Osteoarthritis and Cartilage (2003) **11,** 55–64

1063–4584/03/$30.00/0
doi:10.1053/joca.2002.0869

Osteoarthritis and Cartilage

International Cartilage Repair Society

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Combined effects of insulin-like growth factor-1 and transforming growth factor-β1 on periosteal mesenchymal cells during chondrogenesis *in vitro*§

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Summary

Objective: Periosteum contains undifferentiated mesenchymal stem cells that have both chondrogenic and osteogenic potential, and has been used to repair articular cartilage defects. During this process, the role of growth factors that stimulate the periosteal mesenchymal cells toward chondrogenesis to regenerate articular cartilage and maintain its phenotype is not yet fully understood. In this study, we examined the effects of insulin-like growth factor-1 (IGF-1) and transforming growth factor-β1 (TGF-β1), alone and in combination, on periosteal chondrogenesis using an *in vitro* organ culture model.

Methods: Periosteal explants from the medial proximal tibia of 2-month-old rabbits were cultured in agarose under serum free conditions for up to 6 weeks. After culture the explants were weighed, assayed for cartilage production via Safranin O staining and histomorphometry, assessed for proliferation via proliferative cell nuclear antigen (PCNA) immunostaining, and assessed for type II collagen mRNA expression via *in situ* hybridization.

Results: IGF-1 significantly increased chondrogenesis in a dose-dependent manner when administered continuously throughout the culture period. Continuous IGF-1, in combination with TGF-β1 for the first 2 days, further enhanced overall total cartilage growth. Immunohistochemistry for PCNA revealed that combining IGF-1 with TGF-β1 gave the strongest proliferative stimulus early during chondrogenesis. *In situ* hybridization for type II collagen showed that continuous IGF-1 maintained type II collagen mRNA expression throughout the cambium layer from 2 to 6 weeks.

Conclusion: The results of this study demonstrate that IGF-1 and TGF-β1 can act in combination to regulate proliferation and differentiation of periosteal mesenchymal cells during chondrogenesis.

Key words: IGF-1, TGF-β1, Periosteum, Proliferation, Chondrogenesis.

Introduction

Damaged articular cartilage is well known to have a limited potential for repair or regeneration due to its avascularity and the presence of relatively few cells with low mitotic activity[1,2]. Articular cartilage damage is a major cause of degenerative arthritis. Biological resurfacing of damaged articular cartilage with free periosteal grafts has been shown to be possible in experimental models[3–7] and in clinical studies[8–10]. Periosteum contains undifferentiated mesenchymal stem cells in the cambium layer, which can undergo both osteogenesis and chondrogenesis[11–14]. In normal bone growth, these cells directly differentiate into osteoblasts during intramembranous ossification without passing through a chondrogenic lineage[11,12]. In fracture healing and callus distraction, these cells differentiate into chondrocytes as a part of endochondral ossification[13,14]. However, the factors that regulate periosteal chondrogenesis are relatively unknown. To address this phenomenon, we have established an agarose suspension periosteal organ culture model[15]. In this system, exogenous transforming growth factor-β1 (TGF-β1) and fetal bovine serum (FBS) containing undefined concentrations of growth factors and binding proteins are necessary to induce chondrogenesis[15]. This suggests that growth factors play an important role in inducing periosteal chondrogenesis and maintaining its cartilaginous phenotype. Among these growth factors, insulin-like growth factor-1 (IGF-1) and TGF-β1 are normally present in articular cartilage[16,17] and are among the most potent regulators of proliferation and differentiation of chondrocytes.

IGF-1 has been reported to predominantly stimulate matrix synthesis with a minor effect on mitotic activity in articular chondrocytes[16,18–20]. Kato *et al.*, have also shown that chondrocytes synthesize IGF-1[21,22]. IGF-1 has been found to maintain the steady-state metabolism of proteoglycans in bovine articular cartilage explants[16] and to accelerate proliferation and differentiation of cartilage

Received 22 July 2002; revision accepted 18 October 2002.

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§First presented in part at the 46th Annual Meeting of the Orthopaedic Research Society, 12–15 March 2000. Orlando, FL.

progenitor cells in cultures of neonatal mandibular condyles[23]. Lindhal *et al.*, studied the effect of GH and IGF-1 on colony formation from chondrocytes obtained at different stages of maturation in suspension culture. The growth plate was dissected into three zones (proximal, intermediate, and distal) corresponding to the germinative, proliferative, and hypertrophic cell layers. IGF-1 (100 ng/ml) increased the number of colonies from the proximal zone by 182±18% and in the intermediate zone by 195±12%. There was little or no effect in the distal zone[24].

The effects of TGF-β1 on articular chondrocytes are dependent on the conditions of the target cells. TGF-β1 has been shown to stimulate or inhibit cell proliferation[25,26] and proteoglycan synthesis[27,28] in cultured articular chondrocytes. In cultured periosteal explants, Miura *et al.*, demonstrated that TGF-β1 administration stimulates chondrogenesis in a dose dependent manner[29]. It has also been shown in cultured periosteal explants that the exogenous administration of TGF-β1 up-regulates the endogenous expression of TGF-β1[30,31] and its receptors[31].

These lines of evidence suggest that both IGF-1 and TGF-β1 are potentially important regulators of periosteal chondrogenesis. Therefore, it is essential to understand the effects of these growth factors on periosteal mesenchymal cells. Additionally, such understanding would be relevant to fracture healing or callus distraction to which periosteum contributes. In this study, we report the effects of IGF-1 and TGF-β1 (individually and combined) on proliferation, type II collagen mRNA expression and cartilage formation from periosteal mesenchymal cells during chondrogenesis *in vitro* under serum-free conditions.

Materials and methods

PERIOSTEAL EXPLANTS AND TISSUE CULTURES

All work in this study was conducted with the approval of the Mayo Clinic Institutional Animal Care and Use Committee. One hundred and eleven small (1.5×2 mm) and 96 large (3×4 mm) periosteal explants were harvested by sharp subperiosteal dissection[4] from the medial side of both proximal tibiae of 30 two-month-old male New Zealand White rabbits weighing an average of 1.8 kg. All periosteal explants were obtained within 30 min after death to control for post-mortem effects on the chondrogenic potential[32]. The small explants were used for histomorphometry and the large explants were used for immunohistochemistry and *in situ* hybridization.

