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REGULAR ARTICLE

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Growth factor impact on articular cartilage subpopulations

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Abstract We have examined the effects of growth factor stimulation on superficial and growth zone chondrocyte populations. Zonal articular chondrocytes from 8-month-old Spanish goat distal femurs were plated in monolayer cultures and stimulated by using insulin-like growth factor I (IGF-I), basic fibroblast growth factor (bFGF), and transforming growth factor-β1 (TGF-β1). Gene expression for collagen I and II, aggrecan, and superficial zone protein were evaluated every week for 3 weeks. Finally, proteoglycan and collagen deposition were measured for each experimental group. Major differences existed in the behavior of superficial and growth zone chondrocytes, the most apparent being the higher capacity for protein synthesis by the growth zone population. Variations also existed regarding growth factor treatment. TGF-β1 had the greatest effect on proliferation over 8 days. With respect to differentiation, IGF-I increased average collagen II gene expression in the growth zone populations in comparison with growth zone controls. IGF-I increased aggrecan gene expression for the same groups. Superficial zone populations exhibited lower collagen II, collagen I, and aggrecan gene expression than the growth zone populations under all conditions. However, superficial zone protein expression was dramatically elevated in superficial zone populations by TGF-β1. Collagen I expression showed a general increase under all conditions compared with initial values. Combined biosynthesis results showed that the superficial populations secreted little to no collagen, especially collagen II, in comparison with their growth zone counterparts. Glycosaminoglycan production was also much lower than for the growth zone groups. TGF-β1 and IGF-I increased collagen II production in the growth zone populations. TGF-β1 increased glycosaminoglycan secretions in the superficial zone populations and in the growth zone populations, whereas IGF-I produced an increase in glycosaminoglycan secretion only in the growth zone populations. Thus, growth factors elicit different proliferation, gene expression, and biosynthesis responses from zonal chondrocyte subpopulations.

Keywords Chondrocyte · Zones · IGF · TGF · FGF · Growth · Differentiation · Goat

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Introduction

Mature articular cartilage has a depth-dependent structure that is classified into three zones: superficial, middle, and deep (Benninghoff 1925). Each zone has characteristics that are appropriate to its function. Variations exist in gene expression (Darling et al. 2004), matrix composition (Maroudas 1979), and mechanical properties (Schinagl et al. 1997), findings that have sparked a new trend in articular cartilage engineering—replication of its zonal structure (Kim et al. 2003; Klein et al. 2003; Waldman et al. 2003). Young articular cartilage tissue shows a certain amount of zonal structure, although this is limited to only two zones: superficial and growth (Darling et al. 2004). Whereas replication of the zonal architecture of articular cartilage by using cells from mature tissue would be ideal, past studies have shown that cells from younger cartilage possess greater synthetic abilities that help to increase the rate of tissue formation (Tew et al. 2001; Williamson et al. 2003). Chondrocytes from immature animals (approximately 1–6 weeks old) have been used widely in tissue engineering studies for their proven ability to increase matrix synthesis and produce better mechanical properties (Blunk et al. 2002; Freed et al. 1998; Mauck et al. 2002).

However, immature tissue has little apparent zonal organization. As a compromise, we have used superficial and growth zone chondrocytes from young tissue (approximately 8 months old) to investigate the way in which various growth factor treatments affect proliferation, gene expression, and biosynthesis. The ultimate aim of this work is to engineer a three-dimensional (3D) articular cartilage construct that has multiple zones. Replication of the zonal organization might aid in the production of a functional engineered construct, especially if zone-critical molecules are only expressed and secreted by certain chondrocytes.

Articular chondrocytes can be stimulated in many ways to enhance the production of extracellular matrix molecules. One common method is the application of growth factors, which can positively affect the formation of new cartilage tissue in both explants and engineered constructs (Blunk et al. 2002; Bonassar et al. 2001; Chaipinyo et al. 2002; Guerne et al. 1994; Mauck et al. 2003). Insulin-like growth factor-I (IGF-I) can dramatically increase biosynthesis levels, especially in the presence of mechanical stimulation (Bonassar et al. 2001; Jin et al. 2003). Transforming growth factor-β1 (TGF-β1) increases matrix formation in engineered constructs and stimulates cellular proliferation (Blunk et al. 2002; Darling and Athanasiou 2003; Guerne et al. 1994; van der Kraan et al. 1992). The application of basic fibroblast growth factor (bFGF) stimulates proliferation (Adolphe et al. 1984; Toolan et al. 1996) and biosynthesis (Fujimoto et al. 1999) in chondrocytes cultured under a variety of conditions. Previous studies have determined that growth factors not only influence cell proliferation and differentiation during monolayer expansion, but also affect their redifferentiation potential when transferred to a 3D environment (Jakob et al. 2001; Martin et al. 2001). The current study builds on this work by stimulating zonal articular chondrocytes and investigating the way that the cells react in a non-expansion monolayer environment.

Engineered constructs have to be rapidly produced to provide functional tissue replacements in a timely fashion for clinical use. The stimulation of chondrocytes with bioactive molecules leads to extracellular matrix being deposited more quickly to form the beginnings of an articular cartilage construct (Darling and Athanasiou 2004). However, the secreted proteins are not always characteristic of articular cartilage. The composition of native tissue is primarily collagen II, proteoglycans, and water. Approximately 75%–80% of the wet weight of articular cartilage is water, whereas the dry weight is 15%–30% glycosaminoglycans (GAGs) and 50%–73% collagen II (Athanasiou et al. 2001). A common problem with tissue engineered constructs in vivo is the unintentional formation of fibrocartilage (Peterson et al. 2002), which is characterized by having a large proportion of collagen I in its matrix and a disorganized structure. Since the biomechanical properties of fibrocartilage are inferior to healthy articular cartilage, the longevity of implanted constructs could be compromised when placed in the rigorous native environment (Buckwalter 1998; Mitchell and Shepard 1976). The goal of growth factor treatments should be to stimulate the production of molecules that are characteristic of healthy cartilage without stimulating the secretion of fibroblastic proteins.

