Published in final edited form as:

Am J Sports Med. 2010 July ; 38(7): 1324–1333. doi:10.1177/0363546510361950.

THE POTENTIAL OF HUMAN ALLOGENEIC JUVENILE CHONDROCYTES FOR RESTORATION OF ARTICULAR CARTILAGE

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Abstract

Background—Donor site morbidity, limited numbers of cells, loss of phenotype during *ex vivo* expansion, and age-related decline in chondrogenic activity present critical obstacles to the use of autologous chondrocyte implantation for cartilage repair. Chondrocytes from juvenile cadaveric donors may represent an alternative to autologous cells.

Hypothesis/Purpose—The authors hypothesized that juvenile chondrocyte would show stronger and more stable chondrogenic activity than adult cells *in vitro* and that juvenile cells pose little risk of immunologic incompatibility in adult hosts.

Study Design—Controlled laboratory study.

Methods—Cartilage samples were from juvenile (<13 years old) and adult (> 13 years old) donors. The chondrogenic activity of freshly isolated human articular chondrocytes and of expanded cells after monolayer culture was measured by proteoglycan assay, gene expression analysis, and histology. Lymphocyte proliferation assays were used to assess immunogenic activity.

Results—Proteoglycan content in neocartilage produced by juvenile chondrocytes was 100-fold higher than in neocartilage produced by adult cells. Collagen type II and type IX mRNAs in fresh juvenile chondrocytes were 100- and 700-fold higher, respectively, than in adult chondrocytes. The distributions of collagens II and IX were similar in native juvenile cartilage and in neocartilage made by juvenile cells. Juvenile cells grew significantly faster in monolayer cultures than adult cells ($p = 0.002$) and proteoglycan levels produced in agarose culture was significantly higher in juvenile cells than in adult cells after multiple passages ($p < 0.001$). Juvenile chondrocytes did not stimulate lymphocyte proliferation.

Conclusions—These results document a dramatic age related decline in human chondrocyte chondrogenic potential and show that allogeneic juvenile chondrocytes do not stimulate an immunologic response *in vivo*.

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Clinical Relevance—Juvenile human chondrocytes have greater potential to restore articular cartilage than adult cells, and may be transplanted without the fear of rejection, suggesting a new allogeneic approach to restoring articular cartilage in older individuals.

Key Terms

juvenile human chondrocytes; neocartilage; agarose culture; immunogenicity; serial expansion; cartilage repair; chondrocyte transplantation; aging

Introduction

Surgeons and scientists have developed a wide variety of approaches to restoring cartilaginous articular surfaces with the intention of relieving pain and improving mobility for people with traumatic or degenerative damage to their synovial joints. One of the most promising methods has been the use of transplanted autologous cells. However, prospective randomized studies comparing cell transplantation with microfracture (penetration of subchondral bone to stimulate cartilage repair) have not shown that cell transplantation produced better results^{19, 20, 35}. Potential reasons for the lack of improved results with autologous cell transplantation include the limited number of chondrocytes available for cell therapy and the tendency for chondrocytes to undergo phenotypic change during *ex vivo* expansion in monolayer culture^{10, 39}. Furthermore, there is strong clinical and basic scientific evidence that the chondrogenic potential of chondrocytes and chondrogenic stem cells declines with age^{10, 39, 43}.

For these reasons we decided to explore the potential of human juvenile chondrocytes for treatment of articular surface defects. However, an important consideration in utilizing cells derived from allogeneic donor tissue is the potential for immunogenicity. Fresh allogeneic articular cartilage and isolated chondrocytes have been transplanted successfully for two to three decades with a paucity of adverse events related to rejection^{6–8, 16, 32, 34}. The use of fresh osteochondral allograft tissue to repair contained articular defects has successfully delayed the use of prosthetic implants in young active patients^{6, 7, 37}. Although some instances of delayed immunologic rejection associated with transplantation of fresh osteochondral allografts have been reported, it is believed that these reactions are due to incomplete removal of blood/marrow elements from the osseous component and in no way was attributable to cartilage³⁶. In this article we test the hypotheses that juvenile human chondrocytes have significantly greater potential for restoring articular cartilage than chondrocytes from skeletally mature individuals and that juvenile chondrocytes do not stimulate an immunologic response that would compromise their value as a clinically applicable treatment of articular surface defects.

Methods

Chondrocyte Isolation and Neocartilage Production

Articular cartilage was obtained from deceased human donors following informed consent for use in medical research. Knees en bloc were shipped on wet ice in physiologically buffered solution and stored for no more than 120 hours at 4°C before processing. Only regions of healthy cartilage were used, and those regions of tissue displaying grade 1 or 2 osteoarthritis were avoided in older donors.