The explants were cultured in agarose using a previously described method[15] under serum-free conditions. Immediately after surgical harvesting, the periosteal explants were placed in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Grand Island, NY), with penicillin/streptomycin (50 U/ml and 50 μg/ml) (Life Technologies) and 1 mM proline (Sigma Chemical Co., St. Louis, MO) at 4°C for no more than 1.5 h prior to placement into culture wells. Twenty-four-well flat-bottom culture plates (Corning Glass Works, Corning, NY) were precoated with high T_m agarose gel (Bio-Rad Laboratories, Richmond, CA). The explants were suspended in 1 ml of a 1:1 mixture of 1.0% low T_m agarose gel (Bio-Rad Laboratories) and 2× concentration of DMEM. The final suspension agarose gel contained 0.5% low T_m agarose gel, 1×DMEM, 1 mM proline and penicillin/streptomycin (50 U/ml and 50 μg/ml). The agarose gel was allowed to fully congeal at 4°C for 5 min. Each 1.0 ml of gel layer was covered with 1.0 ml of DMEM containing 1 mM proline, penicillin/streptomycin (50 U/ml and 50 μg/ml), 50 μg/ml ascorbic acid, and 0.1% radioimmunoassay-grade bovine serum albumin (BSA) (Sigma). The medium above the gel layer was replaced every second day. Ascorbic acid was added daily for a final concentration of 25 μg/ml. Cultures were maintained at 37°C and 5% CO_2/95% air for 1, 2, 3, 4, 5 or 6 weeks.

IGF-1 and/or TGF-β1 were added to the medium as follows. Recombinant human insulin-like growth factor-I (IGF-1) (R & D Systems, Minneapolis, MN) was used in concentrations of 5, 10, 50, 100 and 500 ng/ml (to determine the optimal dose) for 6 weeks (added every other day) or for the first 2 days of culture. Ten ng/ml porcine transforming growth factor-β1 (TGF-β1) (R & D Systems) was used only for the first 2 days of culture as previously described[33–35]. The treatment duration of two days was chosen for TGF-β1 because it has been observed that a longer duration, specifically 14 days of TGF-β1, does not result in improved chondrogenesis vs two days of treatment[36]. The growth factors were also added to the DMEM used to make the agarose suspension gel so that the concentration was homogeneously distributed throughout the agarose gel and liquid culture medium.

HISTOMORPHOMETRY

After 6 weeks of culture, a total of 111 small explants were weighed using a Mettler AT261 DeltaRange® balance accurate to 0.01 mg (Mettler-Toledo, Inc. Columbus, Ohio 43240), fixed in 10% neutral buffered formalin, and embedded in paraffin. Three μm thick sections were cut perpendicular to the original cambium surface of the periosteum from the middle and stained with Safranin O/Fast Green. They were analysed by a blinded observer using computerized histomorphometry to determine the percentage of the area that was cartilage in the section[37]. This was performed with a custom-designed software application using the Vidas 2.1 Image Analysis Program from Zeiss (Kontron customized by Carl Zeiss Canada, Don Mills, Ontario, Canada). The analysis of cartilage is based on the uptake of Safranin O stain, with the thresholds and combinations of red, green and blue colors distinguished automatically. With the cartilaginous areas staining red with Safranin O and the non-cartilaginous areas staining blue-green with Fast Green, the computer calculates the cartilage yield (i.e. percent area of cartilage) in each explant by measuring the red-stained region of the histological section and by expressing it as a percentage of the whole section's area[37]. The total amount of cartilage was calculated by multiplying % cartilage by wet weight.

TISSUE PREPARATION FOR IMMUNOHISTOCHEMISTRY AND *IN SITU* HYBRIDIZATION

After 1, 2, 3, 4, 5 and 6 weeks of culture, a total of 96 large explants were fixed in 4% paraformaldehyde/sodium phosphate buffer (pH 7.4) for 4 h at 4°C, and embedded in paraffin. Serial histological sections, 4 μm in thickness, were cut same as above. Each section was stained with Safranin O/Fast Green and analysed by histomorphometry as described above. These Safranin O sections were also used to evaluate the preservation of the cambium layer or cartilage and fibrous layers. Of these original 96 large explants, 90 were used for immunohistochemistry and *in situ* hybridization because they had a well-preserved

cambium layer or cartilage. The remaining six were excluded because of damage to the cambium layer in these explants, which may cause decreased chondrogenic activity[33].

IMMUNOHISTOCHEMISTRY

To detect and localize the cells undergoing proliferation, immunohistochemistry for proliferative cell nuclear antigen (PCNA) was performed. PCNA is an important auxiliary protein for DNA polymerase δ[38], and has been shown to clearly correlate with cell proliferation[39–41]. The sections were deparaffinized with xylene and hydrated with serial concentrations of 100, 95, 80 and 60% ethanol. Endogenous peroxidase activity was blocked with 0.6% hydrogen peroxide in methanol for 1 h. Following three washes in tris-buffered saline (TBS)/0.1% BSA, non-specific binding of antibody was blocked with 1.5% normal horse serum (Vector Laboratories, Burlingame, CA) in TBS/0.5% BSA for 15 min at room temperature. The sections were then incubated with mouse monoclonal antibodies against PCNA (Sigma) diluted at 1:1000 overnight at room temperature in a humidified chamber. After three washes in TBS/0.1% BSA, sections were incubated with biotinylated horse antimouse IgG (Vector Laboratories) for 1 h at room temperature. Following three washes in TBS/0.1% BSA, avidin-biotin complex (Vector Laboratories) was applied for 30 min at room temperature. After extensive washes, the sections were reacted with diaminobenzidine (DAB) (Sigma) for 2 min, then dehydrated and mounted. Mouse IgG (Sigma) was used as a control primary antibody.