Materials and methods

Cell culture

Superficial and growth zone articular chondrocytes harvested from the knee of an 8-month-old female Spanish goat were isolated by using the zonal abrasion technique. This procedure (Darling et al. 2004) involved drawing a scalpel blade firmly across the cartilage surface several times to remove the top 10%–20% of the tissue (approximately 1 mm in total thickness) containing the superficial zone population. The remainder of the articular cartilage layer was then harvested to obtain the growth zone population. Cells were released from their surrounding matrix by standard digestion techniques (Darling et al. 2004). Briefly, the harvested superficial and growth zones were minced separately by using a scalpel and digested overnight in 2 mg/ml type II collagenase (Worthington, Lakewood, N.J.). The resulting cell suspensions were pelleted, and the supernatants removed. Upon resuspension in culture media, superficial and growth zone cells were plated separately on tissue culture plastic. Zonal populations were passaged twice to obtain sufficient cell numbers for this study. For the proliferation experiment, approximately 5×10^4 cells/cm^2 were seeded in 96-well plates with sampling being performed at 1, 2, 4, 6, and 8 days. Gene expression samples were seeded in 48-well plates at approximately 2.5×10^4 cells/cm^2 and analyzed at 7, 14, and 21 days. Biochemical samples were seeded in 6-well plates at approximately 2.5×10^4 cells/cm^2 and were evaluated once at 21 days. The gene expression and biochemical cultures reached confluence within 1 week. The use of higher cell density monolayers has been shown to stabilize the chondrocytic phenotype of seeded cells (Schulze-Tanzil et al. 2002) in addition to reducing the variations in phenotype that occur as chondrocytes proliferate (Darling and Athanasiou 2005). Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 10 mM HEPES, 0.4 mM l-proline, 1% non-essential amino acids, 50 μg/ml ascorbic acid, 1 mM l-glutamine, 100 U/ml penicillin-streptomycin, and 0.25 μg/ml fungizone (Sigma-Aldrich, St. Louis, Mo.) was used in all cell cultures. Growth factors for this study (PeproTech, Rocky Hill, N.J.) included high and low concentrations of insulin-like growth factor-I (10 and 100 ng/ml), basic fibroblast growth factor (10 and 100 ng/ml), and transforming growth factor-β1 (5 and 30 ng/ml). The working concentrations were based on values commonly used for articular cartilage studies (Blunk et al. 2002; Bonassar et al. 2001; Chaipinyo et al. 2002). Half the culture media was replaced with fresh growth factors and media every other day for 21 days.

RNA isolation and real-time reverse transcription/polymerase chain reaction

Primer sequences for all genes were designed by using bovine and human mRNA information published on the website of the National Center for Biotechnology Information. The genes examined in this study were glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen II (Col2), collagen I (Col1), superficial zone protein (SZP), and aggrecan (AGC). Previous validations by means of gel electrophoresis and DNA sequencing showed that the correct primer products were obtained for all genes examined. Table 1 lists the sequence, accession number, product size, and fluorescent dye and quencher for each primer/probe combination. Sample RNA was obtained from zonal population groups at 7, 14, and 21 days (approximately 85,000 cells each). Chondrocytes were lysed, and their RNA was isolated by using an RNAqueous kit (Ambion, Austin, Tex.). DNase-I-treated RNA was used in the reverse transcription (RT) reaction, in which 12.5 μl extracted RNA solution was incubated with 1 mM dNTPs, 1 mM random hexamers, and Stratagene Stratascript RT enzyme (La Jolla, Calif.) for 90 min at 37°C. After cDNA synthesis, real-time polymerase chain reaction (PCR) for GAPDH, Col1, Col2, SZP, and AGC was performed by using the Rotor-gene 3000 real-time PCR machine (Corbett Research, Sydney, Australia). Multiplex PCR master mix (Qiagen, Valencia, Calif.) was used in conjunction with the proper primers (0.2 μM) for each reaction. A 15-min denaturing step was followed by 45 cycles of 15 s at 95°C and 30 s at 60°C. Fluorescence measurements (with fluorophores FAM, ROX, and Quasar 670) were taken every cycle at 60°C to provide a quantitative real-time analysis of the genes analyzed. A test run consisted of measuring either Col1/Col2/GAPDH or AGC/SZP/GAPDH for each experimental condition (IGF-I, bFGF, TGFβ-1 at high/low concentrations and a control).

Relative expression

Gene expressions for Col1, Col2, SZP, and AGC were normalized by using the housekeeping gene, GAPDH. By analyzing gene expression in this manner, variations in the number of cells per sample were rendered insignificant. Unpublished data confirmed that GAPDH levels were consistent between the zonal populations, with and without growth factor treatment, and could therefore be used as a reliable reference gene in this study. Relative expression values were calculated by using the relative expression equation adapted from Pfaffl (2001) in which C_t is the take-off cycle of the gene of interest (GOI) and the housekeeping gene (HKG) and E is the respective primer efficiency as determined from standard curves obtained concurrently with the experiment:

$$R_{GOI} = \frac{(1 + E_{HKG})^{C_{t,HKG}}}{(1 + E_{GOI})^{C_{t,GOI}}}$$

The above equation was modified to account for the use of multiplex PCRs, i.e., GOI and HKG were measured from the same sample tube. This approach simplified the relative expression equation and reduced the error that was inherent in determining relative values by using two different tubes (one for GOI, one for HKG). High relative expression values indicated that the gene of interest was expressed to a greater extent than the housekeeping gene. Since the expression of GAPDH was assumed to be relatively constant from chondrocyte to chondrocyte, comparisons between relative expression values could be made.

