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Type I and II collagen regulation of chondrogenic differentiation by mesenchymal progenitor cells

C.W. Chen [a,1], Y.H. Tsai [b,c,1], W.P. Deng [a], S.N. Shih [e], C.L. Fang [d], J.G. Burch [f], W.H. Chen [a], W.F. Lai [a,b,c,*]

[a] *Institute of Medical Sciences, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan*
[b] *Institute of Biomedical Materials, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan*
[c] *Institute of Cellular and Molecular Biology, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan*
[d] *Department of Pathology, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan*
[e] *Orthopedic Department, Chang-Gung Memorial Hospital, Taoyuan 333, Taiwan*
[f] *Department of Orthodontics, School of Dental Medicine, Nova Southeastern University, Ft. Lauderdale, FL 33328, USA*

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Abstract

Chondrogenic differentiation by mesenchymal progenitor cells (MPCs) is associated with cytokines such as transforming growth factor-beta 1 (TGF-β1) and dexamethasone. Extracellular matrix (ECM) also regulates the differentiation by MPCs. To define whether ECM plays a functional role in regulation of the chondrogenic differentiation by MPCs, an in vitro model was used. That model exposed to dexamethasone, recombinant human TGF-β1(*rh*TGF-β1) and collagens. The results showed that MPCs incorporated with dexamethasone and *rh*TGF-β1 increased proliferation and expression of glycosaminoglycan (GAG) after 14 days. Type II collagen enhanced the GAG synthesis, but did not increase alkaline phosphatase (ALP) activity. When adding dexamethasone and *rh*TGF-β1 MPCs increased mRNA expression of Sox9. Incorporation with type II collagen, dexamethasone and *rh*TGF-β1, MPCs induced mRNA expression of aggrecan and enhanced levels of type II collagen, and Sox9 mRNA. In contrast, incorporation with type I collagen, dexamethasone and *rh*TGF-β1 MPCs reduced levels of aggrecan, and Sox9 mRNA, and showed no type II collagen mRNA. Altogether, these results indicate that type I and II collagen, in addition to the cytokine effect, may play a functional role in regulating of chondrogenic differentiation by MPCs.

Keywords: Chondrogenic differentiation; Collagen; Dexamethasone; Extracellular matrix; Mesenchymal progenitor cells, Sox9; TGF-β1

Introduction

Cartilage has little capacity for self-repair [10]. An injured joint predisposed to continued arthritic degeneration [8]. Current therapies for cartilage regeneration include placement of carbon plugs [5], periosteum [14], or periochondrium [15]. Autologous chondrocyte transplantation [6,31], and subchondral drilling [4,16,26]. Success rates vary. Most methods of therapy have serious clinical limitations.

Structural, chemical, and mechanical properties of regenerated cartilage are definitely not normal [43]. The regenerated tissue does not bond to adjacent tissue [38]. New cartilage undergoes degenerative changes. After one year this new cartilage is similar to that in untreated defects [25]. Thus, most repair methods fail

* Corresponding author. Tel.: +886 2 87800225/27361661x5210; fax: +886 2 27357714.
E-mail address: laitw@tmu.edu.tw (W.F. Lai).
[1] C.W. Chen and Y.H. Tsai contributed equally to this paper.

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to improve cartilage beyond what is found in natural repair of untreated osteochondral defects.

The clinical need for improved treatment options for patients with cartilage injuries has encouraged scientists to investigate in vivo implantation of isolated MPCs. MPCs can undergo differentiation to regenerate either cartilage [13] or bone [7,44] in defects. Implantation of MPCs has been demonstrated to affect tissue regeneration. Then different environmental factors can be introduced to trigger differentiation into specific phenotypes.

Chondrogenic differentiation has been induced in vitro by cytokines TGF-β1 and dexamethasone in MPCs derived from bone marrow of human [46] or animal models [19,27,45]. ECM has been demonstrated to regulate the chondrogenic or osteogenic differentiation induced by MPCs [18,29]. How the mechanisms of ECM affect MPC differentiation are still not well understood. This study was undertaken to further identify how collagen regulated *rh*TGF-β1's and dexamethasone's effects on chondrogenic differentiation. Differential expression of MPC-induced glycosaminoglycan (GAG) and alkaline phosphatase (ALP) exposed to different concentrations of dexamethasone, *rh*TGF-β1, and collagens was measured by spectrophotometry. The levels of mRNA expression of Sox9, aggrecan, and type II collagen were found to be identifiers of certain mechanisms of chondrogenic differentiation by MPCs.