IN SITU HYBRIDIZATION

Rabbit specific degoxigenin (DIG)-labeled antisense and sense cRNA probes for type II collagen were generated from 269 base pair cDNA fragment of rabbit type II collagen[42]. Rabbit type II collagen cDNA was obtained by PCR with degenerate primers based on conserved sequences among species[42,43]. For the antisense probe, the corresponding template had a T7 promoter sequence at the 3′ end of the template provided in the 3′ primer. Similarly for the sense probe, the corresponding template had a T7 promoter sequence at the 5′ end of the template provided in the 5′ primer. Degoxigenin-labeled antisense and sense cRNA probes were transcribed using DIG-RNA Labeling Kit (Roche Molecular Biochemicals, Mannheim, Germany) on the cDNA templates that had T7 promoter sequences.

In situ hybridization was performed according to the method described by Nomura *et al.*[44]. The sections were deparaffinized with xylene, hydrated with serial concentrations of 100, 90, 80 and 70% ethanol followed by two washes in sodium phosphate buffer, and were then permeabilized with 5 μg/ml proteinase K (Roche Molecular Biochemicals) in 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) at 37°C for 7.5 min. The sections were re-fixed in 4% paraformaldehyde/sodium phosphate buffer, rinsed with sodium phosphate buffer, and treated with 0.2 M HCl for 10 min to inactivate endogenous alkaline phosphatase (AP). After a wash in sodium phosphate buffer, sections were acetylated with 0.25% acetic anhydride in 0.1 M Triethanolamine-HCl for 10 min, and dehydrated with ethanol. The sections were then incubated with DIG-labeled antisense or sense probes in hybridization buffer (Amersham, Uppsala, Sweden) at 50°C for 16 h in a humidified chamber. After hybridization, the sections were washed in 5×saline-sodium citrate (SSC) at 50°C, 2×SSC, 50% formamide at 50°C for 30 min, and then treated with 10 μg/ml RNAse A (Sigma) at 37°C for 30 min followed by washes in 2×SSC and two of 0.2×SSC at 50°C for 20 min each. Non-specific binding of antibody was blocked with 1.5% blocking reagent (Roche Molecular Biochemicals) in TBS for 1 h. The sections were incubated with anti-DIG-AP Fab fragment (Roche Molecular Biochemicals) diluted at 1:500 for 30 min. After two washes in TBS for 15 min, AP activity was reacted with 4-Nitro blue tetrazolium chloride (NBT)/5-Bromo-4-chloro-3-iodolyl-phosphate (BCIP) (Roche Molecular Biochemicals).

QUANTIFICATION FOR IMMUNOHISTOCHEMISTRY AND *IN SITU* HYBRIDIZATION

The number of cells expressing PCNA and type II collagen mRNA was assessed using a published cell counting technique[35,45]. Cambium, cartilage and fibrous area in periosteal explants were identified in sections stained with Safranin O/Fast Green. Only cells within the cambium and cartilage area were included. The total number of cells and the number of positive stained cells were counted with three randomly selected microscopic fields (at ×200 magnification) per each section. This analysis was done on 3 to 4 different explants per experimental group. The positive ratio for each field (i.e. proliferation index for PCNA and expression index for *in situ*) was calculated by dividing the number of positive stained cells by the total number of cells.

STATISTICAL ANALYSES

Statistical analyses were performed using 1 factor ANOVA and Duncan's New Multiple Range *post-hoc* testing. All data are expressed as mean±standard error.

Results

Chondrogenesis was determined by measuring the percentage of the tissue in the explants that was cartilage, as well as by calculating the total quantity of cartilage. The addition of IGF-1 (50, 100 or 500 ng/ml), alone for the first 2 days did not significantly increase cartilage formation (data not shown). However, when IGF-1 was added at these doses continually for 6 weeks, there was a marked increase both in the percentage of the tissue that was cartilage and the total amount of cartilage at 6 weeks in a dose-dependent manner compared with controls ($P<0.05$) (Fig. 1). At a dose of 100 ng/ml of IGF-1, 87±5% of the tissue formed was cartilage (vs 13±6% in the unstimulated controls; $P<0.05$) and the explants formed greater total amounts of cartilage (14±3 mg vs 0.6±0.3 mg; $P<0.05$). There was no statistical difference between the total amounts of cartilage formed with 100 versus 500 ng/ml, so the concentration of 100 ng/ml was used as the 'optimal' IGF-1 dose for all subsequent studies involving IGF-1 treatment.

EFFECT OF THE DURATION AND COMBINATION OF GROWTH FACTORS ON PERIOSTEAL CHONDROGENESIS

Under the serum-free conditions used in this study, the cartilage yield was not significantly increased by the

