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Running title: HA up-regulates IL-10 in Synoviocytes

Abstract

Progression to osteoarthritis is a frequent sequela of severe articular fracture, particularly when weight-bearing joints are involved. Prevention from posttraumatic osteoarthritis remains a challenge. Hyaluronan (HA) therapy is reported to represent a safe and effective treatment for patients with osteoarthritis and rheumatoid arthritis. However, the capacity of HA to prevent the occurrence of osteoarthritic changes in fractured joints has not been demonstrated. The present study was undertaken to examine the effects of HA on expression of six osteoarthritis-related proteins in fibroblast-like synoviocytes (FLS) from ten patients with tibia plateau fracture. Osteoarthritis-related factors were quantified using a sandwich enzyme-linked immune-sorbent assay. Regardless of induction of the FLS with interleukin (IL)-1β, HA was found to down-regulate expression of catabolic factors (IL-1β, matrix metalloproteinase-3, and tumor necrosis factor-α) and to up-regulate production of anti-catabolic factors (tissue inhibitors of metalloproteinase-1 and metalloproteinase-2). HA also enhanced expression of interleukin-10, an anti-inflammatory cytokine, in FLS. Our results indicated that HA can promote the expression of both antiinflammatory and structure-protective factors in FLS of patients with tibia plateau fracture.

Keywords: hyaluronan; tibia plateau fracture; synoviocyte

Introduction

In active subjects, traumatic arthritis is a frequent clinical problem manifested initially as synovitis and capsulitis but commonly progressing to osteoarthritis (OA).[1] Activated fibroblast-like synoviocytes (FLS) in the lining layer of the synovium were recently found to contribute significantly to the progression of OA.[2] One of the critical mechanisms is that FLS can produce OA-associated cytokines and enzymes, such as interleukin(IL) 1-β, matrix metalloproteinases (MMPs), and tumor necrosis factor (TNF)- α , all of which cause progressive cartilage disruption.

Hyaluronan (HA), a large glycosaminoglycan composed of repeating disaccharides of D-glucuronic acid and N-acetyl-glucosamine, is a ubiquitous component of the extracellular matrix. Intra-articular injections of HA have been reported to relieve joint pain and improve function in human subjects with OA.[3] However, the utility of HA treatments in preventing the progression to OA following articular fracture has only been addressed using animal models.[4, 5] The feasibility of HA treatments to prevent progression to OA in human subjects with early stage traumatic arthritis remains to be determined.

HA is reported to alter synoviocyte expression of messenger ribonucleic acids (mRNA)s encoding OA-associated cytokines and enzymes.[4,6] However, translation of these mRNAs may be influenced significantly by certain regulatory RNAs such as

microRNA (miRNA) and small interfering RNA (siRNA).[7,8] In other words, the quantity of mRNA may not represent its final expression level, i.e. the amount of the identical protein. The protein is, in fact, the just molecule that induces some biological reactions and interacts with the surrounding tissue. To determine the extent to which HA alters synoviocyte expression of OA-associated cytokines and enzymes, these proteins should be quantified directly.

The present study determined the effects of HA on protein expression of six arthritis-related factors produced by FLS obtained from patients with early stage traumatic arthritis. The objective was to ascertain whether HA would enhance expression of factors associated with improved outcomes in patients at risk of progression to OA. Quantification of cytokines and enzymes was performed using sandwich Enzyme-Linked Immuno-Sorbent Assay (ELISA).

Materials and Methods

Ten patients (four females and six males) with tibia plateau fracture were enrolled in this study. All patients had previously undergone open reduction and internal fixation with plates and screws. Inclusion criteria were: (1) presence of greater than 2-mm residual step-off on the joint surface as shown by immediate postoperative radiography (Figs. 1A and 1B); (2) presence of continuing discomfort, joint limitation, or pain in the injured knee such that daily activities were limited at the time of implant

removal following fracture union (Fig. 1C); and (3) severity of OA of Grade II or less (by Kellgren-Lawrence grading) at the time of plate removal. All patients underwent arthroscopic lavage concurrently with implant removal. Ethical approval was provided by the institution's ethics committee. The average time interval between fracture and implant removal was 73(range, 54-102) weeks. The average patient age was 34.5(range, 25-47) years.

Preparation of FLS

Synovial tissues were harvested at obviously inflamed areas by arthroscopic punch during the arthroscopic procedures (Fig. 2). Harvested synovium was minced to fragments of 1×1 mm in size. Fragments were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1.5 g/L sodium bicarbonate (S6297, Sigma-Aldrich, St Louis, MO), 1 % penicillin-streptomycin-neomycin (P4083, Sigma, USA, St Louis, MO), and 10 % fetal bovine serum (FBS, 04-001-1A, Biological Industries, Grand Island, NY) in the presence of 5 % CO_2 at 37℃ for 3 days in 10 cm dishes. Non-adherent cells were washed out with phosphate buffered saline (PBS). The medium was changed every two days and adherent cells were cultured for an additional two weeks. After 3-6 passages cells, which were exclusively FLS, were employed for phenotypic analysis and subsequent experiments.

