Basic Fibroblast Growth Factor and Insulinlike Growth Factor I Support the Growth of Human Septal Chondrocytes in a Serum-Free Environment

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Objective: To determine if insulinlike growth factor I (IGF-I) and basic fibroblast growth factor (bFGF), individually or in combination, support the growth and viability of human septal chondrocytes in a serum-free medium (SFM) and a serum-enhanced culture medium.

Design: Chondrocytes were recovered from enzymatically digested human septal cartilage and were plated for monolayer culture in a newly developed medium. The medium included Dulbecco modified Eagle medium mixed 1:1 with Ham F12 medium and a supplement of known amounts of 2 growth factors—bFGF (100 ng/mL) and IGF-I (100 ng/mL)—used in combination and separately.

Results: The combination of IGF-I and bFGF enhanced chondrocyte growth and maintained a high degree of viability in SFM and 10% fetal calf serum. After an initial lag, the SFM, augmented with both growth factors, produced a comparable number of viable cells \((4.25 \pm 0.31 \times 10^4)\) to that of the medium with 10% fetal calf serum \((4.64 \pm 0.35 \times 10^4)\) by the seventh day of the experiment. Combined with the 2 growth factors, 10% fetal calf serum provided the greatest proliferation by the end of the experiment. However, the overall mean cell counts for the IGF-I– and bFGF-enhanced SFM were not statistically different.

Conclusions: The combination of IGF-I and bFGF in a serum-free and a serum-supplemented environment supports the growth and viability of human septal chondrocytes in short-term culture. In an SFM, the results obtained approximate those produced in a medium enhanced with 10% fetal calf serum.


SCell culture techniques advance, laboratory-based tissue engineering of human cartilage is slowly evolving into a reality. In 1994, Brittberg et al1 announced that laboratory-cultured autologous articular chondrocytes could be used to repair deep cartilage surface defects in the human knee. In the fall of 1995, Vacanti and Langer awed the public with the televised image of a nude mouse carrying a subcutaneously implanted, tissue-engineered human auricle on its dorsum, an auricle that their laboratories had produced by seeding articular cartilage on a molded bioresorbable scaffold.2,3

Reconstructive surgeons generally agree that native septal cartilage is an optimal implant for repair of cartilaginous defects. Its firmness and nonpliability lend it superior qualities for reconstructive work. Guyuron and Friedman4 reported going as far as banking septal cartilage removed from patients undergoing septoplasty to preserve the excised tissue for future autologous use.

Most investigations of laboratory-based fabrication of cartilage use animal articular chondrocytes. Information is relatively lacking concerning the cellular behavior of human septal chondrocytes compared with that of human and animal articular cartilage. Human septal cartilage is an optimal candidate for tissue engineering because it is so easily available to the reconstructive surgeon.

Growth factors will most likely be important in the tissue engineering of cartilage because they are able to modulate the mitogenesis of chondrocytes and synthesize the cartilaginous extracellular matrix. Bujia et al5 tested the impact of epidermal growth factor, transforming growth factor beta (TGF-beta), and basic fibroblast growth factor (bFGF) on the cellular proliferation of human septal chondrocytes and found that bFGF was the most potent mitogen of the 3. In 1993, however, Quatela et al6 reported that although hu-
MATERIALS AND METHODS

With approval from the Stanford University Institutional Review Board, Stanford, Calif, human septal cartilage specimens were obtained during elective septoplasty (Figure 1, A). Bathed in a sterile isotonic sodium chloride, the samples arrived at the laboratory having been dissected free of periosteum in the operating room. In the laboratory, the samples were processed in sterile fashion within 4 hours of their procurement. To prevent contamination from fibroblasts, all edges of the samples were trimmed free and discarded. The remaining samples were then minced into 1- to 3-mm³ cubes, placed into a spinner flask, and incubated at 37°C in a digestion medium for 18 to 36 hours (Figure 1, B). A modification of a previously described digestion medium¹² was used and consisted of type II collagenase (2.00 mg/mL), hyaluronidase (0.10 mg/mL), and type I deoxyribonuclease (0.15 mg/mL) (all from Worthington Biochem Corp, Freehold, NJ) in Dulbecco modified Eagle medium (DMEM) mixed 1:1 with Ham F12 medium (Gibco, Grand Island, NY). After digestion, the dispersed cells were filtered through a 40-µm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ) to remove any remaining undigested clumps. The cells were then suspended with a phosphate-buffered saline solution (Gibco) and centrifuged at a low speed (1000 g for 7 minutes) twice to remove any remaining enzymes.