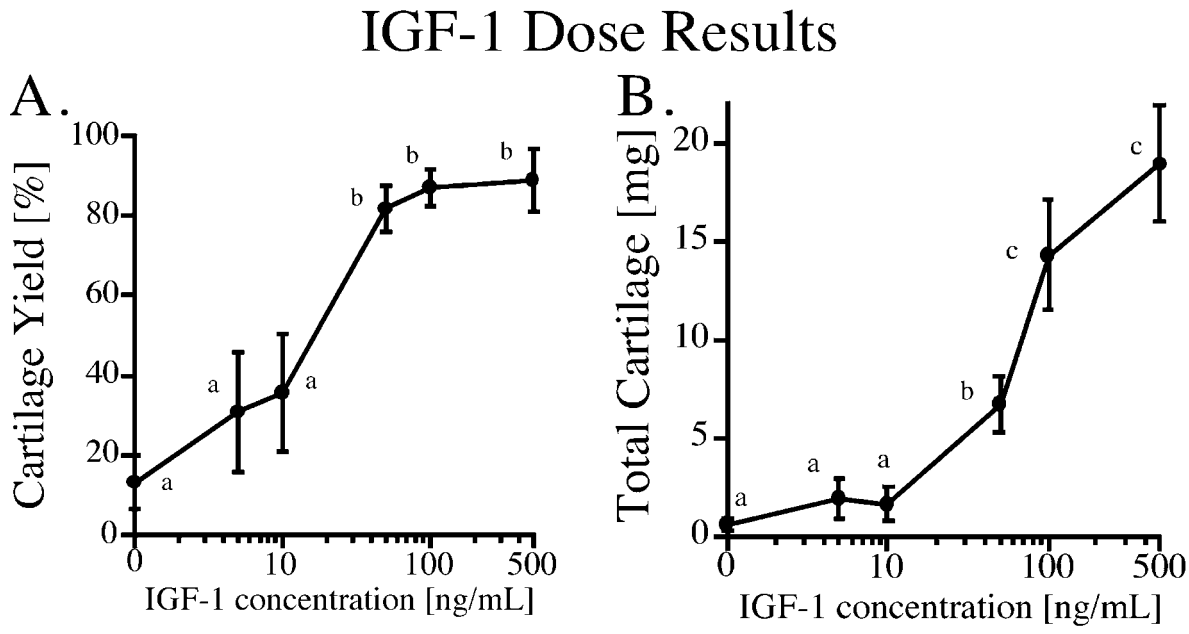

Fig. 1. Dose-dependent effects of IGF-1 on periosteal chondrogenesis. Periosteal explants were cultured with 0, 5, 10, 50, 100 or 500 ng/ml IGF-1 for 6 weeks or for the first 2 days. After 6 weeks of culture, a cartilage yield assay was performed by measuring the % cartilage **(A)** using computerized histomorphometry and the total amount of cartilage **(B)** which was calculated by multiplying % cartilage by wet weight. Lower case letters (i.e. a, b and c) indicate the results of post-hoc testing with Duncan's Multiple Range test at $P<0.05$. Groups with a letter in common are not statistically different from one another. All data are presented ±S.E.

addition of IGF-1 alone for the first 2 days of culture (16±7%) or by IGF-1 and TGF-β combined for the first two days of culture (34±9%) vs the yield in the control group treated with no growth factors (16±5%) [$P>0.05$, Fig. 2(A)). However, the treatment of explants with 6 weeks of IGF-1 alone produced the highest cartilage yield (68±12%,

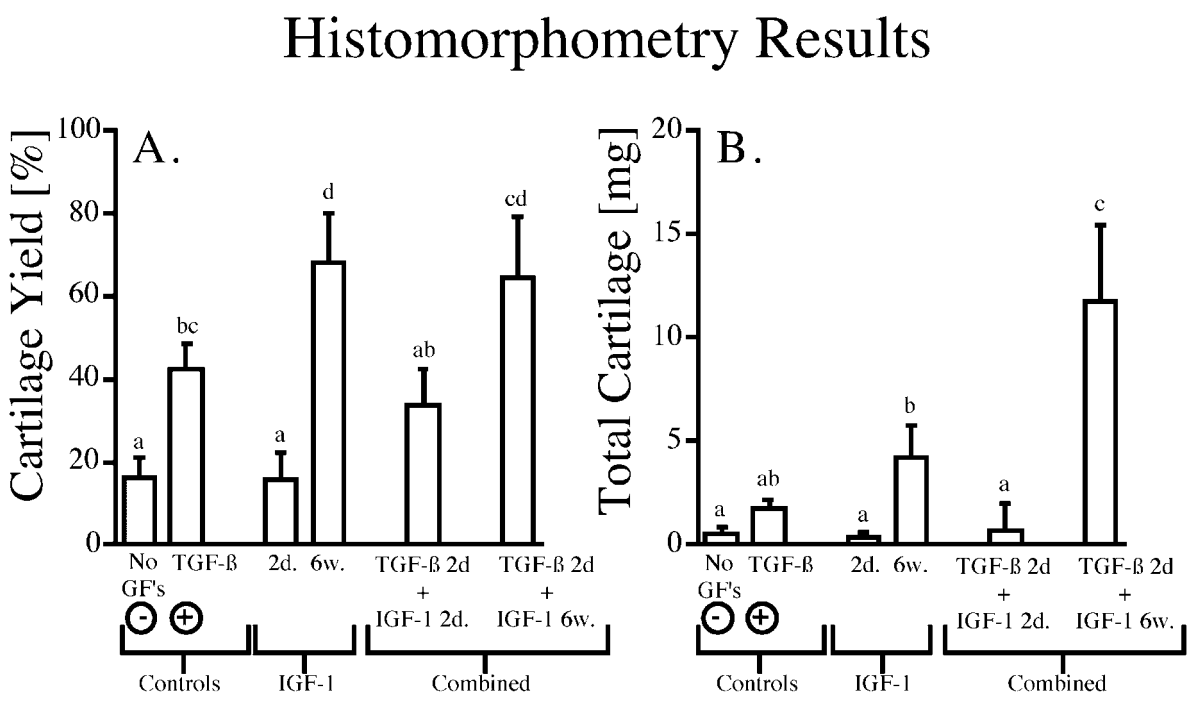

Fig. 2. Effects of the durations and combinations of growth factors on periosteal chondrogenesis. Periosteal explants were cultured with one of the following: (1) no growth factors, (2) 10 ng/mL TGF-β for the first 2 days, (3) 100 ng/ml IGF-1 for the first 2 days, (4) IGF-1 for 6 weeks, (5) TGF-β and IGF-1 for the first 2 days, (6) IGF-1 for 6 weeks with TGF-β for the first 2 days. After 6 weeks of culture, a cartilage yield assay was performed by measuring the % cartilage **(A)** using computerized histomorphometry and the total amount of cartilage **(B)** which was calculated by multiplying % cartilage by wet weight. Lower case letters (i.e. a, b and c) indicate the results of *post-hoc* testing with Duncan's Multiple Range test at $P<0.05$. Groups with a letter in common are not statistically different from one another. All data are presented ±S.E.

Table I
Cartilage yield time course

Time (w)	No growth factors	TGF-β1	IGF-1	TGF-β1+IGF-1
1	0[a]	0[a]	0[a]	6± 5[a]
2	6± 6[a]	12± 5[a]	21±12[a]	56± 8[b]
3	19±12[a]	30± 8[ab]	57±10[ab]	71±20[b]
4	30±15[a]	53±12[ab]	69±10b[c]	99± 0[c]
5	50± 5[a]	54±15[a]	83± 8[b]	97± 1[b]
6	56±21[a]	88± 6[ab]	87± 6[ab]	100± 0[b]

The cartilage yield represents the percent area of the explant that was cartilage as measured by Safranin O staining and computerized histomorphometry. The superscript letters ([a], [b] and [c]) represent grouping based Duncan's new multiple range *post-hoc* statistical testing at $P<0.05$ across treatment groups for each time point. Cartilage yields within a given row with a letter in common, are not statistically different from one another (e.g. at 5 weeks: 50±5[a]≈54±15[a] and 83±8[b]≈97±1[b]).

