ORIGINAL ARTICLE

Injectable Cartilage

Using Alginate and Human Chondrocytes

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Objective: To create engineered cartilage through an injectable medium that could be used as a minimally invasive implant material.

Methods: Human nasal septal chondrocytes, carried in an alginate polymer, were injected and molded percutaneously into nude mice and developed in vivo. The cartilage was harvested from 14 to 38 weeks and analyzed through gross, histological, immunohistochemical, and biochemical analysis.

Results: Of the 15 explants, 14 (93%) resembled native cartilage on gross analysis. The injections maintained their overall appearance with some loss of definition. On histological analysis, 6 of the explants (40%) appeared similar to native cartilage throughout the sample. Eight of the explants (53%) resembled native cartilage; however, there were some areas of fibrous tissue differentiation. The neocartilage stained positive for type II collagen. Explants harvested at week 26 or later and the samples that histologically resembled native cartilage had similar hydroxyproline content to native septal cartilage.

Conclusions: Injectable, autologous cartilage may be the answer to the long search for the ideal implant in facial plastic surgery. Alginate and human chondrocytes can be used to create an injection that may be molded and maintains its overall size and shape, with some loss of definition, for at least 38 weeks after injection.

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IMPLANT MATERIALS HAVE BEEN used to repair various craniofacial contour defects due to acquired or congenital abnormalities. Autograft, allograft, and synthetic materials have been used with varying degrees of success. Tissue engineering, first described by Vacanti et al,[1] offers a promising alternative to implantable materials used in facial plastic surgery. One focus of tissue engineering has been the production of tissue-engineered cartilage (TEC). Researchers have been able to produce viable, autogenous, 3-dimensional grafts from isolated chondrocytes and biodegradable polymer scaffolds.

Traditionally, TEC has been produced by seeding chondrocytes onto a synthetic polymer and implanting this into a host. Some studies have described using an injectable polymer, but this was placed into a mold that was subsequently implanted into the host.[2-4] Expanding this technique to injecting the polymer and cells in vivo would allow a minimally invasive way to augment craniofacial deformities. Few studies have performed injections with nonhuman chondrocytes, and there were no attempts to mold the injection in vivo.[5,6] The ideal injectable polymer should be able to be injected and molded in situ and should maintain its shape, thus creating an engineered cartilage implant.

The goal of this study was to determine if human chondrocytes and an alginate scaffold can be used to create an injectable, moldable TEC that maintains its size and shape and has similar structural and biochemical properties to native cartilage.

METHODS

OVERVIEW

Fifteen human septal cartilage samples were used as the source of chondrocytes. These were collected from surgical procedures (13 samples) performed at the University of Virginia, Charlottesville, or from unrestricted autopsy cases (2 samples). Approval was obtained from the institutional review board of the University of Virginia to use residual cartilage that would otherwise be discarded after surgical procedures.