Immunocytochemistry Staining for Phenotypic Analysis of FLS

Cells in six-well culture plates were fixed with methanol (Merck, Germany) for 10 min, washed twice with PBS, blocked with 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) for 30 min at room temperature and incubated for 1 h with primary antibody to prolyl-4-hydroxylase (clone 5B5; Abcam, Cambridge, UK). Cells were washed twice with PBS and stained with secondary antibody A488 (ALEXA FLUOR 488; Invitrogen-Molecular Probes, Carlsbad, California, U.S.A.) for 30 min. The primary antibody was omitted in negative control samples.

Cell Induction and Cell Treatment

Sub-confluent populations of FLS were incubated in serum-free medium for 24 h, followed by culture in DMEM containing 10% FBS and under the following four conditions: (1) no induction or treatment; (2) treatment with 100 μg/ml HA (600,000-800,000 daltons, Artzdispo, Japan) for 24 h (3) induction with 1 ng/ml IL-1β (4128-10, BioVision, USA) for 24 h; (4) induction with 1 ng/ml IL-1β and treatment with 100μg/ml HA for 24 h. For each condition, FLS were plated at 3 x 10^5/well and with the same contact volume of supernatant. (Supplementary Material 1)

ELISA for Arthritis-related Cytokines and Enzymes

Concentrations of six OA-related factors in culture supernatants of FLS were measured using sandwich binding protein assay kits, including those for IL-1β (88-7010, eBioscience, San Diego, CA), IL-10 (88-7106, eBioscience, San Diego,

CA), TNF-α (88-7340, eBioscience, San Diego, CA), MMP-3 (DY513, R&D system, Minneapolis), tissue inhibitor of metalloproteinase (TIMP)-1 (DY970, R&D system, Minneapolis), and TIMP-2 (DY971, R&D system, Minneapolis). Procedures for quantification of these factors were performed according to the manufacturers' protocols using standard curves obtained from purified recombinant standards. Each sample was performed in duplicate and monitored with an ELISA reader (Sunrise remote, TECAN).

Real-time Quantitative PCR

After treatments, FLS were lysed and total RNA was extracted with Trizol Reagent (Cat. No. 15596-018, Invitrogen, CA, USA). Quantification of total RNA was done by using spectrophotometry. One microgram of total RNA was converted to cDNA using Transcriptor First Strand cDNA Synthesis Kit (Cat. No. 04 896 866 001, Roche, Switzerland), according to the manufacture's protocol. The primer was designed to make the lengths of PCR products ranging between 100 bp and 250 bp. (Table 1) The FastStart Universal SYBR Green Master (Cat. No. 04 913 914 001, Roche, Switzerland) reagents and the Applied Biosystems 7300 Real-Time PCR System were used in all reactions.

Statistical Analysis

Descriptive findings are presented as means ± standard deviation. A two-sample *t*-test was applied for the analyses of IL-1β, IL-10, TNF-α, MMP-3, TIMP-1, and TIMP-2 measurements. The significance level was set as α=0.05.

Results

Morphological and Immunocytochemical Characterization of FLS

When examined by microscopy, cultured FLS were observed to be fibroblast-like and spindle-shaped (Fig. 3). The majority of FLS displayed strong positive immunocytochemical staining for prolyl-4-hydroxylase whereas no response was obtained in the negative control (Figure 4).

Effect of IL-1β Induction on Expression of OA-related Proteins

As to IL-1β, TNF-α, MMP-3,and TIMP-1, the concentrations in culture supernatants were found to increase significantly in response to induction with IL-1β ($p<0.0001$, $p<0.0001$, $p<0.0001$, $p=0.0067$, respectively). By contrast, the concentration of TIMP-2 was reduced significantly after induction ($p<0.0001$). Concentrations of IL-10 in culture supernatants of non-induced or IL-1β-induced preparations were either very low or undetectable (< 2 pg/ml). These findings are detailed in Table 2.

Effect of HA Treatment on non-induced FLS

In the experiments on non-induced FLS, HA significantly reduced the production of

both TNF- α and MMP-3. (p=0.0002 and p=0.003, respectively) On the other hand, HA significantly increased the concentrations of TIMP-1, TIMP-2, and IL-10. (p=0.0003, p=0.0018, and p<0.0001, respectively) IL-1β concentrations were either very low or undetectable in culture supernatants of untreated or HA-treated preparations (< 4 pg/ml). (Table 3)

Effect of HA Treatment on IL-1β induced FLS

In the experiments on IL-1β induced FLS, HA significantly decreased the amount of IL-1β, TNF- α ,and MMP-3 (p <0.0001, p <0.0001, and p =0.0001, respectively). In contrast, HA significantly increased the production of TIMP-1, TIMP-2, and IL-10 (p < 0.0001, p < 0.0001, and p < 0.0001, respectively). (Table 4)

