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Research Article

The Constitutive Expression of Type X Collagen in Mesenchymal Stem Cells from Osteoarthritis Patients Is Reproduced in a Rabbit Model of Osteoarthritis

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The expression of type X collagen (COL X), a late-stage chondrocyte hypertrophy marker in human mesenchymal stem cells (MSCs) from osteoarthritis (OA) patients poses a major setback to current cartilage and intervertebral disc tissue engineering efforts. However, it is not yet clear whether COL X is expressed by all human bone marrow stem cells or if it is related to age, gender, site, disease status, or drug therapy. In the current study, we report that COL X expression is upregulated in MSCs from rabbits in a surgical instability model of OA (anterior cruciate ligament transection (ACLT)) when compared to control rabbit MSCs. Thus COL X expression in OA is a common phenomenon that is due to the disease process itself and not to other environmental factors. It is, therefore, critical to understand MSC phenotype in OA patients, as these cells are essential clinically for biological repair of cartilage lesions using autologous stem cells.

1. Introduction

Osteoarthritis (OA) is characterized by a slow degeneration of articular cartilage and subchondral bone [1, 2]. At the molecular level, degradation of type II collagen (COL II) and proteoglycan components of the extracellular matrix occurs resulting in a loss of both the tensile and compressive strength of articular cartilage [1, 3, 4]. The incidence of OA increases progressively with age [5] and is viewed as an age-related dynamic reaction pattern of a joint in response to insult or injury. Many authors have reported evidence for chondrocyte differentiation in OA including proliferation, cell cloning, expression of type X collagen (COL X) [6],annexins and alkaline phosphatase, parathyroid-hormone-related peptide, matrix calcification, and apoptotic cell death of the terminally differentiated chondrocytes. These cellular changes are also observed in the growth platewhenthegrowth cartilage is converted intobone(endochondralossification) [7]. In line with these findings, we recently identified calcium deposits and COL X in degenerative and scoliotic intervertebral discs (IVD), but not in control discs, and the level of the indicators of calcification potential was consistently higher in degenerative and scoliotic discs than in control discs [8]. If the biological repair of cartilage and IVD is to become a reality, calcification or subsequent bone formation of engineered tissues has to be avoided.

COL X, a short-chain collagen was first isolated from chick hypertrophic chondrocytes and is a specific marker for hypertrophic chondrocytes of the growth plate, the final stage before cartilage is converted to bone [9]. It is synthesized and secreted prior to calcification and interacts with fibrils of COL II throughout the cartilage matrix [10]. It was

shown that COL X was also present in the zone of calcified cartilage of canine cartilage [11]. Furthermore, its synthesis is upregulated by chondrocytes isolated from osteoarthritic cartilage [6]. Osteoarthritic tissue has also been reported to synthesize type I collagen (COL I) in the late stages of degeneration [6, 12].

The clinical repair of articular cartilage lesions continues to evolve. Most protocols address the treatment of full-thickness (injuries that penetrate the subchondral bone plate to the trabecular-bone spaces) rather than partial-thickness defects (those that are confined to the hyaline articular cartilage tissue alone). The reason for this is that partial-thickness defects do not heal spontaneously whereas full-thickness defects below a critical size can heal although transiently [13, 14]. The strategies for repair of full-thickness cartilage defects in humans or experimental animal models are either tissue-based (entailing the grafting of perichondrial, periosteal, cartilage, or bone-cartilage material) or cell-based (utilizing chondrocytes, perichondrial cells or mesenchymal stem cells (MSCs) with or without the assistance of a scaffold matrix, with impregnated bioactive factors, to deliver the cells.

Damaged articular cartilage has very little capacity for spontaneous healing because of the avascular nature of the tissue [15]. As an alternative to enhance repair, tissue engineering has recently been reported [16, 17]. One treatment option is autologous chondrocyte transplantation (ACT) which is based on the isolation of chondrocytes from a minor load-bearing area of the knee, cell expansion, and re-transplantation as cell suspensions [18] or seeded into a biodegradable scaffold before implantation [19, 20]. These chondrocytes are usually from young animals. Employing adult human chondrocytes for cartilage tissue engineering in OA is invasive as it involves 2 surgeries, one to collect and another to implant the construct. Furthermore, there is no benign site for harvesting authentic autologous chondrocytes. It is also preferable to avoid the use of allogeneic donor chondrocytes, as there is a potential risk of transferring infectious agents. Despite degenerative changes in the host's articular cartilage and a hostile inflammatory joint environment in OA, repair of cartilage tissue may be possible, as the use of OA chondrocytes can generate a cartilage-like matrix when grown on hyaluronic acid scaffolds, although the cells significantly synthesized less collagen than what was generated by chondrocytes from individuals without arthritis [21].