Table 1 Primer sequences used for real-time PCR analysis

Primer name (abbreviation, accession number, product size)	Forward sequence (5′ to 3′) Reverse sequence (5′ to 3′) Probe sequence (5′ to 3′), dye/quencher
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, U85042, 86 bp)	ACCCTCAAGATTGTCAGCAA ACGATGCCAAAGTGGTCA CCTCCTGCACCACCAACTGCTT, FAM/BHQ-1
Collagen I (Col1, NM_174520, 97 bp)	CATTAGGGGTCACAATGGTC TGGAGTTCCATTTTCACCAG ATGGATTTGAAGGGACAGCCTGGT, Quasar 670/BHQ-2
Collagen II (Col2, X02420, 69 bp)	AACGGTGGCTTCCACTTC GCAGGAAGGTCATCTGGA ATGACAACCTGGCTCCAACACC, ROX/BHQ-2
Superficial zone protein (SZP, AF056218, 77 bp)	CACCATCAGGATTCACTACACA TCACTTTAACTTCATTATGGAGGA CCCGTCAGAGTCCCTTATCAAGACA, ROX/BHQ-2
Aggrecan (AGC, U76615, 76 bp)	GCTACCCTGACCCTTCATC AAGCTTTCTGGGATGTCCAC TGACGCCATCTGCTACACAGGTGA, Quasar 670/BHQ-2

Biochemical analyses

The deposited matrix from each 6-well plate was digested by using pH buffered solutions of pepsin and elastase. Briefly, 1 ml 0.1 M acetic acid (AcOH) was added to each well, and the matrix sheets were lifted by using a cell scraper. Next, 100 μl pepsin (Sigma; 10 g/l in 0.1 M AcOH) was added to the solution, which was then placed on a rocker, and digestion was carried out overnight at 4°C. The following day, 100 μl 10× TBS (1 M TRIS, 2 M NaCl, 50 mM $CaCl_2$), 50 μl 1 N NaOH, and 100 μl elastase (Sigma; 1 g/l in 1× TBS) were added to the solution, and again digestion was carried out overnight at 4°C. The digest solution was then centrifuged, and the supernatant collected for biochemical analysis.

Cell number

Cell numbers were determined by measuring DNA content with the PicoGreen assay (Molecular Probes, Eugene, Ore.). Briefly, 50 μl of sample was combined with 100 μl of TRIS buffer and 150 μl of PicoGreen solution. Standard curves were run concurrently with the experiment. Results from the fluorescence measurements (excitation: approximately 480 nm; emission: approximately 520 nm) were converted to cell numbers based on a conversion factor of 7.7 pg DNA/cell, a number empirically determined in our laboratory for use in chondrocyte studies.

GAG content

GAG content was tested by using a dimethylmethylene blue (DMMB) dye-binding assay kit (Blyscan kit; Biocolor, Newtownabbey, Northern Ireland, UK). Chondroitin sulfate, provided with the kit, was used as the GAG standard. A 100-μl aliquot from each sample was added to 1 ml DMMB and mixed periodically for 30 min. Solutions were then centrifuged, and the supernatant discarded. The remaining pellet was suspended in 1 ml of the provided dissociation reagent, and absorbance measurements were taken at 656 nm. For analysis and reporting, the GAG content for each 6-well plate was normalized to its respective final cell number (approximately $6.5–9.5\times10^5$).

Collagen content

Collagen I was quantified by an indirect enzyme-linked immunosorbent assay (ELISA), and collagen II was determined by a capture ELISA. Between each incubation step in the ELISAs, plates were washed with phosphate-buffered saline, 0.05% Tween-20. For the indirect ELISA, 96-well plates were coated with standards and samples and incubated overnight at 4°C. The wells were blocked with bovine serum albumin for 2 h and then exposed to a primary antibody, anti-collagen I IgG (Accurate Chemical, Westbury, N.Y.), for 1 h. A secondary antibody, anti-IgG horseradish peroxidase (Chemicon, Temecula, Calif.), was then exposed to the plate for 1 h, after which the results were visualized at 450 nm by using tetramethyl benzidine as a liquid substrate. Collagen II was quantified by using a capture ELISA kit (Chondrex, Redmond, Wash.). Plates

Table 2 Biosynthesis results for zonal chondrocytes treated with growth factors (*CS* control/superficial, *CG* control/growth, *ILS* IGF-I/low concentration/superficial, *IHS* IGF-I/high concentration/superficial, *ILG* IGF-I/low concentration/growth, *IHG* IGF-I/high concentration/growth, *TLS* TGF-β1/low concentration/superficial, *THS* TGF-β1/high concentration/superficial, *TLG* TGF-β1/low concentration/growth, *THG* TGF-β1/high concentration/growth, *FLS* bFGF/low concentration/superficial, *FHS* bFGF/high concentration/superficial, *FLG* bFGF/low concentration/growth, *FHG* bFGF/high concentration/growth)

Growth factor	Zone	Concentration (ng/ml)	Label	Collagen I (μg/10^5 cells)	Collagen II (μg/10^5 cells)	GAG (μg/10^5 cells)
None	Superficial	–	CS	0.042±0.001	0.001±0.001	0.144±0.060
	Growth	–	CG	1.336±0.069	1.187±0.216	0.375±0.115
IGF-I	Superficial	10	ILS	0.039±0.003	Not detectable	0.263±0.064
		100	IHS	0.098±0.024*	0.013±0.002*	0.276±0.028*
	Growth	10	ILG	0.934±0.089*	1.608±0.354	0.301±0.047
		100	IHG	1.992±0.172*	2.832±0.767*	1.350±0.104*
TGF-β1	Superficial	5	TLS	0.185±0.026*	0.048±0.020*	0.265±0.067
		30	THS	1.89±0.618*	0.334±0.134*	0.455±0.056*
	Growth	5	TLG	12.097±3.093*	4.200±0.717*	0.391±0.046
		30	THG	4.406±1.144	2.244±0.413*	0.949±0.156*
bFGF	Superficial	10	FLS	0.024±0.003*	Not detectable	0.139±0.047
		100	FHS	0.018±0.001*	0.009±0.002*	0.260±0.034
	Growth	10	FLG	4.058±0.354*	1.641±0.190	0.164±0.031
		100	FHG	1.333±0.171	0.422±0.171*	0.321±0.047

*$P<0.05$

were coated in capture antibody solution overnight at 4°C. Standards and samples were then exposed to the surface for 2 h at room temperature, followed by the detection antibody solution for another 2 h. Streptavidin peroxidase solution was then added for 1 h, and the results were visualized at 490 nm by using urea-H_2O_2 as a liquid substrate. For analysis and reporting, collagen content for each 6-well plate was normalized to its respective final cell number (approximately $6.5–9.5\times10^5$).

