TISSUE ENGINEERING
Volume 12, Number 10, 2006

The Effect of Synovial Fluid from Injured Knee Joints on *in Vitro* Chondrogenesis

K.G. AUW YANG, M.D., D.B.F. SARIS, M.D., Ph.D., A.J. VERBOUT, M.D., Ph.D., L.B. CREEMERS, Ph.D., and W.J.A. DHERT, M.D., Ph.D.

ABSTRACT

Various *in vivo* and *in vitro* studies suggest that joint homeostasis may have a crucial effect on the quality of regeneration tissue resulting from cartilage tissue engineering techniques. The goal of the current study was to evaluate the effect of synovial fluid (SF) from injured knee joints on *in vitro* chondrogenesis. Chondrocytes were isolated from a healthy human femoral condyle (post-mortem) and expanded in monolayer for 2 passages. Subsequently, the chondrocytes were redifferentiated for 14 days on collagen-coated filters, cultured either in the presence or absence of 10% SF. SF was obtained from 12 injured human knee joints. After 14 days of culture, SF supplementation resulted in a significant downregulation of final proteoglycan (PG) content (7.3 ± 1.8 mg versus 15.6 ± 1.3 mg; $p = 0.0001$), PG content normalized to DNA (0.7 ± 0.5 mg/μg versus 3.0 ± 0.6 mg/μg; $p < 0.05$), relative collagen type II mRNA levels normalized to GAPDH mRNA levels (0.2 ± 0.3 versus 7.0 ± 5.6; $p < 0.001$), and differentiation index (collagen type II/I mRNA ratio; 0.1 ± 0.2 versus 6.0 ± 2.9; $p < 0.001$) as compared to control culture conditions. Additionally, SF-supplemented media resulted in significantly increased cellularity, as reflected by DNA content, compared with control media ($1{,}369 \pm 683$ μg versus 514 ± 72 μg; $p < 0.0001$). Morphology, and collagen type I, X, and aggrecan mRNA levels were not significantly affected. In conclusion, this study demonstrates that SF from injured human knee joints significantly affects *in vitro* chondrogenesis and therefore may provide a viable target for future improvement of ACT by refinement of culture techniques, patient selection, or pretreatment of affected joints to restore joint homeostasis.

INTRODUCTION

OVER THE LAST DECADE, autologous chondrocyte transplantation (ACT) has been increasingly applied for treatment of articular cartilage defects. However, the quality of regeneration tissue resulting from ACT varies greatly, ranging from hyaline cartilage to fibrous or hypertrophic tissues, which have inadequate mechanical properties and are likely to either degenerate over time or lead to more generalized joint destruction.[1–3]

In clinically applied ACT, chondrocytes are generally isolated from small cartilage biopsies and subsequently are expanded in monolayer to obtain sufficient cells for treatment of cartilage defects. During expansion, chondrocytes dedifferentiate and lose their rounded shape, produce less cartilage-specific matrix proteins, such as collagen type II, and behave more like a fibroblastic cell type, producing increased amounts of collagen type I.[4,5] To obtain regenerated tissue with adequate mechanical properties, these cells need to be stimulated to redifferentiate and synthesize appropriate amounts of hyaline cartilage-specific proteins.

In vivo, redifferentiation and extracellular matrix (ECM) synthesis by *in vitro* expanded chondrocytes might be affected by various factors, of which the environment of redifferentiating chondrocytes in ACT might be crucial. This environment is determined by a variety of naturally occurring

Department of Orthopaedics, University Medical Center Utrecht, the Netherlands.

intra-articular components, of which synovial fluid (SF) is frequently proposed as a key mediator. Normal SF is a dialysate of blood plasma with the addition of components synthesized by synovial tissue and catabolic products from the surrounding tissues, for example, proteoglycan (PG) and collagen breakdown products.[6] SF nourishes articular cartilage, contains growth factors and, dependent on the status of the joint, pro-inflammatory and anti-inflammatory cytokines.[7–9] Relatively little is known about the role of SF in cartilage turnover, in particular during cartilage regeneration after trauma. Autologous SF obtained from healthy equine joints is has been demonstrated to support chondrogenic differentiation of equine mesenchymal stem cells.[10] However, various studies suggest that joint homeostasis is disturbed in traumatized joints, which was found to adversely affect chondrogenesis.[11,12] This effect may at least partly be mediated through changes in SF content.

The goal of this study was to investigate the effect of human SF from injured knee joints on *in vitro* chondrogenesis by expanded human chondrocytes in a previously validated culture model.[13] Collagen types I and II and aggrecan mRNA levels were determined to evaluate the effect of SF on the degree of redifferentiation. Collagen type X mRNA levels were measured to detect hypertrophic changes.[14–17] Tissue morphology, PG content, and cell content of the ECM were determined to assess the effect of SF on the final tissue quality.

