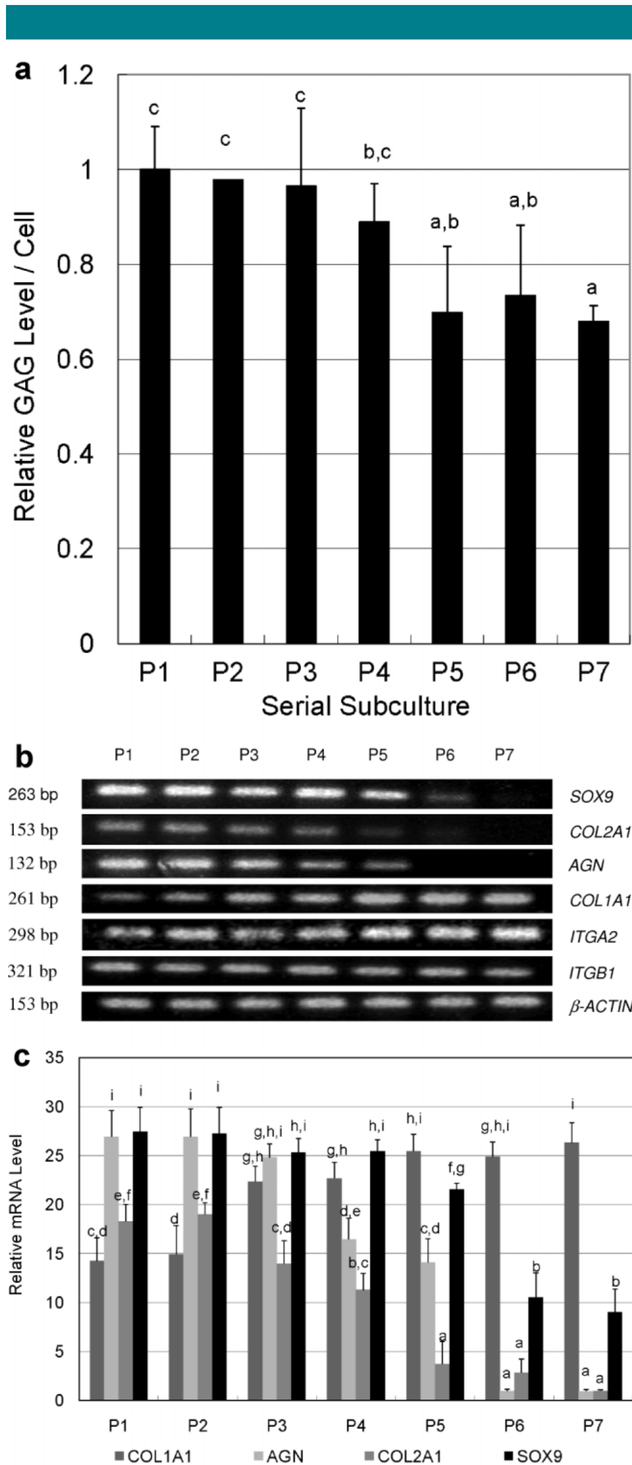

Fig. 2. The GAG levels and marker mRNA expression patterns of serially passaged chondrocytes: (a) The accumulated GAG from each passage of chondrocytes was stained with alcian blue, extracted and quantified at OD600. The quantitative data were normalized with total cell number. b: The quantitative RT-PCR analysis of the expression levels of *COL2A1*, *SOX9*, *AGN*, and *COLIA1* mRNAs in chondrocytes from passage 1 to passage 7 were summarized. c: The bars represent the mean expression level for each marker mRNA in the chondrocyte of each passage after quantified and normalized with β-actin internal control. The statistical significance of each group was analyzed by one-way ANOVA and Duncan's multiple range test. The data represent means ± SD (n = 3).

primary chondrocytes lost their native phenotype during extensive in vitro expansion.