$P<0.05$). IGF-1 for 6 weeks with TGF-β1 for the first 2 days of culture did not further enhance cartilage yield (64±15%).

As shown in Fig. 2(B), the total amount of cartilage produced in the explants (i.e. % cartilage area×wet weight) was also calculated. Significant increases in total cartilage were only observed in the explants treated with IGF-1 (alone or with TGF-β) for 6 weeks ($P<0.05$) when compared to the control group (no growth factors). Although the total amount of cartilage produced in the group treated with IGF-1 for 6 weeks [Fig. 2(B)] was not as high as it was in the dose response experiment, it was still significantly higher than that seen in the control (no growth factors) group ($P<0.05$). In this agarose culture system, the differences in the magnitude of the chondrogenic response sometimes differ from experiment to experiment. However, the trends of chondrogenic responses between treatment groups within any one experiment are reproducible. As can be seen in Fig. 2(b), the mean total cartilage produced in the explants treated with IGF-1 alone for 6 weeks (4±2 mg) was significantly higher than in the explants from the control group (no growth factors) (1±0.2 mg), IGF-1 alone for 2 days (0.4±0.2 mg) and IGF-1 for 2 days with TGF-β for 2 days (1±0.3 mg) groups ($P<0.05$). The mean total cartilage produced in the explants treated with IGF-1 alone for 2 days (0.4±0.3 mg) was not significantly different from the total cartilage seen in the TGF-β alone for 2 days group (2±0.4 mg). The highest ($P<0.05$) total cartilage production was observed in the explants treated with IGF-1 for 6 weeks plus TGF-β for 2 days. The mean total cartilage in this group (12±4 mg) was three times higher than in the IGF-1 alone for 6 weeks group (4±2 mg), six times higher than the TGF-β alone for 2 days group (2±0.4 mg), and over twelve times higher than the remaining 3 groups [$P<0.05$, Fig. 2(B)].

EFFECT OF THE COMBINATION OF GROWTH FACTORS ON PROLIFERATION AND TYPE II COLLAGEN EXPRESSION

Based on these day 42 cartilage yield and total cartilage results, a time course for the localization within the cambium layer of proliferative activity (PCNA immunostaining), type II collagen expression (*in situ* hybridization), and overall Safranin O staining were done on explants treated with either no growth factors, TGF-β1 for the first 2 days of culture, IGF-1 for all 6 weeks of culture or IGF-1 for all 6 weeks of culture *with* TGF-β1 for the first 2 days of culture. These treatment groups were evaluated at 1, 2, 3, 4, 5, and 6 weeks using four explants per group per time point.

Examination of the histological sections stained with Safranin O revealed a gradual increase in positive staining matrix over time in all of the experimental groups. Automated histomorphometry and two-factor ANOVA of the resulting data revealed that culture period and growth factor treatment had a significant effect on cartilage yield ($P<0.0001$). The mean cartilage yields for each treatment group over time are summarized in Table I. At two weeks, the earliest significant increase in cartilage yield was induced by treatment with TGF-β1+IGF-1 ($P<0.05$, Fig. 3). At two weeks, the cartilage yield in the TGF-β1+IGF-1 group was 56±8%. This was significantly higher ($P<0.05$) than the cartilage yields seen with no growth factors (6±6%), TGF-β1 alone (12±5%) or IGF-1 alone (21±12%) groups. By 28 days, the cartilage yield in the TGF-β1+IGF-1 group had reached a maximum while the cartilage yields continued to increase in the other treatment groups up to day 42. To examine the reasons behind this elevated and accelerated cartilage yield in the TGF-β1+IGF-1 group, we looked to the proliferation (i.e. PCNA) and type II collagen expression (i.e. *in situ* hybridization) data.

The proliferative index means for each treatment group are summarized in Table II. Examination of the cambium layer of the PCNA immunostained sections indicated an increased level of proliferation in the explants treated with IGF-1 in combination with TGF-β1 [Fig. 4(A)]. Early proliferative indices were maximal with IGF-1+TGF-β1. This treatment induced highest level of proliferation at 1 week [$P<0.05$, Fig. 4(B)]. At one week, the proliferative index in the TGF-β1+IGF-1 group was 49±6%. This was significantly higher ($P<0.05$) than the proliferative indices seen with no growth factors (15±3%), TGF-β1 alone (29±4%) or IGF-1 alone (31±6%) groups. Although statistical significance was not achieved at *every* time point, elevated levels of PCNA staining were seen in both the IGF-1 alone and the TGF-β1+IGF-1 groups from two weeks onward (Table II). In the explants treated with TGF-β1 alone, the proliferative index rose steadily for the first 3 weeks. Then, from weeks 4 to 6, the proliferative index of the TGF-β1 group decreased to levels that were not significantly different from those seen in the no growth factor control group ($P>0.05$). This is in contrast to what was observed in both of the IGF-1 treated groups that exhibited proliferative indices that were significantly higher than those seen in the no growth factor control and the TGF-β1 alone groups at 6 weeks ($P<0.05$).