Statistics

A sample size of n=4 was used for each experimental group. Graphs are depicted as mean±standard deviation. Analyses of variance (ANOVAs) were conducted to determine whether significance existed at $P<0.05$. For the proliferation and gene expression tests, a two-factor ANOVA was used to determine whether significance existed. A single-factor ANOVA was used for the biosynthesis tests (Table 2). If significance existed, a post-hoc analysis was performed with Student's t-test, adjusted with the Bonferroni correction to compare individual experimental groups with their respective zonal controls. By using this test, significance between an experimental group (i.e., 10 ng/ml IGF-I, growth population) and its control (i.e., control, growth population) was set at $P<0.05/6=0.0083$, since 6 comparisons were made (control versus each treatment).

Results

Morphology

Morphological differences among the four treatment groups were apparent throughout the study, but no major differences in cell shape were seen between superficial and growth populations (Fig. 1). Control groups at 21 days showed a normal confluent appearance for growth zone cultures (Fig. 1a). At the same time point, 100 ng/ml IGF-I had induced a rounded swollen morphology in growth chondrocytes (Fig. 1b). The 30-ng/ml TGF-β1 treatment stimulated matrix synthesis in the growth populations so effectively that the monolayer sheets began rolling up on themselves after only 9 days (Fig. 1c) and eventually formed small pellets. At 9 days, the 100-ng/ml bFGF treatment had elicited an elongated cell shape with clear spaces between chondrocytes (Fig. 1d). After 3 weeks in culture, however, the morphology of the bFGF-treated samples was similar to that of controls, with densely packed cells being embedded in a monolayer matrix.

Proliferation

Growth factor treatments appeared to have only minimal effects on zonal chondrocyte proliferation (Fig. 2; for simplicity, no error bars were included on the graph, but most data points had standard deviations of 5%–15%). All treatments showed significant increases ($P<0.004$) in cell numbers from day 1 to day 8. Proliferation did not vary significantly until day 6, at which point most treatments showed increased levels of cellular expansion. Comparisons at day 8 were made between each treatment and its zonal control to determine which condition most benefited proliferation. For superficial chondrocytes, cell numbers were 116% higher than controls for the 5 ng/ml TGF-β1 treatment and 145% higher ($P<0.0001$) for the 30-ng/ml TGF-β1 treatment. A 68% increase ($P<0.002$) was recorded for 100 ng/ml IGF-I. In the growth zone populations, significant increases over controls were measured for the 5-ng/ml TGF-β1 (55%, $P<0.005$) and 30-ng/ml TGF-β1

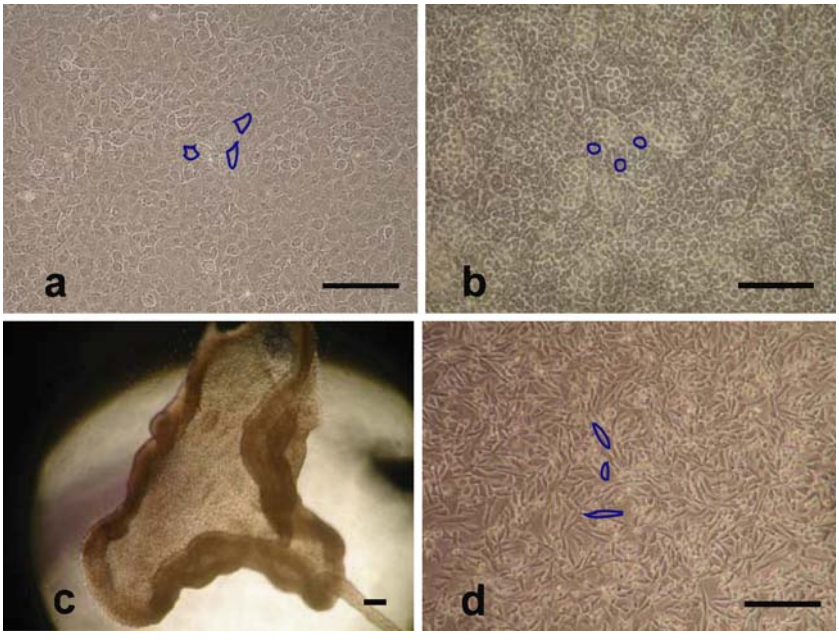

Fig. 1 Gross morphology of monolayer cultures treated with control medium (**a**), IGF-I (**b**), TGF-β1 (**c**), or bFGF (**d**). Although no major differences in cell shape existed between superficial and growth zone chondrocytes, clear differences were observed among the growth factor conditions. Control cultures contained flattened confluent monolayers (growth, 21 days). IGF-I induced a rounded shape (growth, 21 days), and bFGF promoted an elongated shape (growth, 9 days). TGF-β1 rapidly caused its monolayer sheet to curl up (growth, 9 days), most probably because of elevated proliferation and high levels of collagen deposition. Some cells are outlined (*blue*) to emphasize the morphologies typical of the various treatments