Table 1. Demographic Data of Synovial Fluid Samples

Patient No.	*Patient age [Years]*	*Defect age [Days]*	*Diagnosis*
1	27	5	Knee distortion with pain and swelling: no further tissue damage demonstrated
2	43	7	Knee distortion with pain and swelling: no further tissue damage demonstrated
3	23	8	Cartilage defect Medial Femoral condyle
4	47	9	Knee distortion with pain and swelling: no further tissue damage demonstrated
5	28	14	Medical meniscus rupture
6	36	14	Cartilage defect Medial Femoral condyle
7	20	31	Cartilage defect Medial Femoral condyle and anterior cruciate ligament rupture
8	33	39	Cartilage defect Medial Femoral condyle
9	46	254	Cartilage defect Medial Femoral condyle
10	46	341	Cartilage defect Medial Femoral condyle and medial meniscus rupture
11	44	885	Cartilage defect Medial Femoral condyle
12	46	>1000	Cartilage defect Medial Femoral condyle

MATERIALS AND METHODS

Synovial fluid collection

SF samples were collected from 12 patients, either during arthroscopy or in the outpatient clinic. Demographic and clinical data of these samples are summarized in Table 1. Defect ages were determined from patient history. Diagnoses were determined either by magnetic resonance imaging or arthroscopically. The donors had no clinical history of inflammatory or degenerative joint disorders and did not use corticosteroids (systemically or intra-articularly). After aspiration, the samples were spun at 300 *g* to remove debris and stored in small aliquots at -80°C until further use.

Culture methods

Articular cartilage was harvested post mortem (within 24 h after death) from a femoral condyle of a 48-year-old human female donor. The donor had no clinical history of degenerative, inflammatory, or crystalline joint disorders and did not use corticosteroids (systemically or intra-articularly).

Within 12 hours after biopsy, chondrocytes were isolated by a 3-h 0.1% pronase (Roche, Mannheim, Germany) digestion at 37°C followed by an O/N 0.04% collagenase (Sigma, St. Louis, MO) digestion at 37°C. The chondrocytes were plated at a cell density of 5,000 cells/cm^2 and were expanded in monolayer at 37°C and 5% carbon dioxide (CO_2) for 2 passages. The culture media consisted of DMEM (Gibco, Carlsbad, CA) containing L-glutamine, 4.5 mg/mL glucose, 25 mmol HEPES buffer, 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 ng/mL basic fibroblast growth factor (bFGF; R&D, Minneapolis, MN). No bFGF was added to the culture media during the first 3 days. The culture medium was renewed every 3 days. At confluency, the cells were trypsinized using 0.25% trypsin/EDTA. Viable cells were counted using a Bürker-Turk hemocytometer after staining with Trypan blue.

After 2 passages, the expanded chondrocytes were seeded at a cell density of 1.6×10^6 cells/cm^2 on Millicell filters (Millipore Co., Bedford, MA) precoated with collagen type II (Sigma; redifferentiation culture). The filters were cultured in DMEM (Gibco) containing, L-glutamine, 2% human serum albumin (Equitech-Bio, Kerrville, TX), $1\times$ ITSx (Gibco), 5 ng/mL transforming growth factor (TGF)-β2 (R&D), and 0.4 mmol ascorbic acid (control medium). The culture media of the experimental group were supplemented with 10% SF (100 µL per sample). In both treatment groups, the growth factors were added to the culture media

after all other components were mixed to ensure identical amounts TGF-β supplemented to the culture media. The cultures were incubated at 37°C in 5% CO_2. Culture media were renewed at 7 and 10 days of culture. The samples were harvested after 14 days of culture. For each analysis method, 3 samples per SF donor were cultured. The results of the present study were confirmed by repeating the experiment with chondrocytes isolated from a 51-year-old human male donor. Results that were not consistent with results observed in the first experiment are mentioned explicitly in the Results section.

Analytical methods

Histologic analysis. Samples were fixed in 10% buffered formalin and cut at 5 μm for histologic evaluation (Safranin O/Fast green) after 14 days of redifferentiation culture. Before embedding the filters, they were first rinsed in PBS and subsequently halved. One half was embedded for histology and the other half was used for PG and DNA analysis. Safranin O staining was performed as described previously by Rosenberg.[18] The histologic quality of the tissue was analyzed by 4 blinded observers using a scoring system specifically designed to evaluate cartilage synthesized *in vitro*. This scoring system is hereafter referred to as the Bern score.[19] This scoring system evaluates 3 parameters: (1) the darkness and uniformity of the Safranin O staining, (2) the amount and organization of the ECM, and (3) the morphology of the cell. Each item can be scored with a minimum of 0 points (worse) and a maximum of 3 points (best); thus, the maximum score is 9 points (histologically resembling hyaline cartilage).