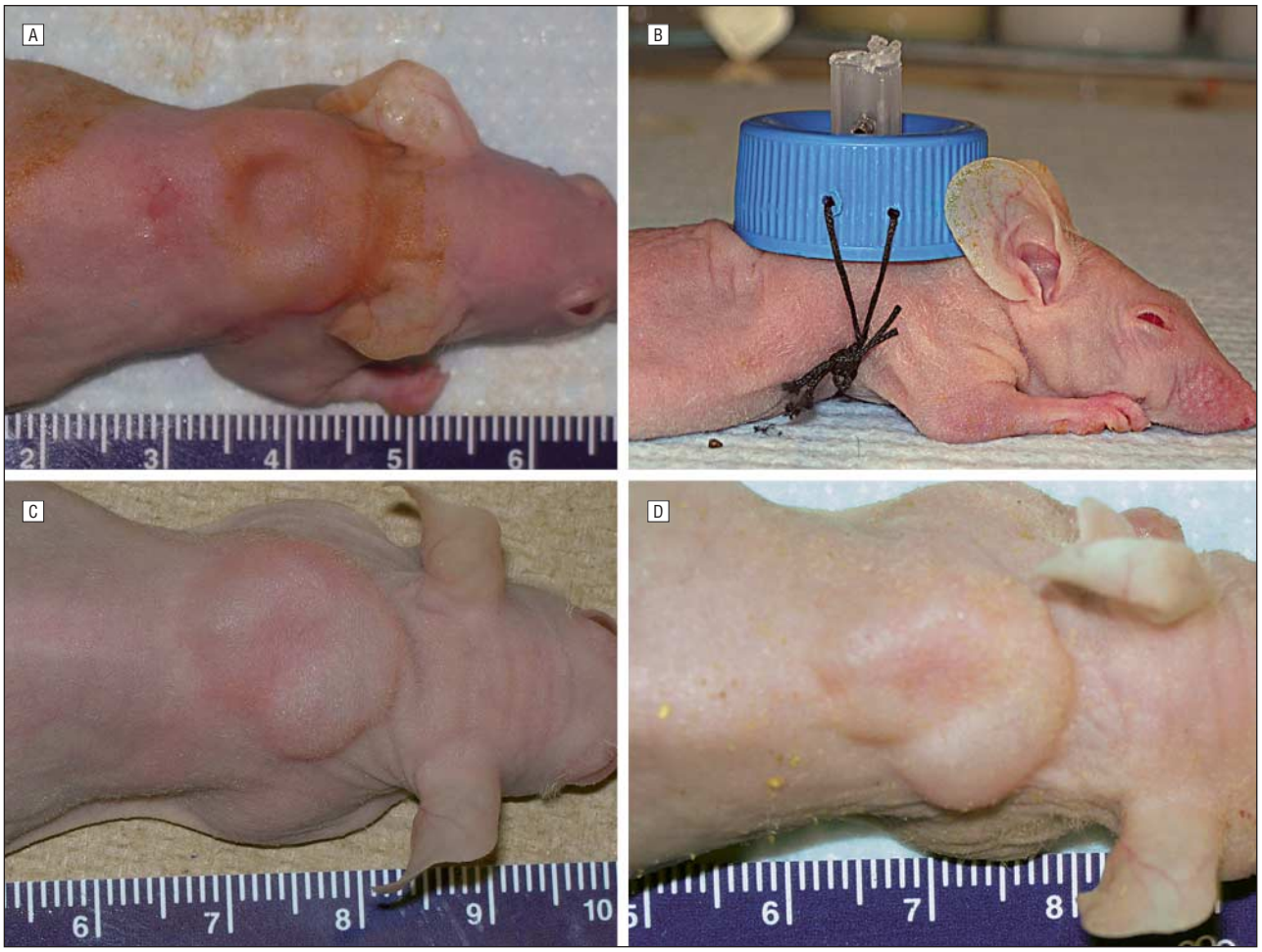

Figure 1. Appearance of injection. Photograph of a nude mouse immediately after the injection of alginate and chondrocytes, with a central depression created with a syringe cap (A) and of the mold placed after an injection (B). The injection area is shown at 8 weeks (C) and 20 weeks (D). The ruler is in centimeters.

The chondrocyte and alginate mixture was injected into the dorsal subcutaneous tissue of athymic mice. The animal protocol was reviewed and approved by the University of Virginia Animal Care and Use Committee. All animals were housed and received veterinary care in the vivarium at the University of Virginia. The explanted cartilage was harvested and analyzed for gross appearance and histological, immunohistochemical, and biochemical properties.

HARVESTING CHONDROCYTES

The perichondrium was removed, and the septal cartilage was diced into small pieces (1-2 mm^3). These pieces were then digested with 0.05% hyaluronidase (Worthington Biochemical Corp, Lakewood, New Jersey) for 30 minutes. The supernatant was aspirated, and the cartilage was washed with phosphate-buffered saline (PBS) with 1% penicillin-streptomycin solution. The cartilage pieces were then further digested with 0.5% collagenase (Crescent Chemical, Hauppauge, New York) and 0.5% trypsin (Worthington Biochemical Corp) for 2 to 3 hours. The resulting suspension was filtered to separate chondrocytes from undigested cartilage. The collagenase-trypsin digest was repeated up to a total of 3 incubations if any undigested cartilage remained. The cells were then washed and resuspended in PBS with 1% penicillin-streptomycin solution. Cell number and viability were determined using the trypan blue exclusion method. All samples had viability greater than 90%.

The isolated chondrocytes were resuspended in complete medium (Dulbecco modified Eagle medium [DMEM] containing 4500 mg/L of glucose, 10% fetal calf serum, 10mM HEPES, 0.1mM nonessential amino acids, 0.4mM L-proline, 50 mg/L of L-ascorbic acid, 100 U/mL of penicillin G sodium, 100 µg/mL of streptomycin, and 25 µg/mL of amphotericin B) and incubated in vitro for 7 to 10 days to confluence. The medium was changed every 48 to 72 hours. The cells were then trypsinized using TryPLE Express (Invitrogen, Carlsbad, California) for 10 to 15 minutes and then placed in PBS with 1% penicillin-streptomycin solution and washed.