Tissue engineering of cartilage using MSCs induced to differentiate into a chondrocyte phenotype has been considered as an alternative treatment for cartilage degeneration. Differentiation into chondrocytes first appears to be necessary for successful repair as MSCs directly implanted into cartilage defects in animal models [22] resulted in a fibrocartilaginous repair. MSCs have been shown to undergo chondrogenesis *in vitro* using a high-cell-density pellet culture system [23–25], which mimics the cellular condensation requirements for embryonic mesenchymal chondrogenesis and provides the physical and biochemical environmental factors conducive to cartilage formation [24]. Treatment with transforming growth factor-beta (TGF-β) superfamily members, TGF-β1 [24, 26–29] or TGF-β3 [23, 25], and dexamethasone (Dex) [24, 25, 30, 31] are key requirements for the *in vitro* chondrogenic differentiation of MSCs. Other growth factors, such as basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1) [32], and parathyroid hormone [33], have been found to enhance chondrogenesis.

We recently discovered that a major drawback of current cartilage tissue engineering strategies is that human MSCs harvested from OA patients express COL X [34–37], a marker of late-stage chondrocyte hypertrophy [7, 38–40] that is usually implicated in endochondral ossification and normally absent from hyaline cartilage. The expression of this marker in chondrocytes is considered to reflect an undesirable degenerative phenotype. Reports that freshly isolated OA chondrocytes synthesize predominantly COL X while control chondrocytes from healthy cartilage synthesized mostly COL II suggest focal premature chondrocyte differentiation to hypertrophic cells in OA cartilage [6].

It was not clear whether our observations with COL X message expression in OA MSCs were a universal phenomenon with human bone marrow stem cells or whether they are related to variables such as age, gender, site, disease status (osteoarthritis/rheumatoid arthritis), or drug therapy. Murphy et al. reported an altered phenotype in MSCs isolated from OA patients [41]. They observed that their proliferation rates were reduced, and differentiation profile was altered, and although chondrogenic and adipogenic capacity was reduced, osteogenic capacity was augmented. This enhanced osteogenesis would fit with our gene expression profile (COL X expression) in these cells. Furthermore, she reported that these effects were systemic and not only in MSCs cultured from bone marrow at the OA site.

Animal models of OA provide an important opportunity to address some of these issues [42, 43]. To date, there have been no reports of studies in animal models of OA examining this crucial question. One of the most widely used experimental models of OA is the anterior cruciate ligament transection (ACLT) model that creates a joint instability and progressive degeneration of the joint. The use of experimental ACLT models is of particular clinical relevance, since rupture of the ACL occurs in humans and also leads to the development of OA [44]. Rabbit ACLT is increasingly being used in OA studies [42, 43, 45, 46] because the disease onset is rapid.

The objective of our study was to determine whether the expression of COL X could be reproduced in MSCs from rabbits in a standard rabbit ACLT model of OA. These observations help provide more insight into the complex interrelationships of the expression of COL X during OA.

2. Materials and Methods

2.1. Rabbit Experimental Model of Osteoarthritis. Fifteen skeletally mature (9-month-old) female New Zealand white rabbits (Charles River, Saint Constant, QC, Canada) weighing a mean ± SD of 4.28 ± 0.39 kg were employed in this experiment. Radiographs of both femorotibial joints were taken to exclude animals with joint pathology at the study outset. 5 unoperated rabbits served as controls, and

Table 1: Scores employed for macroscopic articular cartilage changes.