The methods for chondrocyte isolation and neocartilage production were essentially as described previously.¹ Briefly, whole knees from 65 different donors were processed aseptically in accordance with current Good Tissue Practices to harvest articular cartilage from the proximal femur and distal tibia. Minced cartilage was disaggregated by sequential

enzymatic dissociation in pronase (0.2%), followed by overnight digestion using a mixture of 0.07% collagenase (Worthington, Lakewood, New Jersey) and 0.04% hyaluronidase (Sigma-Aldrich, St Louis, Missouri) in HL-1 chemically defined, serum-free medium (Lonza, Walkersville, Maryland). Of the tissues collected for analysis, 19 were obtained from subjects ranging in age from 13 to 72 years (adult), with the remaining 46 tissues derived from subjects spanning birth to 13 years of age (juvenile). The general distribution of donor tissues was 63% male and 37% female. Neocartilage was produced by seeding 0.5 to 1.0×10^6 nonexpanded chondrocytes per cm^2 in HL-1 medium or a proprietary chemically defined medium displaying growth characteristics similar to HL-1. Cultures were supplemented with $50 \mu\text{g/mL}$ ascorbate after day 3 of culture, with medium exchange occurring every 3 to 4 days. Cultures were maintained at 37°C in a humidified environment consisting of 5% CO_2 . Neocartilage was harvested for characterization and/or used in animal studies between days 44 and 63 of culture.

Population growth and differentiation in culture

Human articular chondrocytes were isolated from 5 juvenile donors (each < 1 year old) and 9 adult donors (14, 22, 29, 42, 52, 65, 67, 75, and 78 years old) by overnight digestion of chopped cartilage in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, California) containing 0.25 mg/mL collagenase type I and 0.25 mg/mL pronase E (Sigma-Aldrich). The cells were counted, plated in culture flasks, and grown for 5 to 10 days before passaging.

Cells isolated from each cartilage sample were serially passaged in monolayer culture for up to 104 days. The cells were allowed to grow to ~80% confluence before trypsinization and replating (1:3 split). Population doublings (PDs) were tracked through time based on cell counts at each passage. The growth rate of each culture was calculated by regression analysis of data collected during the linear phase of the growth (5–20 PDs). The Student *t* test was used to evaluate the significance of the difference between mean growth rates for juvenile and adult chondrocytes.

At various times during serial monolayer culture (indicated in figures), chondrocytes were suspended at a density of 1×10^7 cells/mL in 1% (w:v) low-temperature melting agarose (FMC Bioproducts, Rockland, Maine) in phosphate-buffered saline. The agarose/cell suspensions were cast in 4-mm–diameter cylinders, which were subsequently cut into 3-mm–tall disks using a scalpel. Individual disks were cultured in 48-well plates in DMEM/10% FBS supplemented with insulin (0.1 units/mL) and cortisone ($0.04 \mu\text{g/mL}$) (Sigma-Aldrich). The disks were incubated for 2 weeks at 37°C in an atmosphere consisting of 5% CO_2 , 5% O_2 , and 90% N_2^{26} with medium changes every second day. The disks were cryoembedded, sectioned, and mounted on slides, and stained with safranin O, fast green, and Wiegert's hematoxylin after fixation in 10% neutral-buffered formalin (Fisher Scientific, Indianapolis, Indiana). Safranin O–stained sections were scanned at high resolution (10× objective) using a Prior motorized stage (Prior Scientific, Rockland, Massachusetts) and ImagePro (MediaCybernetics, Bethesda, Maryland) software. The total number of nuclei associated with safranin O staining of the pericellular/territorial matrix were counted using a custom MATLAB-based image analysis program (The MathWorks, Natick, Massachusetts) and the percentage of safranin O–positive cells was determined.²⁷ Two sections from each of 3 disk cultures were analyzed for each treatment group. One-way analysis of variance on ranks with Dunn test for multiple comparisons was used to evaluate the significance of differences among the median values ($P < .001$).

After growing in monolayer culture through multiple passages up to 35 population doublings, chondrocytes from 3 juvenile donors (<1 year old) and 4 adult donors (22, 43, 53, and 75 years old) were suspended in agarose (1×10^7 cells/mL). After 2 weeks, the agarose

cultures were digested in papain and assayed for total sulfated glycosaminoglycans (S-GAGs) using the dimethylmethylene blue assay with chondroitin-6 sulfate (Calbiochem, La Jolla, California) as a standard.¹³ Results are reported as S-GAG content per volume of agarose ($\mu\text{g}/\text{mm}^3$). The difference in mean S-GAG content between juvenile and adult cultures was evaluated over various intervals of monolayer culture times by *t* test.