INJECTION OF CALCIUM ALGINATE–CHONDROCYTE SUSPENSION

Twelve million cultured cells were resuspended in 300 µL of complete media and then mixed with 300 µL of filtered (0.22 µm) 4% sodium alginate (NovaMatrix, Sandvika, Norway) mixed with DMEM without calcium. This created a suspension of 20×10^6 cells/mL in 2% alginate. Then, 500 µL of the suspension was injected subcutaneously on the back of a nude mouse, creating a raised circular shape. Filtered (0.22 µm) calcium chloride mixed in complete media was then injected subcutaneously into the alginate-chondrocyte suspension in a circular shape. Approximately 150 µL of calcium chloride was injected to initiate the cross-linking process. A syringe cap was used to create a central depression in the injection to represent molding the injection (**Figure 1**A). Another 150 µL of

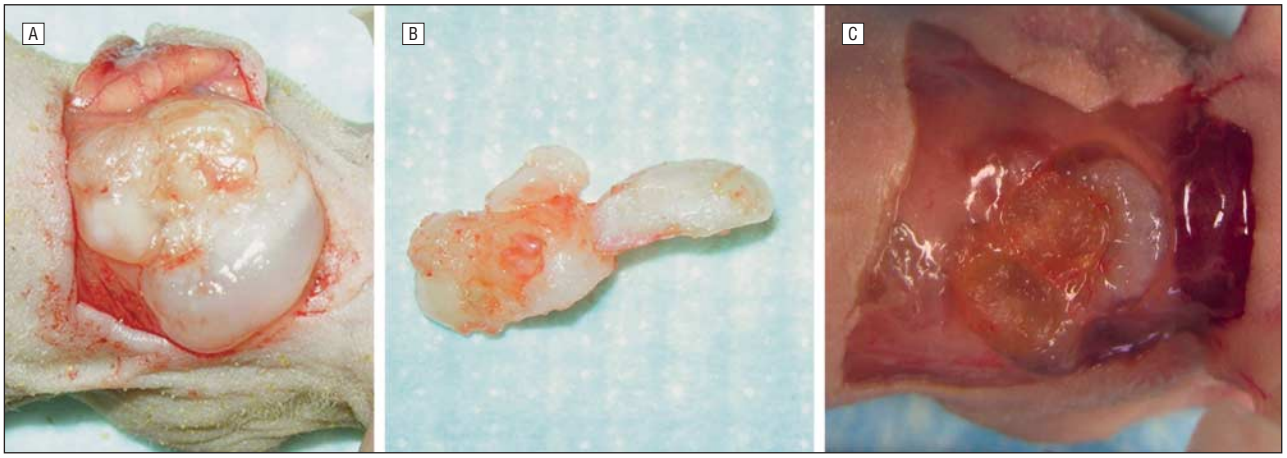

Figure 2. An example of explanted neocartilage. A and B, The explanted cartilage from the injection shown in Figure 1, harvested at 20 weeks. C, A photograph of the one injection that did not resemble native cartilage throughout the sample at harvest.

calcium chloride was injected into the suspension. A mold with a syringe cap was then placed over the injection and left in place for 48 to 72 hours (Figure 1B). The mold was placed to keep the depression in the central portion of the injection while the gel solidified completely. The injection totaled 800 µL; however, some of the calcium chloride solution leaked back out while creating the depression. The calcium was likely cross-linked at this point, and the remaining solution seeped out of the gel. A total of 15 injections of the alginate-chondrocyte suspension and 8 control injections of alginate alone were performed.

HARVESTING OF CARTILAGE

The experimental injections were incubated in the nude mice for 14, 20, 26, 32, and 38 weeks. Four of the control injections were harvested at postinjection day 1 to determine the mean mass of injection. The remaining control injections were harvested at weeks 14, 26, and 32.

ANALYSIS OF EXPLANTED INJECTIONS

Gross Analysis

The gross appearance, size, shape, and mass of the engineered cartilage and the control injections were recorded at the time of explantation and compared with the size and shape at the time of injection. Photographs were taken at the time of injection and the time of harvest. The mice were observed weekly in the vivarium, and representative photographs were taken at various time points during incubation.

Histological Analysis

The experimental and control injections, as well as native septal cartilage, were analyzed by frozen-section histological staining. Approximately half of each sample was used for histological analysis. Hematoxylin-eosin–stained sections were examined at original magnifications of ×4 and ×10 and evaluated for graft architecture.

Immunohistochemical Analysis

Unstained frozen section slides were stained for type II collagen using a staining kit (Chondrex Inc, Redmond, Washington). The slides were developed with diaminobenzidine (DAB) chromogen, resulting in a brown stain for positive samples. Alginate-only injections, as well as mouse skin and muscle, were used a negative controls.