Proteoglycan content of tissue samples. The PG content after 14 days of culture was analyzed using a previously described Alcian blue assay.[20,21] To this end, the samples were digested in 12% papain dissolved in 50 mmol phosphate buffer, 2 mmol *N*-acetylcysteine, and 2 mmol Na_2-EDTA, pH 6.5, at 65°C for 2 h. Part of the digest was used to measure the DNA content. From the remaining digest, GAGs were precipitated and stained with an Alcian blue dye solution (Alcian blue 8GX, Sigma, saturated in 0.1 mol sodium acetate buffer, containing 0.3 M $MgCl_2$, pH 6.2) for 30 min at 37°C. The blue staining was quantified spectrophotometrically from the change in absorbance at 620 nm. Chondroitin sulphate (Sigma, St. Louis, MO) was used as a reference.

DNA content. After 14 days of culture, the DNA content of the samples was determined as a measure of cellularity. In the papain digest samples, DNA was stained with the fluorescent dye HOECHST 33258 as described previously and fluorescence was measured on the Cytofluor.[22] Calf thymus DNA (Sigma) was used as a reference.

Total RNA isolation. After 14 days of redifferentiation culture, total RNA was extracted. To this end, redifferentiated chondrocytes were lysed in RNeasy lysis buffer (RLT buffer, RNeasy Mini Kit) and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.[23] RNA concentration was measured spectrophotometrically at 260 nm and sufficient RNA purity was assumed if the 260/280 nm ratio was between 1.6 and 2.0. Subsequently, RNA samples were DNase treated and 500 ng of RNA was reverse transcribed using the iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA).

Semiquantitative real-time polymerase chain reaction analysis. Collagen type I, II, X, and aggrecan mRNA levels were determined using a Taqman assay on the ABI prism 7900HT sequence detection system. Primers and probes were generously provided by Dr. H. Jahr and Dr. G.J.V.M. van Osch (Department of Orthopaedics, Rotterdam, the Netherlands). Collagen and aggrecan primer and probe sequences were previously published by Mandl *et al.*[24] and Martin *et al.*[25] GAPDH primer and probe endogenous control mix for the Taqman assay is commercially available (Applied Biosystems, Foster City, CA). The expression levels were normalized to endogenous GAPDH expression levels and were calculated using the $2^{-\Delta\Delta CT}$ formula (ABI Prism 7700 sequence Detection System User bulletin #2). Because collagen type II is one of the typical structural proteins synthesized by differentiated chondrocytes in hyaline cartilage, as compared with collagen type I that is normally synthesized by dedifferentiated chondrocytes, we defined the ratio of collagen type II and collagen type I mRNA levels as *differentiation index*.

Statistical analysis

The effect of SF supplementation compared to no supplementation was analyzed using a separate-variance *t*-test. If nonnormal distribution or unequal variance was observed among the treatment groups, that specific variable was analyzed by the Wilcoxon rank-sum test. The graphs show average values in either treatment group; $p < 0.05$ was considered statistically significant. Statistical calculations were done with the JMP (Cary, NC) version 5.0 software package.

RESULTS

Tissue morphology

Cultures with and without SF supplementation resulted in the synthesis of fibrocartilaginous tissue by chondrocytes. The ECM stained positive with Safranin O in all samples and most chondrocytes had a rounded appearance, although a substantial part of the cells had a fibroblastic appearance and lacked lacunae, typical for native chondrocytes in articular cartilage (Fig. 1A, B). No differences in tissue morphology between both treatment groups were observed. To objectify this finding, 3 blinded observers scored the

morphological quality of the tissue synthesized according to the Bern score. No statistically significant differences were found between the treatment groups; tissue synthesized under control conditions scored 4.9 ± 0.7 points and tissue synthesized in SF-supplement culture media scored 4.6 ± 0.8 points (Fig. 1C).

Proteoglycan synthesis and DNA content

ECM synthesis as measured by the final PG content of the tissue was significantly affected by the supplementation of SF to the culture media. Chondrocyte redifferentiation culture under control conditions resulted in a significantly higher amount of PGs synthesized after 14 days of culture compared to SF-supplemented culture conditions (15.6 ± 1.3 mg versus 7.3 ± 1.8 mg, respectively; $p = 0.0001$; Fig. 2A). PG content normalized to DNA content showed a similar significant downregulation by SF-supplemented culture conditions compared to control culture conditions (0.7 ± 0.5mg/μg versus 3.0 ± 0.6 mg/μg, respectively; $p < 0.05$; Fig. 2B).

Chondrocyte redifferentiation under SF-supplemented culture conditions resulted in a significantly higher DNA content after 14 days of culture compared to control media ($1{,}369 \pm 683$ μg versus 514 ± 72 μg, respectively; $p < 0.0001$; Fig. 2C).

Post-redifferentiation mRNA levels

SF supplementation resulted in significant downregulation of relative collagen type II mRNA levels (0.2 ± 0.3 versus 7.0 ± 5.6, respectively) and the differentiation index (the collagen type II/I mRNA ratio; 0.1 ± 0.2 versus 6.0 ± 2.9, respectively) as compared with control media (Fig. 3B, C).