Biochemical Analysis of Neocartilage

Neocartilage cultures prepared from freshly isolated juvenile and adult articular chondrocytes were submitted for biochemical analysis to compare the rate of matrix synthesis during *in vitro* culture. The S-GAG content of individual grafts was determined *via* colorimetric analysis of papain digested material as described previously.¹³ The DNA content was estimated by fluorometric analysis of the papain digest using Hoechst dye and calf thymus DNA as standard²¹. Samples were assayed in triplicate.

Isolation of RNA and Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis of Gene Expression

Total RNA was extracted from disrupted neocartilage using the FastPrep Instrument and RNeasy Pro Solution (both from Qiagen, Inc., Carlsbad, CA), followed by RNeasy Mini Kit column purification using the DNase/RNase-free kit from Qiagen, Inc. (Valencia, CA). The concentration of RNA was measured with an ultraviolet spectrometer, and 260/280 ratios ranged between 1.8 and 2.1. Primers for PCR were designed using the Beacon Designer 4 program of the Premier Biosoft International software package (Palo Alto, CA) or as supplied by Applied Biosystems (Foster City, CA). Polymerase chain reaction primers and TaqMan-labeled probes used for real-time PCR analysis are presented in Table 1. Genes used for analysis include SOX9, the master regulator of chondrogenesis^{22, 23}, as well as genes encoding the cartilage-specific extracellular matrix proteins, COL2A1 and COL9A1. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used to normalize data. Real-time PCR reactions were carried out using an iCycler iQ instrument (Bio-Rad, Hercules, CA) according to manufacturer's instructions. Reactions were conducted in triplicate to generate a standard curve using RNA-specific primers and TaqMan probes in separate non-multiplexed reactions. Chondrocyte RNA was introduced into the reaction wells at eight concentrations: 20 ng, 10 ng, 5 ng, 2 ng, 1 ng, 0.5 ng, 0.2 ng and 0 ng. The cycling conditions consisted of the following step-wise procedure: Cycle 1, 50°C 20 min (for Reverse Transcription [RT]); Cycle 2, 95°C for 5 min (to destroy RT and Denature cDNA:RNA); Cycle 3, 95°C for 10 sec, then 58°C for 1min 10 sec for 50 repeats (for PCR); and Cycle 4, hold at 4°C. The calculated efficiency of real-time PCR reactions for each of the collagen genes and GAPDH was greater than 95%, while that of SOX9 was no less than 85%. Values of gene expression were estimated relative to the mean of control values obtained for the reactions spiked with the lowest amounts of RNA. From these relative gene expression values, a mean relative expression value was calculated. One-way analysis of variance (ANOVA) was used to determine the statistical significance of age-related differences in gene expression of chondrocytes obtained from juvenile and adult articular cartilage. The criterion for significance was $P < 0.05$.

Histologic Characterization and Immunostaining of Neocartilage

Neocartilage specimens were fixed for 24h in 10% neutral buffered formalin, rinsed three times in phosphate-buffered saline (PBS), dehydrated in a graded series of ethanol and stored in 70% ethanol at 4°C before processing by routine paraffin embedding. 5 μm sections were cut and stained for sulfated glycosaminoglycan content using Safranin-O/fast green. Immunohistochemical staining of collagen type II (Clone CII1, Developmental Studies Hybridoma Bank [DSHB]), type VI (Clone 5C6 [DSHB]) and type IX (Clone D1-9, [DSHB]) was carried out as follows except where noted below: Slides were deparaffinized

in xylene and rehydrated in a graded series of ethanol before treatment with trypsin-EDTA (0.05% HBSS Sigma) at 37°C for 30 min. Slides were then rinsed in TBS and treated for 20 min with 3% H₂O₂ in methanol. After rinsing in TBS, slides were blocked for 1 h in 1% FBS, and primary antibody was applied (1:200) for overnight incubation at room temperature using a humidified chamber. Slides were rinsed, and secondary antibody (1:100 rabbit polyclonal anti-mouse IgG-HRP conjugated; Dako) was applied for 1 hour. Slides were rinsed in TBS and stained 10 min with diaminobenzidine (BD Pharmingen, San Diego, California). Cell nuclei were counterstained with Mayer's acid hemalum (Sigma-Aldrich) and embedded with Gelmount (Biomed, Foster City, CA). Normal human articular cartilage derived from a 10 year-old male was used as a positive control. Staining for collagen type IX was performed using a 1:50 dilution of the primary antibody, and in contrast to native tissues, the unmasking of antigens from tissue engineered neocartilage required 30 minute treatment in pronase E (1 mg/mL in TBS at 22°C) after deparaffinization.