Materials and methods

Subjects

Consenting bone marrow donors were selected from patients admitted to the Orthopedic Section of Taipei Municipal Chung-Hsin Hospital, Taipei, Taiwan. None had endocrine disease or was receiving hormone replacement therapy. Bone marrow was obtained from femur fracture site by proximal femur aspiration during surgical treatment procedures.

Isolation and cultivation of MPCs

MPCs were isolated from rabbit and human bone marrow. They were mixed with sodium-heparin, and diluted with equal five volumes of PBS. The cell suspension was fractionated on a Percoll gradient (40% initial density, Phamacia). The MPC-enriched interface fraction was collected and cultured in Dulbecco's modified Eagle medium with 1 g/ml glucose (DMEM/LG, Sigma D5523), 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone under normal conditions. The medium was changed every four days. During the primary culture, adherent cells formed colonies that were passaged when cells proliferated subconfluently. Second- and third-passage cells were selected to identify the mechanisms of differentiation.

mRNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA harvested from subconfluent monolayer cultures (approximately10^6 MPCs) was extracted using TRIzol® Reagent (Invitrogen life technologies, Carlsbad, CA). Extracted RNA was dissolved in sterilized ddH_2O and stored at −80 °C. Reverse transcription was performed with SuperScript™ III (Invitrogen life technologies) and Oligo $d(T)_{12-18}$ primer. Four micrograms of RNA was added into a final solution of 21 μl 10 mM dNTP mix, 10 X RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, RNase Inhibitor and RNase H. Six μg of RT products were used in PCR amplification in a final 50 μl solution containing 2.5 mM dNTP, 25 mM $MgCl_2$, upstream/downstream primers and *Taq* DNA polymerase (Invitrogen life technologies). Following an initial denaturation at 95 °C for 5 min, the DNA was amplified in the Touchgene Gradient (TECHINE, UK). Thirty-five cycles of 1 min at 94 °C were applied for denaturation and 1 min at 72 °C for extension. A final extension was applied at 72 °C for 5 min. PCR products were visualized on 1% agarose gel (Agarose I, AMRESCO, Ohio) and stained with EtBr. PCR products were confirmed by size verification and analyzed using FloGel-I (Fluorescent Gel Image System, TOPBIO). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Primer sets were following:

(1) Sox9-5′-GGCAGCTGTGAACTGGCCA-3′ (sense primer) and 5′-GCACACGGGGAACTTGTCC-3′ (antisense primer) which gives a 408 bp product;
(2) COL2A1-5′-CACGCAGAAGTTCACCAAGAA-3′ (sense primer) and 5′-CTGCAGGATCAGCCATGGTAGA-3′ (antisense primer) which gives a 501 bp products;
(3) Aggracan-5′-TGAGGAGGGCTGGAACAAGTACC-3′ (sense primer) and 5′-GGAGGTGGTAATTGCAGGGAACA-3′ (antisense primer) which gives 466 bp product;
(4) GADPH-5′-GCTCTCCAGAACATCATCCCTGCC-3′ (sense primer) and 5′-CGTTGTCATACCAGGAAATGAGCTT-3′ (antisense primer) which gives 346 bp product.

Preparation of type I and II collagens

Type I and II collagens were prepared as previously described in this laboratory [20,21]. Collagen was heat-denatured at 95 °C for 30 min, and analyzed using SDS-PAGE. After breaking the peptide chain of α-subunits, the triple-helical structure of collagen was denatured.

Analysis of chondrogenic differentiation in monolayer culture

Rabbit and human MPCs were seeded into six-well plates with 10% FBS and 50 μg/ml ascorbic acid in DMEM/LG. After four days of cultivation, the medium of rabbit MPCs was changed with dexamethasone (10^{-7} M, Sigma D2915, St. Louis), *rh*TGF-β1 (0.1, 1, and 10 ng/ml, R & D Systems 240-B, Minneapolis, MN), and collagen (10, and 100 μg/ml). Six wells were employed for each condition. Differentiation was analyzed by GAG synthesis and ALP activity after 14 days. GAG synthesis and ALP activity were measured using spectrophotometry of the absorption of OD_{600} and OD_{405} respectively after alcian-blue staining [1]. The medium of human MPCs, after four days of cultivation, was changed with dexamethasone (10^{-7} M), *rh*TGF-β1 (1 ng/ml), and collagen (100 μg/ml). Three wells were employed for each condition. The mRNA for aggrecan, type II collagen, and Sox9 was analyzed using RT-PCR as described above.

