Original Article

Chondrocyte source for cartilage regeneration in an immature animal: Is iliac apophysis a good alternative?

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ABSTRACT

Background: Autologous articular cartilage at present forms the main source of chondrocytes for cartilage tissue engineering. In children, iliac apophysis is a rich and readily accessible source of chondrocytes. This study compares the growth characteristics and phenotype maintenance of goat iliac apophysis growth plate chondrocytes with those sourced from goat articular cartilage, and thereby assesses their suitability for autologous chondrocyte transplantation in immature animals for growth plate and articular cartilage regeneration.

Materials and Methods: Four sets of experiments were carried out. Cartilage samples were harvested under aseptic conditions from goat iliac apophysis and knee articular cartilage. The chondrocytes were isolated in each set and viable cells were counted and subsequently cultured as a monolayer in tissue culture flasks containing chondrogenic media at 2.5×10^3 cells/cm^2. The growth was periodically assessed with phase contrast microcopy and the cells were harvested on 8th and 15th days for morphology, cell yield, and phenotype assessment. Student's *t*-test was used for comparison of the means.

Results: Confluence was reached in the iliac apophysis growth plate chondrocytes flasks on the 10th day and the articular cartilage chondrocytes flasks on the 14th day. Mean cell count of growth plate chondrocytes on the 8th day was 3.64×10^5 (SD = 0.601) and that of articular cartilage chondrocytes was 1.40×10^5 (SD = 0.758) per flask. The difference in the means was statistically significant ($P = 0.003$). On the 15th day, the mean cell number had increased to 1.35×10^6 (SD = 0.20) and 1.19×10^6 (SD = 0.064) per flask, respectively. This difference was not statistically significant ($P = 0.26$). The population doubling time on the 8th day of cell culture was 3.18 and 6.24 days respectively, for iliac apophyseal and articular cartilage chondrocytes, which was altered to 3.59 and 3.1 days, respectively, on the 15th day. The immunocytochemistry showed 100% retention of collagen 2 positive and collagen 1 negative cells in both sets of cultures in all samples.

Conclusion: Iliac apophysis is a rich source of chondrocytes with a high growth rate and ability to retain phenotype when compared to articular cartilage derived chondrocytes. Further *in vivo* studies may determine the efficacy of physeal and articular repair in children with apophyseal chondrocytes.

Key words: Articular cartilage, collagen 1, collagen 2, growth plate chondrocyte, immunocytochemistry, physeal bar, physis

Introduction

Cartilage tissue engineering in humans for focal articular cartilage defect is a standard therapy offered in many centers in the world.[1-4] The sources of cells for this are mainly articular cartilage and autologous mesenchymal stem cells.[4] Chondrocytes are the logical cells to be used as a source for cartilage tissue engineering. These are obtained in the adults from the non weight bearing articular cartilage of the joints by an arthroscopic procedure at the time of diagnosing joint pathology.[1] When considering articular cartilage or growth plate tissue engineering in a child, iliac crest apophysis forms a rich and readily accessible source of chondrocytes.[5] The growth and culture characteristics of chondrocytes isolated from the Iliac apophysis have not been extensively studied. Additionally, its efficacy as a rich and proliferative source of chondrocytes in monolayer cultures has not been compared to the usual source of autologous chondrocytes, i.e. articular cartilage.

The aim of this study was to compare the growth characteristics and phenotype maintenance of chondrocytes sourced from articular cartilage with those sourced from iliac apophysis. This could in turn form the basic groundwork for future studies aimed at cartilage regeneration using chondrocytes isolated from iliac apophysis. The beneficiaries

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of this would be children with cartilage defects in the articular surfaces and growth plate.[6,7]

Materials and Methods

This study was carried out at the basic science laboratory as part of the preliminary work leading to development of use of iliac crest chondrocytes for growth plate regeneration in goats. This was an Institutional Review Board approved study funded by the Department of Biotechnology, Government of India. Four paired sets of experiments were designed, comparing iliac crest derived chondrocytes with articular cartilage derived cells from freshly slaughtered goats.

