Age Dependence of Cellular Properties of Human Septal Cartilage

Implications for Tissue Engineering

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Background: The persistent need for cartilage replacement material in head and neck surgery has led to novel cell culture methods developed to engineer cartilage. Currently, there is no consensus on an optimal source of cells for these endeavors.

Objectives: To evaluate human nasal cartilage as a potential source of chondrocytes and to determine the effect of donor age on cellular and proliferation characteristics.

Subjects: Nasal cartilage specimens were obtained after reconstructive surgery from 46 patients ranging in age from 15 to 60 years.

Methods: Specimens were weighed and chondrocytes were isolated by digestion in 0.2% collagenase type II for 16 hours. Cells were maintained in primary cultures until confluency, then seeded onto polylactic acid-polyglycolic acid scaffolds. Seeding efficiency was determined by quantification of DNA content of seeded constructs by means of Hoechst dye 33258. Specimen weights, cell yields, cell content, and doubling time were also measured and correlated to donor age.

Results: Mean (±SD) cartilage mass obtained (648±229 mg) is higher than from typical biopsy specimens of auricular cartilage, and the cellular characteristics show a higher proliferation rate than auricular chondrocytes. Cell yield increased with age, while doubling time decreased with age in samples from patients ranging from 15 to 60 years old.

Conclusions: The use of nasal septal cartilage as a source of cells for tissue engineering may be valid over a wide range of patient ages. The large tissue yield and consequent cell yield make this tissue a potential starting source of chondrocytes for large-volume tissue-engineered implants.


Tissue engineering has recently become a viable method for generating material for the replacement of cartilage defects in the head and neck. It has been demonstrated that different types of chondrocytes are capable of forming cartilage specimens in vitro and in vivo with the help of resorbable polymer scaffolds. Several studies have documented the use of bovine articular chondrocytes1-4 as well as porcine5 and canine6 auricular cells in the generation of tissue-engineered cartilage. The translation of this technology to clinical applications necessitates that human cells be characterized as thoroughly to determine their suitability for applications of tissue engineering. Human septal chondrocytes have also been demonstrated to be capable of forming cartilage specimens, closely resembling the native cartilage from which they were derived.7-9 However, the optimal source of tissue for harvest of cartilage still needs to be determined.

Little is known about the specific characteristics of different cartilage types for tissue engineering.10 This information is of critical importance in the effort to translate tissue engineering into clinical practice. The density of cells in the tissue, as well as the efficiency with which they are isolated and the in vitro growth rates, are important design constraints for planning the size of tissue needed for harvest and the time necessary for cell expansion. Furthermore, it is still unknown whether these characteristics and others such as cell-scaffold interactions are dependent on patient age.

Studies investigating growth characteristics of the human septum in the context of midfacial development11,12 report an increasing or constant number of chondrocytes in the human nasal septum in dependency of the location within the sep-
MATERIALS AND METHODS

HARVEST OF CARTILAGE AND CELL ISOLATION

Human septal cartilage was obtained after reconstructive septorhinoplasty in accordance with the guidelines of the University of Massachusetts Medical Center, Worcester, and Englewood Hospital, Englewood, NJ. Donor age ranged from 15 to 60 years, with a mean (±SD) age of 32.9 ± 12.9 years. Samples were obtained from 46 patients, 27 female and 19 male. Immediately after surgery, samples were placed in Dulbecco minimum essential medium (Life Technologies, Grand Island, NY) containing penicillin G, 100 U/mL; streptomycin, 100 µg/mL; and amphotericin B, 0.25 µg/mL (Life Technologies) for 4 to 16 hours before processing. Specimens were freed of surrounding perichondrium, bone, or connective tissue, and the wet weights were determined. A 6-mm punch biopsy was obtained, weighed, and frozen at −20°C until biochemical testing. The remaining cartilage sample was exposed to 0.2% collagenase type II (Worthington, Lakewood, NJ) for 10 to 14 hours to isolate the chondrocytes as described previously.1

CELL CULTURE

Chondrocytes were seeded at a density of 8000 cells/cm² and maintained at 37°C in a 5% carbon dioxide atmosphere in primary monolayer culture until confluence (approximately 12-14 days). Dulbecco minimum essential medium supplemented with penicillin G, 100 U/mL, streptomycin, 100 µg/mL, and amphotericin B, 0.25 µg/mL; ascorbic acid, 25 µg/mL; and 10% fetal bovine serum served as culture medium, with fresh medium added every 2 to 3 days. Cells were released from culture flasks by treatment with 0.05% trypsin-EDTA (Life Technologies) and counted with a hemocytometer using trypan blue exclusion. Chondrocytes were seeded onto polyglycolic acid (PGA) (Albany International, Mansfield, Mass) disks coated with 0.5% polyactic acid (PLA) (Polysciences Inc, Warrington, Pa) at a density of 30 × 10⁶ cells per milliliter. The disks had a diameter of 10 mm and a thickness of 2 mm. The PLA coating was achieved by immerision of prefabricated PGA disks into a 0.5% solution of PLA in methylene chloride (Sigma-Aldrich Corp, St Louis, Mo).14