Structural effects of collagen on chondrogenic differentiation by MPCs

To further identify the regulatory effect of collagen's structure on chondrogenic differentiation, native and denatured collagens were tested in cell cultivation. Each collagen solution (2 mg/ml) was heated at 95 °C for 30 min to denature the peptide chains of the collagen subunits. Degradation of collagen and the triple-helical structure was analyzed using 5% SDS-PAGE. Native or denatured collagen was individually added to the medium at a concentration of 100 μg/ml each. The expression of GAG by MPCs in each condition was measured by alcian-blue assay.

Statistical analysis

Expressions of each GAG and ALP from chondrocyte cultures exposed to different concentrations of dexamethasone, *rh*TGF-β1, and collagens were analyzed using Mann–Whitney test. Dexamethasone-,

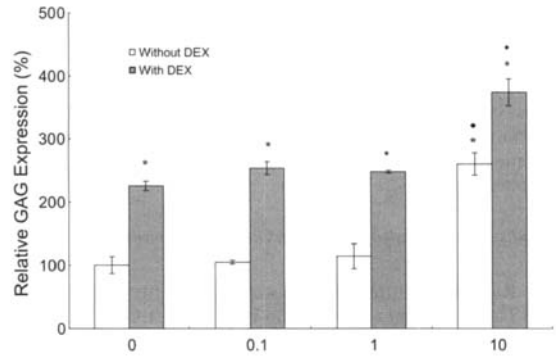
*rh*TGF-β1-1, and collagen-treated and untreated control cultures at different concentrations were also compared. Data are reported as the mean ± SD. $P < 0.05$ was considered statistically significant.

Results

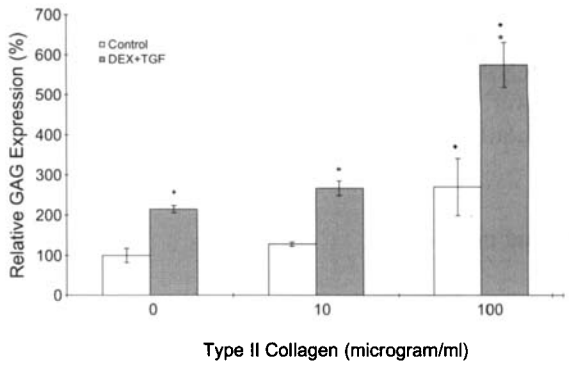
*Type I and II collagen regulated **rh**TGF-β1 and dexamethasone effects on GAG expression of rabbit MPCs*

Significantly increased GAG expression was found in the culture supplemented with 10 ng/ml *rh*TGF-β1. GAG expression was 2.6 ± 0.2 times greater than those of untreated cells. However, GAG synthesis did not increase with 0.1 and 1 ng/ml concentrations of *rh*TGF-β1. Treatment with 10^{-7} M dexamethasone alone increased GAG expression 2.25 ± 0.18 times in rabbit MPCs compared to the untreated group. The increased rate of GAG expression remained the same as in the untreated group after adding 0.1 and 1 ng/ml concentrations of *rh*TGF-β1. A synergistic effect was found when incorporated with both 10 ng/ml *rh*TGF-β1 and 10^{-7} M dexamethasone. The maximal GAG expression was 3.75 ± 0.32 times higher than that of control cells (Fig. 1(A)).