HA significantly up-regulate the protein level of IL-10

In this study, our results show that HA significantly up-regulates IL-10 expression in protein level. (p<0.0001) To ascertain whether HA treatment increased transcription of the gene encoding IL-10, we further compared the m-RNA levels of IL-10 in FLS between pre-HA treatment and post-HA treatment conditions in two patients by using real-time quantitative polymerase chain reaction. The result also demonstrated that HA promotes the gene expression of IL-10 in FLS. (Figure 5 and Supplementary Material 2)

Discussion

HA injections are now licensed worldwide for the treatment of OA. Several clinical studies proved that HA injection into OA joints effectively reduces patient's pain and improves their function.[3,9] However, the efficacy of HA for patients with articular fracture has not been established. In the present report, production of MMP-3 by FLS was found to be significantly suppressed by HA regardless of induction with IL-1β. MMP-3, also known as stromelysin 1or progelatinase, is intimately concerned with the breakdown of extracellular matrix and with the degradation of collagens and proteoglycans in cartilage and also serves to activate other MMPs [10]. The observed suppression of MMP-3 production by HA therefore supports the concept that HA possesses structure -protective properties for joints with traumatic arthritis. Additionally TIMP-1 and TIMP-2, inhibitors of MMPs, were increased by HA under both induced and non-induced conditions despite the insignificant increase of TIMP-1 in the non-induced condition. These findings further support the structure-protective properties of HA in human traumatic arthritis.

The findings of the present report are in agreement with those of previous studies employing animal models. Using a rabbit partial medial meniscectomy model, Hulmes et al[11] observed that intraarticular injections of HA prevented the deteriorations in proteoglycan content in articular cartilage. Amiel *et al.*[12], who examined the long-term effects of HA on OA progression in a rabbit anterior cruciate

ligament transection model, also concluded that HA decreases the degree of cartilage degeneration.

In the present study, production of the proinflammatory cytokines IL-1 and TNF-α was reduced by HA under IL-1β-induced conditions. In addition to their established roles in the initiation of inflammatory reactions, these cytokines function importantly to alter extracellular matrix cartilage turnover to induce pain and promote joint destruction.[13,14] Furthermore, we also found that HA can promote the creation of IL-10, an anti-inflammatory cytokine, in FLS. IL-10, also known as human cytokine synthesis inhibitory factor, blocks nuclear factor (NF)-κB activity and is involved in the regulation of the c-jun NH_2-terminal kinase (JNK)- signal transducers and activators of transcription signaling pathway.[15] Hiramitsu *et al.*[13] recently reported that HA suppresses IL-1β-induced MMP production in rheumatoid synovial fibroblasts by downregulating (NF)-κB, p38, and JNK. Yasuda [16] also provided evidence that HA inhibits cytokine production through down-regulation of NF-κB. The finding of the present report that HA upregulates IL-10 expression in FLS explains the observed down-regulation NF-κB by HA. Further support for IL-10 involvement is provided by Kontoyiannis *et al.*[17] who observed that IL-10 represses translation of mRNA for TNF by suppressing activation of the p38/MAPK-activated protein kinase-2 pathway. In addition, Qin *et al.* [18] reported that IL-10 can induce expression of the suppressor of

cytokine signaling (SOCS)-3, a negative regulator of inflammation capable of suppressing cytokine-mediated signal transduction associated with the acute inflammatory process. [19] Up-regulation of IL-10 therefore appears to mediate the anti-inflammatory actions of HA by suppressing inflammatory pathway itself and to mediate the structure-protective effects of HA through reduction of MMP production.

In the present study, IL-10 was found to be significantly up-regulated at the protein level in FLS treated with HA. However, the mRNA for IL-10 was observed to increase only 1.2-fold increase in response to HA; this increase was considered statistically insignificant. The discrepancy between the increases in IL-10 protein and IL-10 mRNA values may be attributable to specific regulatory modifications that occur during or following the translational process. For example certain regulatory mRNAs, such as miRNA and siRNA, may influence translation of IL-10 mRNA.[7,8] or post-translational modifications of the protein may affect the degree to which IL-10 is expressed in the presence of HA.[20-22] Future studies are planned to test these possibilities.

Although CD 44 is considered the principal HA receptor, the hyaluronan-mediated motility (RHAMM) and intercellular adhesion molecule-1 receptors are also reported to function as receptors for HA.[13,16,23] In the present study, the HA receptor of FLS was not characterized. Additionally, the pathways through which HA exerts its

beneficial effects on FLS were not defined. Further studies are planned to address these issues. Regardless of these limitations, this study is the first to examine the effects of HA on FLS from human subjects with traumatic arthritis. Considering the finding that HA enhances the expression of both anti-inflammatory and structure-protective factors in FLS of patients with tibia plateau fracture, clinical investigations to ascertain whether HA possesses efficacy against traumatic arthritis of the knee are warranted.