SF supplementation appeared to result in upregulation of collagen type I mRNA levels in both donors, although this effect was not consistently significant (donor 1, 28.3 ± 61.7 versus 10.6 ± 3.9, respectively, $p = 0.31$; donor 2, 10.2 ± 9.1 versus 4.5 ± 1.7, respectively, $p = 0.03$; Fig. 3A). Aggrecan mRNA levels were not significantly different between culture conditions (control 5.1 ± 1.7 versus 10% SF 8.6 ± 13.8; Fig. 3D). Collagen type X was below detection level in all samples.

DISCUSSION

To optimize the quality of tissue formed after clinically applied ACT, cartilage tissue engineering has been studied extensively, both *in vitro* and *in vivo*. However, few studies have aimed at elucidating the effect of the *in vivo* environment on chondrogenesis. The current study clearly demonstrates that *in vitro* chondrogenesis is significantly affected by supplementation of culture media with SF obtained from injured knee joints. After 14 days of culture, SF supplementation resulted in a significant decrease of final PG content, significant increase of final cellularity, and a significant inhibition of chondrocyte redifferentiation at the mRNA level.

The observed decrease in final PG content by chondrocyte redifferentiation in SF-supplemented culture media may be caused either by a higher anabolic activity of chondrocytes cultured in control media, a higher catabolic activity of chondrocytes cultured in SF-supplemented culture media, or a combination of these 2 factors. Either way,

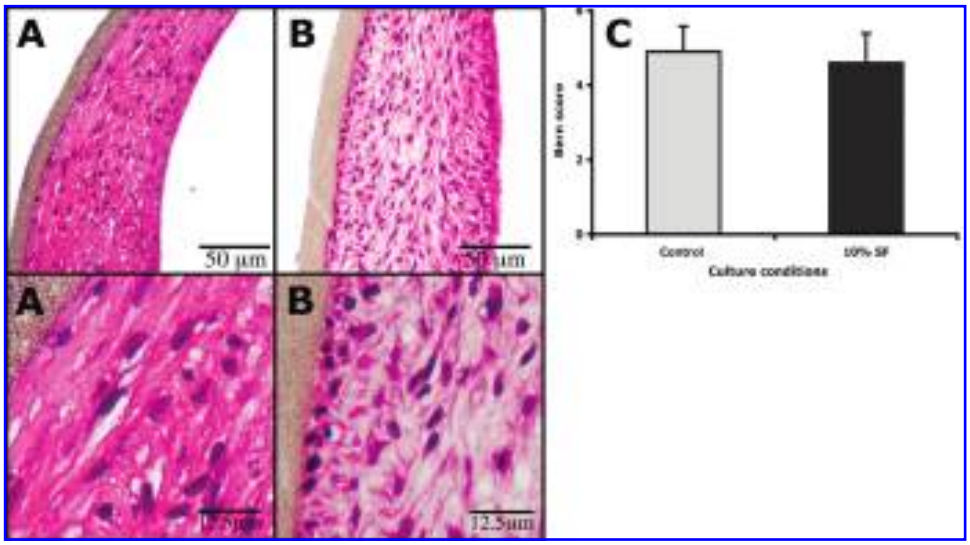

FIG. 1. Five-micrometer histologic sections with safranin O/fast green staining after 14 days of culture of tissue synthesized by chondrocytes redifferentiated in (**A**) control media and (**B**) culture media supplemented with 10% SF. Original magnification × 40. (**C**) Bern histologic score after 14 days of culture. Bars represent average ± SD. Color images available online at www.liebertpub.com/ten.