Technique

Goat legs and hindquarters (taken from freshly slaughtered goats) were collected from the slaughter house. In the laboratory, outer skin from the goat leg and the iliac bone was removed using scalpel, and the leg and iliac bone were wrapped in sterile tissue paper and soaked in 70% ethanol for 1 hour. Under sterile condition, cartilage samples from iliac apophysis and articular surface of the knee were dissected out. Perichondrium and other surrounding tissues were removed from the cartilage by sharp dissection using a knife. The cartilage specimens were transferred to the Dulbecco's modified Eagle Media/Hams f12 (DMEM/F12; Sigma Aldrich, St. Louis, MO, USA). All cartilage sampling was processed within 4 hours of slaughter.

Chondrocyte isolation

The cells were isolated from the cartilage by mincing into small pieces and transferred into the DMEM/F12 media containing 2 mg/ml of collagenase type II (Worthington, Lakewood, NJ, USA). The tubes were incubated in a 37°C water bath shaker overnight. After incubation, the collagenase action was diluted by adding 20 ml of DMEM/F12 media into 10 ml of sample and the digested cartilage sample filtered through a 100 µm cell strainer (BD Biosciences, San Jose, CA, USA) and centrifuged at 2400 rpm for 10 minutes at 25°C. The supernatant was discarded and the cells were resuspended with 5 ml of media and centrifuged. The pellet was again suspended in media and then 100 μl of cell suspension mixed with Trypan Blue was placed in a hemocytometer and viable cells were counted to arrive at the cell yield.[8]

Cells were then cultured as a monolayer in T-25 cm^2 flask containing DMEM/F12 medium at 2.5×10^3 cells/ cm^2 density. Culture flasks were incubated for 15 minutes in a CO_2 incubator, and then 10% fetal bovine serum (Sigma) and 62 μg/ml ascorbic acid (Sigma) were added. Monolayer cultured chondrocytes were harvested on 8th and 15th days for the assessment of morphology, phenotype by immunocytochemistry [Figure 1a–f], and population doubling time (PDT). Phase-contrast microscopy was done to assess cell growth, morphology, and time to confluence [Figure 2 a-d].