To sum up, the sandwich ELISA method was employed to examine the effects of HA on the expression of six OA-related proteins in FLS from patients with tibia plateau fracture. The effects of HA are promising in terms of suppressing the catabolic factors, i.e.IL-1, TNF-α, MMP-3, and promoting the anticatabolic factors, i.e. TIMP-1,TIMP-2, IL-10. Further studies are anticipated to clarify the pathways and to verify the effects in clinical use.

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Legends

Figure 1. A 36-year-old woman with a tibia plateau fracture. (A) Preoperative radiograph revealing a left tibia plateau bicondylar fracture classified as Schatzker type five. (B) Immediate postoperative radiograph displaying more than 2 mm residual step-off on the articular surface. (C) Fracture consolidation achieved after 72 postoperative weeks.

Figure 2. Prominent synovitis observed during arthroscopic surgery and use of a punch forceps to obtain the inflamed synovial tissue.

Figure 3. Light microscopy revealing the homogeneous fibroblast-like and spindle-shaped morphology of the fibroblast-like synoviocyte (FLS) preparation.

Figure 4. Light microscopy revealing strongly positive immunocytochemical staining for prolyl-4-hydroxylase for the majority of FLS (upper two photographs). No staining was observed for cells present in the negative control preparation (lower two photographs).

Figure 5. Expression of IL-10 mRNA in FLS in response to treatment with hyaluronan (HA). FLS preparations obtained from two different subjects were treated for 24 h without or with HA (100 µg/ml). Findings are expressed as fold changes in expression as compared to the corresponding control preparation (no HA treatment).

Table 1: Primer sequences in real-time quantitative PCR

Target gene	Forward primer (5'-3') (450-471)	Reverse primer (5'-3') (575-595)
IL-10	5'-CCCTGTGAAAACAAGAGCAAGG-3'	5'-TCAGTTTCGTATCTTCATTGTC-3'

Gene Bank accession number: NM_000572

Table 2: Efficacy of IL-1β induction on FLS

Cons (pg/ml)	IL-1β	TNF-α	IL-10	MMP-3	TIMP-1	TIMP-2
Non-Induced	3.7±1.01	9.3±1.63	L	28.2±8.70	46.5±3.75	14.57±0.83
Induced	22.4±1.53	22.8±2.89	L	125.4±14.98	52±4.24	8.22±1.25
p value	<0.0001	<0.0001		<0.0001	0.0067	<0.0001

Cons: Concentration in supernatant L: Concentration in supernatant < 2 pg/ml

Table 3: Effect of HA on non-induced FLS

Cons (pg/ml)	IL-1β	TNF-α	IL-10	MMP-3	TIMP-1	TIMP-2
Control	3.7± 1.01	9.3± 1.63	1.1±0.41	28.2±8.70	46.5±3.75	14.6±0.83
HA Tx	2.4± 0.48	6.2± 1.06	11.7±3.58	14.8±8.79	54.8±4.42	16.8±1.59
p value	0.0027	0.0002	<0.0001	0.0030	0.0003	0.0018