A modification of the modified Webber medium of Rosseto et al³ that was developed for chick postembryonic growth-plate chondrocytes was used. The main differences were that the 2 growth factors—bFGF and IGF-I (R & D Systems, Minneapolis, Minn)—were used as components; that ascorbic acid was a constant supplement; and that 4 instead of 18 amino acid supplements were used. The contents of the medium used are listed in Table 1.

Primary cultures were established by seeding cells freshly recovered from enzymatic digestion into a 25-cm² tissue culture flask (Falcon, Franklin Lakes) with DMEM supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, Utah); penicillin, streptomycin, and amphotericin (Gibco); levoglutamide (Gibco); and ascorbic acid (25 µg/mL; Sigma Chemical Co, St Louis, Mo). This and all subsequent cultures were incubated at 37°C in a 5% carbon dioxide atmosphere. The cells were subcultured 4 times. Once they neared confluency during their fourth subculture, they were removed from their flask by trypsination, suspended in phosphate-buffered saline solution, and centrifuged at 1000g for 10 minutes 3 times to rinse off any remaining enzymes. Cells were then suspended in the respective experimental media to produce an optimal seeding density, as determined by pilot studies, of 2.2 × 10⁴ cells/mL. The 6 seeding suspensions were delivered in aliquots of 1 mL per well to a single 24-well culture plate (2 cm² per well), as shown in Figure 2. The cell culture media were renewed every 48 hours.

Duplicate cell counts were made with a Neubauer hemocytometer for each well measured. Viability was assessed via trypan blue dye exclusion. Cell morphologic features were examined by light and electron microscopy. Normally distributed data were evaluated by analysis of variance. The Student t test was used to compare data between groups. Differences at the 5% level were considered significant.

man auricular chondrocyte mitogenesis increased in the presence of bFGF and TGF-β, septal chondrocytes did not display increased proliferation in the presence of either bFGF or TGF-β.

Of particular relevance to this study is the combination of insulinlike growth factor I (IGF-I) and bFGF, which act synergistically on bovine growth-plate chondrocytes. When the 2 are combined, cell proliferation is more than that of the growth factor–free control cultures.⁷ One purpose of the present study is to resolve the conflicting data offered by Quatela et al⁶ and Bujia et al⁵ by determining whether human septal chondrocytes respond mitogenically to growth factors and in particular to bFGF, which is a known potent mitogen.⁶⁻¹⁰ Insulinlike growth factor I was selected for its demonstrated synergy with bFGF.⁷,¹¹

With this in mind, this study was designed to develop a reliable and serum-free method for the ex vivo expansion of human septal chondrocytes. With the eventual goal being autologous reimplantation, such a technique would allow manipulation of mitogenesis and cellular synthetic functions. This would enable optimization of the cartilage’s biomechanical properties and reduce the likelihood of implant rejection. A serum-free environment would also enable further and more specific identification of growth factor functions and interactions by eliminating the presence of uncharacterized serum growth factor modulators.