Score	Structure	Length of the lesion
1	Intact surface: surface normal in appearance and does not retain India ink	
2	Fissures	0 mm to ≤4 mm
3	Fissures	>4 mm to ≤8 mm
4	Fissures	>8 mm
5	Full-depth erosion	0 mm to ≤2 mm
6	Full-depth erosion	> 2 mm to ≤5 mm
7	Full-depth erosion	>5 mm

the remaining 10 underwent unilateral ACLT under general anesthesia as described previously with some minor modifications [47]. The animals in this study were part of another larger unrelated study. The Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, University of Montreal, approved all the procedures.

2.2. Surgical Procedure. The surgery was performed by a board-certified veterinary surgeon under sterile conditions. Trimethoprim-sulfamide (Tribissen 24%, Schering-Plough Animal Health, Pointe Claire, QC, Canada), 15 mg/kg SC, was administered immediately preoperatively. Rabbits were then premedicated, and anesthesia was induced with xylazine (Novopharm Limited, Animal Health, Toronto, Canada) 3 mg/kg, IM glycopyrrolate (Sabex Inc, QC, Canada) 0.01 mg/kg IM, and ketamine (Bioniche, Animal Health Canada Inc.) 17 mg/kg IM. Anesthesia was maintained with 2–2.75% isoflurane (Baxter Corporation, Mississauga, ON, Canada) via an orotracheal tube.

The left ACL was exposed by a medial parapatellar skin incision, the patella was subluxated laterally, and the knee was placed in full flexion. The anterior cruciate ligament was transected, and the incision was sutured in a routine manner. Analgesia was achieved with the administration of buprenorphine (Schering-Plough Ltd, Welwyn Garden City, UK) 0.03 mg/kg SC, preoperatively and 12 hours later. Fentanyl (Novo-Fentanyl 25, Fentanyl Transdermal System, Novopharm, Markham, ON, Canada), 25 μg/hour, was applied to the lumbar region for 72 hours. Following surgery, rabbits were housed separately and permitted free cage activity until the termination of the study.

2.3. Postmortem Harvest of Bone Marrow Aspirate. Animals were humanely euthanized 12 weeks after surgery**.** Bone marrow aspirates were aseptically harvested from the distal femur, following transection, by injecting DMEM with a syringe, followed by aspiration of the bone marrow similar to methods described previously [48]. The samples were then transported to the laboratory for separation and culture.

2.4. Macroscopic Cartilage Assessment. Both femur compartments (medial femoral condyle (MFC) and lateral femoral condyle (LFC)) were assessed for the extent of gross morphologic changes of the articular cartilage surface including fibrillation and ulceration of cartilage that are characteristic features of OA. The mean score of both compartments represented a femur macroscopic score that reflected the disease burden at the articular surface (ranging from 1 for a normal joint surface to 7 with very severe disease). Details of the score attribution are provided in Table 1 [42]. Scoring was performed after the application of Indian ink that highlights areas of fibrillation.

2.5. Histological Assessment of Cartilage. Femoral articular surfaces were fixed in 10% neutral buffered formalin. Tissue blocks were decalcified with 20% EDTA (Fisher Scientific, Nepean, ON, Canada) in 10 mM phosphate buffer (pH 7.4), dehydrated through graded alcohols, cleared with 100% toluol, and embedded in paraffin. Following paraffin embedding, 5 μm sections were cut from the articular surface (through the most severe lesion observed macroscopically) and stained with Safranin O/fast green. Histological sections were obtained from each of the femur compartments described above. The histologic sections from each site were evaluated using a modified Mankin's grading system illustrated in Table 3 [42, 47]. The mean histological score from both compartments represented the femur cartilage pathology score.

2.6. MSC Culture. Bone marrow aspirates were processed, and MSCs were cultured as previously described [34]. Briefly, each aspirate was transferred to a 50 mL sterile tube and diluted 1 : 1 with Dulbecco's modified eagle medium (DMEM; Hyclone, Chateauguay, QC) supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin (1% PS; Hyclone). The tube was centrifuged at 600 ×g for 10 minutes. After two washes, the cells were resuspended in 20 mL MSC complete medium (DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% PS and cultured under preconfluent monolayer conditions in 150 mm culture dishes, at 37°C with 5% humidified CO_2. After 72 hours, nonadherent cells were discarded, and the adherent ones were thoroughly washed twice with DMEM (low glucose) and cultured for 2 passages in DMEM (low glucose) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 ug/mL streptomycin as described previously [34].

Table 2: Primers used for PCR amplification.