Type II collagen-induced GAG re-deposition and re-expression of cartilaginous marker mRNAs

The re-expression of down regulated cartilaginous marker in the serially passaged chondrocytes (passage 7) in responding to exogenous type II collagen were investigated. The concentrations of supplemented type II collagen were ranging from 50 to 200 μg/ml. The non-treated groups were treated with same volume of solvent 5 mM acetic acid as control. The GAG deposition levels of P7 chondrocytes were elevated dose dependently after 12 days of treatment with type II collagen. The GAG deposition levels were up to 2.5-fold in the 200 μg/ml type II collagen group as compared with that of the control group (Fig. 3a). The mRNA expression patterns were also determined by 12 days of treatment with type II collagen. After normalized with the respective internal β-actin level, the expression of cartilaginous marker mRNAs were all increased in dose dependent manners. At 200 μg/ml of type II collagen, *SOX9*, *COL2A1*, and *AGN* mRNA levels in the P7 chondrocytes were significantly re-expressed as compared with those in the non-treated control cells (Fig. 3b,c). On the contrary, the elevated type I collagen mRNA expression in the P7 chondrocytes was down-regulated in a dose dependant manner after exposed to type II collagen (Fig. 3b). By two-way ANOVA, a significant main effect between type II collagen concentrations and gene expression levels was also found ($P < 0.001$), indicating that the type II collagen concentration affects the expression level of various genes. Combined with data stated above, it confirms that type II collagen alone is capable of restoring the typical expression pattern of cartilaginous markers in primary chondrocytes after extensive monolayer expansion.

The effects of chondroitin sulfate and hyaluronic acid

The effects of exogenous CS (chondroitin sulfate) and HA (hyaluronic acid) on the re-expression of down regulated cartilaginous marker mRNAs in the P7 chondrocytes were examined. After 12 days of treatment, the patterns of cartilaginous marker mRNAs were analyzed using realtime PCR and normalized with the internal control β-actin mRNA. When HA was included in the P7 chondrocyte culture, ranging from 0.3 to 300 μg/ml, the *COL1A1* expression level was not affected significantly. However, in the presence of increasing concentrations of HA, both *SOX9* and *AGN* mRNAs were suppressed in dose dependant manners (Fig. 4a). On the other hand, when CS was included in the chondrocyte culture at concentrations ranging from 10 μg/ml to 1 mg/ml, neither *COL1A1*, *SOX9* nor *AGN* expression level was affected (Fig. 4b). Likewise, two-way ANOVA showed no interaction between the gene expression levels and chondroitin sulfate concentrations ($P = 0.547$) or hyaluronic acid concentrations ($P = 0.162$), indicating that chondroitin sulfate concentration nor hyaluronic acid concentration did not significantly affect the expression level of various genes. The results from this study indicates that HA and CS did not significantly trigger the phenotype re-expression of primary chondrocytes after extensive monolayer expansion.

Type II collagen-induced activation of MAPK signaling

The short-term effect of exogenous type II collagen on the activation of FAK and MAPK signaling was examined in serially passaged chondrocytes by analyzing the phosphorylated FAK, ERK1/2, JNK and SOX9. The P7 chondrocytes were treated with 200 μg/ml type II collagen and collected at 15, 30, 60, and 90 min after treatment. The cell lysates were subjected to western blot analysis. FAK was sequentially phosphorylated at