Control: Uninduced and untreated control; HA Tx: Treated with HA

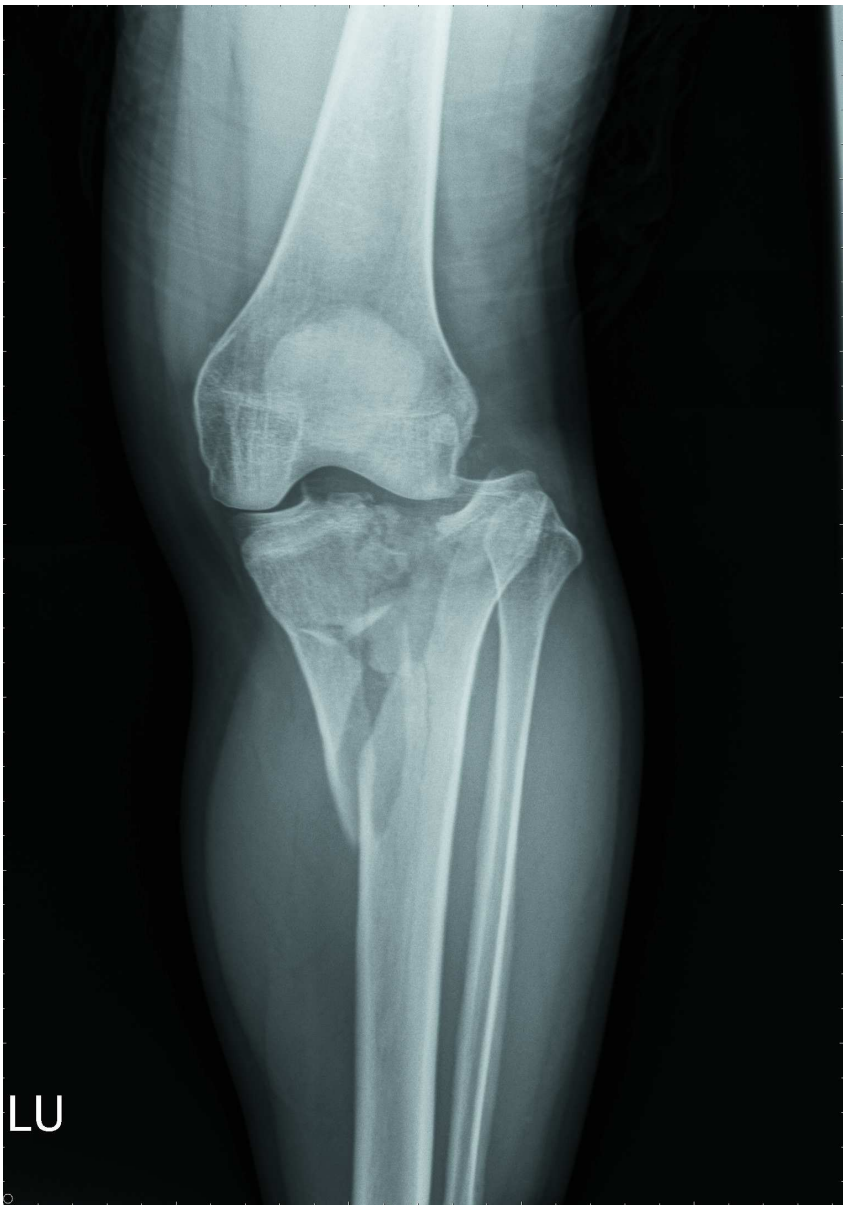
Cons: Concentration in supernatant

Table 4: Effect of HA on IL-1β induced FLS

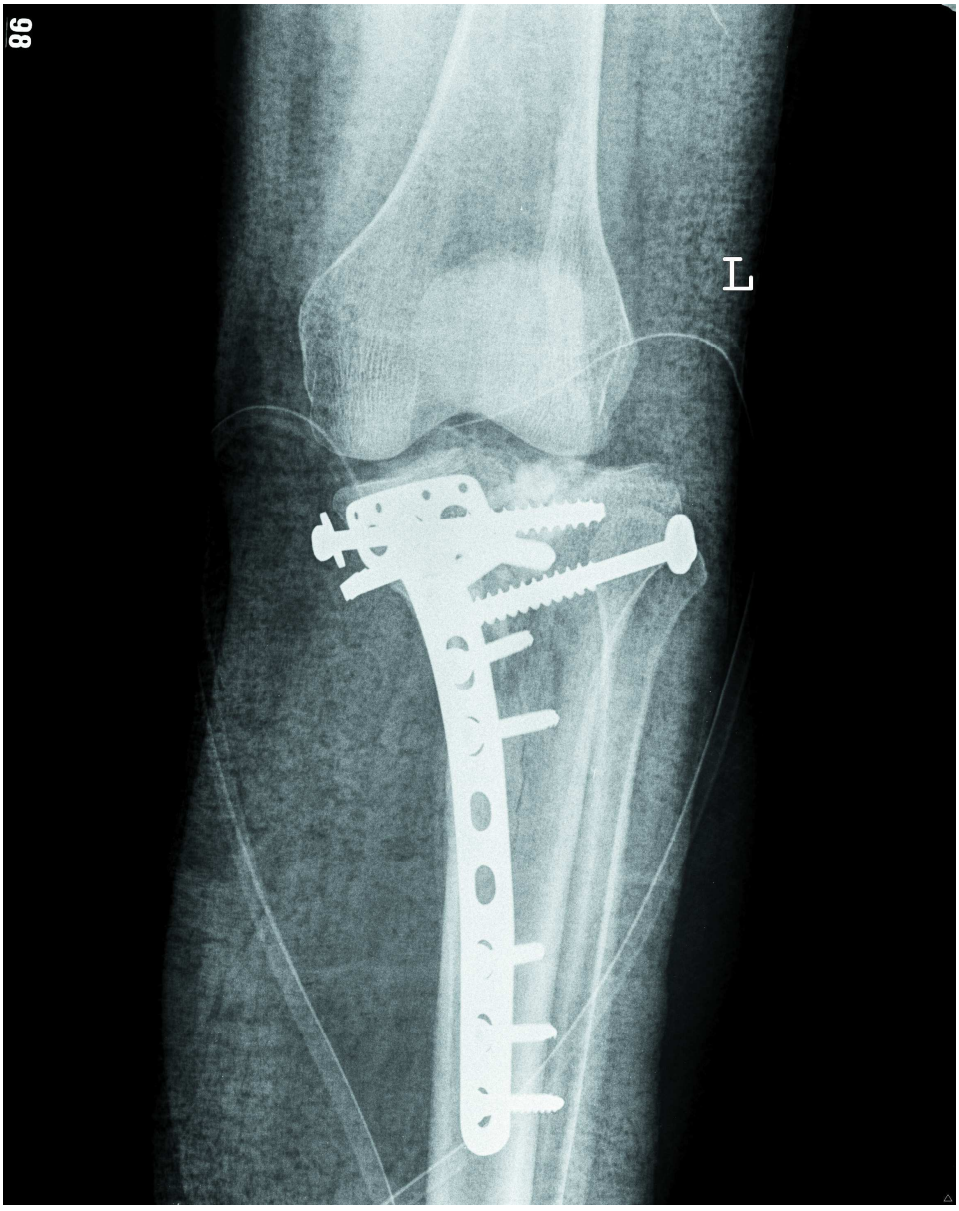
Cons (pg/ml)	IL-1β	TNF-α	IL-10	MMP-3	TIMP-1	TIMP-2
Induced	22.4±1.53	23.8±2.89	1.92±0.27	125.4±14.98	52.0±4.24	8.2±1.25
HA Tx	8.0±0.71	10.5±2.35	10.6±0.69	95.5±8.97	69.3±5.98	19.5±0.96
p value	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	<0.0001