In Vitro Assessment of Lymphocyte Priming to Transplanted Neocartilage

A study evaluating potential immune responses to implanted neocartilage was performed in adult goats (n = 3), with an endpoint at 12 weeks post-implantation. A full-thickness chondral defect, approximately 6 mm in diameter, was created in the medial femoral condyle using a stainless steel coring device and a curet via an open parapatellar approach. Human neocartilage xenografts (n = 3; day 63 of culture in HL-1 complete serum-free medium using P1 cells), derived from a single juvenile donor (male, 7 weeks of age), were affixed to subchondral bone using commercial fibrin sealant in 3 separate animals and the stifle joints were closed in layers. Pre- and post-transplant blood was collected from goats receiving neocartilage xenografts. Lymphocytes were isolated from peripheral blood using histopaque gradients, washed, and cryopreserved. Chondrocytes used to generate the neocartilage for the animal study were cryopreserved (P1 cells, 3.8 PDs) for subsequent use in the mixed lymphocyte/chondrocyte reaction (MLCR). Co-cultures of human chondrocytes and goat lymphocytes were established within 3 months after lymphocyte cryopreservation. The number of viable lymphocytes used as responder cells in MLCR assays was held constant at 2×10^5 per well, and the stimulatory capacity of irradiated chondrocytes was tested at 1×10^5 cells per well. On day 5 of culture, assay plates were pulsed with 1 μ Ci³H-thymidine during the final 18 hours of culture. Cells were then harvested, and radiolabel uptake was assessed by liquid scintillation counting. Raw data were reported as the mean counts per minute $\pm$ standard error of the mean (SEM) of quadruplicate samples. To simplify interpretation, these data were converted to stimulatory index (i.e., fold-increase over background counts generated by responders alone). Human peripheral blood leukocytes (PBLs) derived from healthy donors were used in the MLCR for comparative purposes to demonstrate that antigen presenting cells of human origin could drive proliferation of goat lymphocytes.

Results

Juvenile Articular Cartilage Chondrocytes Demonstrate Superior Biologic Activity as Compared to Adult Chondrocytes

Early in the development of neocartilage, it was observed that traditional culture methods, in particular supplementation of basal medium with 10% fetal bovine serum, markedly reduced the ability of cultured human articular chondrocytes to deposit an insoluble hyaline extracellular matrix *in vitro*.¹ Consequently, a variety of novel defined serum-free media formulations were developed and tested to optimize matrix production in the absence of exogenous growth factors by chondrocytes derived from articular cartilage. Functional studies were performed *in vitro* to measure *de novo* synthesis of cartilage matrix from

chondrocytes that had been isolated from more than 68 cartilage donors of increasing age. Figure 1 illustrates the effect of donor age on chondrocyte matrix production during static culture *in vitro*. When maintained under defined conditions, chondrocytes derived from juvenile articular cartilage were found to be two orders of magnitude more efficient in producing laboratory grown neocartilage than adult articular chondrocytes. An exponential decline in chondrocyte matrix synthesis, as measured by sulfated glycosaminoglycan (S-GAG) content, was observed to occur at pubescence and showed no gender dependence. These results suggested that there may be a fundamental difference in the biologic activity of juvenile and adult articular chondrocytes that is reflected at the molecular level.

Subsequent work aimed at understanding potential mechanisms regulating the age-dependent loss of chondrocyte matrix synthesis suggested that a decline in collagen gene expression may limit the amount of tissue ultimately formed by adult articular chondrocytes during *in vitro* culture. Cartilage is comprised of a collagen framework into which aggregates of aggrecan are entrapped. Because type IX and type II collagen interact to form fibrillar crosslinks to which newly synthesized proteoglycan macromolecules are bound, we examined whether loss of matrix production by adult chondrocytes may be associated with direct changes in the level of gene expression for type II and/or type IX collagen.