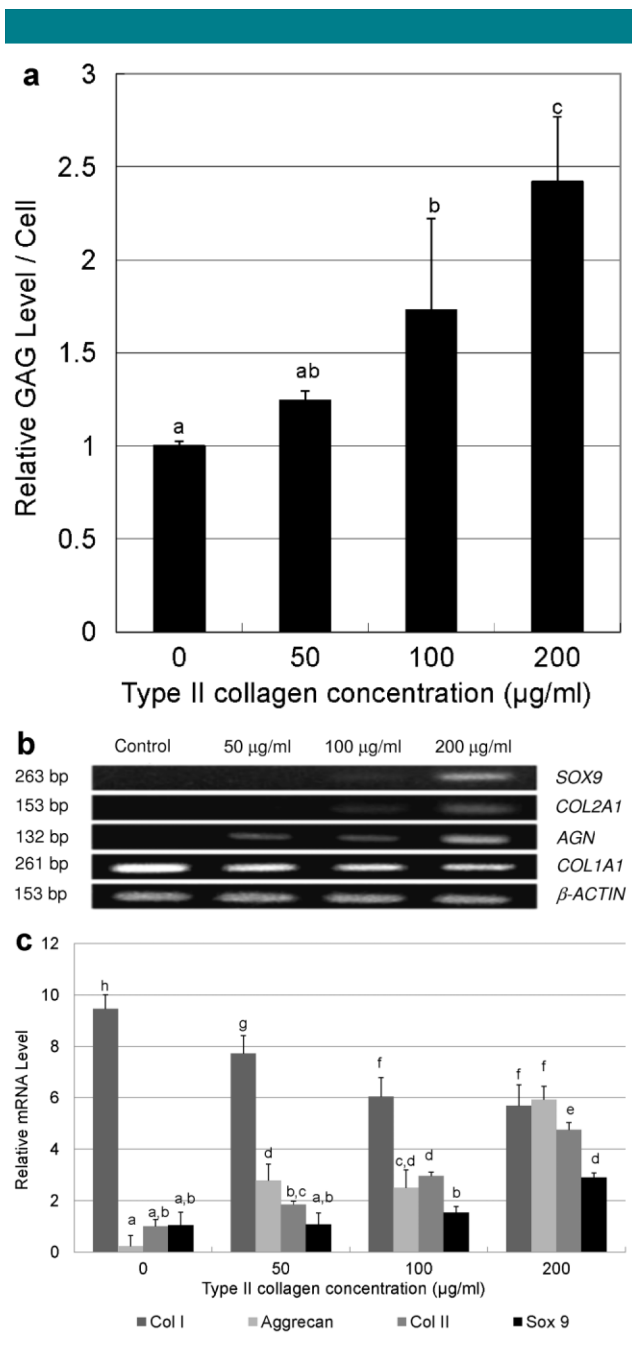

Fig. 3. The accumulated GAG levels and marker mRNA expression patterns of P7 chondrocytes treated with various concentrations of type II collagen: (a) The effect of type II collagen on GAG level was performed as described in Figure 2; a dose dependent relationship was observed. b: The quantitative RT-PCR analyses of mRNA expression levels in P7 chondrocytes after treated with various amounts of type II collagen were presented. c: The bar graphs represent relative mRNA levels of cartilaginous marker mRNAs after normalized with β-actin internal control. The statistical significance of each group was analyzed by one-way ANOVA and Duncan's multiple range test. The data represent means ± SD (n = 3).

Tyr-397 (peak at 15 min), −576/577 (peak at 60 min), and −925 (peak at 90 min). The phosphorylated CrkII and JNK were observed at 15 and 30 min, while the phosphorylation of c-JUN was also detected at 15 min and peaked at 30 min of type II collagen treatment. The phosphorylation of p38 was gradually elevated and peaked at 60 and 90 min. However, a robust up-