Biochemical Analysis

The remaining half of each sample was stored in 50-µg aliquots at –80°C to be used for biocompositional assays. These samples were analyzed for DNA content and hydroxyproline content (which is indicative of collagen content). The samples were diced and placed in sterile papain (Sigma-Aldrich Corp, St Louis, Missouri) buffer solution at 60°C for 24 hours. DNA content was analyzed using the Hoechst 33258 dye (Sigma-Aldrich Corp) assay with a calf thymus DNA standard.[7] For the hydroxyproline assay, an aliquot of the papain digest was hydrolyzed in 6N hydrochloric acid for 16 hours at 110°C using the dimethylaminobenzaldehyde (DMBA)–chloramine T (Sigma-Aldrich Corp) colorimetric assay with hydroxy-L-proline (Sigma-Aldrich Corp) as a standard.[8]

STATISTICAL ANALYSIS

Statistical analysis was performed using the unpaired *t* test to compare the weight, DNA, and hydroxyproline content. The values for the explanted alginate-chondrocyte injections were grouped into less than 26 weeks and 26 weeks or more for statistical analysis.

RESULTS

GROSS ANALYSIS

A mold was placed at the time of injection to maintain the central depression while the gel solidified completely during the first days after the injection. The mold tended to become displaced while the mice moved around in the cage, and therefore many samples lost the definition of the central depression by the time that the mold was removed on postinjection day 2 or 3. The appearance of the injection and the definition of depression that was present when the mold was removed remained constant throughout the remainder of the incubation (Figure 1C and D). The size of the injection did not change during the incubation.

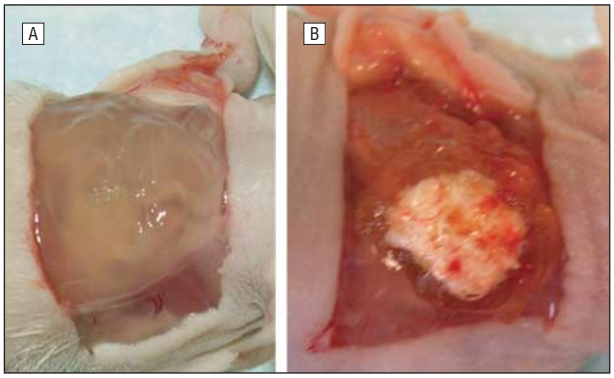

Figure 3. Control injections. Alginate-only injections are shown at day 1 (A) and week 36 (B). At day 1, the injection was a clear gel that was easily malformed. During incubation the injection became smaller, and a portion of the gel calcified.

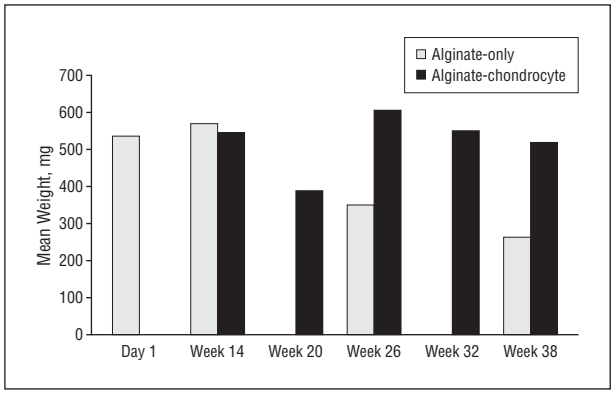

Figure 4. Mean weight of injections at harvest. The weight of the alginate-only injections decreased over time. The weight of the alginate-chondrocyte injections remained stable over time.

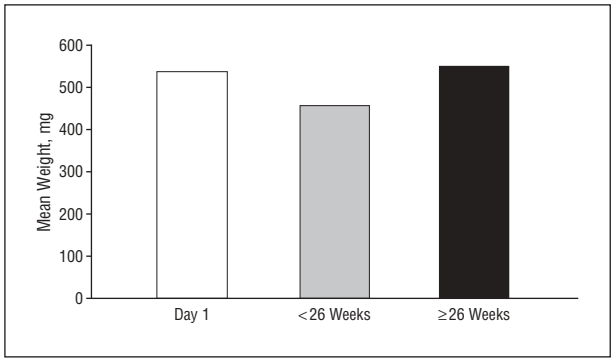

Figure 5. Mean weight of alginate-chondrocyte injections grouped into less than 26 weeks and 26 weeks or more. There was no significant difference between the experimental groups and the control injections harvested at day 1. There was also no significant difference between the 2 experimental groups.