Induced: Induced with IL-1β; HA Tx: Induced with IL-1β and treated with HA

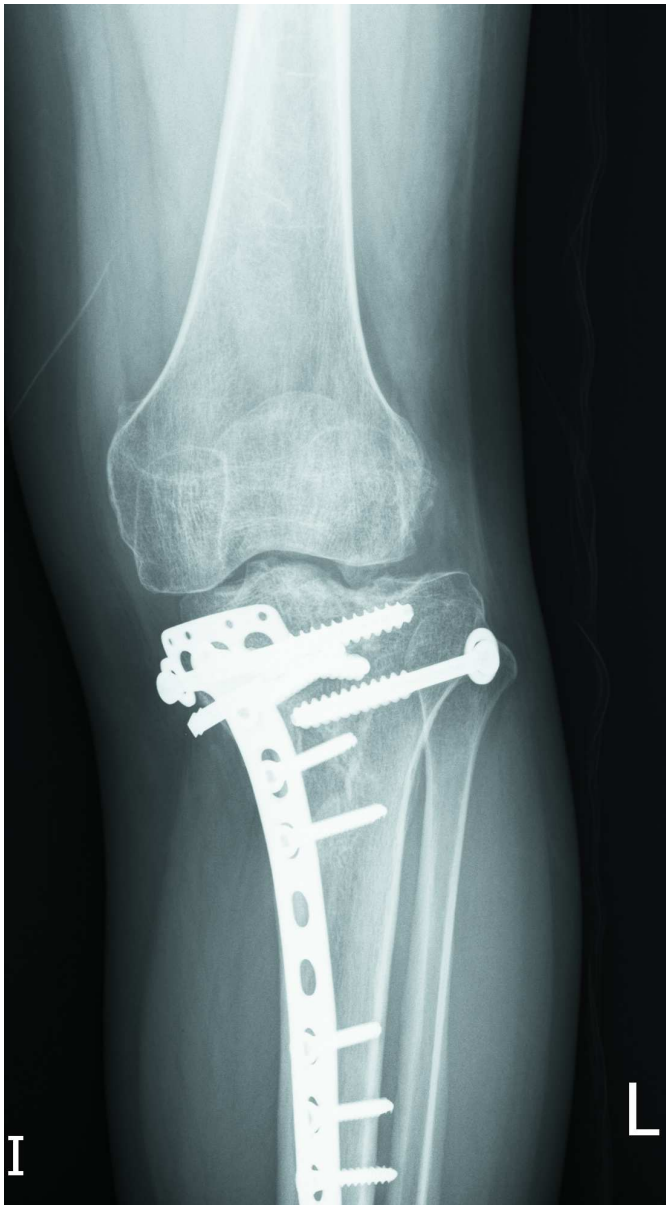
Cons: Concentration in supernatant



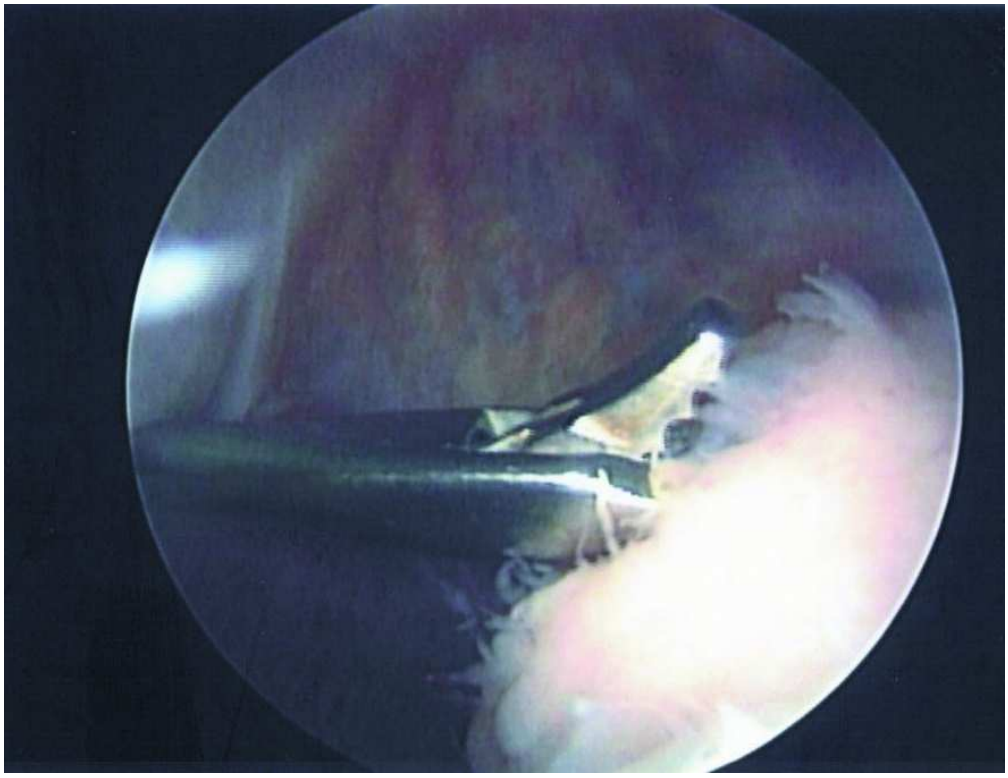
A 36-year-old woman with a tibia plateau fracture. (A) Preoperative radiograph revealing