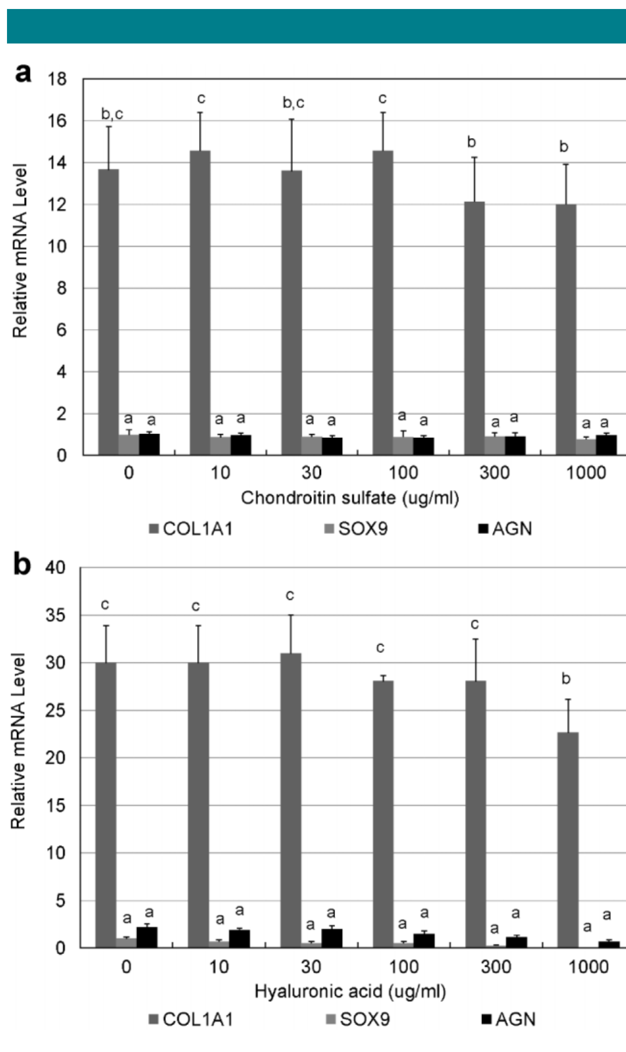

Fig. 4. The expression patterns of cartilaginous marker mRNAs in P7 chondrocytes treated with various concentrations of CS (chondroitin sulfate) or HA (hyaluronic acid) using realtime PCR analysis: (a) P7 chondrocytes were treated with various concentrations of CS ranging from 0.01 to 1 mg/ml for 12 days, neither *COL1A1*, *SOX9* nor *AGN* expression levels was affected. b: P7 chondrocytes were exposed to various concentrations of HA ranging from 0.003 to 0.3 mg/ml for 12 days; *COL1A1* mRNA levels were not affected. However, at the increased concentrations of HA, *SOX9* and *AGN* mRNA expression levels were suppressed in a dose dependant manner. All the statistical significance of each group was analyzed by one-way ANOVA and Duncan's multiple range test. The data represent means ± SD (n = 3).

regulation of phosphorylated ERK1/2 was found at 15 min and then declined after 30 min. Most importantly, the phosphorylated SOX9 was greatly elevated, reached peak state within 15 min, remained till 60 min, and down regulated by 90 min of exposure to type II collagen (Fig. 5). Combined with the above data, it is likely that type II collagen induces the re-expression of cartilaginous makers through the activation of ERK1/2 and JNK signalings in chondrocytes after extensive monolayer expansion.

The effects of MAPK inhibitors and integrin blocking peptide on type II collagen-induced GAG expression

To further clarify the possible downstream signaling pathways activated by type II collagen, GFOGER integrin blocking peptide (Knight et al., 2000; Xu et al., 2000), MEK, p38 or JNK inhibitors