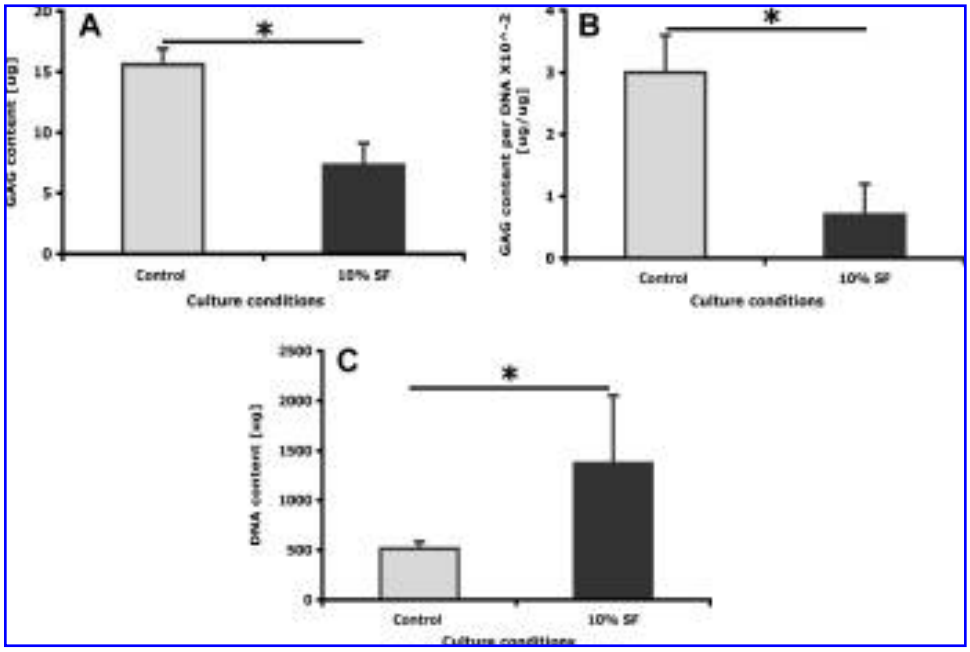

FIG. 2. (**A**) Final GAG content. (**B**) Final GAG content normalized to DNA content. (**C**) Final DNA content. Bars represent average ± SD. *$p < 0.05$. Note that SF supplementation to redifferentiation media significantly inhibits the final PG content of the tissue synthesized and stimulates the cellularity compared to control redifferentiation conditions.

these results in combination with decreased redifferentiation suggest that SF from injured knee joints is detrimental to *in vitro* chondrogenesis. Possible factors responsible are chemokines, inflammatory cytokines, and matrix metalloproteinases. These mediators have been described to be induced as a physiologic reaction to damage in a variety of tissues and are known to be crucial for cutaneous wound healing, as an example.[26–29] Likewise, joint injuries might

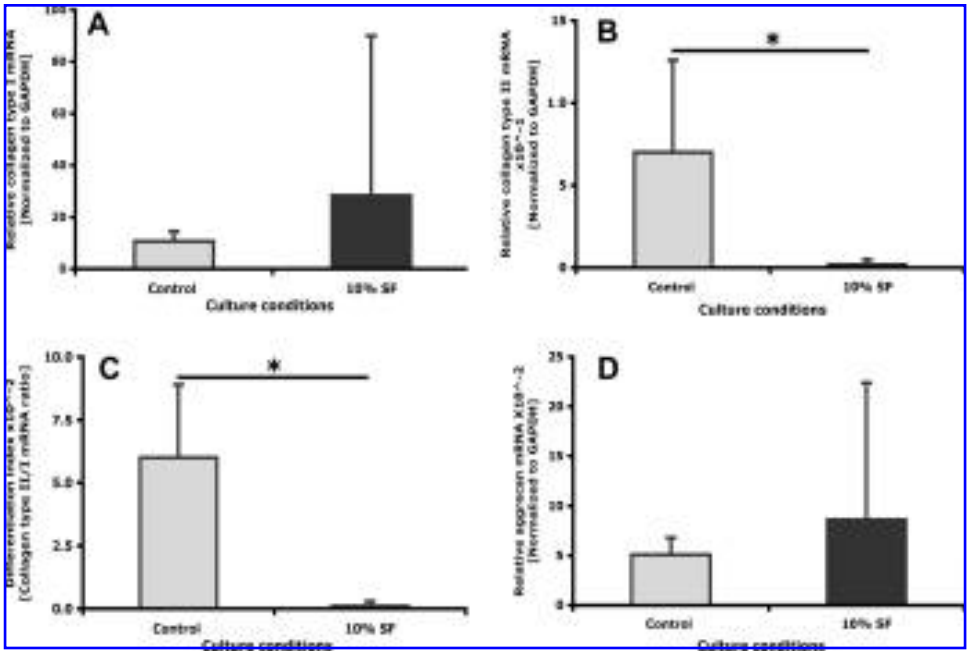

FIG. 3. Relative mRNA levels of (**A**) collagen type I, (**B**) collagen type II, (**C**) the collagen type II/I ratio (differentiation index), and (**D**) aggrecan in tissue synthesized by expanded chondrocytes that were redifferentiated on collagen-coated filters for 14 days. The mRNA levels shown in these graphs are relative expression levels after normalization to GAPDH as endogenous control. *$p < 0.05$. Note that SF supplementation results in significantly decreased collagen type II mRNA levels and differentiation index compared to control redifferentiation conditions.

result in the induction of such mediators.[30,31] However, in the context of cartilage biology, these mediators are suggested to be involved in osteoarthritis development and to be detrimental to the ECM of articular cartilage.[7,32] Therefore, they might be responsible for the decreased final PG content in SF-supplemented cultures by inducing increased PG degeneration rate. Future studies should aim at elucidating the factors responsible for the observed effects, because these might provide a possible target for improvement of clinically applied ACT, for example, by desensitizing chondrocytes for specific cytokines using siRNA or restoring a beneficial joint homeostasis for chondrogenesis by pretreatment of joints. Similar treatments have been studied in patients with rheumatoid arthritis, namely, tumor necrosis factor-α inhibitors.[33,34]