Septal chondrocyte cultures maintained a monolayer throughout the experiment. The cell counts are displayed in Table 2, and the growth curves are displayed in Figure 3. All cell counts decreased from their initial plating count of 2.2 × 10⁴ cells/mL 24 hours after initiation. This reduction was noticeably less for the cultures that were enhanced with FCS. The latter cultures also experienced a noticeably shorter lag phase. After an initial lag, the combination of IGF-I and bFGF in serum-free medium (SFM) produced comparable mean cell counts for the experiment duration to those of 10% FCS and those of IGF-I and bFGF in 10% FCS; there were no significant statistical differences among these 3 groups.
Insulinlike growth factor I and bFGF in SFM yielded mean cell counts that were statistically significantly higher than those of unsupplemented SFM (P<.05). No episodes of bacterial or fungal contamination occurred.

The mean percent viability of septal chondrocytes is shown in Figure 4. Viability remained greater than 89% during the experiment except for the IGF-I–enhanced culture 72 hours after initiation (mean ± SD, 84.7% ± 0.9%).

All chondrocytes displayed a fusiform appearance (Figure 5). Chondrocytes exposed to IGF-I displayed a more cuboidal appearance, especially as they neared confluency (Figure 6).

**COMMENT**

The data generated from this study clearly show that it is possible to grow human septal chondrocytes in a serum-free environment augmented with growth factors. Most tissue culture typically involves the use of FCS to support cellular growth and metabolism. Hence, a serum-free formulation represents a significant reduction in the antigenicity of the culture medium and in the chances of immunologic rejection of reimplanted tissue-engineered cartilage. A current limitation to our method is the presence of human lactalbumin and human transferrin in its formulation. The optimal formulation will be entirely devoid of any human blood products. This laboratory is currently testing a subsequent formulation that does not include either transferrin or lactalbumin.

The reduced antigenicity of SFM is advantageous in the realm of tissue engineering, but there is yet another use that is perhaps even greater. The actions of growth factors are well known to be heavily modulated by their environment and other cytokines. A serum-free model is additionally beneficial because it allows the study of endogenous and exogenous growth factors without the effect of as-of-yet uncharacterized agents found in serum. Serum introduces an unknown number of variables. A serum-free model has already been developed by this laboratory for the in vitro study of keloid fibroblasts.14 Such a model enables the evaluation of individual endogenous growth factors and specific combinations and sequences of growth factors without these confounding variables. This is beneficial because the eventual goal is to gain a better understanding of the functions of growth factors and the cellular physiologic mechanisms of chondrocytes.

Our data corroborate with the findings of Bujia et al1 that the mitogenic actions of growth factors are further enhanced in the presence of FCS. The final cell counts from the growth factor–enhanced 10% FCS medium were

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**Table 1. Composition of Serum-Free Medium and Concentrations of Individual Components**

<table>
<thead>
<tr>
<th>Concentration</th>
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<tbody>
<tr>
<td><strong>Base</strong> Ham F12: Dulbecco modified Eagle medium 1:1</td>
</tr>
<tr>
<td><strong>Supplements</strong></td>
</tr>
<tr>
<td>Dexamethasone 100 ng/mL</td>
</tr>
<tr>
<td>Human transferrin 1 µg/mL</td>
</tr>
<tr>
<td>Ascorbic acid 25 µg/mL</td>
</tr>
<tr>
<td>Sodium selenite 1 µg/mL</td>
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<tr>
<td>Lactalbumin hydrosylate 2 µg/mL</td>
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<tr>
<td>Sodium bicarbonate 44 mmol/L</td>
</tr>
<tr>
<td>Penicillin 100 U/mL</td>
</tr>
<tr>
<td>Streptomycin 100 µg/mL</td>
</tr>
<tr>
<td>Amphotericin 250 µg/mL</td>
</tr>
<tr>
<td>Levoglucosamine 292 µg/mL</td>
</tr>
<tr>
<td>Tryptophan 7.0 µg/mL</td>
</tr>
<tr>
<td>Threonine 40.8 µg/mL</td>
</tr>
<tr>
<td>Cysteine hydrochloride 122 ng/mL</td>
</tr>
<tr>
<td><strong>Variable supplements</strong></td>
</tr>
<tr>
<td>Basic fibroblast growth factor 100 ng/mL</td>
</tr>
<tr>
<td>Insulinlike growth factor I 100 ng/mL</td>
</tr>
<tr>
<td>Fetal calf serum 10%</td>
</tr>
</tbody>
</table>