a left tibia plateau bicondylar fracture classified as Schatzker type five.
101x144mm (380 x 380 DPI)



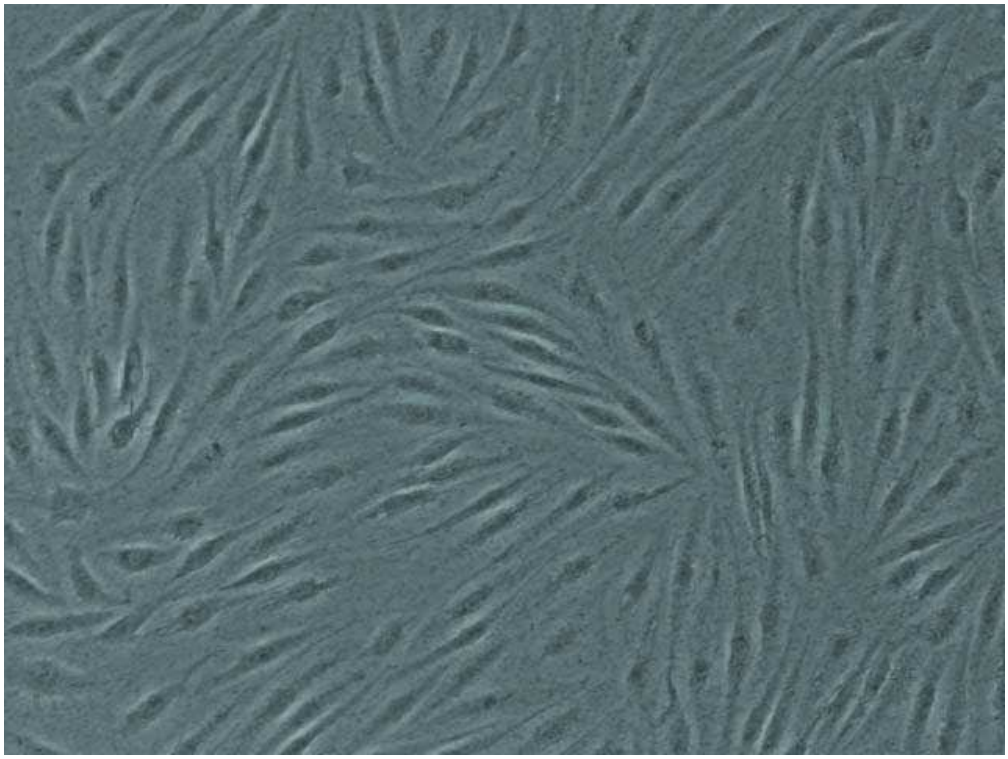
(B) Immediate postoperative radiograph displaying more than 2 mm residual step-off on the articular surface.
101x127mm (580 x 580 DPI)