In contrast with our results, Hegewald *et al.*[10] demonstrated that autologous SF obtained from healthy equine joints is supportive for *in vitro* chondrogenic differentiation and PG synthesis by equine mesenchymal stem cells. Furthermore, Skoog *et al.*[35] presented similar supportive effects for *in vitro* chondrogenesis from perichondral tissue harvested from rabbit rib cartilage. These discrepancies may be explained by the fact that, in the current study, SF samples were harvested from human injured knee joints in contrast to described studies, which used SF obtained from healthy joints. This hypothesis appears supported by the fact that SF from ''chronically'' injured knee joints has indeed been demonstrated to inhibit the PG synthesis rate in a chick limb bud assay.[11] However, in the same study, PG synthesis rate was stimulated by SF from ''acutely'' injured knee joints. Likewise, periosteal transplantation in joints with longer existing cartilage defects has been demonstrated to result in regeneration tissue with significantly worse morphology and to affect PG turnover of the treated joint negatively compared to periosteal transplantation in joints with ''fresh'' cartilage defects.[12] These studies suggest that the effect of SF on chondrogenesis is related to the age of injury of the joint from which the SF samples are harvested. Such trauma-age–related effects were not observed in the current study. This may be due to various factors, such as the culture model used, differences in chondrocyte species, the limited number of SF samples, or the outcome parameter used; the described studies determined PG turnover parameters rather than final PG content, and the latter appears more relevant because this has a direct relation to mechanical properties of the tissue. These studies do confirm that joint injury may result in disturbed joint homeostasis, which may be detrimental for the quality of regenerating tissue after ACT.

Interestingly, SF supplementation resulted in significantly higher cellularity of tissue formed after 14 days of culture. These results are in accordance with a recently published study in which cartilage injuries were demonstrated to result in bFGF release.[36] Besides being a potent mitogen, bFGF is known to be a potent inhibitor of the anabolic effects of both IGF-1 and OP-1; bFGF significantly downregulated the PG synthesis rate of culture chondrocytes induced by these growth factors.[37] A similar mechanism might be responsible for the observed effects of SF supplementation in the current study. However, in an animal study Fukuda *et al.*[38] demonstrated that treatment of osteochondral defects by matrix induced autologous chondrocyte implantation resulted in macroscopically, morphologically, and mechanically improved tissue regeneration when scaffolds were impregnated with bFGF. These apparently contrasting results may be explained if bFGF-induced proliferation precedes redifferentiation rather than occurring simultaneously. A similar process was previously suggested to occur during *in vitro* periosteal chondrogenesis.[39] Although this hypothesis seems to be supported by the downregulation of redifferentiation parameters in our study, future experiments with extended culture periods should investigate whether this hypothesis holds true. Joint-injury–induced upregulation of intra-articular bFGF levels might actually result in increased amounts of regeneration tissue, rather than detrimental as the results of the current study suggest.

Altogether, various parameters of ECM quality and chondrocyte redifferentiation appear to be strongly affected by SF supplementation to redifferentiation media. However, pinpointing the exact factors involved in the effects found is difficult, as various limitations should be considered in this study. All SF tested were obtained from injured knee joints. Therefore, the observed effects cannot be assigned with certainty to the fact that the joints, from which the SF samples were harvested were injured. Unfortunately, human SF samples from healthy joints are sparsely available. Furthermore, tissue quality was determined at only 1 time point, limiting the possibility of temporal interpretations of the current data. Whether decreased PG content combined with increased cellularity actually represent detrimental effects induced by SF supplementation, or rather reflects increased amounts of tissue that may be synthesized, remains to be elucidated in future studies with extended culture periods. Finally, SF samples that were supplemented to the redifferentiation media were obtained from different donors than the chondrocytes, which suggests that the adverse effects induced by SF supplementation may be due to immunologic rejection. However, the significantly increased final cellularity induced by SF supplementation contradicts this hypothesis.

In conclusion, SF supplementation to culture media significantly affects *in vitro* chondrogenesis, suggesting that caution is warranted when investigating *in vitro* chondrocyte redifferentiation without SF supplementation to culture media. Moreover, this study suggests that SF from injured knee joints has a detrimental effect on redifferentiation and ECM synthesis. Therefore, factors present in the SF of injured joints may, once identified, provide a viable target for future improvement of ACT by refinement of culture techniques, patient selection, or pretreatment of affected joints to restore joint homeostasis.

ACKNOWLEDGMENTS

Dr. Auw Yang is supported by the Anna Foundation and the Foundation "De Drie Lichten" in the Netherlands. Dr. Saris is supported by the Netherlands Organisation for Health Research and Development (NWO). The authors thank Dr. P. Westers from the center of Biostatistics (University Utrecht) for his statistical assistance with this study, Dr. G. van Osch and Dr. H. Jahr (Erasmus Medical Center, Rotterdam, the Netherlands) for providing PCR primes, and the Dutch Bone Foundation (BIS, Leiden, the Netherlands) for providing cartilage.