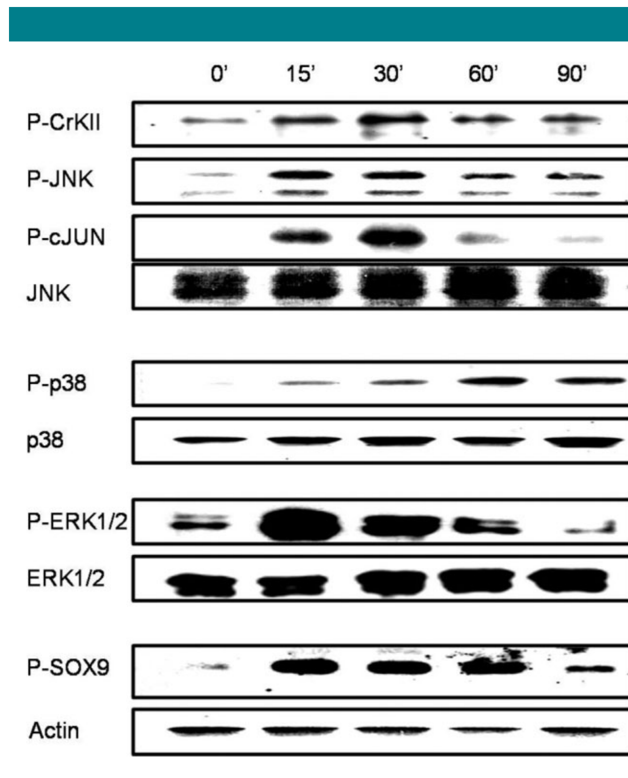

Fig. 5. Activation of FAK, JNK and ERK signaling in P7 chondrocytes by type II collagen treatment: P7 chondrocytes treated with 200 µg/ml type II collagen were collected at designated time points up to 90 min. FAK was sequentially phosphorylated at Tyr-397, Tyr-576/577, and Tyr-925. The phosphorylation of CrkII and JNK were up-regulated at 15 and 30 min, while the phosphorylation of c-JUN was elevated at 15 min peaked at 30 min. An up-regulation of ERK1/2 phosphorylation was observed at 15 min and gradually decreased from 30 min on. The phosphorylation of SOX9 was also elevated within 15 min, maintained till 60 min, and then down-regulated by 90 min post-treatment with type II collagen.

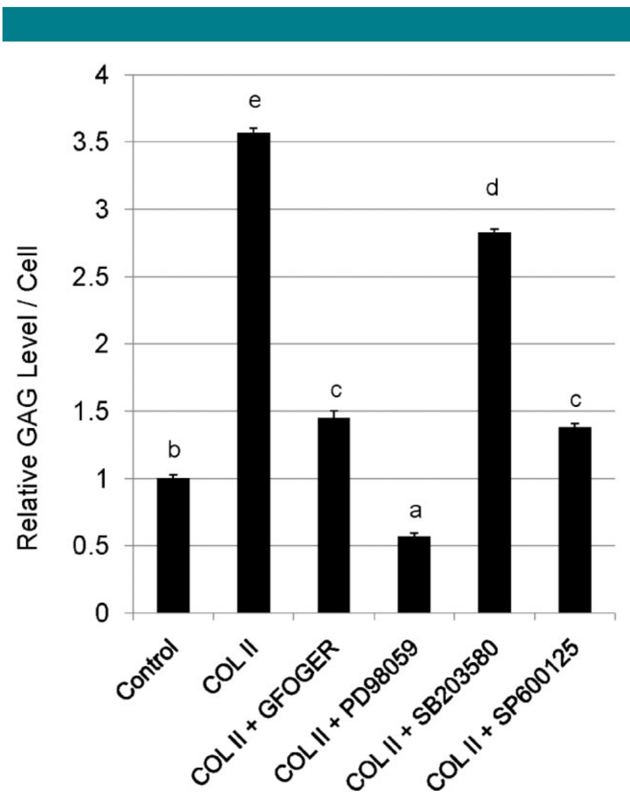

Fig. 6. The effects of various MAPK inhibitors and integrin blocking peptide on GAG level of human chondrocytes: Human P7 chondrocytes were cultured with or without type II collagen in the presence of GFOGER integrin blocking peptide, MEK inhibitor, p38 inhibitor, or JNK inhibitor for 12 days. Control: P7 chondrocytes cultured without type II collagen; COL II: P7 chondrocytes cultured with 200 µg/ml type II collagen; COL II + GFOGER: P7 chondrocytes cultured in the presence of 200 µg/ml type II collagen and 20 µg/ml of GFOGER integrin blocking peptide; COL II + PD98059: P7 chondrocytes cultured in the presence of 200 µg/ml type II collagen and 10 µM of PD98059; COL II + SB203580: P7 chondrocytes cultured in the presence of 200 µg/ml type II collagen and 10 µM of SB203580; COL II + SP600125: P7 chondrocytes cultured in the presence of 200 µg/ml type II collagen and 10 µM of SP600125. The statistical significance of each group was analyzed by one-way ANOVA and Duncan's multiple range test. The data represent means ± SD (n = 3).