Real-time PCR analysis revealed marked differences in mRNA level for each of these genes in freshly isolated chondrocytes from adult as compared to juvenile human articular cartilage. The relative level of gene expression for collagen types II and IX were reduced by a factor of 100-fold and 700-fold, respectively, in adult relative to juvenile donor tissue (Figure 2). Assuming that mRNA levels measured by real-time PCR reflect the amount of protein synthesized *in vitro*, it is plausible that a reduction in collagen gene expression directly affects the ability of adult articular chondrocytes to form neocartilage tissue *in vitro* and to initiate repair *in vivo*. Surprisingly, the mRNA levels of SOX9, considered by many to be the master regulatory gene critical for maintenance of chondrocyte phenotype^{22, 23}, were nearly two-fold higher in adult chondrocytes than juvenile chondrocytes. The ratio of COL2A1/SOX9 was reduced by a factor of 200-fold in adult chondrocytes as compared to juvenile articular chondrocytes, supporting the conclusion that SOX9 mRNA levels do not control type II collagen expression in juvenile and adult articular chondrocytes³.

Juvenile Articular Cartilage Chondrocytes Generate Hyaline Articular Cartilage In Vitro

Neocartilage has been characterized previously, and based on its collagen and glycoprotein content, closely resembles juvenile hyaline cartilage¹. To confirm the hyaline phenotype of neocartilage in the present study, collagen type II, VI and IX was identified by immunohistochemical localization (Figure 3A, 3C and 3E), using native articular cartilage derived from a 10-year-old male donor as positive control (Figure 3B, 3D, 3F). As expected, the extracellular matrix of neocartilage showed rather uniform staining for both type II collagen (Figure 3A) and type IX collagen (Figure 3C). Type VI collagen staining of neocartilage (Figure 3E) appeared to localize to the pericellular matrix surrounding chondrocytes, confirming our earlier observation by transmission electron microscopy of beaded filaments in direct association with the chondrocyte plasma membrane of neocartilage¹.

Growth and differentiation of juvenile and adult chondrocytes

Growth rates were calculated for chondrocyte cultures derived from donors of various ages (<1 year old to 78 years old, mean = 34 years). Linear regression analysis revealed a weak correlation between age and population doublings ($r = 0.58$) (Figure 4). However, the mean growth rate for juvenile cells was significantly greater than for adult cells ($P = 0.002$).

Extracellular matrix production

Safranin-O staining of agarose disk cultures after 2 weeks of incubation revealed age- and passage-related differences in extracellular matrix accumulation (Figure 5A–5D). Quantitative image analysis showed that after 2 passages in monolayer culture nearly all chondrocytes ($89 \pm 6\%$) from juvenile cartilage samples were positive (Figure 5E). After 3 passages there was statistically insignificant reduction in the percentage of positive cells in cultures from juvenile cartilage ($74 \pm 6\%$). In second passaged cultures from adult cartilage there were significantly fewer positively stained chondrocytes ($47 \pm 7\%$) than in cultures from juvenile cartilage. By the third passage the number of positive cells in cultures from adult cartilage declined to $9 \pm 4\%$. This was significantly lower than in juvenile cells at the same passage and a significant reduction relative to adult cells at passage 2. S-GAG assays showed growth-dependent declines in proteoglycan content in both juvenile and adult cells (Figure 6). However, the decline was more dramatic in adult cell cultures than in juvenile cell cultures, which produced significantly more proteoglycan at 5 to 15 PDs ($P=0.030$), at 15 to 25 PDs ($P=0.008$).

Allogeneic Juvenile Chondrocytes and Cell-Mediated Immunogenicity

Because the technology used to generate neocartilage is amenable to large-scale manufacturing and allows treatment of multiple subjects from a single allogeneic donor, it is important to understand the immunological consequences of transplantation of allogeneic neocartilage. A potential technical hurdle that would limit the use of allogeneic neocartilage for tissue repair is the possibility that chondrocytes are immunogenic and may stimulate an anti-graft immune rejection response. Although the immunology of cartilage in general, and chondrocytes in particular, remains incompletely understood, earlier studies utilizing the mixed lymphocyte/chondrocyte reaction (MLCR) suggested that juvenile chondrocytes display unique immunological properties that may minimize graft rejection². Chondrocytes did not stimulate T cell proliferative responses *in vitro*, and they suppressed, in a contact-dependent fashion the proliferation of activated T cells.

To address the question in goats of whether host subjects become sensitized to neocartilage derived chondrocytes after neocartilage transplantation, the proliferative activity of host lymphocytes was measured *in vitro* using goat peripheral blood leukocytes (PBL) as responder cells both pre-transplantation and at 12 weeks post-neocartilage transplantation. As described in the Methods section, the study design utilized xenografts of human origin that were affixed to the central aspect of the medial femoral condyle of adult goats. Incubation of irradiated human chondrocytes either with pre- or post-transplant goat PBLs did not result in PBL proliferation, suggesting that T-lymphocyte priming to human chondrocytes did not occur following neocartilage transplantation (Figure 4). These findings support previous histological data demonstrating the absence of lymphocytic infiltration into neocartilage-treated defects as well as the surrounding synovial tissue^{1,15, 24}. Human PBL were used as a positive control stimulator cell population. In contrast to human chondrocytes, co-incubation of irradiated human PBL with goat PBL resulted in lymphocyte proliferative responses ranging from 7-to 65-fold above background, indicating that goat T cells can be stimulated by xenogenic antigens expressed on human cells. These data further support the concept that transplantation of human neocartilage into goats did not result in the priming of host T cells and suggest that transplanted neocartilage will not elicit T cell mediated host anti-graft immune rejection responses.