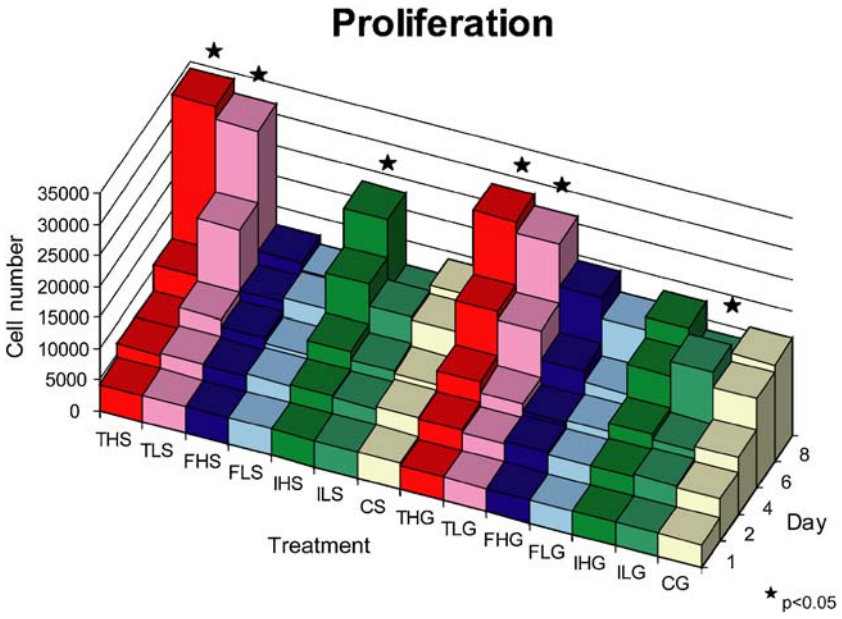

Fig. 2 Proliferation attributable to growth factor stimulation (for an explanation of the abbreviations, see Table 2). All groups showed statistically significant increases in cell numbers between days 1 and 8. When compared with zonal controls at day 8, significantly higher cell numbers ($P<0.05$) were seen in populations treated with 5 or 30 ng/ml TGF-β1. Higher superficial cell numbers were also observed for the 100 ng/ml IGF-I treatment. The only group to have significantly fewer cells than its zonal control was the 10-ng/ml IGF-I treatment group of growth zone chondrocytes

(85%, $P<0.003$) treatments. The 10-ng/ml IGF-I treatment of the growth zone populations was the only condition to produce significantly fewer cells at day 8 than its control (21%, $P<0.004$). However, an increase had occurred since day 1.

Gene expression

Variations in relative gene expression between the superficial and growth zones were apparent for all genes except Col1. No overall statistical significance was evident for

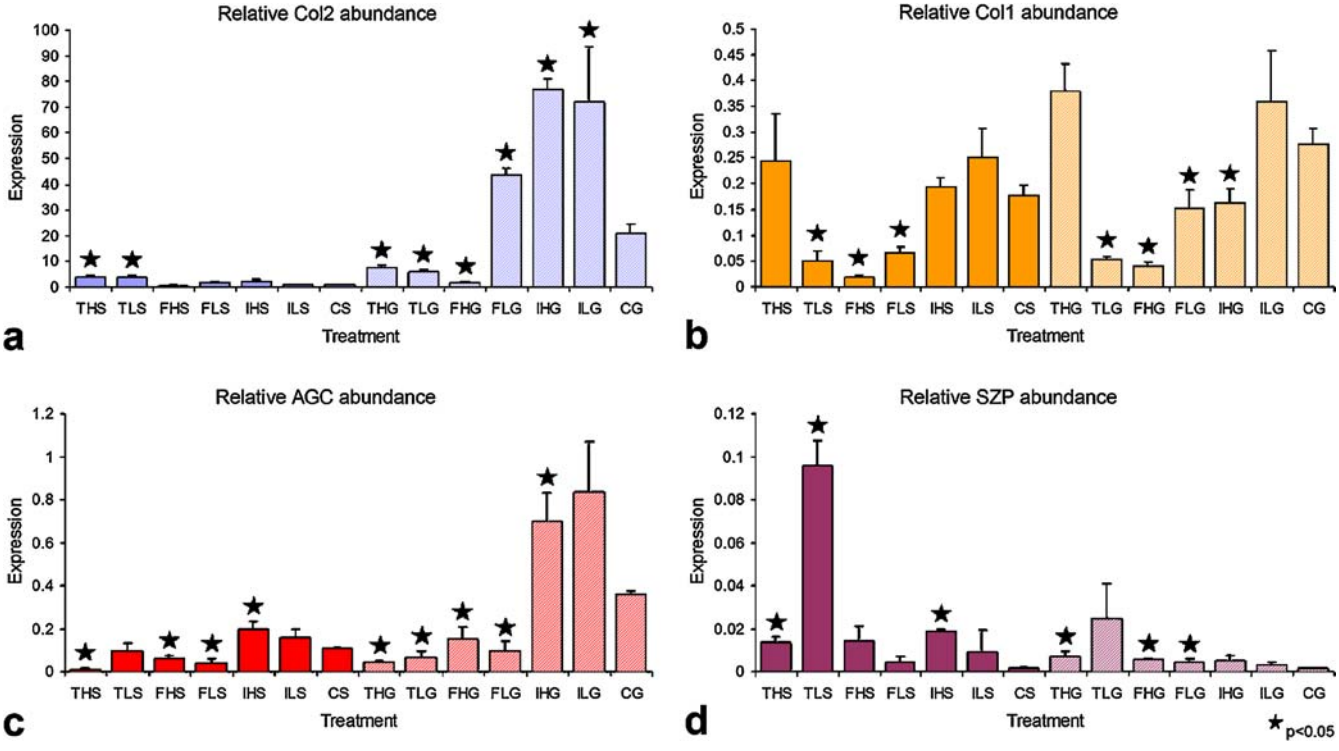

Fig. 3 Gene expressions for Col2 (**a**), Col1 (**b**), AGC (**c**), and SZP (**d**) measured by real-time PCR (for an explanation of the abbreviations, see Table 2). Average gene expressions (from days 7, 14, and 21 combined) revealed that zonal variations existed for all genes except Col1. The highest values for Col2 and AGC were seen in growth zone populations treated with IGF-I. TGF-β1 showed a small increase in Col2 expression for superficial populations but decreased expression in growth zone populations. However, TGF-β1 did show an increase in SZP expression that was much larger than that of the IGF-I groups. Significance ($P<0.05$) was determined by comparing treatment groups with their corresponding zonal controls