were employed to examine their effects on the type II collagen-induced GAG accumulation in P7 chondrocytes. Cells were cultured with either control solution (5 mM acetic acid), GFOGER (20 µg/ml), 200 µg/ml type II collagen, or 200 µg/ml type II collagen in the presence of MEK inhibitor (PD98059, 10 µM) JNK inhibitor (SP600125, 10 µM), or p38 inhibitor (SB203580, 10 µM). After 12 days of culture, cells were fixed and evaluated for GAG deposition. The data show that type II collagen stimulated GAG level up to 3.5-fold over the control group. Both GFOGER peptide and JNK inhibitors, especially MEK inhibitor (down to 55% of control), greatly suppressed the type II collagen-elevated GAG deposition level in the P7 chondrocytes. However, p38 inhibitor only reduced GAG deposition by 20% (Fig. 6). These data suggest that the effect of type II collagen on P7 chondrocytes is mediated through integrins. Mainly ERK1/2, and partially JNK signaling pathways are also involved in the type II collagen-induced increase in GAG deposition. Together with the data stated above, it is concluded that the type II collagen-induced GAG accumulation in P7 chondrocytes were majorly mediated through both integrin-related FAK-ERK1/2 and FAK-JNK signalings.

P7 chondrocytes in 3D constructs fabricated with collagen

The effects of type II collagen matrix on the cartilaginous phenotype expressions of P7 chondrocytes were also examined in a 3D culture condition. Figure 7 shows the HE staining (a–d) and Safranin O staining (e,f) of 14 day-cultured P7 chondrocytes embedded in 3D collagen constructs. More fibrous ECM structures were observed in the chondrocyte-type II collagen construct groups than those in type I collagen construct. In the presence of growth factor, the P7 chondrocytes in type II collagen constructs showed a more mature cartilaginous feature as compared with those cells in the type I collagen construct. A rounder, clustered chondrocyte phenotype was observed in the section of P7 chondrocyte-type II collagen constructs. More clustering cells with lacunae-like structure (arrows), which represent the secreted cartilaginous matrices by the embedded chondrocytes, were observed in the 3D construct made of type II collagen than in those of type I collagen constructs. Furthermore, Safranin O staining revealed much more and thicker proteoglycan fibers in the sliced section of P7 chondrocyte-type II collagen construct than in that of the type I construct. These data indicate that significant richer proteoglycan secretion and matrices deposition occurred in P7 chondrocytes embedded in type II collagen than those in the type I collagen. These results suggest that, in 3D culture condition, the combination of type II collagen with TGF-β_1 and IGF-I induces a synergistic effect on phenotype re-expression in the senescent chondrocytes.