At the time of harvest, all of the injections were easily removed from a thin layer of surrounding fibrous tissue. Of the 15 alginate-chondrocyte injections, 14 (93%) resembled native human cartilage on explantation. The explants exhibited an irregular pearly white appearance and were firm but flexible on palpation (**Figure 2**A). In cross-section, the majority of the explants had a solid firm consistency throughout the entire sample. Some explants had small areas that fractured on incision. Of the 15 injections, 1 (7%) did not form cartilage throughout the injection area (Figure 2B). There was a small portion at the edge of the injection that resembled cartilage; however, the remainder of the sample resembled a flat, dried gel. All 4 alginate-only injections that were harvested on day 1 were clear, gelatinous, and easily malformed on palpation (**Figure 3**A). The remaining 4 alginate-only injections were harvested after longer incubations. These explants fractured on palpation and had a soft gelatinous consistency in some portions and a gritty, calcified consistency in other portions of the explants (Figure 3B).

The 4 alginate-only samples collected on day 1 had a mean weight of 536 mg, which was used to compare the change in weight over time. The mean weight of the TEC samples remained stable over time, and the weight of the alginate-only samples decreased over time (**Figure 4**). There was a decrease in the mean weight at week 20, and this corresponds to the 1 injection that did not form engineered cartilage. The samples were then grouped for statistical analysis, and there was no significant difference between the weight of the samples harvested before week 26 and those harvested at 26 weeks or more when compared with the samples harvested at day 1 (**Figure 5**). There was also no difference in the weight of the samples between the 2 experimental groups (<26 weeks vs ≥26 weeks).

HISTOLOGICAL ANALYSIS

Histological evaluation of the alginate-chondrocyte samples using hematoxylin-eosin showed varying density of lacunae and extracellular matrix (**Figure 6**). None of the explants showed vascular ingrowth or ossification. Of the 15 alginate-chondrocyte explants, 6 (40%) had an overall appearance similar to that of native cartilage throughout the entire sample. There were some slight differences from native cartilage, including a decreased number of lacunae that appeared larger and had an increased number of cells per lacunae when compared with native cartilage (Figure 6A and B). The surrounding extracellular matrix appeared similar, and there was no fibrous tissue ingrowth. The samples having an appearance similar to native cartilage and no fibrous tissue growth did not appear to be dependent on the time of incubation. There was 1 sample at each of the 5 time points. Of the 15 explants, 8 (53%) resembled native cartilage throughout most of the sample; however, they contained other areas that resembled fibrous tissue (Figure 6C). There were lacunae within the cartilage that contained fibroblasts and fibrous tissue within the lacunae.

Of the 15 injections, 1 (7%) did not form any significant amount of cartilage. There were a few areas of fibrous tissue, and the remainder of the sample resembled alginate alone. The alginate samples showed basophilic staining with no rare cells and fibrous tissue.

Of the 15 samples, 14 (93%) contained type II collagen on immunohistochemical stains. There appeared to be a direct correlation between the amount of type II collagen within the sample and the amount of the extracel-

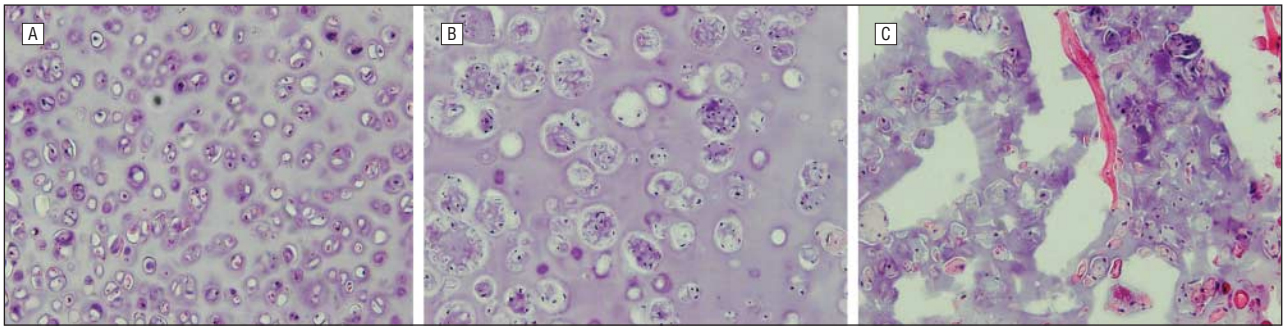

Figure 6. Hematoxylin-eosin staining of native and engineered cartilage (original magnification ×10). A, Representative of native septal cartilage; B, representative of the 6 explants that resembled native cartilage throughout the sample and no fibrous tissue; and C, representative of the remaining explants that had areas of fibrous tissue both adjacent to the extracellular matrix and within the lacunae.