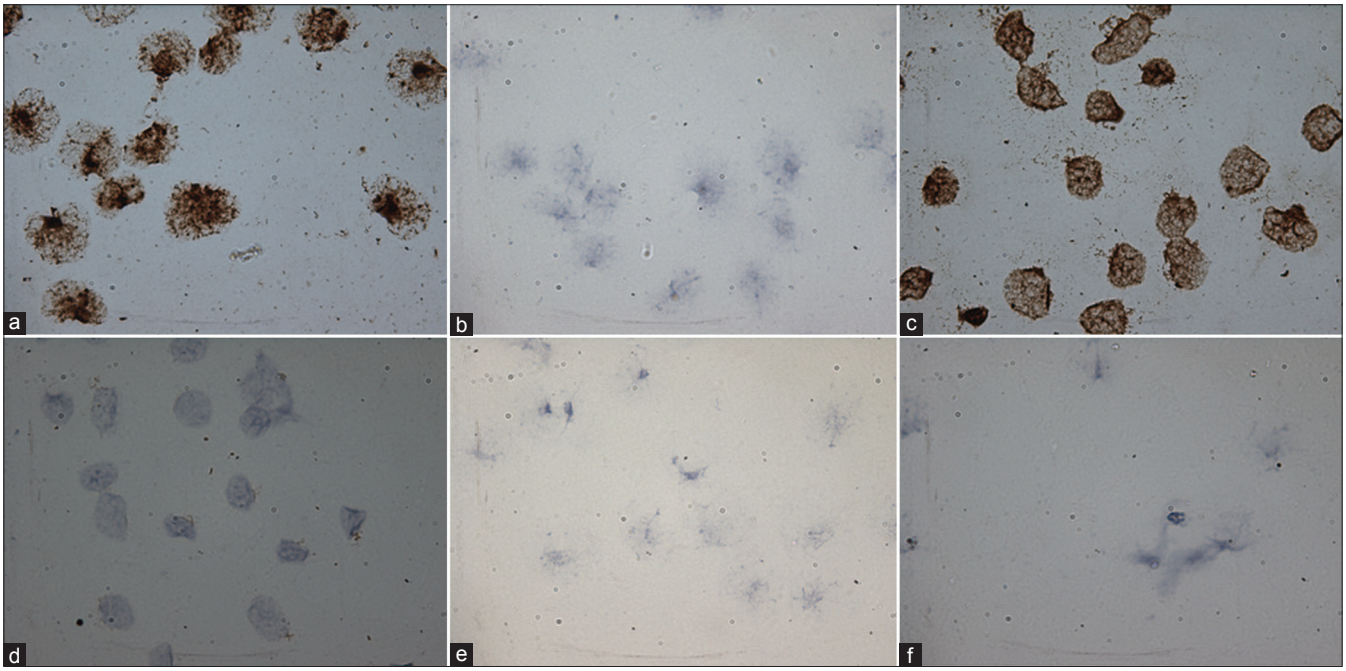

Figure 1: (a–f) Photomicrograph of growth plate and articular chondrocytes from 15th day culture. Immunocytochemistry showing growth plate and articular cartilage derived and expanded cells to be collagen II positive and collagen I negative, expressing the chondrocyte phenotype. Collagen I and II stains were developed by the horseradish peroxidase-conjugated secondary antibody that produced brown color. Cells were counterstained with H and E (magnification, 40×). Growth plate chondrocyte showing (a) 100% collagen II positive and (b) 100% collagen I negative cells. Articular chondrocytes showing (c) 100% collagen II positive and (d) 100% collagen I negative cells. Control for immunocytochemistry using growth plate chondrocyte without adding primary antibody (e) negative control for collagen II and (f) negative control for collagen I

PDT can be calculated given two measurements of a growing quantity, q_1 at time t_1 and q_2 at time t_2, and assuming a constant growth rate, the doubling time is calculated as:

$$PDT = (t_2 - t_1) \times \log (2 - q_2/q_1)$$

The PDT was determined by using an online calculating software.[9] Iliac and articular chondrocytes phenotype on 8th and 15th days was assessed by immunocytochemistry using anti-collagen type I and II antibodies following the protocol by Marlovits *et al.*[10]

The statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software ver. 16. Student's *t*-test was used to test the significance of difference of means.

Results

Of the four sets of cultures done, the articular cartilage culture from the second set of cultures was found to be contaminated after the 8th day and had to be discarded, and hence the 15th day results for all the criteria for this were not available for analysis.

Confluence was reached in the growth plate chondrocyte flasks on the 10thday [Figure 2a–d]. In the articular cartilage chondrocyte flasks, this was seen on the 14th day.

Each flask was plated with 62,500 cells. At the end of 8th day, the mean number of cells in the iliac crest growth plate chondrocyte flask was 3.64×10^5 (n=4, SD=0.601) and in the articular cartilage chondrocytes was 1.71×10^5 (n=4, SD=0.758). On the 15th day, the mean number of cells had gone up to 1.35×10^6 (n=3, SD=0.20) for growth plate flask and 1.19×10^6 (n=3, SD=0.064) for articular chondrocyte flask [Table 1]. This difference was not statistically significant (P=0.26) using Student's *t*-test. Cell yield and viability of the harvested cells were assessed by dye exclusion test using hemocytometer. Cell suspension was mixed with equal volume of (0.4%) Trypan Blue dye. 10 μl of this suspension was loaded on both sides of hemocytometer using a fine pipette. Viable cells were counted and the cell yield was determined using the following formula:

Cells/ml = (Number of viable cells) / (Number of squares counted × dilution factor x 10^4)

Total cell yield = (Cells/ml) × (Total volume of cell suspension)

Based on the 8th day cell culture counts, the mean doubling time for growth plate chondrocytes was 3.18 days (SD=0.317) and for articular cartilage chondrocytes was 6.24 days (SD=2.27). The difference in means was significant (P=0.043).