Gene	Primer sequence	Size (bp)
COL I	Forward: CGGTTACCCTGGCAATATTG Reverse: TCCAGTGCGACCATCTTT	116
COL X	Forward: ACAGGAATGCCTGTGTCTGCTTTACT Reverse: CATTGGGAAGCTGGAGCCACACCTGGTC	331
GAPDH	Forward: GCTCTCCAGAACATCATCCCTGCC Reverse: CGTTGTCATACCAGGAAATGAGCTT	346

Table 3: Histochemical/histological assessment of the articular cartilage changes in the rabbit model of osteoarthritis.

Safranin O—fast green staining
0 = uniform staining throughout articular cartilage
1 = loss of staining in superficial zone of hyaline cartilage ≤50% the length of the condyle or plateau
2 = loss of staining in superficial zone of hyaline cartilage ≥50% the length of the condyle or plateau
3 = loss of staining in the upper 2/3s of hyaline cartilage ≤50% the length of the condyle or plateau
4 = loss of staining in the in the upper 2/3s hyaline cartilage ≥50% the length of the condyle or plateau
5 = loss of staining in all the hyaline cartilage ≤50% the length of the condyle or plateau
6 = loss of staining in all the hyaline cartilage ≥50% the length of the condyle or plateau
Structure
0 = normal
1 = surface irregularities
2 = fissures in ≤50% surface
3 = fissures in ≥50% surface
4 = erosion 1/3 hyaline cartilage ≤50% surface
5 = erosion 1/3 hyaline cartilage ≥50% surface
6 = erosion 2/3 hyaline cartilage ≤50% surface
7 = erosion 2/3 hyaline cartilage ≥50% surface
8 = full-depth erosion hyaline cartilage ≤50% surface
9 = full-depth erosion hyaline cartilage ≥50% surface
10 = full-depth erosion hyaline and calcified cartilage to the subchondral bone ≤50% surface
11 = full-depth erosion hyaline and calcified cartilage to the subchondral bone ≥50% surface
Chondrocyte density
0 = no decrease in cells
1 = focal decrease in cells
2 = multifocal decrease in cells
3 = multifocal confluent decrease in cells
4 = diffuse decrease in cells
Cluster formation
0 = normal
1 = <4 clusters
2 = ≥4 but < 8 clusters
3 = ≥8 clusters

2.7. Gene Expression. Pellets were digested with proteinase K, and total RNA was extracted using Trizol (Invitrogen). Reverse transcription reaction was performed using Superscript II RNase H-RT (Invitrogen) and then amplified by PCR using gene-specific primers for COL I and COL X (Table 2). Relative gene expression with reference to GAPDH was determined and expressed as before [37]. PCR products were visualized by ethidium bromide staining on 2% agarose gels.

2.8. Statistical Analysis. A Wilcoxon-signed rank test was employed to assess differences in the femoral articular cartilage macroscopic and histological scores between ACLT and control groups of animals. An analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) post hoc test using Stat view (SAS Institute Inc., Cary, NC) was employed to assess gene expression. Results are presented as the mean ± standard deviation. Differences were considered statistically significant at $P < 0.05$.