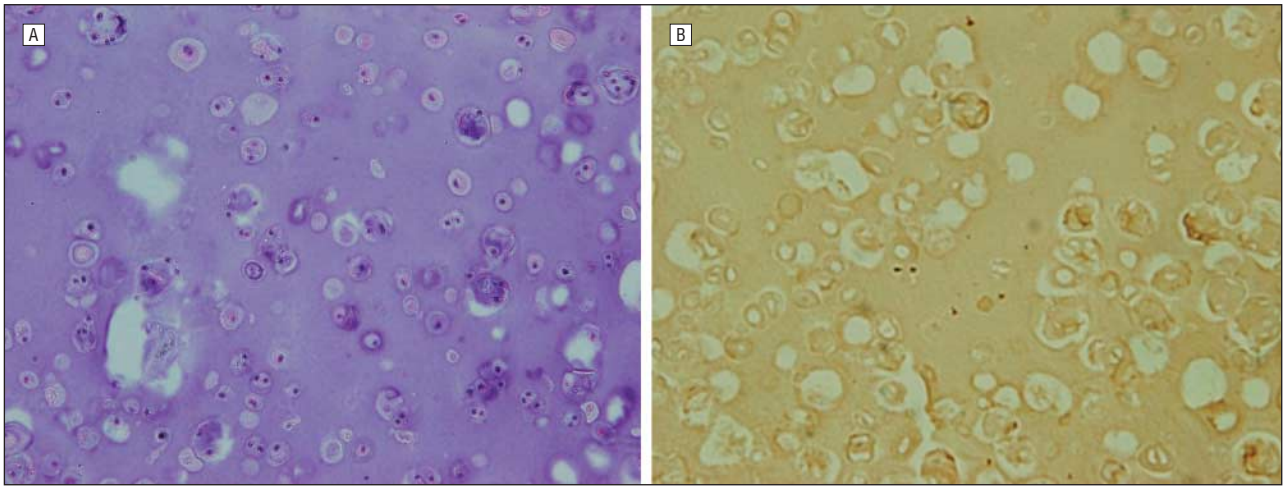

Figure 7. Immunohistochemical stain for type II collagen (original magnification ×10). Hematoxylin-eosin (A) and type II collagen immunohistochemical stain (B) on an alginate-chondrocyte sample harvested at week 26. The extracellular matrix noted on hematoxylin-eosin stains was positive for type II collagen.

lular matrix seen on hematoxylin-eosin staining (**Figure 7**). The alginate controls, mouse skin, and mouse muscle did not stain for type II collagen.

BIOCHEMICAL ANALYSIS

The alginate-chondrocyte samples were grouped as less than 26 weeks and 26 weeks or more for statistical analysis. There was no difference in DNA content between these 2 groups. There was no difference when compared with native septal cartilage as well. The explants harvested before 26 weeks had significantly less hydroxyproline content than native cartilage ($P=.02$). Explants harvested at week 26 or more had a hydroxyproline content that was similar to native cartilage (**Figure 8**). The difference in the hydroxyproline between the 2 experimental groups was not significant ($P=.13$).

The hydroxyproline content for each explant was analyzed and compared with the histological architecture (**Figure 9**). The explants that had histological features similar to native cartilage without evidence of fibrous tissue had a higher hydroxyproline content than explants with mixed histological features that had areas of fibrous tissue growth and decreased cartilage ($P=.02$). The native cartilage samples had higher hydroxyproline content than the explants with mixed histological features as well ($P=.008$). There was no difference in hydroxyproline content between the native cartilage samples and those explants with histological features similar to native cartilage without evidence of fibrous tissue differentiation.

COMMENT

The ideal injectable polymer should be able to be injected and molded in situ and should maintain the shape and size while it develops into engineered cartilage. Two main types of injectable polymers have been used to generate TEC: natural and synthetic. Injectable synthetic polymers include polyethylene oxide hydrogel and pluronic F-127 (polyethylene oxide and polypropylene oxide). In one study using pluronic F-127, the resulting TEC was amorphously shaped, possibly owing to mechanical leakage.[9] Pluronic F-127 was used to successfully produce TEC using autogenous swine chondrocytes injected into a skinfold channel created with sutures.[10] It is not clear, though, how long it took for the suspension to solidify or if it has the ability to form cartilage if it is not injected into a skinfold channel.