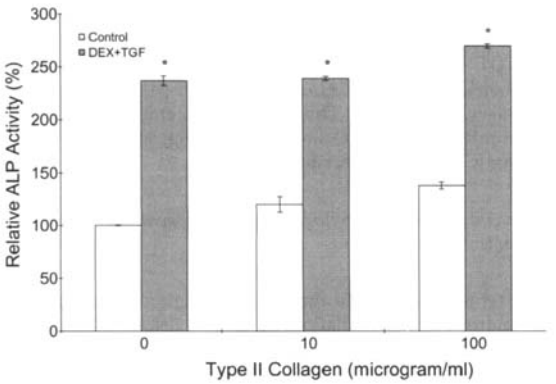
Type II collagen alone at the concentration of 10 μg/ml did not significantly change GAG expression, whereas at the concentration of 100 μg/ml, GAG expression increased 2.5 times. Synergistic increases in GAG expression by MPCs to be up to 5.8 times when adding 100 μg/ml type II collagen (Fig. 1(B)). When adding either 10 μg/ml or 100 μg/ml concentration of type II collagen (10 or 100 μg/ml), the osteogenic marker ALP activity of MPCs did not significantly increase. Increases in ALP activity were only 1.2 and 1.4 times, respectively, higher than the control. With treatment of 10 ng/ml TGF-β1 and 10^{-7} M dexamethasone, ALP expression increased 2.4 times. Further addition of type



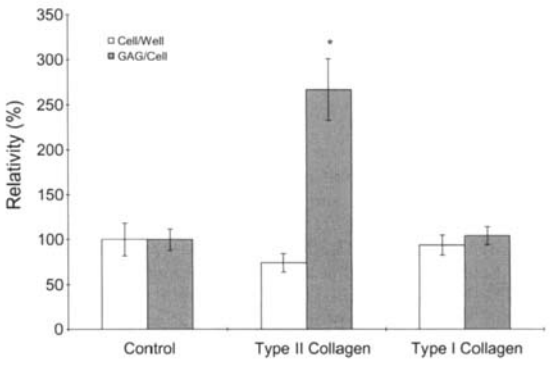
(A) GAG, glycosaminoglycan; DEX, dexamethasone; TGF, *rh*TGF-β1



(B) GAG, glycosaminoglycan; DEX, dexamethasone; TGF, *rh*TGF-β1



(C) ALP, alkaline phosphatase; DEX, dexamethasone; TGF, *rh*TGF-β1



(D) GAG, glycosaminoglycan

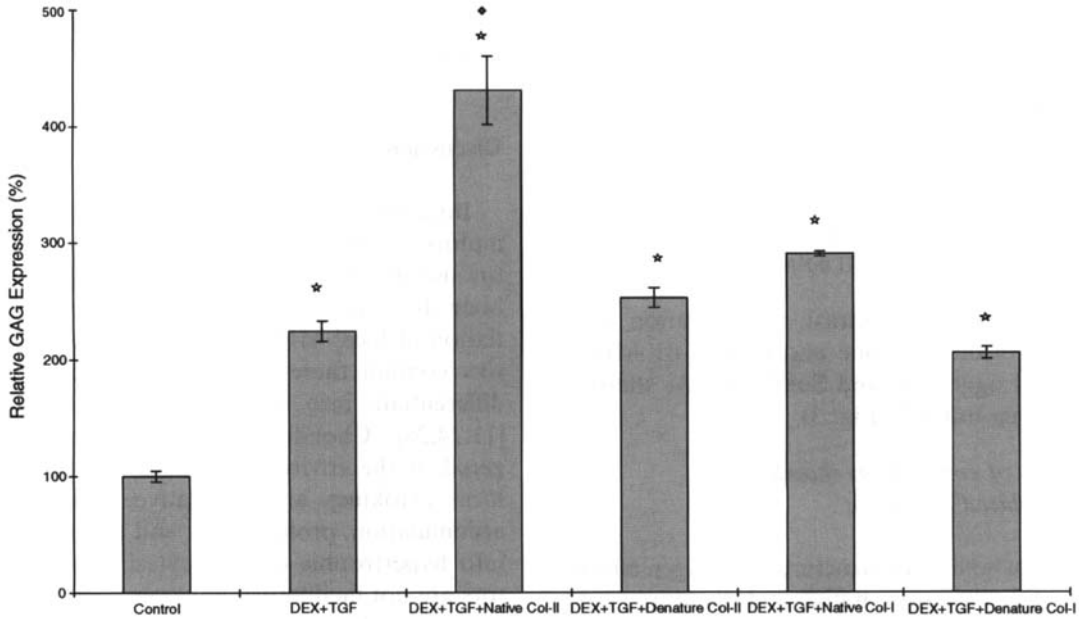
Fig. 1. GAG and ALP expression of MPCs exposed to varying concentrations of *rh*TGF-β1, DEX, and collagen, measured by spectrophotometry. Means ± S.D; ($n = 6$). (A) TGF-β1 and dexamethasone induced an increase in GAG by MPCs. An asterisk indicates a significant ($p < 0.05$) difference in DEX compared to the untreated control. A diamond indicates a significant ($p < 0.05$) difference among different TGF-β1 concentrations. (B) Type II collagen up regulated GAG synthesis with and without dexamethasone. An asterisk indicates a significant ($p < 0.05$) difference in DEX compared to the untreated control. A diamond indicates a significant ($p < 0.05$) difference among different type II collagen concentrations. (C) Type II collagen, in contrast to dexamethasone, did not significantly increase ALP activity by mesenchymal progenitor cells. An asterisk indicates a significant ($p < 0.05$) difference in TGF-β1 and DEX compared to the untreated control. (D) Only type II collagen significantly increased GAG synthesis, but not cell proliferation, whereas type I collagen did not change GAG synthesis or cell proliferation. An asterisk indicates a significant ($p < 0.05$) difference in type II collagen compared to type I collagen and the untreated control.