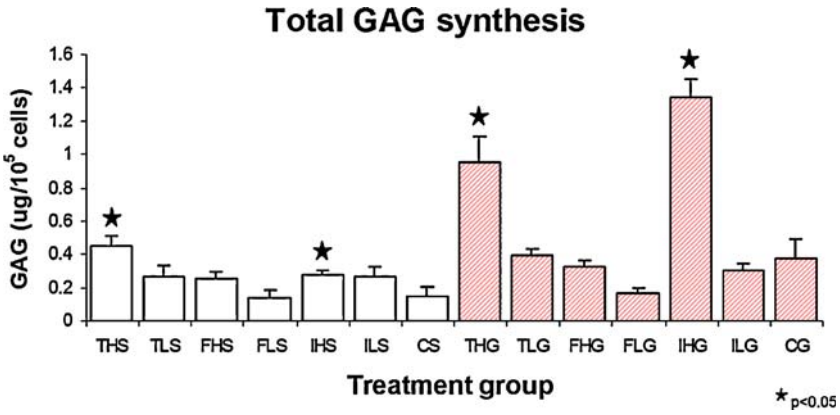

Fig. 4 GAG synthesis measured by the DMMB assay. Increases in GAG production over zonal controls were significant ($P<0.05$) for the high concentrations of IGF-I and TGF-β1 after 21 days (for an explanation of the abbreviations, see Table 2). In these groups, the growth zone populations (*red bars*) secreted several times more GAGs than the superficial populations (*open bars*)

gene expression changes over time. However, a few groups did show major changes at time points during the study (data not shown). IGF-I (10 and 100 ng/ml) increased Col2 and AGC expression in growth zone populations from 7 to 21 days. Over the same period, 30 ng/ml TGF-β1 decreased Col2 expression in growth zone populations and SZP expression in superficial populations. The 5-ng/ml TGF-β1 treatment also decreased Col2 expression in growth zone cells (from day 14 to 21) but produced an increase in SZP expression (from day 7 to 21). For growth zone populations treated with 10 ng/ml bFGF, AGC expression increased from day 7 to 14, whereas growth zone populations treated with 100 ng/ml bFGF showed an increase in Col2 expression from day 14 to 21.

Although temporal changes might indicate the time point at which proteins are secreted, average expression values (from days 7, 14, and 21) might give a better indication of the cumulative effects of growth factors. These data are presented in Fig. 3. From these values, Col2 gene expression was significantly higher than in controls (270% increase, $P<0.0001$) for growth zone chondrocytes treated with 100 ng/ml IGF-I (Fig. 3a). The relative expression of Col1 showed major variations within treatments (Fig. 3b). The largest change, however, was for cultures treated with 100 ng/ml bFGF, which showed decreases to only 14% of growth zone control values ($P<0.0001$). Relative AGC expression in growth zone chondrocytes was enhanced by IGF-I to a greater degree than in other treatments (Fig. 3c). The 10-ng/ml treatment induced a 130% increase over growth zone controls ($P<0.0001$), and the 100-ng/ml treatment induced a 95% increase ($P<0.002$). TGF-β1 had the opposite effect on AGC expression in growth zone cells, decreasing expression levels to 18% ($P<0.0001$) and 13% ($P<0.0001$) of controls for the 5-ng/ml and 30-ng/ml concentrations, respectively. However, TGF-β1 showed significant stimulation of SZP gene expression (Fig. 3d). In the superficial populations, the 5-ng/ml treatment induced a 72-fold increase ($P<0.0001$), and the 30-ng/ml treatment induced a 9.4-fold increase over superficial controls ($P<0.0001$). IGF-I at 100 ng/ml also increased SZP expression in the superficial cells (13-fold, $P<0.0001$). An additional finding of this study was that gene expression was correlated qualitatively with most changes observed in matrix synthesis.

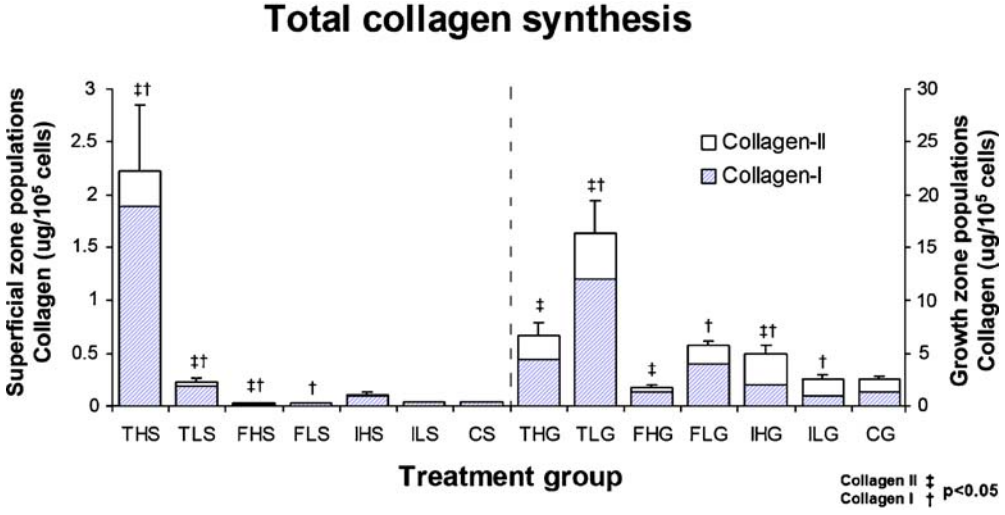

Fig. 5 Collagen I and II synthesis measured by ELISA. Overall collagen production was stimulated to the greatest extent by TGF-β1 after 21 days (for an explanation of the abbreviations, see Table 2). Synthesis of collagen I was especially high for these groups in comparison with the others. However, the chondrocytic phenotype was characterized by having a predominant amount of collagen II. In contrast to TGF-β1, 100 ng/ml IGF-I elicited only slight increases in collagen I synthesis, while significantly ($^{\dagger,\ddagger}P<0.05$) elevating collagen II production. Note the different scales for the superficial and growth zone populations

GAG synthesis

Results indicated that no zonal variations in GAG secretion existed between the untreated controls. However, in two experimental groups, the growth zone populations showed a much higher level of GAG secretion than their respective superficial zone populations. TGF-β1 and IGF-I had a large effect in upregulating GAG synthesis at 21 days. Significant differences from control values were found for the 100-ng/ml IGF-I and 30-ng/ml TGF-β1 treatments (Fig. 4). IGF-I elicited a 92% increase ($P<0.008$) in synthesis for the superficial population and a 260% increase ($P<0.0001$) for the growth zone population. TGF-β1 also showed increases in GAG production. The superficial population produced 220% more GAG than the controls ($P<0.0003$), and the growth zone population produced 150% more GAG than the controls ($P<0.003$).