Discussion

In terms of extracellular matrix production we found that juvenile-derived chondrocytes significantly outperformed chondrocytes from adult donors in two very different culture

systems (serum *versus* serum-free media, agarose *versus* scaffold-free). Strong matrix accumulation ($>30 \mu\text{g}/\text{mm}$ S-GAG) was still seen in agarose cultures of juvenile-derived cells grown to 15 PD, a stage when S-GAG in adult cell cultures dipped below $25 \mu\text{g}/\text{mm}^3$. These results provide evidence of the strongly enhanced chondrogenic potential of juvenile chondrocytes *versus* adult chondrocytes. Moreover, data from our lymphocyte proliferation assays suggest that juvenile chondrocytes may not be immunogenic in adult hosts, supporting the possibility that such cells may be used as allografts for cartilage regeneration *in vivo*.

The treatment of full-thickness articular cartilage defects in the knee remains a significant clinical challenge. Unlike vascularized tissues, adult articular cartilage displays a limited capacity for repair and once damaged does not heal¹⁷. Furthermore, if the subchondral bone is also damaged, a fibrous repair response emanating from the marrow space results in the formation of a fibrocartilagenous tissue incapable of duplicating the biochemical composition and functional properties of native hyaline cartilage^{9, 20, 35}. Consequently, chondral and osteochondral lesions arising from acute traumatic injury may progress to premature osteoarthritis, particularly in young active subjects²⁵.

Cell-based orthobiologic strategies aimed at replacing cartilage damaged by acute injury or repeated minor trauma may delay the onset and progression of degenerative joint disease, thereby reducing the dependence upon arthroplastic reconstruction and increasing patient quality of life. The development of novel cell-based therapeutic approaches to cartilage repair requires the identification and utilization of cells that can be manipulated *ex vivo* to produce a tissue displaying hyaline characteristics. The resulting implant must retain sufficient synthetic activity to integrate with surrounding host tissues and remodel to form a functional replacement tissue.

Unlike adult osteochondral grafts (of either autologous or allogeneic origin), grafts prepared from juvenile joints were reported to show complete integration of cartilage at the graft/host junction in adult animals³⁷. Comparable findings have been reported in animals with implantation of cell-based constructs prepared using juvenile articular chondrocytes^{4, 30, 31}. In fact, Namba et al. demonstrated that $100\mu\text{m}$ deep lacerations created in the superficial surface of fetal articular cartilage undergo spontaneous healing within twenty eight days after injury, suggesting that juvenile chondrocytes may retain sufficient biologic activity to stimulate integrative repair without scar formation²⁹.

To overcome the limitations of autologous chondrocyte implantation (ACI), currently the only approved cell-based therapy for articular cartilage repair⁴⁰, it may be necessary to harness the biologic activity of juvenile articular chondrocytes. In recent years, an improved understanding of chondrocyte biology has led to the development of novel *in vitro* techniques resulting in reproducible manufacturing of neocartilage allografts, three-dimensional cartilagenous tissues produced independently of bioresorbable scaffolds¹. As shown previously¹ and in the current study, the biochemical composition and morphological features of tissue engineered neocartilage are consistent with those of hyaline articular cartilage derived from juvenile subjects. Comparative studies evaluating the biosynthetic capacity of freshly isolated juvenile and adult chondrocytes revealed that cells derived from juvenile donor tissue were 100-fold more active than their adult counterparts at producing cartilagenous tissue *in vitro*.