II collagen ALP caused the same increased expression of 2.4 times (at 10 μg/ml concentration) and 2.7 times (at 100 μg/ml concentration). Thus, type II collagen did not significantly regulate the ALP activity of MPCs with or without TGF-β1 and dexamethasone (Fig. 1(C)).

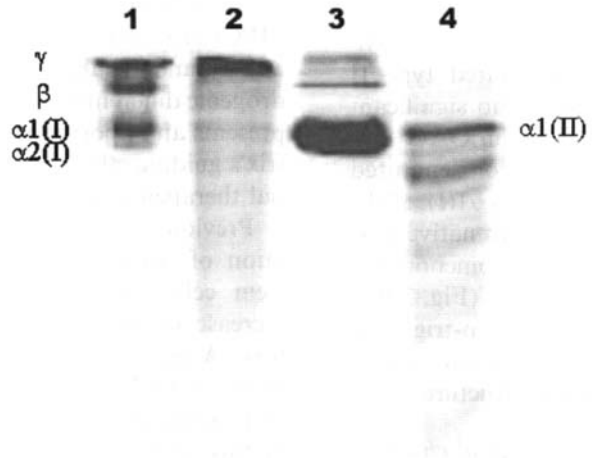
Incorporated with 100 μg/ml of type II collagen, the population of MPCs was reduced by 40%. However, GAG expression increased 2.5 times. In contrast, with the addition of 100 μg/ml of type I collagen, the population and GAG expression of MPCs appeared to not significantly change (Fig. 1(D)).

Type I and II collagen regulated rhTGF-β1 and dexamethasone effects on aggrecan, Sox9 and collagen mRNA expression

RT-PCR using RNA obtained from human MPCs showed no signal for aggrecan, type II collagen, and Sox9. An mRNA expression of type II collagen and Sox9 was induced when treated with dexamethasone. With dexamethasone and *rh*TGF-β1 MPCs increased mRNA expression of type II collagen and Sox9. In addition to dexamethasone and *rh*TGF-β1, type II collagen induced mRNA expression of aggrecan and enhanced



(A) GAG, glycosaminoglycan; DEX, dexamethasone; TGF, *rh*TGF-β1



(B)

Fig. 2. Structural effects of collagen on chondrogenic differentiation by MPCs. (A) Native type II collagen remarkably increased 4.3 times GAG synthesis, the increase of GAG was reduced to 2.9 times with the denatured type II collagen. Noted no significant difference of GAG synthesis among groups of native type I collagen, denatured type I and II collagens, and dexamethasone and *rh*TGF-β1 treated. (B) SDS-polyacrylamide gel electrophoresis of native and denatured collagens. Note the clearly identifiable bands of type I native collagen in lane 1 and of type II collagen in lane 3. The denatured type I collagen showed a smear phenomenon in lane 2, and small fragments of denatured type II collagen was showed in lane 4.