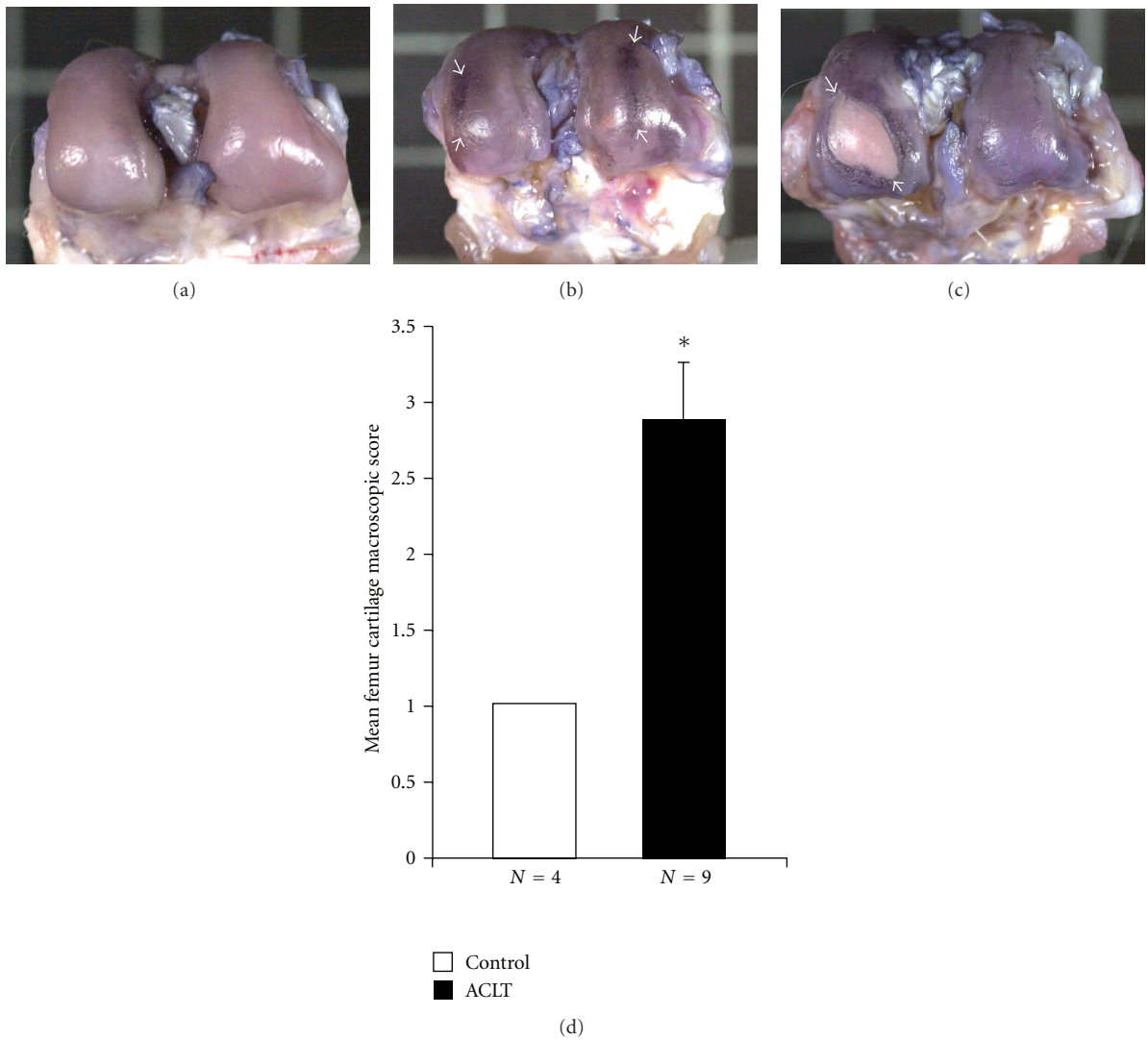

Figure 1: Macroscopic articular cartilage lesions of the distal femur observed in the rabbits. (a) Intact cartilage surface, (b) fibrillation (enhanced uptake of ink between arrows), (c) complete ulceration of cartilage (between the arrows) on the femoral condyle. (d) The mean femur cartilage macroscopic score reflecting the disease burden in the control and OA (ACLT) joints. *Indicates $P < 0.05$. One animal was deleted from each group to give $n = 4$ in the control group and $n = 9$ in the ACLT group because of problems encountered with cell culture from these animals (see methods).

3. Results

3.1. Macroscopic and Histologic Assessment of Articular Cartilage. Postmortem inspection revealed that ACLT was complete in all rabbits included in the study. Macroscopic cartilage scores are illustrated in Figure 1. In the control rabbit joints (Figure 1(a)), the mean femur articular cartilage macroscopic grade was 1. In the ACLT rabbit joints (Figures 1(b) and 1(c)), the mean femur articular cartilage macroscopic score was significantly increased (Figure 1(d)) when compared to the control joints.

Histological scores and representative lesions are presented in Figure 2. In the OA group, the induced OA lesions (fibrillation and erosion of articular cartilage) were focal and occurred at the same sites in all ACL-transected joints (Figures 2(b), 2(c) and 2(d)). Typical fibrillation and erosion of the articular cartilage, hallmarks of OA, were observed in the ACLT joints but not in the control joints (Figure 2). The mean femur articular cartilage histological score was significantly higher in the ACLT animals when compared to the controls.