Natural polymers that have been used to successfully engineer cartilage include fibrin glue and alginate. Stud-

ies that have used fibrin glue have produced TEC that resembles native cartilage; however, there have been unreliable changes in the size of the resultant engineered cartilage. Studies have found that the injection of swine chondrocytes from various sources of cartilage resulted in an increased or decreased TEC size, depending on types of chondrocytes used.[4,11] In another study using autogenous rabbit chondrocytes, the injection failed to maintain the size and contour over time.[12]

Alginate is a natural anionic polysaccharide that solidifies in the presence of multivalent cations such as Ca^{2+}. Alginate has been used to produce successful TEC in animal models using nonhuman chondrocytes as a direct injection or by injecting into a mold with subsequent implantation.[2,3,5,13,14] These studies resulted in cartilage that resembled native cartilage on gross and histological analysis with stable mass through incubation. Some of these studies examined the seeding density of the chondrocytes and determined that alginate-chondrocyte injections with increased density ($20\text{-}40 \times 10^6$ cells/mL) showed higher-quality cartilage formation.[2,5,13]

Alginate has been used in immunocompetent animal hosts as well. These studies have shown evidence of cartilage formation with no signs of inflammation[14] and biochemical and biomechanical properties similar to that of native cartilage.[3]

One group has published results using alginate for injecting human chondrocytes[15]; however, they were unable to prove that these injections resulted in the formation of TEC. In 2001, Caldamone and Diamond[15] published their results for injecting a human chondrocyte and alginate suspension into the ureters of children with vesicoureteral reflux. They described a 70% (32 of 46 ureters) success rate at 1 year in preventing reflux. Only 4 of the patients had the injected material removed, and the findings from histological analysis showed calcified alginate without evidence of viable chondrocytes. The injections for the other patients were not analyzed. The chondrocytes were cultured for 6 weeks, which increases the likelihood of dedifferentiation and failure to produce TEC.

Taken together, these studies suggest that alginate could be the optimal polymer to produce autologous, injectable cartilage. Alginate has been shown to be an effective injectable scaffold that is biodegradable and nonimmunogenic. To our knowledge, however, there have been no studies that have used human chondrocytes and alginate to produce injectable TEC with subsequent harvest and analysis of all of the injections.

In the present study, we were able to successfully produce engineered cartilage using human chondrocytes and alginate with an injectable model that allowed for in situ molding of the injection. The overall size and shape of the injections in this study remained stable over time. There was some loss of definition of the central depression that occurred over the first days after the injection. We believe that this was due to the inability to prevent displacement of the mold while the mice moved around in their cage. There was minimal change in appearance after the removal of the mold and complete solidification of the gel during the first week. In humans, a mold would be able to be secured during this crucial solidification phase.

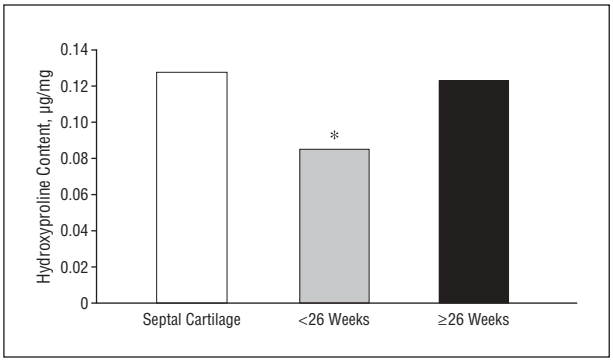

Figure 8. Hydroxyproline content of the alginate-chondrocyte injections grouped into less than 26 weeks and 26 weeks or more. There was a significant difference between native septal cartilage and samples harvested at less than 26 weeks. There was no significant difference between native septal cartilage and samples harvested at 26 weeks or more or between experimental groups. *P=.02.

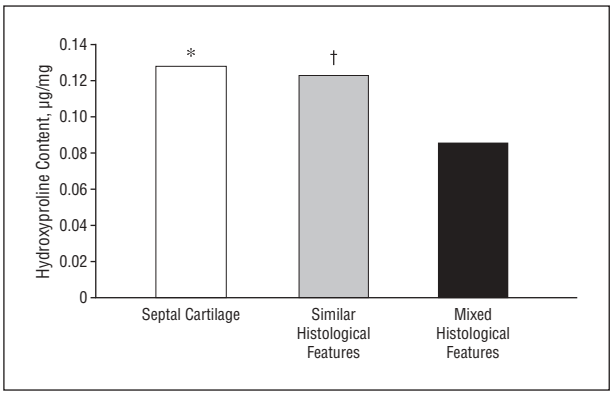

Figure 9. Hydroxyproline content of the alginate-chondrocyte injections based on histological appearance. The samples with mixed histological features had significantly less hydroxyproline than both the native septal cartilage samples and the samples that resembled native septal cartilage. There was no difference between native septal cartilage and the experimental samples that resembled native cartilage. *P=.008. †P=.02.

The mass of the resultant engineered cartilage remained stable through the various incubation time points, up to week 38. On histological analysis, 93% of the samples formed cartilage, and 40% of the explants had histological features similar to native cartilage throughout the sample and had no evidence of fibrous tissue growth. The remaining explants had some areas of fibrous tissue within the sample.