Collagen synthesis

Collagen synthesis occurred almost exclusively in the growth zone populations (Fig. 5). Significant increases in collagen II compared with controls were measured for superficial (244-fold, $P<0.003$) and growth zone (1-fold, $P<0.004$) chondrocytes exposed to 30 ng/ml TGF-β1. TGF-β1 also stimulated synthesis at 5 ng/ml, inducing a 34-fold ($P<0.004$) and 2.5-fold ($P<0.0005$) increase for the superficial and growth zone populations, respectively. The 100-ng/ml IGF-I treatment increased production 8.7-fold ($P<0.0001$) for superficial cells and 1.4-fold ($P<0.007$) for growth zone cells.

Similar to the collagen II results, collagen I synthesis was limited primarily to the growth zone populations (Fig. 5). Large increases in production were observed for the 5-ng/ml and 30-ng/ml TGF-β1 treatments. The 5-ng/ml treatment induced a 3.4-fold ($P<0.0001$) and 8.1-fold ($P<0.002$) collagen I increase in the superficial and growth zone populations, respectively. At 30 ng/ml, the superficial cells secreted 44-times ($P<0.001$) more collagen I than superficial controls and 2.3-times (p=0.01, not significant) more collagen I than growth zone controls. For comparison, 100 ng/ml IGF increased collagen I synthesis 1.4-fold ($P<0.004$) in superficial groups but only increased synthesis by 50% ($P<0.003$) in growth zone groups.

Discussion

The combined results of this study indicate that superficial and growth zone chondrocytes respond differently to the biochemical stimuli provided by growth factors. The gene expression and biosynthesis data suggest that the growth zone is the more active producer of extracellular matrix proteins. Although these changes might be different when translated to a 3D environment (Jakob et al. 2001; Martin et al. 2001), engineered constructs should be designed taking into account the findings of this study in order that the cartilage replacements replicate the zonal synthesis characteristics of native tissue.

To obtain sufficient cell numbers initially, we utilized second passage, zonal articular chondrocytes. Whereas monolayer expansion could cause a loss of phenotype, previous work has shown that second passage cells still express chondrocytic genes to a limited extent (Darling and Athanasiou 2005). Furthermore, tissue engineering and cell implantation procedures involving cells obtained from biopsies require cellular expansion to at least the second passage in order to attain sufficient cell numbers for experimentation, thereby laying the foundation for the use of expanded cells in this study (Brittberg et al. 1994).

The proliferative response of superficial and growth zone chondrocytes was altered dramatically by the TGF-β1 treatments over 8 days. The 100-ng/ml IGF-I treatment also showed a statistically significant increase in superficial populations but not to the same extent as TGF-β1. The other treatment groups showed proliferation responses that were similar to the controls, indicating no major impact from IGF-I and bFGF. When analyzing the proliferation results globally, zonal differences were apparent. The growth zone populations had more cells by day 8 than the superficial populations under the same conditions, except for 5 ng/ml and 30 ng/ml TGF-β1 and 100 ng/ml IGF-I. Previous findings from untreated zonal populations agree with these results, showing that middle/deep zone cells have higher proliferative capabilities than superficial cells (Aydelotte and Kuettner 1988; Siczkowski and Watt 1990). We hypothesize that some growth factors affect superficial and growth zone cells differently. Since TGF-β1 has a noticeable effect on gene expression in superficial cells (elevated SZP levels), it may also increase proliferation in these populations disproportionately to that in growth zone cells.

Cellular morphologies varied over the course of the study. All experimental groups began with cells uniformly covering the plastic well surfaces after being seeded. However, the TGF-β1-treatment groups rapidly proliferated to form dense, collagenous sheets. After 1 week in culture, these monolayers started folding up and eventually became dense pellets. This morphology was most likely indicative of major collagen deposition early in the treatment, a hypothesis supported by the biochemical data. The IGF-I samples exhibited swollen cell shapes, possibly indicative of elevated synthesis levels within the cells. This also agrees with the biosynthesis data that were collected. The bFGF samples showed an elongated fibroblastic morphology early on, but this quickly changed to a monolayer appearance similar to that of the control cells. The gene expression and synthesis data for these cells indicated that the fibroblastic phenotype was not strongly stimulated. One explanation for this result is that the cells lacked space to spread out, which is a condition characteristic of fibroblasts, but additional investigation is warranted to determine why bFGF did not have a more dramatic effect.

Gene expression differences existed between the two zones of young articular cartilage, as shown previously (Darling et al. 2004). The results of this study further

illustrated the individuality of the superficial and growth zone populations. We found that, in general, gene expression did not vary significantly over time. The monolayer cultures reached a confluent state within 1 week of seeding and did not appear to change dramatically from days 7 to 21. The low density of these cultures might be one possible reason for the lack of gene expression changes, since higher cell densities have been shown to help stabilize the expression and synthetic characteristics of chondrocytes (Watt 1988). However, a few treatment groups did show major changes with respect to time, which might help explain some of the protein synthesis results. The TGF-β1-treated populations exhibited elevated levels of collagen secretion throughout the study. The decrease in Col2 gene expression over time could reflect the change from collagen II synthesis to collagen I synthesis, although no major upregulation in Col1 was measured. Although qualitative comparisons between gene expression and protein synthesis were possible, the results of this study highlight the difficulty of making this comparison quantitatively.