To understand at the molecular level why adult and juvenile chondrocytes display marked differences in their ability to produce neocartilage tissue *in vitro*, quantitative TaqMan analysis of a select group of anabolic gene products characteristic of the chondrocyte phenotype was performed. This analysis revealed an inverse correlation between

chondrocyte age and mRNA levels for collagen 2A1 and 9A1 of cells freshly isolated from human articular cartilage. While the levels of mRNA for collagen 2A1 were reduced in adult chondrocytes by a factor of 100 as compared to juvenile chondrocytes, those of type 9A1 were reduced by 700-fold. The potential significance of this observation is underscored by the following rationale. First, collagen comprises 2/3 of the dry weight of articular cartilage, forming a meshwork into which proteoglycan aggregates are entrapped¹². Thus, the observed reduction in collagen mRNA expression appears to correlate with the *in vitro* loss of chondrocyte function for cells harvested from adult articular cartilage. Secondly, experimental evidence strongly suggests that type IX collagen plays an important role in the assembly and maintenance of normal cartilage structure. Transgenic mice with a truncated form²⁸ or complete deletion of the $\alpha 1$ (IX) chain¹⁴ are predisposed to microscopic degenerative changes in cartilage structure suggestive of osteoarthritis. As a prototypical *facit* collagen, type IX collagen is reported to form cross-links both with type II collagen and with itself at a minimum of six different sites¹¹. Finally, it has been shown that type IX collagen interacts with other extracellular matrix proteins such as cartilage oligomeric matrix protein and matrilin-3 and that this interaction is critical to cartilage matrix assembly^{5, 38}. In light of the observed 100–700-fold reduction in collagen gene expression, our findings provide new evidence that adult chondrocytes may not provide an ideal source of chondrogenic cells capable of restoring cartilage damage arising from traumatic injury or disease. Surprisingly, SOX9 gene expression by chondrocytes from adult articular cartilage was nearly 2-fold greater than that measured for juvenile cartilage. Although this transcription factor is believed to control chondrocyte matrix synthesis, the reduced level measured for juvenile human articular cartilage did not appear to be predictive of enhanced chondrogenic potential. We speculate that this observation may be attributed to cells undergoing mitogenesis as these tissues were harvested from individuals younger than 2 years of age.

To the best of our knowledge this is the first report correlating marked differences in chondrocyte functional activity to gene expression for human articular chondrocytes harvested from juvenile and adult articular cartilage. This age-dependent decrease in chondrocyte type IX collagen mRNA levels is reminiscent of the changes in gene expression reported to occur following serial monolayer culture of bovine chondrocytes and may be a harbinger of the differentiation state of articular chondrocytes⁴². From the data presented, it is clear that juvenile articular chondrocytes offer a significant biologic advantage over cell therapies comprising adult articular chondrocytes. Furthermore, use of juvenile articular chondrocytes derived from allogeneic cadavers would eliminate donor site morbidity and the requirement for two surgical procedures associated with therapeutic application of autologous chondrocyte implantation.

To address the immunological consequence of transplanting non-autologous chondrocytes on the host immune response, *in vitro* assessment of lymphocyte proliferation was assessed using lymphocytes recovered from goats. Our findings indicate that, unlike professional antigen presenting cells, chondrocytes do not stimulate proliferation of allogeneic or xenogeneic lymphocytes². These observations are consistent with those of Jobanputra et al. who demonstrated that chondrocytes fail to evoke lymphocyte alloproliferation even after induction of class II antigens with cytokine treatment¹⁸.

In conclusion, allogeneic chondrocytes derived from juvenile articular cartilage may represent an improved source of cells for cartilage repair. These cells are amenable to large-scale manufacturing and display an inherent enhanced ability to synthesize cartilage matrix when compared to adult chondrocytes. Finally, the *in vivo* immunogenicity studies described here suggest that allogeneic juvenile chondrocytes, when transplanted with neocartilage, will not promote graft rejection immune responses in unrelated recipients.

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Figure 1.

Chondrocytes from juvenile human articular cartilage (<10 years) show significantly greater matrix synthesis potential than chondrocytes isolated from skeletally mature individuals. Neocartilage was grown under defined serum-free conditions as described previously.²⁸ Matrix proteoglycan content of newly synthesized neocartilage was measured colorimetrically using dimethylmethylene blue after papain digestion of day 44 to 60 grafts. Data are expressed as the average daily amount of proteoglycan deposited in the tissue matrix after normalization to DNA content. The rate of total sulfated glycosaminoglycan (S-GAG) synthesis for juvenile chondrocytes was more than 100-fold greater than that of adult chondrocytes. For reasons that remain unclear, this potent biologic activity appears to decrease with age and may be lost at pubescence.

Figure 2.