3.2. MSC Culture. Marrow aspirates were obtained from 5 control and 10 operated groups. One sample from each group was eliminated because no cells attached after 72 hours in culture, giving a total of $n = 4$ controls and $n = 9$ ACLT animals. MSCs were successfully expanded from the remaining aspirates forming colonies in primary culture that were characteristically spindle-shaped with a fibroblastic morphology (data not shown). MSCs from ACLT animals

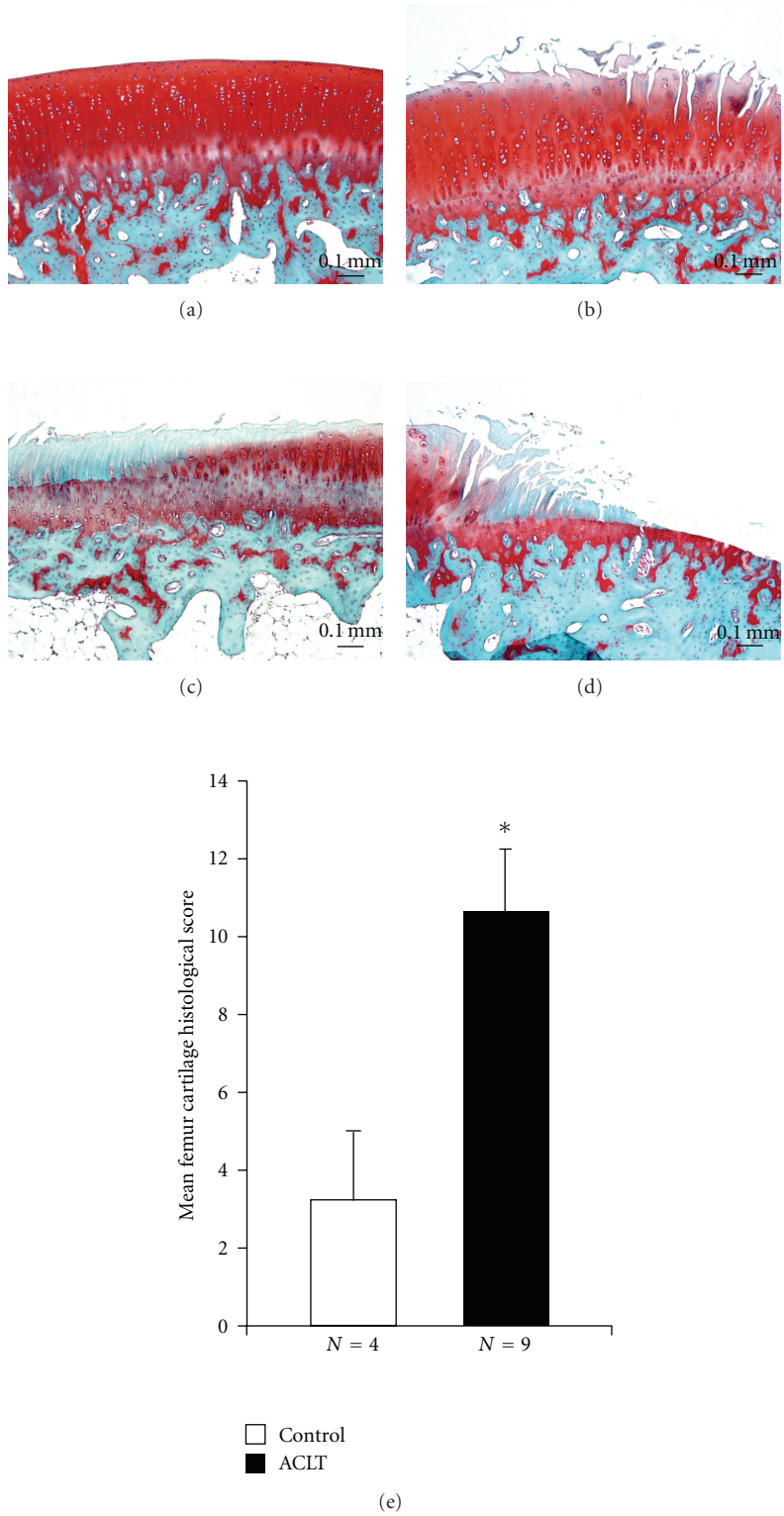

Figure 2: Safranin-O-stained sections from the distal femur illustrating representative OA lesions in this model. (a) Intact articular cartilage, (b) fissures of the articular cartilage with some loss of Safranin O stain from the surface, (c) extensive loss of Safranin O stain, Focal chondrocyte