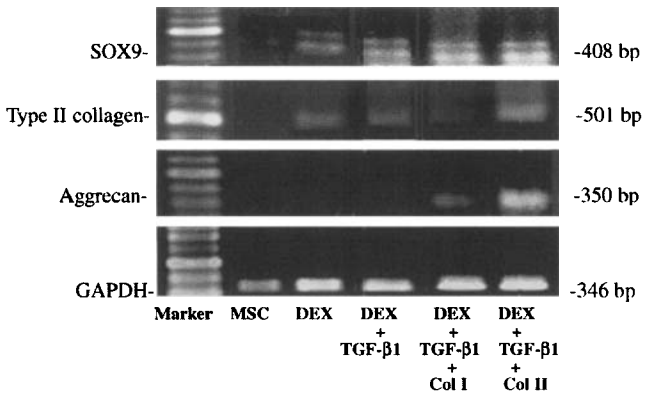


Fig. 3. Type I and II collagens regulated *rh*TGF-β1 and dexamethasone effects on aggrecan, Sox9 and collagen mRNA expression. Total RNA was isolated from MPCs and used for PT-PCR to detect expression of type II collagen and aggregan. Expression of GADPH was used as control. Lane 1: DNA standards; lane 2: MPCs showed no signal for aggrecan, type II collagen, and Sox9; lane 3: With dexamethasone MPCs induced mRNA expression of type II collagen and Sox9; lane 4: With dexamethasone and *rh*TGF-β1 MPCs increased mRNA expression of type II collagen and Sox9; lane 5: Cultured with type I collagen, dexamethasone and *rh*TGF-β1 MPCs showed no type II collagen mRNA; lane 6: Cultured with type II collagen, dexamethasone and *rh*TGF-β1 MPCs induced mRNA expression of aggrecan and enhanced levels of Sox9 and type II mRNA.

levels of Sox9 mRNA. In contrast, incorporation with type I collagen, dexamethasone and *rh*TGF-β1 MPCs reduced levels of aggrecan, and Sox9 mRNA, showed no type II collagen mRNA (Fig. 3).

Structural effects of collagen on chondrogenic differentiation induced by MPCs

To determine whether the structure of collagen affects chondrogenic differentiation, native and denatured collagens were introduced to MPCs. With pretreatment using *rh*TGF-β1 and dexamethasone, MPCs increased GAG synthesis 4.3 times after adding native type II collagen. The enhancement of GAG synthesis was downgraded to 2.9-fold increase, when denatured type II collagen was added instead. In contrast, no significant difference was noted between the increase in GAG synthesis in the presence of native (2.5 times) or denatured type I collagen (2.1 times) (Fig. 2(A)). Intact α1(I), α2(I), and α1(II) bands were clearly identified with native type 1 and II collagens. Contrarily, a smear phenomenon was noted with denatured type I and II collagens (Fig. 2(B)). These results indicate that type II collagen-triggered chondrogenic differentiation is type-specific, and correlates with collagen's native triple-helical structure.

Type II and I collagen effects on morphological changes in MPCs treated with dexamethasone and rhTGF-β1 in monolayer cultures

MPC cultures of all six wells showed a phenomenon of contact inhibition with fibroblast-like morphology after 14 days (Fig. 4(A)). A mild pile-up was noted in MPCs of all six wells when supplemented with *rh*TGF-1 and dexamethasone (Fig. 4(D)). In all cultures incorporating type I collagen, cells showed a shift to elongated spindle-shaped fibroblastic appearance independent of the absence or presence of TGF-β1 and dexamethasone (Fig. 4(B) and (E)).

Incorporated with type II collagen, MPCs of all six wells showed more cuboidal-, and less spindle-shaped morphology (Fig. 4(C)). Semi-transparent fibrous like substance was noted on cells. Cells predominantly appeared rectangular after pretreatment with type II collagen, dexamethansone and *rh*TGF-β1. Cell-collagen matrix aggregates were found in four of six wells (Fig. 4(F)). Abundant GAG, which found in the cartilage-like cell-collagen matrix aggregate was then identified by alcian-blue staining (Fig. 4(G)).