The mean doubling time for the growth plate cartilage was 3.59 days (SD=0.306) and for articular cartilage chondrocyte was 3.1 days (SD=0.219) when calculated based on 15 days of culture. The difference in means was significant for 95% confidence intervals (P=0.095), but significant within 90% confidence intervals.

The immunocytochemistry was done on all samples except for articular cartilage sample of set 2. The tests done on day 0, 8, and 15 were positive for collagen II and negative for collagen I, showing that cells in both groups maintained their phenotype [Figure 1a–f]. Articular cartilage sample of set 2 was discarded because of contamination.

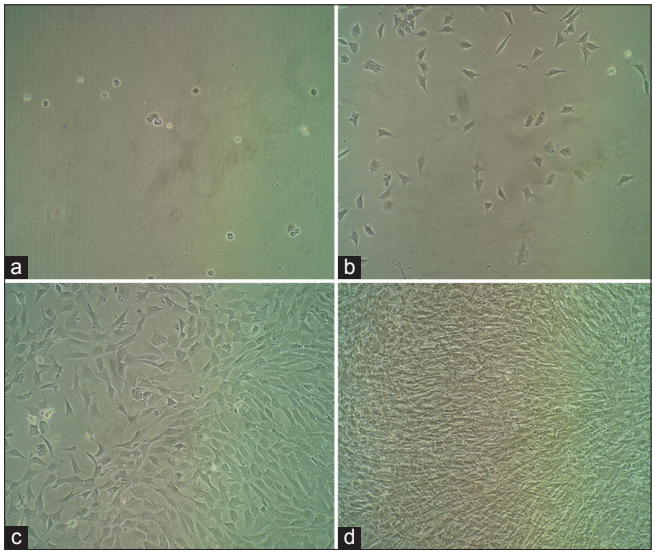

Figure 2: (a–d) Phase-contrast microscopic images show morphology of growth plate chondrocytes on (a) day 1, (b) day 3, (c) day 7, (d) day 10 when they reach confluence. Note the change of morphology from spherical/polygonal to elongated fibroblast-like phenotype due to adherence to the flask (magnification, 10×);

Discussion

The source of chondrocytes for articular cartilage regeneration has been extensively investigated in the last two decades.[4,11,12] In the initial stages of autologous

Table 1: Number of chondrocytes harvested on 8th and 15th days from a seeding density of 6.25 × 10⁴ per T-25 cm flask

	Day 8 culture		Day 15 culture	
	Iliac apophysis	Articular	Iliac apophysis	Articular
Set 1	3.04×10^5	1.18×10^5	1.15×10^6	1.24×10^6
Set 2	3.46×10^5	5×10^4	Not done, contamination	
Set 3	3.6×10^5	1.64×10^5	1.56×10^6	1.22×10^6
Set 4	4.47×10^5	2.3×10^5	1.35×10^6	1.12×10^6