The expression pattern of type II collagen mRNA is summarized in Table III. *In situ* hybridization revealed that

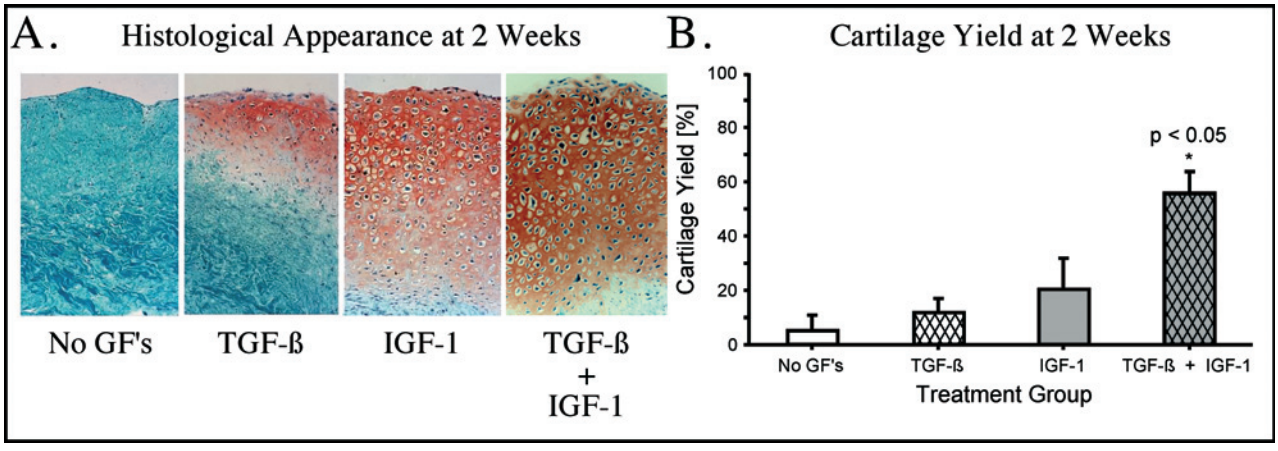

Fig. 3. Cartilage formation at 2 weeks. Periosteal explants were cultured with no growth factors (No GFs), 10 ng/ml TGF-β1 for the first 2 days (TGF-β), 100 ng/ml IGF-1 for 6 weeks (IGF-1), or 10 ng/ml TGF-β1 for the first 2 days with 100 ng/ml IGF-1 for 6 weeks (TGF-β+IGF-1). **(A)** Representative Safranin O/Fast Green histology of periosteal explants in each of the aforementioned groups at 2 weeks. (magnification=×200). **(B)** Cartilage yield analysis at 2 weeks via histomorphometry (n=4 explants per time point per group) demonstrating that that the most cartilage was produced in the TGF-β+IGF-1 group at this early time point (P<0.05). All data are presented ±S.E.

Table II
PCNA immunostaining proliferative index

Time (w)	No growth factors	TGF-β1	IGF-1	TGF-β1+IGF-1
1	15± 3^{a}	29±4^{ab}	31±6^{b}	49±6^{c}
2	15± 0^{a}	61±4^{b}	84±1^{c}	81±1^{c}
3	49±11^{a}	76±1^{b}	86±0^{b}	83±2^{b}
4	61± 3^{a}	61±1^{a}	81±1^{b}	77±1^{b}
5	61± 0^{a}	65±2^{a}	74±3^{ab}	68±4^{b}
6	40± 0^{a}	44±0^{a}	63±4^{b}	67±4^{b}

The proliferative index represents the percent of the cambium cells that demonstrated positive immunostaining for PCNA (proliferating nuclear cell antigen). The superscript letters (^{a}, ^{b} and ^{c}) represent grouping based Duncan's new multiple range *post-hoc* statistical testing at P<0.05 across treatment groups for each time point. Proliferative indices within a given row with a letter in common, are not statistically different from one another (e.g. at 4 weeks: 61±3^{a}≈61±1^{a} and 81±1^{b}≈77±1^{b}).

type II collagen expression was seen in all four treatment groups at 3-6 weeks in the cambium layer (Fig. 5A). In the growth-factor-treated explants, type II collagen expression was seen starting at 2 weeks. In the explants treated with IGF-1, the level of expression remained elevated throughout the culture period (Fig. 5A). With TGF-β1 alone, type II collagen expression was seen in the juxtaosseous region of the cambium layer (i.e. the region originally closest to the bone[35]) at 2 weeks, then from 4–6 weeks, showed a similar pattern as seen in the control group (no growth factors). Detailed quantitative and statistical analysis revealed that, at week 1 there was no significant difference in the type II expression indices between the treatment groups. At weeks 2 and 3, all of the growth-factor-treated groups had type II expression indices that were significantly higher than the unstimulated controls [Fig. 5(B)]. The type II collagen expression index for the TGF-β1 group decreased to the

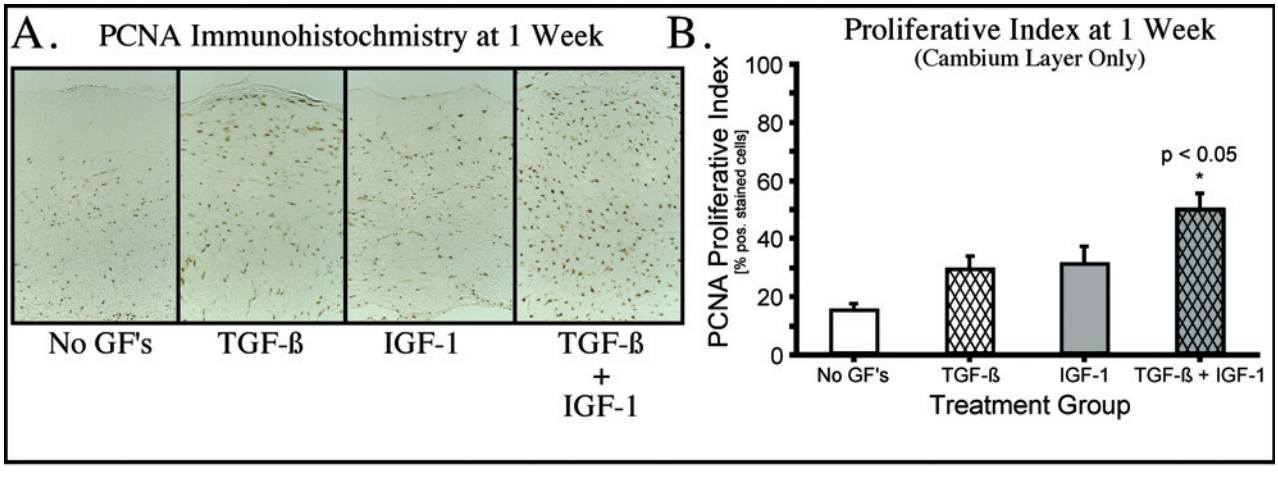

Fig. 4. Immunohistochemistry for proliferative cell nuclear antigen (PCNA) at 1 week. Periosteal explants were cultured with no growth factors (no GFs), 10 ng/ml TGF-β1 for the first 2 days (TGF-β), 100 ng/ml IGF-1 for 6 weeks (IGF-1), or 10 ng/ml TGF-β1 for the first 2 days with 100 ng/ml IGF-1 for 6 weeks (TGF-β+IGF-1). **(A)** Representative photomicrographs of the PCNA immunostained periosteal explants in each of the aforementioned groups at 1 week. (magnification=×200). **(B)** Proliferation index analysis at one week via cell counting showing that the highest level of proliferation at one week was induced when the explants were treated with TGF-β+IGF-1 (P<0.05). All data are presented±S.E. (four explants were assessed per time point per group).