Real-time polymerase chain reaction analysis demonstrates 100-fold and 700-fold greater amounts of mRNA for type II (A) and type IX (B) collagen, respectively, in freshly isolated chondrocytes derived from juvenile versus adult human articular cartilage. Gene expression profile analysis of chondrocyte-specific genes was performed as described in the Methods section using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for data normalization. A marked reduction in collagen gene expression of adult chondrocytes was observed independent of SOX9 expression (C), the master chondrogenic transcription factor of cartilage, which was expressed at approximately the same order of magnitude in juvenile and adult chondrocytes (approximately 83% greater in adult). The ratio of COL2A1/SOX9 mRNA (D) was reduced 200-fold in adult chondrocytes as compared with juvenile articular chondrocytes (n = 5 for juvenile chondrocytes, mean age = 5.6 months, range = 1 day-19 months; n = 3 for adult chondrocytes, mean age = 50.7 years, range = 35–70 years).

Figure 3.

Immunohistochemical staining of neocartilage collagen. Formalin-fixed paraffin-embedded sections of human neocartilage (A, C, and E) and native human articular cartilage (B, D, and F) were stained as described in the Methods section for collagen type II (A and B), collagen type IX (C and D), and collagen type VI (E and F). Uniform staining of neocartilage extracellular matrix was observed for collagen types II and IX, whereas weaker staining for type VI collagen demonstrated pericellular localization, similar to what was observed for native articular cartilage obtained from a 10-year-old individual. Original magnification 100 $\times$.

Figure 4.

Chondrocyte growth rate. A, growth rate (population doublings [PDs] per day) was calculated using cell count data from serially passaged monolayer cultures of chondrocytes from donors of various ages (<1 year old to 78 years old, mean = 34 years). B, the columns show the mean growth rates for chondrocytes derived from juvenile (n = 5) and adult donors (n = 9). Error bars indicate standard deviations. The growth rate for juvenile cells was significantly greater than adult cells ($P = .002$).

Figure 5.

Extracellular matrix accumulation in agarose cultures. Representative areas of sections of agarose-cultured cells derived from juvenile (A and B) or adult donors (C and D) are shown after 2 passages or 3 to 5 population doublings (A and C) or 3 passages or 5 or 6 population doublings (B and D). The black bar in D is 125 μm in length. Percentage of safranin O positive cells were counted microscopically as described in the Methods section for cultures of adult chondrocytes (stippled columns) and juvenile chondrocytes (solid gray columns) at the indicated passages (E). Lines and asterisks above the columns indicate statistically significant differences ($P < .001$). Error bars represent standard deviations.

Figure 6.

Effect of population growth on proteoglycan production. Total sulfated glycosaminoglycan (S-GAG) content was measured in agarose cultures initiated after up to 35 population doublings in monolayer culture. Data are shown for 3 juvenile cell populations and 4 adult cell populations. Error bars show standard deviations of replicate S-GAG assay measurements for each sample (n = 6).

Figure 7.

Mixed lymphocyte/chondrocyte reaction (MLCR) culture analysis for potential priming of host lymphocytes after human neocartilage transplantation demonstrates a lack of sensitization 12 weeks after implantation. Cell-based immunity against the implanted grafts was screened 12 weeks after implantation using the MLCR. Goat lymphocytes collected before and after implantation were used as responder cells for analysis as described in the Methods section. No evidence of lymphocyte priming by the implanted neocartilage-derived chondrocytes was observed, despite the fact that goat lymphocytes displayed potent proliferative responses to human peripheral blood leukocytes (PBLs) used as the positive control (ranging from 7-fold to 65-fold above background).

TABLE 1

Primer Sequences Used For Real-Time Polymerase Chain Reaction Analysis

Gene	Gen Bank Reference	TaqMan Probe Sequence (5' to 3')	Sense Primer	Anti-Sense Primer
GAPDH	NM_002046	5' VIC labeled 3' MGB labeled, exon 3 probe proprietary sequence; amplifies only mRNA; primers in separate exons	Probe/primer combination	ABI # 4326317E
COL2A1	NM_033150.1	5' FAM labeled, 3' BHQ-1 labeled; CCCTGAG TGGAAGAGTGGAGACTACTGGAT; probe spans exon boundary; primers in separate exons	GAACCCTGC TCGCACCTG	GACGCAAGTC TCGCCAGTCT
COL9A1	BC015409	5' FAM labeled, 3' BHQ-1 labeled; CCAACTAGGAA TTTATATCCCAGTGGACTGCCTG; probe spans exon;exon junction and primers in separate exons	CTTACAAGTT GGGAAATAAT GTAGACTTC	CTGAATCTGCC AAATGTTCCAGTTC
SOX-9	Z46629	5' HEX labeled, 3' BHQ-1 labeled; AGCACTCGGG GCAATCCCAGGGC; probe spans exon boundary; primers in separate exons	CACTCCTCCT CCGGCATGAG	CCCCTCTCGCT TCAGGTCAG