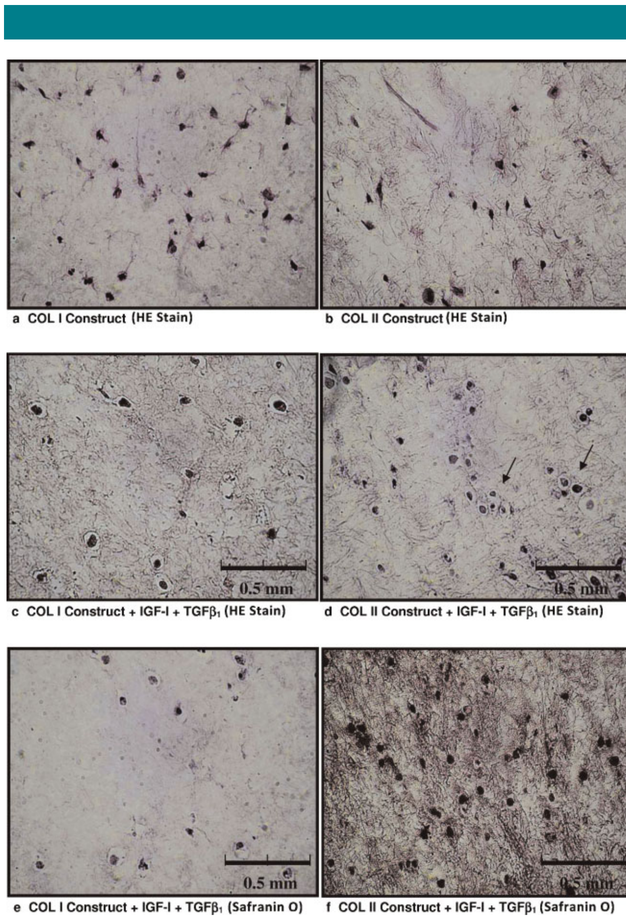

Fig. 7. Hematoxylin–eosin (a–d) and Safranin O (e,f) stained sections of three-dimensional culture of P7 chondrocyte-collagen constructs: P7 chondrocytes were cultured in three-dimensional collagen gel for 14 days with or without growth factor supplement (10 ng/ml TGF-β_1 and 100 ng/ml IGF-I). To compare with the cartilaginous effect of type II collagen (COL II) in 3D culture, type I collagen (COL I) from rat tail tendon was used to fabricate the chondrocyte-type I collagen constructs. The chondrocytes in either type I or type II collagen constructs cultured with growth factors (c,d) showed a much rounded cell shape in morphology as compared to these cells in constructs cultured without growth factor supplement (a,b). Comparing the histological feature of chondrocytes in type II collagen fabricated constructs with those in type I collagen constructs cultured in the presence of growth factors (c,d), more lacunae-like cartilaginous feature and clustering chondrocytes (arrows) were found in the sliced section of type II collagen fabricated group. Further comparison of the proteoglycan accumulation in these growth factor treated groups (e,f), Safranin O staining showed stronger and thicker red-brown fibers of cartilaginous proteoglycan deposition in type II collagen fabricated group (f) than in the type I collagen fabricated group (e).

Discussion

As reported by Benya et al. (1978) and Benya and Shaffer (1982), after a long period of serial expansion in monolayer cultivation, chondrocytes in our culture condition also rapidly lost their phenotype as characterized by decreased capacity of biosynthesis and deposition of cartilage-specific extracellular matrix, such as type II collagen and proteoglycans. The collagen typing of the serially passaged progenies also shift from type II to type I collagen during the course from rapid proliferation to quiescence. In this study, the serially expanded chondrocytes indeed lost their original round or polygonal appearance and become fibroblast-like cells, and gradually become fully extended, flattened on the substratum during monolayer expansion. In addition, less uniform aggrecan as well as functional linking proteins, and altered responsiveness to anabolic growth factors, such as TGF-β1 and IGF-I (Martin et al., 1997; Martin and Buckwalter, 2000, 2003), were also reported in these cells. This kind of chondrocytes with reduced functions and altered morphology were referred as "dedifferentiated" since it no longer possesses the general phenotypic features of freshly harvested chondrocytes (Benya et al., 1978; Benya and Shaffer, 1982). Numerous factors, including the seeding density, culture medium, and age of the cell donor affect the extent and rate of this process (Watt, 1988; Lefebvre et al., 1990).

To understand the effects of different ECM components on serially expanded human chondrocytes, we treated the P7 cells with various ECM components. Only type II collagen among the tested ECM molecules exhibited an effective cartilaginous-restoring effect on serially passaged chondrocytes. After treated with different concentrations of exogenous type II collagen, the serially passaged P7 human chondrocytes showed increased expression levels of cartilaginous mRNAs and GAG deposition. On the contrary, CS and HA either showed no effect on or inhibited, respectively, the gene expression of *SOX9* and *AGN*. HA has been shown to affect cell migration and adhesion (Shiedlin et al., 2004). Bansal et al. (1986) showed that rat chondrocytes in suspension culture responded to high concentrations of exogenous HA by reducing proteoglycan synthesis. In this study, HA inhibited *AGN* and *SOX9* mRNA expression in serially passaged P7 chondrocytes. Furthermore, CS is also a component of glycosaminoglycan. In our system, the inclusion of CS did not affect *AGN* and *SOX9* mRNA expression in P7 chondrocytes. Therefore, the detail function of HA and CS on chondrogenic phenotype requires further study.