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**Figure 1. Schematic of the tissue engineering of cartilage. A, A small septal biopsy sample is excised. B, Within hours of procurement, the specimen is minced, placed in digestion media, and incubated at 37°C to release chondrocytes from their native extracellular matrix (ECM). Unconfined cells then go to either step C or step D. C, Chondrocytes are monolayer cultured in a growth factor–enhanced serum-free medium (SFM) for rapid proliferation. D, The chondrocytes are seeded onto a premolded biodegradable scaffolding either directly after digestion or after having been grown in monolayer culture (C). Attached to the scaffolding, cells presumably assume their native morphologic shape and synthesize an ECM, all while remaining in an antigen-free SFM. E, Once the appropriate physical characteristics are achieved, the laboratory-grown tissue is removed from the SFM and minor adjustments in shape are made. F, The autologous reimplantation corrects the original cartilaginous defect.**

**Figure 2. The arrangement of a 24-well culture plate. Human septal chondrocytes were seeded at an initial density of 2.2 × 10^4 viable cells/well (1.1 × 10^4 viable cells/cm²) in 1 mL of culture medium. The culture media were renewed every 48 hours. Column 1 represents base medium with no additives; column 2, base medium with insulinlike growth factor I (IGF-I; 100 ng/mL); column 3, base medium with basic fibroblast growth factor (bFGF; 100 ng/mL); column 4, base medium with IGF-I and bFGF (100 ng/mL of each); column 5, base medium with 10% fetal calf serum (FCS); and column 6, base medium with 10% FCS and IGF-I and bFGF (100 ng/mL of each).**

**Figure 3. Electron micrograph of human septal chondrocytes after 7 days in culture.**

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nearly 3 times as great as those of the FCS-enhanced medium and the combined bFGF- and IGF-I–supplemented SFMs. It is unclear at this point whether this is because of the presence of facilitating cofactors in the serum, enhanced stability of growth factors in serum, greater up-regulation of growth factor receptors in the chondrocytes, or enhanced receptor function in the presence of serum.

This accelerated growth factor–induced mitogenesis in the presence of FCS has led to the proposition of using autologous patient serum as a supplement to culture medium. This would avoid the exposure of human cells to animal serum components and, in theory, retain the advantage of increased growth factor–catalyzed mitogenesis. However, further investigation is needed to determine whether autologous human serum will indeed provide a similar augmentation of growth factor action.

A considerable disadvantage of this approach centers

Table 2. Human Septal Chondrocyte Cell Counts

<table>
<thead>
<tr>
<th>Medium</th>
<th>Mean (SD) Cell Counts, ×10⁴ Cells/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>Day 3</td>
</tr>
<tr>
<td>Base medium</td>
<td>0.82 (0.17)</td>
</tr>
<tr>
<td>IGF-I</td>
<td>0.97 (0.25)</td>
</tr>
<tr>
<td>bFGF</td>
<td>1.13 (0.11)</td>
</tr>
<tr>
<td>IGF-I and bFGF</td>
<td>1.41 (0.32)</td>
</tr>
<tr>
<td>10% FCS</td>
<td>1.99 (0.08)</td>
</tr>
<tr>
<td>10% FCS, IGF-I and bFGF</td>
<td>1.72 (0.13)</td>
</tr>
</tbody>
</table>

*IGF-I indicates insulin-like growth factor I; bFGF, basic fibroblast growth factor; and FCS, fetal calf serum.
around blood-borne pathogens such as the human immunodeficiency and hepatitis viruses; a strict system of labeling biopsy tissue and patient serum would need to be in place to avoid cross-contamination and the possible infection of a patient on reimplantation. Considering the inherent problems of controlling autologous serum, and the immunologic problems of allogenic supplementation, a growth factor–enhanced SFM may be the best current option for tissue engineering of reimplantable cartilage.