REFERENCES

1. Horas, U., Pelinkovic, D., Herr, G., Aigner, T., and Schnettler, R. Autologous chondrocyte implantation and osteochondral cylinder transplantation in cartilage repair of the knee joint. A prospective, comparative trial. J Bone Joint Surg Am **85A,** 185, 2003.
2. Knutsen, G., Engebretsen, L., Ludvigsen, T.C., Drogset, J.O., Grontvedt, T., Solheim, E., Strand, T., Roberts, S., Isaksen, V., and Johansen, O. Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial. J Bone Joint Surg Am **86,** 455, 2004.
3. Peterson, L., Minas, T., Brittberg, M., Nilsson, A., Sjogren-Jansson, E., and Lindahl, A. Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. Clin Orthop **374,** 212, 2000.
4. Schnabel, M., Marlovits, S., Eckhoff, G., Fichtel, I., Gotzen, L., Vecsei, V., and Schlegel, J. Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. Osteoarthritis Cartilage **10,** 62, 2002.
5. Benya, P.D., and Shaffer, J.D. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell **30,** 215, 1982.
6. Swann, D.A. Macromolecules of synovial fluid. In: Sokoloff, L., ed. The Joints and Synovial Fluid. London: Academic Press, 1978, p. 407.
7. Fernandes, J.C., Martel-Pelletier, J., and Pelletier, J.P. The role of cytokines in osteoarthritis pathophysiology. Biorheology **39,** 237, 2002.
8. Neidel, J., Schulze, M., Sova, L., and Lindschau, J. [Practical significance of cytokine determination in joint fluid in patients with arthroses or rheumatoid arthritis]. Z Orthop Ihre Grenzgeb **134,** 381, 1996.
9. Okazaki, R., Sakai, A., Uezono, Y., Ootsuyama, A., Kunugita, N., Nakamura, T., and Norimura, T. Sequential changes in transforming growth factor (TGF)-beta1 concentration in synovial fluid and mRNA expression of TGF-beta1 receptors in chondrocytes after immobilization of rabbit knees. J Bone Miner Metab **19,** 228, 2001.
10. Hegewald, A.A., Ringe, J., Bartel, J., Kruger, I., Notter, M., Barnewitz, D., Kaps, C., and Sittinger, M. Hyaluronic acid and autologous synovial fluid induce chondrogenic differentiation of equine mesenchymal stem cells: a preliminary study. Tissue Cell **36,** 431, 2004.
11. Rodrigo, J.J., Steadman, J.R., Syftestad, G., Benton, H., and Silliman, J. Effects of human knee synovial fluid on chondrogenesis in vitro. Am J Knee Surg **8,** 124, 1995.
12. Saris, D.B.F., Dhert, W.J.A., and Verbout, A.J. Joint homeostasis: the discrepancy between old and fresh defects in cartilage repair. J Bone Joint Surg Br, **85B,** 1067, 2003.
13. Auw Yang, K.G., Saris, D.B.F., Geuze, R.E., van der Helm, Y.J.M., van Rijen, M.H.P., Verbout, A.J., Dhert, W.J., and Creemers, L.B. Impact of expansion and redifferentiation conditions on chondrogenic capacity of cultured chondrocytes. Tissue Eng (in press).
14. Gack, S., Vallon, R., Schmidt, J., Grigoriadis, A., Tuckermann, J., Schenkel, J., Weiher, H., Wagner, E.F., and Angel, P. Expression of interstitial collagenase during skeletal development of the mouse is restricted to osteoblast-like cells and hypertrophic chondrocytes. Cell Growth Differ **6,** 759, 1995.
15. Johansson, N., Saarialho-Kere, U., Airola, K., Herva, R., Nissinen, L., Westermarck, J., Vuorio, E., Heino, J., and Kahari, V.M. Collagenase-3 (MMP-13) is expressed by hypertrophic chondrocytes, periosteal cells, and osteoblasts during human fetal bone development. Dev Dyn **208,** 387, 1997.
16. Uusitalo, H., Hiltunen, A., Soderstrom, M., Aro, H.T., and Vuorio, E. Expression of cathepsins B, H, K, L, and S and matrix metalloproteinases 9 and 13 during chondrocyte hypertrophy and endochondral ossification in mouse fracture callus. Calcif Tissue Int **67,** 382, 2000.
17. Wu, C.W., Tchetina, E.V., Mwale, F., Hasty, K., Pidoux, I., Reiner, A., Chen, J., Van Wart, H.E., and Poole, A.R. Proteolysis involving matrix metalloproteinase 13 (collagenase-3) is required for chondrocyte differentiation that is associated with matrix mineralization. J Bone Miner Res **17,** 639, 2002.
18. Rosenberg, L. Chemical basis for the histological use of safranin O in the study of articular cartilage. J Bone Joint Surg Am **53,** 69, 1971.
19. Grogan, S.P., Barbero, A., Winkelmann, V., Fitzsimmons, J.S., O'Driscoll, S.W., Martin, I., and Mainil-Varlet, P. Development of a visual histological grading system to evaluate tissue engineered cartilage. Annual meeting of the Orthopaedic Research Society, Washington, DC, February 19–22, 2005.
20. Lafeber, F.P., Vander Kraan, P.M., Van Roy, J.L., Huber-Bruning, O., and Bijlsma, J.W. Articular cartilage explant culture; an appropriate in vitro system to compare osteoarthritic and normal human cartilage. Connect Tissue Res **29,** 287, 1993.
21. Whiteman, P. The quantitative measurement of Alcian Blue-glycosaminoglycan complexes. Biochem J **131,** 343, 1973.
22. Kim, Y.J., Sah, R.L., Doong, J.Y., and Grodzinsky, A.J. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Anal Biochem **174,** 168, 1988.
23. Gehrsitz, A., McKenna, L.A., Soder, S., Kirchner, T., and Aigner, T. Isolation of RNA from small human articular cartilage specimens allows quantification of mRNA expression levels in local articular cartilage defects. J Orthop Res **19,** 478, 2001.
24. Mandl, E.W., Jahr, H., Koevoet, J.L., Van Leeuwen, J.P., Weinans, H., Verhaar, J.A., and Van Osch, G.J. Fibroblast growth factor-2 in serum-free medium is a potent mitogen and