Table III
In situ *collagen II gene expression index*

Time (w)	No growth factors	TGF-β1	IGF-1	TGF-β1+IGF-1
1	0± 0[a]	0±0[a]	0±0[a]	29±16[a]
2	0± 0[a]	78±3[b]	80±7[b]	76± 3[b]
3	40±19[a]	78±2[b]	81±2[b]	78± 3[b]
4	44± 4[a]	32±2[a]	73±6[b]	66± 5[b]
5	24± 2[a]	31±2[a]	74±3[b]	63± 7[b]
6	25± 1[a]	26±1[a]	73±5[b]	58± 3[c]

The *in situ* collagen II gene expression index represents the percent of the cambium cells that demonstrated positive *in situ* hybridization for Type II collagen mRNA. The superscript letters ([a], [b] and [c]) represent grouping based Duncan's new multiple range *post-hoc* statistical testing at $P<0.05$ across treatment groups for each time point. Gene expression indices within a given row with a letter in common, are not statistically different from one another (e.g. at 4 weeks: $44\pm4^{a}\approx32\pm2^{a}$ and $73\pm6^{b}\approx66\pm5^{b}$).

same level as the controls for weeks 4–6 while the expression in the IGF-1-treated explants remained elevated [$P<0.05$, Fig. 5(B)].

Discussion

Subperiosteal injection of TGF-β1 has been known to stimulate the proliferation of periosteal mesenchymal cells and induce chondrogenic differentiation[46,47]. Previous studies have reported that TGF-β1 stimulates periosteum-derived cells, as well as other mesenchymal cells[48,49], toward chondrogenic differentiation[50,51]. We have previously observed that TGF-β1 significantly increases ^{3}H-thymidine incorporation into periosteal explants early during chondrogenesis *in vitro*[52]. The effect of TGF-β1 on periosteal cell proliferation may depend on the conditions of the target cells[53,54].

We currently use 2 days of TGF-β as our standard culture model because we have observed that 14 days of TGF-β1 treatment is not significantly better than 2 days of TGF-β1 treatment[36]. Miura *et al.*[55] showed that even very brief exposures of high doses of TGF-β1 (100 ng/mL for 30 min) had a dramatic effect on the amount of cartilage produced in this culture system. Separate gene expression studies by Davis *et al.* and Mizuta *et al.* suggest that a 2 day exposure to TGF-β1 increases periosteal chondrogenesis over 6 weeks because it directly or indirectly up-regulates not only the expression of cartilage related genes (aggrecan and collagen type II), but also the expression of endogenous TGF-β1 and its receptors[30,31].

We have previously observed induction of chondrogenesis in periosteal explants when cultured in agarose in the presence of FBS without growth factors[15,29]. In this study, explants were cultured in serum-free conditions to eliminate any possible effects of serum. The cartilage yield (i.e. % area cartilage) results with and without TGF-β1 in this study using 0.1% bovine serum albumin as a media supplement are comparable to those reported previously reported using 10% fetal bovine serum in this culture model[5,33,55,56]. The Type II Collagen *in situ* data are also comparable to those reported by Ito *et al.* using 10% fetal bovine serum[35].