cloning, (d) complete erosion of the hyaline cartilage, (e) the mean femur cartilage histological score reflecting the disease burden in the control and OA (ACLT) joints. *Indicates $P < 0.05$. One animal was deleted from each group to give $n = 4$ in the control group and $n = 9$ in the ACLT group because of problems encountered with cell culture from these animals (see methods).

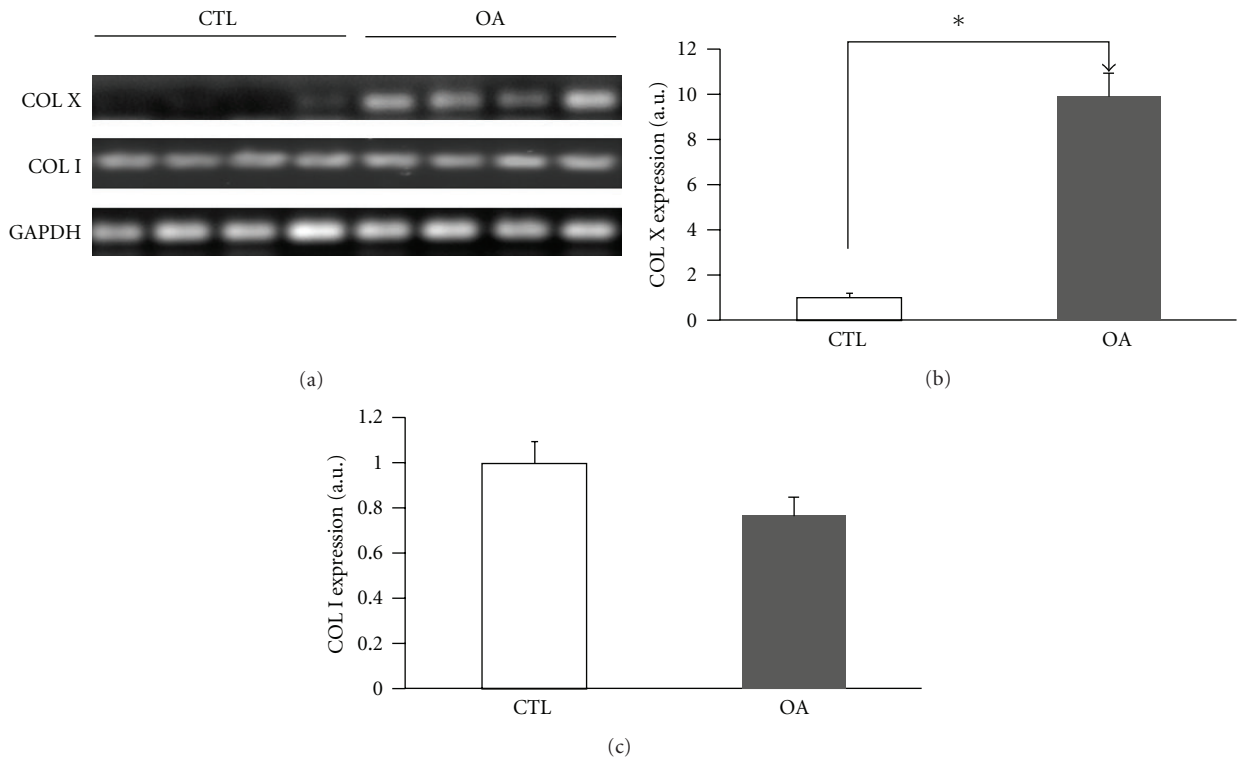

Figure 3: Expression of COL X mRNA and COL I mRNA in MSCs from experimental OA rabbits. COL I mRNA level did not vary significantly in both groups of rabbits. Expression of COL X was enhanced in MSCs from OA rabbits. *Signifies $P < 0.05$.

were morphologically indistinguishable from the cultures of normal controls, and the cell yield was comparable at the end of primary cultures.