Discussion

Bone marrow-derived MPCs are highly proliferative, multipotential cells that have been considered ideal cells for use in repair of injured cartilage and fractures of bone. It is known that repair tissue arises from differentiation of local MPCs. Both periosteum and bone marrow contain these cells, which preserve the ability to differentiate into both chondrocytes and osteoblasts [11,24,28]. Chondrogenic differentiation can be triggered, if the environmental factors such as ECM and local cytokines are facilitative. Local MPCs enable accumulation, proliferation, and terminal differentiation into hypertrophic chondrocytes. If environmental factors are not facilitative; however, local MPCs differentiate into fibrochondrocytes and form fibrocartilage [9,38].

In addition to the potential for multidifferentiation, MPCs are relatively easy to extract from bone marrow and expand in culture. Development of an in vitro chondrogenic differentiation model of marrow-derived MPCs presents an opportunity to explore the extracellular matrix's guiding effects on chondrogenesis that is of potential therapeutic utility.

Previous study showed that TGF-β1 induces proliferation of osteoblasts, chondrocytes, and mesenchymal stem cells [40]. The present study also showed an increase in the proliferation of mesenchymal progenitors. Additionally, other study have shown that synthesis of ECM was found to be enhanced by TGF-β1 [39]. Similarily, this study also showed a consistency in this regard, in that TGF-β1 increased synthesis of GAG in MPCs. TGF-β1 controls the morphology and differentiation of epithelial cells [47, 3,30]. The morphological changes in MPCs induced by TGF-β1 in this research were elongation and extension of cell shapes.