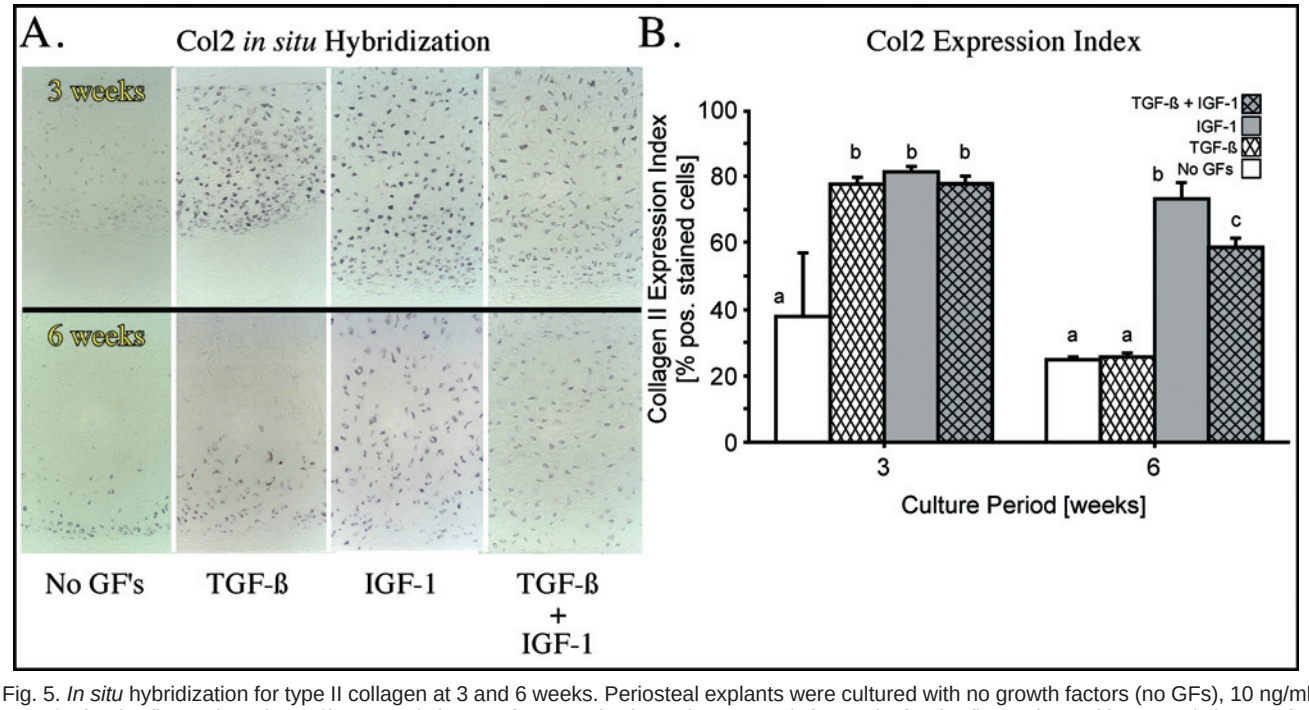

Fig. 5. *In situ* hybridization for type II collagen at 3 and 6 weeks. Periosteal explants were cultured with no growth factors (no GFs), 10 ng/ml TGF-β1 for the first 2 days (TGF-β), 100 ng/ml IGF-1 for 6 weeks (IGF-1), or 10 ng/ml TGF-β1 for the first 2 days with 100 ng/ml IGF-1 for 6 weeks (TGF-β+IGF-1). **(A)** Representative photomicrographs of the periosteal explants after *in situ* hybridization for type II collagen in each of the aforementioned groups at 3 and 6 weeks. (magnification=×200). **(B)** Type II Collagen expression index analysis at 3 and 6 weeks cell counting demonstrating that the Col2 expression was elevated in all of the growth factor treated explants ($P<0.01$) and was maintained in the explants exposed to IGF-1 ($P<0.01$). The lowercase letters (a, b, or c) represent the results of means contrast testing. Bars with letters in common are not statistically different from one another ($P<0.03$). All data are presented±S.E. (four explants were assessed per time point per group).

The data from this study demonstrate that IGF-1 stimulates periosteal chondrogenesis increasing both the relative and absolute amounts of cartilage in the periosteal explants. However, unlike TGF-β1, in order to cause such an effect, IGF-1 had to be continuously present in the media. IGF-1 has been reported to have effects on the mitogenic activity and matrix synthesis of articular and growth plate chondrocytes[20,57–60]. In addition, Bohme *et al*. reported that IGF-1 stimulated proliferation and matrix synthesis in embryonic sternal chondrocytes cultured in agarose gel under serum-free conditions[61]. Also, with continuous IGF-1 treatment, the chondrocyte phenotype was maintained and cells did not progress to the hypertrophic stage[61]. However, the presence of FBS counteracted this effect and elicited hypertrophy[61].

While the effects of IGF-1 on undifferentiated mesenchymal cells in periosteum are not well known, our results are consistent with prior reports. During bone development, IGF-1 mRNA is expressed in the periosteum at the early cartilage stage[62]. Izumi *et al*. reported that IGF-1 stimulated DNA synthesis and type II collagen mRNA expression in periosteum-derived cells[63]. Recently, Nixon *et al*. observed that exogenous IGF-1 stimulation of bone marrow-derived mesenchymal stem cells enhanced chondrogenesis in full-thickness articular defect *in vivo* [64]. In separate *in vitro* studies, Gooch *et al.*[65], and Madry *et al.*[66], demonstrated via exogenous administration[65] and gene transfection[66], that long-term exposure to IGF-1 has a beneficial effect on cartilage matrix production in bovine chondrocytes.

In our study, long term IGF-1 supplementation resulted in elevated and sustained PCNA staining. While the proliferative indices may be overestimates of active proliferation in the periosteum because of the relatively long half-life of PCNA[67], it is clear that IGF-1 treatment increased PCNA synthesis, and therefore, increased proliferation throughout periosteal chondrogenesis. The addition of IGF-1 also resulted in sustained type II collagen expression. The latter observation supports the notion that IGF-1 plays a role in the maintenance of cartilaginous phenotypic expression.

Several studies have reported the interactions of IGF-1 and TGF-β1 on proliferation and differentiation of chondrocytes[59,68–70]. In this study, IGF-1 in combination with TGF-β1 enhanced total cartilage production compared with IGF-1 alone. It is interesting that there was an increase in the total cartilage and no statistical difference in cartilage yield with TGF-β1 and IGF-1 compared to IGF-1 or TGF-β1 alone. The earlier expression of type II collagen mRNA and early induction of proliferation observed in the TGF-β1 and IGF-1 combined treatment group may explain the observed differences in wet weights that resulted in the increased total cartilage production in the combined treatment group compared to IGF-1 alone.

Tsukazaki *et al*. showed that TGF-β increased the number of IGF-1 receptors in chondrocytes without changing their affinity[69]. O'Keefe *et al*. reported that IGF-1 enhanced the effect of TGF-β1 on DNA synthesis in chondrocytes[58]. Although it has not been ruled out that TGF-β1 can stimulate both the induction and the progression/maintenance of chondrogenesis, these lines of evidence suggest that IGF-1 and TGF-β1 may interact to stimulate the early induction of periosteal chondrogenesis, which is then maintained with IGF-1 in the later stage. The histomorphometry data in this study suggests that the addition of IGF-1 and TGF-β1 results in a dramatic increase in overall growth in both the cartilaginous and non-cartilaginous regions of the explants. It is likely that complex interactions involving many growth factors at different stages of periosteal chondrogenesis are responsible for its regulation. Further examination of their interactions may reveal how periosteal chondrogenesis may be enhanced not only for articular cartilage repair but also for fracture healing.

The present study has demonstrated the combined effects of TGF-β1 and IGF-1 on chondrogenesis in periosteal explants. TGF-β1 acted early to induce chondrogenesis, while IGF-1 enhanced and maintained proliferation, increasing total cartilage formation. These observations indicate that these factors could be useful to enhance tissue-engineered articular cartilage repair.

Acknowledgments

This work was supported by grants from the National Institute of Health (AR43890), American Home Products, Inc, and Mayo Foundation, Rochester, Minnesota.

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