chondrocyte transplant for focal chondral defects, the source of chondrocytes was the non weight bearing cartilage harvested from the affected joint expanded *in vitro* and transplanted as a monolayer.[1] In the recent past, there have been two major directions in research. One of these is to explore the role of scaffolds in maintaining the phenotype.[1] The second one is to find an alternative source of chondrocytes because of the diminished growth potential and availability in the adult human.[4,11,12] The chondrogenic differentiation of mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord has been explored.[4,11,12] One of the major issues in chondrocyte expansion is the quality of the chondrocytes grown *in vitro*, mainly its ability to retain its phenotype in culture, if hyaline cartilage regeneration is to be achieved.[4,11-13] There is a good source of autogenous chondrocyte in the iliac apophyseal growth plate in children, which can be potentially used for treating growth plate abnormality and articular cartilage defects.[14] In this study, we focused on the growth rates of the harvested cells as evidenced by their doubling time and time to reach confluence when seeded in a uniform manner. Our results showed that the growth rate of the cells from iliac apophysis was much higher as compared to the articular cartilage chondrocytes, as demonstrated by the significantly higher total number of cells on the 8th day culture, a shorter doubling time on the 8th day, and earlier confluence in each flask (10th vs. 14th day). This implies that iliac apophysis chondrocytes divide much faster than articular cartilage chondrocytes. We posit the paradoxical slowing of growth rate on 15th day of iliac apophyseal chondrocytes to the fact that the confluence was reached much earlier in the iliac apophyseal chondrocytes, and thus, due to factors like contact inhibition, space, and nutrition limitation, there was slowing of expansion in their number.[15] We are unable to find a similar study in the literature which has compared the growth rates of chondrocytes derived from different sources. The previous studies comparing chondrocytes from growth plate with those from articular cartilage have focused on the expression of markers to distinguish chondrocytes from articular cartilage from those derived from the growth plate.

The second aim was to study the ability to retain morphology and phenotype of the chondrocytes, which determines their ability to form hyaline cartilage when transplanted. The phenotypical assessment done using markers for collagen I and collagen II on the 8th and 15th days showed maintenance of chondrocyte phenotype, proving growth plate to be satisfactory in this respect with suitability for transplantation up to 15th day. The collagen II expression as compared to collagen I expression in *in vitro* culture is time dependent and has been shown to decrease from peak values of 215- to 430-fold higher in the first week of human articular chondrocyte culture and slowly decreases from day 10 onward.[10] The changes toward collagen I expression are associated with a more fibroblastic phenotype which is not suitable for transplantation as it forms fibrocartilage *in vivo*. While we have not done any quantitative assay, the comparable retention of 100% of the cells as collagen I negative and collagen II positive in growth plate cultures up to 15 days suggests that they are suitable for transplantation. Another study has shown that porcine growth plate chondrocytes show comparable phenotype maintenance in the first week of monolayer culture, but show progressively increased collagen I expression on continued culture, similar to articular chondrocytes.[16]

There are a number of limitations in this study. Growth and doubling time of chondrocyte would have been better assessed by using more number of time points.[8] Once confluence is achieved, it becomes more difficult to assess the number of cells in the culture because of the voluminous extracellular matrix produced by the chondrocytes. This needs to be digested and cells need to be released without lysis for accurate cell counts.

One of the important features of growth plate chondrocyte is its ability to undergo hypertrophy and cell death as an initial step toward endochondral ossification.[17] These differences can be best investigated by looking for hypertrophy markers such as collagen X, parathyroid hormone receptor, and Indian hedgehog.[18] While hypertrophy is desirable in the growth plate reconstitution, this feature is not advantageous when undertaking articular cartilage regeneration. These hypertrophy markers were not tested in this experiment as they were outside the scope of this study. However, literature shows that collagen X, a hypertrophy marker, is expressed in higher quantity in the growth plate chondrocyte as compared to articular chondrocyte in monolayer and 3-D culture.[16] There is also evidence to suggest that *in vitro* growth plate derived tissue engineered cartilage in 3-D pellet culture gradually loses hypertrophy markers and expresses markers which are common with articular cartilage.[18] More *in vivo* studies would also be warranted to understand the behavior of the growth plate chondrocytes when subjected to normal mechanical stresses of the joint and studied for articular replacement therapy.

Conclusion

Iliac apophysis is a rich source of chondrocytes with a very high growth rate and comparable ability to retain the phenotype. We posit that this is suitable as a chondrocyte source for growth plate regeneration.

This has clinical use in children and adolescents, who have an immature iliac apophysis, as a source of autologous chondrocytes for treatment of articular cartilage defects

such as osteochondritis dissecans and traumatic osteochondral defects, and repairing and replacing physeal defects following infection and trauma. We expect the harvesting procedure, if eventually translated, to be simpler and less morbid when compared to arthroscopic harvesting from articular cartilage for autologous cartilage transplantation.

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