CELL SEEDING AND DETERMINATION OF SEEDING EFFICIENCY

Seeding was performed by using a pipetting technique, in which a volume corresponding to the scaffold volume was slowly pipetted onto the prewetted polymer disks. Cells were allowed to seed for 2 hours before addition of medium. Twenty-four hours after the initial cell seeding procedure, scaffolds were digested with papain, 0.125 mg/mL, and digests were assayed for DNA with Hoechst dye 33258.15 The number of cells in the constructs was calculated by means of an assumed amount of 7.7 pg of DNA per chondrocyte.15

STATISTICS

Linear regression analysis was used to evaluate the age dependence of cell density, isolation efficiency, doubling time, and seeding efficiency. The t test was used to evaluate sex differences in cell density, isolation efficiency, doubling time, and seeding efficiency. By means of experimentally determined variances, the powers of age and sex analyses were greater than 0.8. Unless otherwise indicated, data are given as mean ± SD.

RESULTS

The average mass of specimens of human nasal cartilage obtained by reconstructive (septo)rhinoplasty was 648.1 ± 229.4 mg. The mass was not dependent on donor age or sex (Table). The average mass of samples from male donors was 690.3 ± 249.9 mg, and from female donors, 614.6 ± 227.6 mg.
Isolation efficiency increased from 10% in tissue from 15-year-old patients to 14% in tissue from 60-year-old patients (Figure 4), but that this change was not statistically significant (.05 < P < .1). The isolation efficiency did not vary with donor sex (Table).

The average doubling time of human septal chondrocytes was 2.6 ± 0.98 days. This variable showed a significant dependence on donor age, with cells from older patients proliferating more rapidly (P < .02) (Figure 5). Linear regression analysis indicated that doubling time decreased from more than 3 days for cells from 15-year-old patients to less than 2 days for cells from 60-year-old patients. Proliferation was independent of sex (Table).

The seeding efficiency, calculated as the number of cells that adhered to the scaffold after 24 hours normalized to the number of cells placed on the scaffold, averaged 25.1% (Table). The seeding efficiency did not vary with donor age (Figure 6) or sex (Table).

### Group and Sex Averages of Cell Culture Variables*

<table>
<thead>
<tr>
<th></th>
<th>Average</th>
<th>Female</th>
<th>Male</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size, mg</td>
<td>648.1 ± 229.4</td>
<td>614.6 ± 227.6</td>
<td>690.3 ± 249.9</td>
<td>.29</td>
</tr>
<tr>
<td>Cell yield per wet weight, No./mg</td>
<td>4.45 × 10^3 ± 2.28 × 10^3</td>
<td>4.85 × 10^3 ± 2.39 × 10^3</td>
<td>4.18 × 10^3 ± 2.40 × 10^3</td>
<td>.39</td>
</tr>
<tr>
<td>Cell content per wet weight, No./mg</td>
<td>4.29 × 10^4 ± 1.87 × 10^4</td>
<td>4.33 × 10^4 ± 1.84 × 10^4</td>
<td>4.28 × 10^4 ± 1.55 × 10^4</td>
<td>.68</td>
</tr>
<tr>
<td>Isolation efficiency, %</td>
<td>11.9 ± 6.9</td>
<td>11.5 ± 6.6</td>
<td>13.0 ± 7.0</td>
<td>.47</td>
</tr>
<tr>
<td>Doubling time, d</td>
<td>2.6 ± 1.0</td>
<td>2.8 ± 1.0</td>
<td>2.5 ± 0.5</td>
<td>.56</td>
</tr>
<tr>
<td>Seeding efficiency, %</td>
<td>25.1 ± 13.5</td>
<td>26.7 ± 14.1</td>
<td>21.1 ± 12.5</td>
<td>.45</td>
</tr>
</tbody>
</table>

*Values are given as mean ± SD.
The goal of this study was to characterize the suitability of human septal cartilage for tissue engineering on the basis of cellular characteristics. The results indicated that isolation and cellularity of septal chondrocytes are independent of age over a large age span. Doubling time decreases with increasing age, indicating more rapid proliferation, and isolation efficiency increases slightly. Although significant age trends exist, the data from the current study suggest that the use of nasal septal cartilage as a source of cells for cartilage tissue engineering remains viable for all patient ages sampled.