Type II collagen has been reported to modulate chondrocyte specific gene expression at transcriptional level and subsequent changes in its internal status, display of surface receptors, and extracellular matrix (ECM) molecules (Qi and Scully, 2003; Schneiderbauer et al., 2004). Moreover, matrix deteriorations, such as reduced type II collagen accumulation and increased metalloproteinase activity, are often associated with the pathological state of the cartilage (Martin and Buckwalter, 2000, 2003). It has also been addressed that direct type II collagen stimuli increased type II collagen transcription activity in chondrosarcoma cell line (Schneiderbauer et al., 2004). This study further demonstrated that exogenous type II collagen alone is enough to restore the re-expression of type II collagen, aggrecan and SOX9 mRNAs in serially expanded, dedifferentiated human chondrocytes which have lost their cartilaginous feature. Furthermore, the exogenous type II collagen treatment induced the phosphorylation of FAK, MAPK and SOX9 in P7 chondrocytes. This activation of FAK by direct type II collagen stimuli may cause the down-stream gene activation through either JNK or ERK1/2 signaling. Our study showed the activation of ERK1/2 and JNK by 15 min of type II collagen treatment. It has been reported that phosphorylated ERK1/2 physically interacts with SOX9, and IGF-I promoted such molecular interaction (Shakibaei et al., 2006). The reported direct interaction between phosphorylated ERK1/2 and SOX9 and our data suggest a possible mechanism that linking integrin-FAK-ERK signaling axis to SOX9 activation. In addition, the involvement of MAPK signalings in the effect of type II collagen was evaluated by using specific inhibitors and integrin blocking peptide. Type II collagen treatment significantly enhances the GAG deposition of P7 chondrocytes. GFOGER integrin blocking peptide, MEK inhibitor (PD98059) and JNK inhibitor (SP600125), not p38 inhibitor (SB203580), significantly reduced the type II collagen-induced GAG deposition level. All together, it is concluded that the effect of type II collagen on P7 chondrocytes is integrin-dependant, and mainly mediated through FAK-ERK1/2 and FAK-JNK signaling pathways.

Many investigators have reported that culturing chondrocytes in a three-dimensional (3D) environment, such as in gels of agarose, collagen or alginate (Benya and Shaffer, 1982; Thenet et al., 1992; Bonaventure et al., 1994; Lemare et al., 1998), maintains a better expression of cartilaginous marker mRNAs. These preserved cellular properties in 3D culture condition are probably resulted from the outside–in signaling which is capable of retaining the chondrocyte functions by expression normal phenotypic features.

IGF-I was reported to extend the chondrogenic potential of primary human chondrocytes through promoting the molecular interaction between ERK1/2 and SOX9 (Shakibaei et al., 2006). The combination of type II collagen with TGF-β_1 led to a synergistic increase in the phosphorylation of Smad 2 and 3 (Schneiderbauer et al., 2004). However, in the extensively expanded chondrocytes, altered responsiveness to anabolic growth factors, such as TGF-β_1 and IGF-I were observed (Martin et al., 1997; Martin and Buckwalter, 2003). In this study, the coupling effects of TGF-β_1 and IGF-I in addition to type II collagen on restoring chondrocyte phenotypic features in 3D condition were performed. The combination of these growth factors with type II collagen synergistically restored the expression of cartilage-specific GAG deposition in the serially expanded P7 chondrocytes. The observation to some extent resembles that reported by other investigators in their studies on the freshly isolated chondrocytes (Yaeger et al., 1997; Qi and Scully, 2003).

Type II collagen is the most abundant extracellular molecule in the cartilage and plays important roles in cartilaginous basic framework. Our study demonstrate that exogenous type II collagen alone appears sufficient to initiate the re-expression of cartilaginous features of the serially expanded P7 chondrocytes both in monolayer and 3D culture condition. Furthermore, the direct activation of MAPK signaling in P7 chondrocytes by type II collagen implies that the possible downstream signaling axis following integrin-FAK-MAPK activation may be responsible for the re-expression of cartilaginous features in the P7 chondrocytes.

Conclusion

This study demonstrates that exogenous type II collagen alone is sufficient to restore the re-expression of *SOX9*, *COL2A1*, and *AGN* mRNAs in the serially expanded P7 human chondrocytes. The study provides evidence that type II collagen alone can promptly induce FAK-ERK phosphorylation in these cells. Thus, in turn, restores/maintains the phenotypic expression of chondrocytes. The intimate ECM-cell interaction between type II collagen and chondrocytes and the essential proper microenvironment for residing chondrocytes play a pivotal role in maintaining or restoring the integrity and health of the cartilage.

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