reduces dedifferentiation of human ear chondrocytes in monolayer culture. Matrix Biol **23,** 231, 2004.
25. Martin, I., Jakob, M., Schafer, D., Dick, W., Spagnoli, G., and Heberer, M. Quantitative analysis of gene expression in human articular cartilage from normal and osteoarthritic joints. Osteoarthritis Cartilage **9,** 112, 2001.
26. Gailit, J., and Clark, R.A. Wound repair in the context of extracellular matrix. Curr Opin Cell Biol **6,** 717, 1994.
27. Gillitzer, R., and Goebeler, M. Chemokines in cutaneous wound healing. J Leukoc Biol **69,** 513, 2001.
28. Saarialho-Kere, U.K. Patterns of matrix metalloproteinase and TIMP expression in chronic ulcers. Arch Dermatol Res **290**(suppl), S47, 1998.
29. Saarialho-Kere, U.K., Kovacs, S.O., Pentland, A.P., Olerud, J.E., Welgus, H.G., and Parks, W.C. Cell-matrix interactions modulate interstitial collagenase expression by human keratinocytes actively involved in wound healing. J Clin Invest **92,** 2858, 1993.
30. Tchetverikov, I., Lohmander, L.S., Verzijl, N., Huizinga, T.W., TeKoppele, J.M., Hanemaaijer, R., and DeGroot, J. MMP protein and activity levels in synovial fluid from patients with joint injury, inflammatory arthritis, and osteoarthritis. Ann Rheum Dis **64,** 694, 2005.
31. Chan, P.S., Schlueter, A.E., Coussens, P.M., Rosa, G.J., Haut, R.C., and Orth, M.W. Gene expression profile of mechanically impacted bovine articular cartilage explants. J Orthop Res **23,** 1146, 2005.
32. Goldring, M.B. Osteoarthritis and cartilage: the role of cytokines. Curr Rheumatol Rep **2,** 459, 2000.
33. Barrera, P., Joosten, L.A., den Broeder, A.A., van de Putte, L.B., van Riel, P.L., and van den Berg, W.B. Effects of treatment with a fully human anti-tumour necrosis factor alpha monoclonal antibody on the local and systemic homeostasis of interleukin 1 and TNFalpha in patients with rheumatoid arthritis. Ann Rheum Dis **60,** 660, 2001.
34. Maini, R.N., Elliott, M.J., Brennan, F.M., Williams, R.O., Chu, C.Q., Paleolog, E., Charles, P.J., Taylor, P.C., and Feldmann, M. Monoclonal anti-TNF alpha antibody as a probe of pathogenesis and therapy of rheumatoid disease. Immunol Rev **144,** 195, 1995.
35. Skoog, V., Widenfalk, B., Ohlsen, L., and Wasteson, A. The effect of growth factors and synovial fluid on chondrogenesis in perichondrium. Scand J Plast Reconstr Surg Hand Surg **24,** 89, 1990.
36. Vincent, T., Hermansson, M., Bolton, M., Wait, R., and Saklatvala, J. Basic FGF mediates an immediate response of articular cartilage to mechanical injury. Proc Natl Acad Sci U S A **99,** 8259, 2002.
37. Loeser, R.F., Chubinskaya, S., Pacione, C., and Im, H.J. Basic fibroblast growth factor inhibits the anabolic activity of insulin-like growth factor 1 and osteogenic protein 1 in adult human articular chondrocytes. Arthritis Rheum **52,** 3910, 2005.
38. Fukuda, A., Kato, K., Hasegawa, M., Hirata, H., Sudo, A., Okazaki, K., Tsuta, K., Shikinami, Y., and Uchida, A. Enhanced repair of large osteochondral defects using a combination of artificial cartilage and basic fibroblast growth factor. Biomaterials **26,** 4301, 2005.
39. O'Driscoll, S.W. Articular cartilage regeneration using periosteum. Clin Orthop **367**(suppl), S186, 1999.

Address reprint requests to:
D.B.F. Saris
Department of Orthopaedics
University Medical Center Utrecht
PO Box 85500
3508 GA Utrecht
The Netherlands

E-mail: d.saris@umcutrecht.nl