We analyzed the histological appearance based on incubation time to determine if the duration of incubation influenced the differences that were noted on histological analysis (the presence of fibrous differentiation). The histological appearance did not appear to be dependent on the length of incubation because there was 1 explant at each time point that had histological features similar to native cartilage. There were samples with fibrous tissue at all time points as well. On biochemical analysis, there seemed to be a partial relationship between time of incubation and hydroxyproline content. The injections that were harvested at less than 26 weeks had statistically significantly less hydroxyproline than native cartilage. The injections harvested at 26 weeks or more had hydroxyproline content similar to native cartilage. There

was not a statistically significant difference, however, between the 2 experimental groups ($P = .13$). It seems that a strong relationship between time of incubation and successful generation of engineered cartilage would result in more explants that were histologically similar to native cartilage at later time points. One would also expect a significant difference in hydroxyproline between the 2 groups (<26 weeks vs ≥26 weeks).

There was, however, a strong correlation between histological appearance and hydroxyproline content. The samples of native cartilage and the experimental samples with histological features similar to native cartilage without fibrous tissue differentiation both had statistically significantly greater amounts of hydroxyproline than the explants that resembled native cartilage but contained areas of fibrous tissue growth.

It seems that the presence of fibrous tissue in the engineered cartilage influenced collagen content more than time of incubation of the injection. Samples without fibrous tissue growth within the sample had similar appearance and collagen content as native cartilage. The fibrous tissue that was noted in the remaining samples may have been due to fibrous ingrowth from surrounding tissues; however, in many of these samples there was fibrous tissue and fibroblasts within lacunae. The presence of fibroblasts within the lacunae likely represents dedifferentiation of the chondrocytes. The lack of complete replacement of the alginate with neocartilage, due to dedifferentiation of these chondrocytes, may have left vacant areas in the injection that were susceptible to fibrous tissue ingrowth. Thus, the presence of fibrous tissue may be a result of both dedifferentiation and ingrowth.

In this study, we cultured the cells to confluence in order to perform injections with a high concentration of cells (20×10^6 cells/mL) and increase the likelihood of successful formation of engineered cartilage. We did not passage the cells and limited the culture to 10 days with the intention of limiting dedifferentiation of the chondrocytes. As such, relatively large pieces of septal cartilage were required to isolate enough cells to achieve the desired density for injection. This limited the number and size of injections that we were able to perform in this study.

To successfully produce a minimally invasive injectable cartilage to be administered on a human subject, one would require a way to generate a large number of chondrocytes with smaller amounts of cartilage. One would ideally be able to perform a biopsy of cartilage in the clinic and then grow the cells in culture to develop a desirable concentration of chondrocytes for injection. There are promising studies by Chia et al[16,17] that have shown the ability to decrease dedifferentiation of chondrocytes through expansion in culture in alginate beads. The chondrocytes were subsequently recovered from the alginate culture beads and then used to generate engineered cartilage in vitro that resembled native cartilage. Culture techniques such as those described by Chia et al[16,17] may help to provide the necessary concentration of nondifferentiated chondrocytes required to successfully create injectable cartilage from a small piece of harvested native cartilage.

Future studies should attempt to culture human chondrocytes with methods such as those described by Chia et al[16,17] and then recover the chondrocytes with subsequent injection and development of engineered cartilage in vivo as described in this study. This would allow one to use smaller pieces of septal cartilage to produce an injection with a sufficient density of chondrocytes. These culture methods should also reduce the chance of dedifferentiation. Other studies should attempt to perform this type of injection method and molding with autologous chondrocytes in a larger animal, in which the mold could be secured for the first week with limited movement.

CONCLUSIONS

Injectable, autologous cartilage may be the answer to the long search for the ideal implant in facial plastic surgery. Using alginate and human chondrocytes, we were able to produce engineered cartilage using a minimally invasive injectable model that allowed for in situ molding of the injection. The resultant injection remained stable in size and shape for at least 38 weeks of incubation in vivo, with some loss of definition of the molded central depression. We were able to successfully generate cartilage throughout the explant in 93% of the injections. There was some variation in the degree of similarity to native cartilage on histological and biochemical analysis based on the presence or absence of fibrous tissue within the engineered cartilage.

With time and continued refinement of chondrocyte-culturing techniques, the injection technique described in this study may provide a way to develop successful injectable TEC. This would allow a minimally invasive way to augment or recontour craniofacial irregularities in such procedures as medialization laryngoplasty, rhinoplasty (dorsum, tip, and valve), or refinement with microtia repair.

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