An important measure in assessing the suitability of a certain type of tissue for application in tissue engineering is the amount of tissue available at initial harvest. No consensus exists on which type of cartilage is best suited for certain applications. Several studies have used bovine articular cartilage\(^1,4\) to demonstrate feasibility, as it is readily available in large amounts, thereby omitting the problem of tissue selection and cell amplification. However, for clinical applications this source is not suitable.

Common locations for cartilage harvests in clinical settings are the outer ear, the rib, and the nasal septum. The mass of recovered cartilage (about 650 mg) in our study was large compared with tissue gained by surgery on the outer ear (about 50 mg).\(^1,6\) This is of great relevance, as was large compared with tissue gained by surgery on the rib. However, for clinical applications this source is not suitable.

Figure 5. Doubling time vs donor age for 22 patients. Values of correlation coefficient (\(R^2\)) and significance level \((P)\) were calculated by linear regression analysis.

Figure 6. Seeding efficiency (number of cells attached normalized to number of cells seeded) vs donor age for 18 patients. Values of correlation coefficient \((R^2)\) and significance level \((P)\) were calculated by linear regression analysis.

The volume of the tissue for harvest should be determined before the procedure by correlation with the desired volume of the tissue-engineered construct. To reconstruct large defects like, for example, a whole adult human ear, the necessary cell number is in the range of \(1 \times 10^8\) to \(5 \times 10^8\) cells. The primary aim, therefore, is to obtain a sufficient amount of tissue, allowing amplification in monolayer culture for 2 to 3 weeks without extensive cell dedifferentiation. The large variation in the efficiency of the isolation procedure (Figure 3) suggests that conservative estimates must be used when procedures are planned for harvest of nasal cartilage for tissue engineering. However, our data show that this expansion of cell number could take place entirely in primary culture, which would minimize the risk of dedifferentiation and loss of chondrocyte cell function.

The doubling time of human nasal septal chondrocytes (2.6±1.0 days) is shorter than that reported for human ear chondrocytes (3.4 days).\(^1,6\) This is of significant concern, since doubling time would directly limit the time between tissue harvest and reimplantation of an engineered construct. Given the average cell yield (approximately 12%) and the doubling time (2.6 days), it would take approximately 8 days of culture (3 doublings) to obtain the same number of cells present in the original tissue. Further culture beyond this time would allow for expansion of cell population beyond that originally harvested (eg, 13 days of total culture time would provide 4 times the amount of cells originally harvested, or enough to make approximately 2.5 cm\(^2\) of tissue).

While the phenomenon of dedifferentiation limits the possible level of expansion of the cell population, data from the current study begin to allow for estimation of the volume of tissue that can ultimately be fabricated by these techniques. Previous work\(^18\) has demonstrated that placement of cells into scaffolds after second passage preserves chondrocyte phenotype markers such as proteoglycan and type II collagen production. A conservative estimate is that each passage will allow for an 8-fold expansion, such that 2 passages will convey a 64-fold expansion of the cells obtained with a 6% efficiency from the current study begin to allow for estimation of the possible level of expansion of the cell population.
(12% × 64) increase in tissue mass, or approximately 4.5 cm³ of implant. This would be of great utility in a variety of procedures in craniofacial reconstruction.

The slight increase in isolation efficiency as well as the decrease in doubling time might be explained by changes in cellular characteristics into a more fibroblast-like cell type. This is consistent with previous reports that the proteoglycan content of human nasal cartilage decreases with age, while the collagen content increases. Embedded in a slightly more fibrous tissue matrix, isolation would be facilitated and the fibroblast-like cell would tend to proliferate more rapidly than hyaline chondrocytes. If this is true, one may expect decreased matrix synthesis from cells in these tissues as well, but this has not been established.

The current study did not demonstrate any characteristics to exclude any patient aged 15 to 60 years from such procedures. The seeding efficiency was also independent of donor age. However, with the use of the classic pipette seeding technique, the efficiency with PLA-coated PGA scaffolds was relatively low, with an average of 25%. Interestingly, this is consistent with data obtained by seeding of bovine articular chondrocytes on PGA scaffolds, coated with different concentrations of PLA, thus indicating a comparable behavior of bovine articular and human nasal septal chondrocytes with regard to seeding characteristics. Other groups have demonstrated seeding efficiencies of 100% with more complex procedures requiring specialized equipment.

In summary, septal cartilage appears to be a suitable source for tissue harvest for engineering of cartilage in vitro. It is easy to obtain with low donor site morbidity and offers sufficient cell numbers. Many cellular characteristics seem to be independent of donor age for the span from 15 to 60 years, thus defining the suitable patient collective. Further studies on the characteristics of the tissue-engineered cartilage of these specimens are currently being conducted.

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