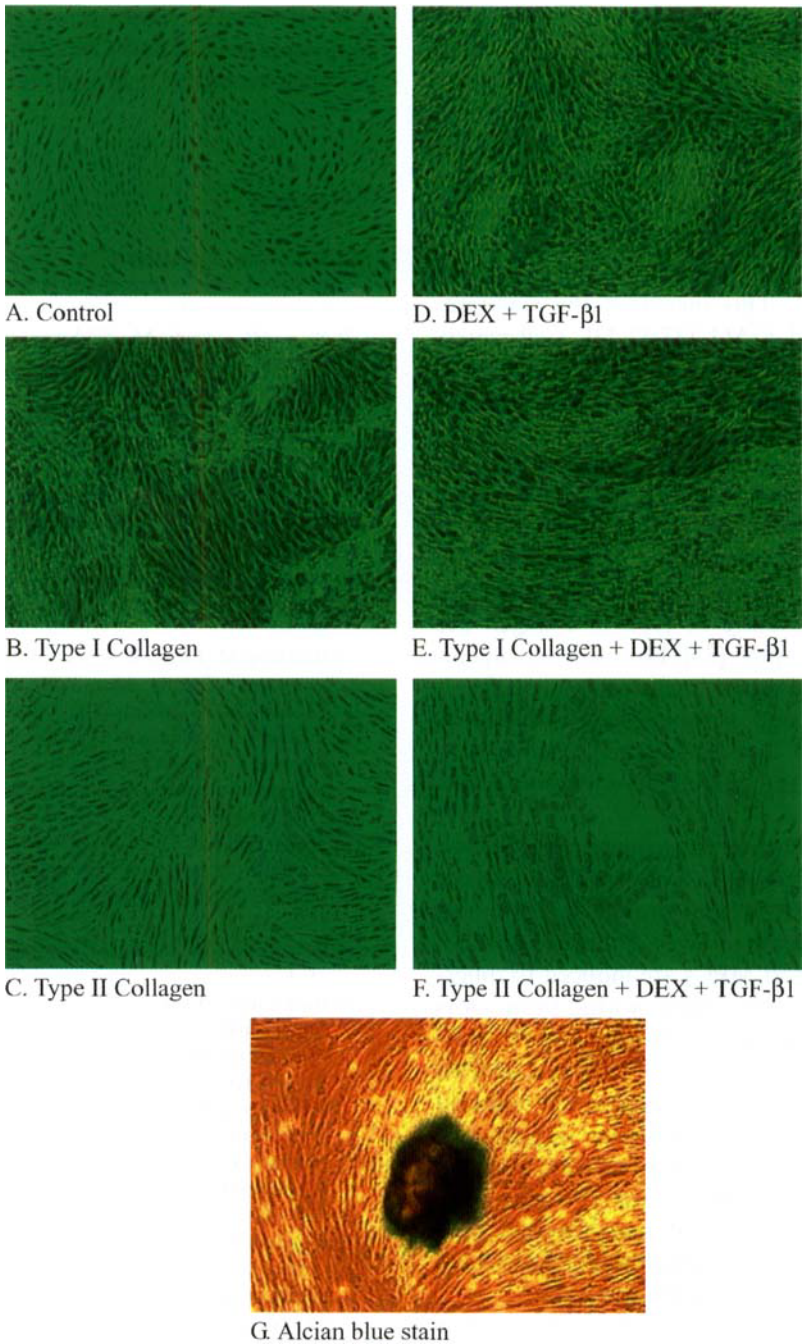


Fig. 4. Type I and II collagen effects on morphological changes in MPCs treated with dexamethasone and *rh*TGF-β1 in monolayer culture (100×). (A and D) MPCs appeared fibroblast-like morphology and mild pile-up 14 days after cultured with dexamethasone and TGF-β1. Incorporation with extracellular type I collagen cells showed an elongated spindle-shaped fibroblastic appearance in the absence (B) or presence (E) of *rh*TGF-β1 and dexamethasone. (C) Cells showed more cuboidal-, and less spindle-shaped morphology after pretreatment with type II collagen. (F) Cells appeared predominantly rectangular after pretreated with type II collagen in addition to dexamethansone, and *rh*TGF-β1. (G) Note abundant GAG in the cartilaginous-like cell-collagen matrix aggregate (alcian-blue staining).

Chondrogenesis of chondroprogenitors can be stimulated in the presence of the dexamethasone [12]. Chondrogenesis was induced by dexamethasone. That was revealed by increased GAG expression. There was a synergistic effect with TGF-β1. This study imply that dexamethasone may be the fundamental factor that triggers chondrogenic differentiation, whereas TGF-β1 further enhances the process of the synthesis of extracellular matrix. Levels of mRNA for type II collagen and Sox9 were also increased when treated with dexamethasone in this study. This was correlated with previous study that an enhancement of Sox mRNA by dexamethasone was showed in chondrocytes [36].

The regulatory effect of collagen seems to be correlated with its triple-helical structure. Our data showed that enhancement of GAG synthesis by native type II collagen (4.3 times) was significantly downgraded when using denatured type II collagen (2.9 times). These findings agree with previous studies that denatured type II collagen dimilished native type II collagen effect on chondrocyte regulation [33,41,42].

Exogenous type II collagen could maintain the phenotype of chondrocytes. This increased the syntheses of type II collagen and GAG [37,32,33]. Cell-matrix interactions via cell surface receptors transduce extracellular signals inwards to regulate the cell phenotype [34]. In this research by adding type II collagen the semi-transparent fibrous-like substance became noted on MPCs. This indicated the type II collagen receptor might be induced on cell surfaces. Integrin $\alpha 2\beta 1$, a major receptor for type II collagen, plays important roles during chondrogenic differentiation by MPCs [17,23]. Binding affinity and signaling of type II collagen are mediated by integrin receptor [35]; however, the signaling of specific domains of α or β integrins for type II collagen is not well understood. Further study will identify the specific receptor for type II collagen, their downstream signals, and how it regulates chondrogenic differentiation of MPCs.

Type I collagen, in contrast to cartilage-specific type II collagen, exists ubiquitously in bone, tendon, cornea, and skin and acts as a structural protein in mammals [22]. During cartilage repair of large osteochondral defects, bone marrow-derived MPCs are induced to differentiate into fibroblasts or osteoblasts within fibrin clots containing fibronectin and type I collagen [2,38]. Consistent with previous research, type I collagen treated MPCs did not increase GAG expression, but induced an elongated spindle-shaped fibroblastic appearance. Therefore, type I collagen matrix may provide the suitable microenvironment, which is better for fibrogenic differentiation. This matrix-guided mesenchymal progenitor cell differentiation in situ predominantly induces regeneration towards fibro-cartilage formation.

The current study substantiates the concept that chondrogenic differentiation by MPCs can be synergistically triggered by cytokines and further regulated by the ECM. A comprehensive understanding of the regulation of chondrogenic differentiation by MPCs may enable the intentionally engineering of cartilage development in vitro. Subsequent results may yield further information for the purpose of a direct repair of large cartilage defects.

Acknowledgments

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