3.3. Effect of OA on Collagen Gene Expression. Message expression for COL I and COL X genes was analyzed after culturing MSCs from control or the experimental OA rabbits. After culturing the cells for 7 days, MSCs from normal rabbits show weak COL X message (Figure 3). Of special interest here was that OA led to an increase in COL X expression (Figure 3). COL I message is present in MSCs from normal and OA rabbits and did not vary significantly in both groups of rabbits. COL II was not expressed, and OA had no effect on its expression (data not shown).

4. Discussion

This study was conducted to determine whether the expression of COL X could be reproduced in MSCs from rabbits in a standard rabbit ACLT model of OA. In recent years, surgically induced instability models of OA are frequently used to assess the therapeutic efficacy of compounds targeted for OA treatment, because the procedure induces cartilage lesions similar to those observed in humans with OA [46, 49, 50]. The ACLT is a reproducible and effective model of OA [49]. The cartilage lesions in this model, and their response to therapy can be graded according to an adapted histological and histochemical grading system [42].

We showed that human MSCs from OA patients express COL X [34–37]. Our attempts to investigate whether the expression of COL X can be reproduced in MSCs from rabbits in a surgical instability model of OA using established methodology clearly revealed that OA upregulates COL X expression in MSCs. These observations are contrary to what one would expect, as COL X is only supposed to be expressed by chondrocytes when they become hypertrophic [40, 51, 52]. It is tempting to suggest that COL X may play a role in the bone marrow stromal microenvironment, as mice deficient in COL X show defects in hematopoiesis [53].

The present study suggests that the expression of COL X in OA is a universal phenomenon that is due to the disease process itself and not other environmental factors. Results of the present study show for the first time that OA modulates the expression of COL X expression. In this system, COL X message expression may not be a good marker for chondrocyte hypertrophy during early MSC differentiation.

It is possible that COL X has a serendipitous expression but with no functional consequence. However, we have shown that MSCs from OA patients express not only COL X but also osteogenic differentiation markers such as alkaline phosphatase, bone sialoprotein, osteocalcin, and the transcription factor RUNX2 [36, 37]. Therefore, the risk of calcification and subsequent bone formation in cartilage repair is real. Previously we have shown that COL I but not COL II is expressed by MSCs from OA patients [34, 37]. Similar findings were observed in this study.

Other factors such as age, gender, or drug therapy [54] may still have an influence on the ability of MSCs to differentiate. Finding ways to suppress this expression of COL X is important for future tissue engineering strategies to succeed in OA patients [33, 36, 37, 55, 56]. The sites assessed were adjacent to the injury so we suspect that the injury environment altered their gene expression. An additional issue, worth exploring, would be whether MSCs, remote from the site of injury, in other bones could also have this altered expression (a systemic effect) as previously observed by Murphy et al. [41]. Equally, although less plausible, the MSCs with altered COL X expression may represent cells that have homed to these sites. The latter hypothesis is unlikely as these were young adult rabbits, and it would be surprising if they did not have an ample population of stem cells locally at the site of injury, but this also requires confirmation. An additional area of future study, related to the findings reported herein, would be the effect of OA on MSC chondrogenesis. Previously it was shown that these cells had a reduced proliferative capacity, chondrogenic and adipogenic activity compared with that in normal cultures although the CFU-F formation capacity was similar [41]. Our studies showed that MSCs from OA patients cultured in defined chondrogenic medium supplemented with transforming growth factor (TGF-) β1 or β3 and dexamethazone expressed aggrecan constitutively, and COL X was expressed before COL II [34].

Although we did not analyze the surface markers on these cells, MSCs from OA patients stained strongly for CD90 (Thy-1), and they were mildly to highly positive for CD73 and CD44 (HCAM), three markers of MSCs [56]. The observed wide range depended upon the particular donors. The expression range for CD73 extended from 52% to 100%, with the median at 94%. CD44 expression range was from 11% to 100%, with the median at 65%. Cells stained predominantly negatively for CD34 and CD45, two markers of hematopoietic stem cells. Further studies are, however, necessary to better understand the effect of OA on chondrocyte hypertrophy.

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