The chondrocytic phenotype is characterized by high Col2 and AGC expression in the growth zone population, high SZP expression in the superficial zone population, and low Col1 expression for both populations (Darling et al. 2004). In this study, Col2 gene expression was overwhelmingly higher in growth zone chondrocytes, especially when treated with IGF-I, and was much lower in superficial chondrocytes. TGF-β1 was an exception, however, with an increase in expression occurring for the superficial zone but not the growth zone. The increases were small compared with the effect of IGF-I, as can be seen in Fig. 3a. AGC expression also showed a zonal dependency, with IGF-I stimulating growth chondrocytes to a greater extent than superficial chondrocytes. For replication of the native chondrocytic phenotype, SZP expression should be higher in the superficial cells. Whereas most treatments showed elevated levels of SZP in the superficial groups with respect to their growth zone counterparts, these differences were not statistically significant, except under two conditions. This loss of zonal phenotype has been noted previously for monolayer-cultured chondrocytes (Darling and Athanasiou 2005). For the 5-ng/ml TGF-β1 and 100-ng/ml IGF-I treatments, SZP expression in the superficial zone was significantly enhanced compared with that of controls or of the corresponding growth zone populations. This is important because it shows that growth factors can have an impact on zonal gene expression in addition to affecting the major extracellular matrix molecules. Although the role of SZP is not fully known, it is believed to function partly in lubricating the joint surface (Flannery et al. 1999). Finding the means to stimulate its production is important for the creation of engineered constructs that have the ability to replicate native surface properties. Col1 expression for all groups was higher than initial values (data not shown), with the greatest increases occurring for groups treated with 30 ng/ml TGF-β1. Significant expression decreases, relative to controls, were seen in superficial and growth zone populations treated with 100 ng/ml bFGF.

Despite the finding that more chondrocytic genes were expressed after some growth factor treatments, the levels for SZP and Col1 never reached initial harvest values (data not shown). The highest SZP expression was still only 3.7% of native levels, and the lowest Col1 expression was more than 1500-fold higher than freshly harvested chondrocytes. Although these shortcomings are discouraging, the translation of the growth factor treatments to a 3D culture environment could produce similar increases when starting at an elevated basal level nearer to that of native chondrocytes. A positive sign is that Col2 and AGC expression levels are the same or better than that of native chondrocytes (6.5% increase and 150% increase, respectively). Based solely on gene expression data, IGF-I or TGF-β1 would appear to be “optimal” for the superficial zone, with only IGF-I being “optimal” for the growth zone.

Biochemical synthesis was also heavily dependent on zone and corresponded qualitatively with the gene expression data. Drawing a direct quantitative correlation between gene expression and protein synthesis is however still not feasible. The main discrepancies between the expression and synthesis data are thought to exist because of temporal differences in matrix deposition. For example, AGC expression was lower for the TGF-β1 groups than in the controls, but actual GAG synthesis was higher. However, an early accumulation of matrix occurred in the TGF-β1 samples and might have helped retain proteoglycan macromolecules, whereas the control samples had little significant matrix to help retain GAGs. The differences between Col1 gene expression and synthesis could likewise be explained by morphological changes. The 5-ng/ml TGF-β1 group rapidly formed a collagenous sheet during the first week of culture; this sheet folded up on itself by day 9. Gene expression data for day 7 indicated a high level of Col1, but this expression decreased sharply at days 14 and 21 (data not shown). The average gene expression over the course of the study was therefore low in relation to the amount of collagen I deposited.

In general, the growth zone populations showed a greater capacity for matrix secretion than the superficial zone populations, especially for collagen production. In some cases, the synthesis response of the superficial and growth zone populations was different for the same growth factor treatment. For example, GAG synthesis in both zones was stimulated by 100 ng/ml IGF-I and 30 ng/ml TGF-β1, but the superficial population underwent its highest increase in GAG synthesis following TGF-β1 treatment, whereas the growth zone population exhibited its highest increase after treatment with IGF-I. Collagen synthesis was almost nonexistent for the superficial populations, except when stimulated by TGF-β1. For collagen II production, 5 ng/ml TGF-β1 and 100 ng/ml IGF-I had the most noticeable effect on the growth zone populations. However, TGF-β1 also elicited the highest overall production of collagen I in the same groups. Of the total collagen secreted, TGF-β1 produced 70% collagen I, whereas IGF-I produced only 39%. The presence of collagen I is undesirable because it is indicative of fibrocartilage formation (Buckwalter 1998). These results, coupled with the

GAG synthesis data, indicate that IGF-I may have a more chondrocytic effect on matrix synthesis.

The engineering of a functional articular cartilage construct might require the utilization of zonal chondrocyte subpopulations. These cells could be manipulated in vitro to elicit responses characteristic of native superficial and growth zone chondrocytes. The results of this study have shown that growth factors can significantly affect the proliferation, gene expression, and biosynthetic responses of zonal chondrocytes. Furthermore, superficial and growth zone populations respond differently to growth factor treatments, often showing "optimal" conditions that differ based on zone. During the fabrication and culture of zonal constructs, these data should therefore be taken into account in order to increase the likelihood of success. One possible scenario would be to use IGF-I on growth zone chondrocytes to enhance GAG and collagen II synthesis without the production of large amounts of collagen I. For the superficial zone chondrocytes, TGF-β1 could be used to increase SZP expression and GAG and collagen synthesis. Based on our experimental results, collagen I seems to be the primary collagen produced in the superficial zone. However, previous studies have shown that monolayer culture promotes collagen I expression (Darling and Athanasiou 2005), and so, if the cells were placed in a 3D environment, then collagen II might be secreted to a much greater extent (Murphy and Sambanis 2001). Replicating the zonal structure of articular cartilage might be a possible means of producing a more functional engineered construct, and an awareness of the different responses of superficial and growth zone chondrocytes could help in establishing the conditions that stimulate rapid tissue formation, while retaining important zonal characteristics. Future studies will investigate the way that these results can best be used to create an organized zonal structure in engineered cartilage.

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