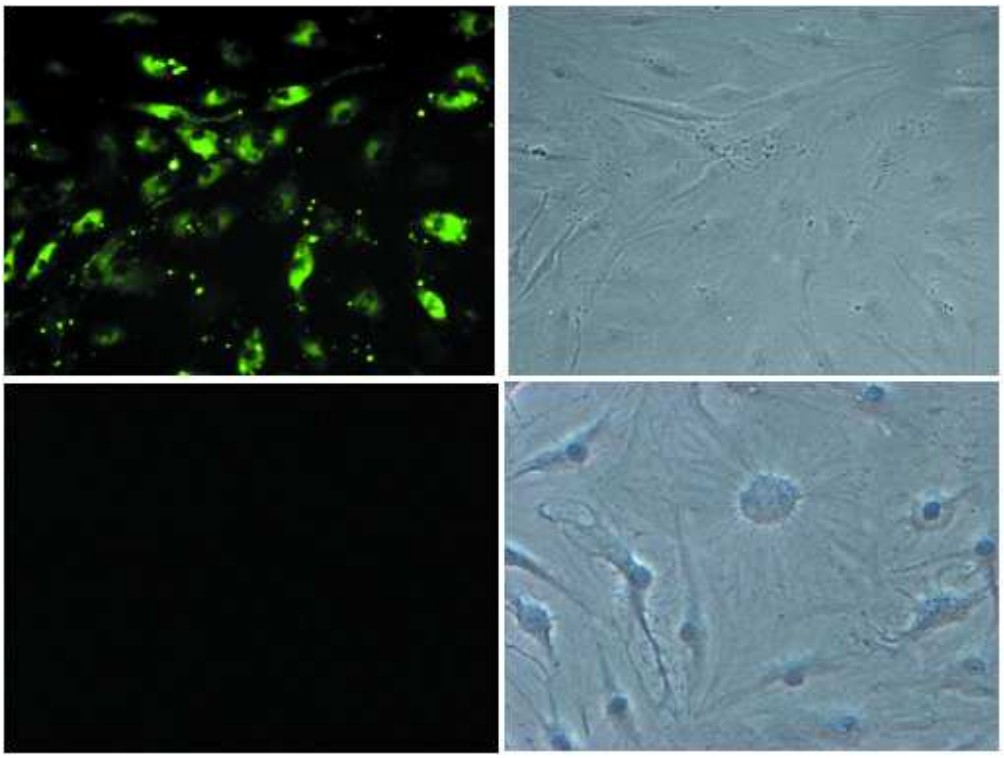
(C) Fracture consolidation achieved after 72 postoperative weeks.
88x160mm (313 x 313 DPI)



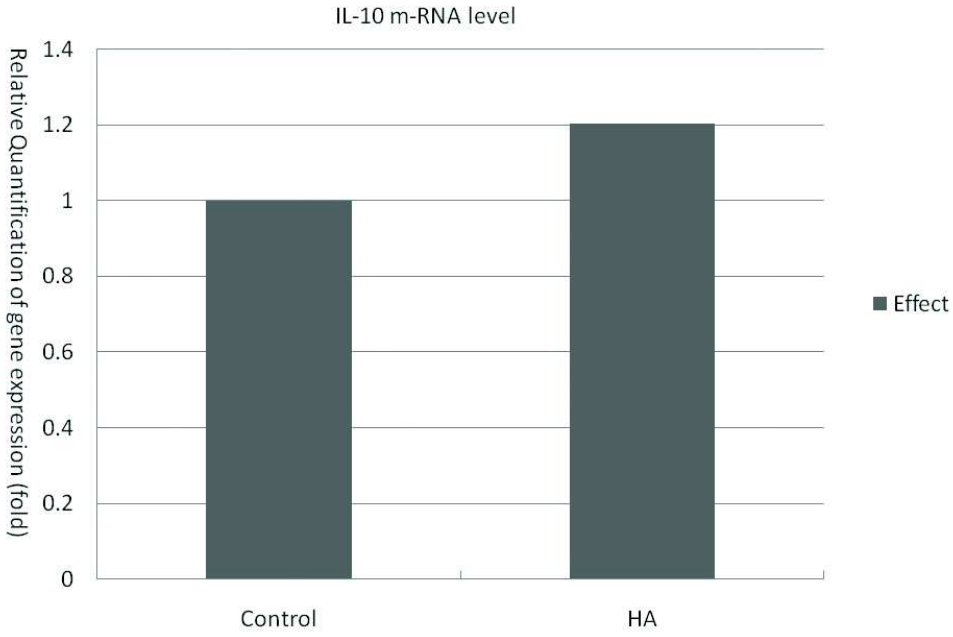
Prominent synovitis observed during arthroscopic surgery and use of a punch forceps to obtain the inflamed synovial tissue.
73x56mm (304 x 304 DPI)



Light microscopy revealing the homogeneous fibroblast-like and spindle-shaped morphology of the fibroblast-like synoviocyte (FLS) preparation.
53x39mm (305 x 305 DPI)



Light microscopy revealing strongly positive immunocytochemical staining for prolyl-4-hydroxylase for the majority of FLS (upper two photographs). No staining was observed for cells present in the negative control preparation (lower two photographs).
43x32mm (301 x 301 DPI)



Expression of IL-10 mRNA in FLS in response to treatment with hyaluronan (HA). FLS preparations obtained from two different subjects were treated for 24 h without or with HA (100 µg/ml). Findings are expressed as fold changes in expression as compared to the corresponding control preparation (no HA treatment).
81x60mm (300 x 300 DPI)