Our study differs from those by Bujia et al and Quatela et al in 1 important regard. Both of their studies used fully differentiated human chondrocytes. Chondrocytes from avian and mammalian species alter their native round or polygonal shape to flattened and fusiform within a month of monolayer culture or within 4 passages. They also switch their production of collagen from the cartilaginous type II to the genetically distinct type I. After 2 weeks of culture, about 50% of the synthesized collagen is type I. This process of losing a phenotypic profile is known as dedifferentiation and is reversible. Dedifferentiation should not be confused with a carcinogenic process because dedifferentiated chondrocytes do not exhibit abnormal growth and display normal contact inhibition in culture. Dedifferentiation in culture may be a response to flattened morphologic shape because dedifferentiated chondrocytes reexpress their differentiated phenotype when suspended and cultured in firm gel agarose, an environment in which they resume their native round or polygonal morphologic shape.

For purposes of tissue engineering, the number of chondrocytes obtained from a biopsy sample can be expanded in either of 2 forms. They can be grown in a format that will maintain their differentiated state, for example, by directly seeding them onto a scaffold where they will presumably resume their native shape and production of type II collagen. Alternatively, they can be grown in a format that will promote their dedifferentiation, such as the monolayer culture technique (Figure 1, C). The latter would undoubtedly result in a population of predominantly dedifferentiated chondrocytes because of the considerable time required to grow a sufficient number of cells for reconstructive purposes, especially from a small biopsy sample.

It would be wise to address the effects of dedifferentiation of the cultured chondrocytes within the context of tissue engineering. Not only do dedifferentiated chondrocytes have a different synthetic profile but differentiated chondrocytes from human articular cartilage also respond differently to cytokines than when in a differentiated state. This may present an advantage in that there may be a symbiotic association between mitogenesis and dedifferentiation of the chondrocytes. Basic fibroblast growth factor has inhibitory effects on rat rib growth-plate chondrocyte differentiation, and its mitogenic stimulation coincides with a decrease in collagen type II. Evidence suggests that bFGF stimulates chondrocyte proliferation by preventing terminal differentiation. The association of bFGF and the prevention of differentiation of chondrocytes then begs the question: Will dedifferentiated cells respond more vigorously than differentiated chondrocytes to the mitogenic effects of bFGF and other growth factors?

In contrast to bFGF, IGF-I up-regulates collagen type II. If IGF-I is used in high enough concentrations, it may prevent the chondrocyte-dedifferentiating effect of bFGF without inhibiting bFGF’s up-regulation of DNA synthesis. The process of redifferentiating may be, at least in part, modulated by cytokines in articular cartilage. Although IGF-I was selected in this experiment purely for its augmentation of bFGF’s mitogenicity, it may be possible to adequately manipulate the redifferentiation of dedifferentiated chondrocytes with growth factors alone. This possibility and the effects of suspension culture are currently being evaluated.

In the laboratory setting, which sequence and combination of growth factors will most aptly choreograph, in order, the mitogenesis and redifferentiation of chondrocytes and then augment their production of cartilaginous extracellular matrix in such a manner as to optimize the mechanical properties of the cartilage? A larger and more detailed, long-term, serum-free culture study is currently under way to examine the effects of growth factors on human septal chondrocytes and the behavior of dedifferentiated cells.

CONCLUSIONS

In the realm of reconstructive surgery, a sound understanding of how to grow reimplantable, autologously derived septal cartilage in vitro would have tremendous benefit. Although tissue engineering is young as a science, there is an overwhelming amount of evidence that growth factors may play a crucial role in the laboratory-based manipulation of cells because of their potent ability to regulate both cell metabolism and mitogenesis. This newly developed serum-free model supports the growth of septal chondrocytes and allows the evaluation of growth factor optimization.

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REFERENCES