Resurfacing of articular cartilage explants with genetically-modified human chondrocytes in vitro

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Summary

Objective: We are attempting to genetically-modify chondrocytes transplanted to cartilage in vitro as a prelude to gene therapy trials in patients with osteoarthritis.

Design: With human cartilage and chondrocytes, we have explored the duration of binding of chondrocytes to cartilage in vitro and the expression of the β-galactosidase gene introduced into the chondrocytes through infection with an adenoviral vector both before and after transplant of the chondrocytes to cartilage.

Results: Transplanted chondrocytes continued to bind to cartilage explants at 45 days in our longest trial. We could successfully infect chondrocytes with adenovirus at least 35 days after we transplanted the chondrocytes to cartilage. Expression of the β-galactosidase gene continued throughout the duration of each trial.

Conclusions: These results raise the possibility of repairing and rebuilding cartilage by resurfacing the cartilage with genetically modified chondrocytes. The ability to infect chondrocytes well after transplant raises the possibility of repeated infections of surface chondrocytes as an alternative to repeated injections of chondrocytes into the joint space.

Key words: Cartilage, Chondrocytes, β-galactosidase, Adenovirus.

Introduction

Osteoarthritis (OA) is the most prevalent chronic disorder affecting humans. This disease places a huge economic burden on society and often has severe adverse consequences for individual patients. The incidence of OA steadily escalates with advancing age to become the most prevalent disease adversely affecting quality of life.

Despite the medical and economic consequences of OA, therapies that can reverse the degenerative changes that occur in articular cartilage and restore cartilage to normal have not been forthcoming. At present, all forms of treatment including medical, physical and surgical approaches are focused on treating the symptoms of the disease rather than on retarding or reversing the OA disease process itself.

In the past decade there have been tremendous advances in our understanding of the basic pathologic processes that contribute to degeneration of articular cartilage. In healthy adult cartilage chondrocytes maintain an equilibrium of matrix synthesis and breakdown [1, 2]. Injury to cartilage initiates a specific reparative process that increases the production of proteoglycans and collagen [2–9]. The absence of progressive breakdown results from a compensatory anabolic response by chondrocytes to maintain normal amounts of extracellular matrix despite increased activities of matrix degrading enzymes [2, 8]. In OA, the equilibrium between synthesis and loss of matrix macromolecules is eventually lost in favour of net catabolism and cartilage degeneration ensues.

With improved understanding of these fundamental mechanisms, there has been increasing interest in evolving new therapeutic approaches that can repair or regenerate cartilage. Potential therapeutic strategies for 'healing'
cartilage include decreasing chondrocytic catabolic activity to prevent degeneration of the constituent components of cartilage or alternatively increasing the anabolic activity of chondrocytes to promote cartilage repair. In promoting anabolic activity, the production of proteoglycans and collagen by native chondrocytes can be increased. An alternative or complementary strategy may be the introduction of additional chondrocytes. In the absence of these types of intervention, proteoglycan and collagen synthesis continue to rise in response to increased cartilage catabolism until the OA disease process is far advanced and proteoglycan and collagen synthesis fall off sharply.

The purpose of the present paper is to explore the potential for combining these various strategies in a multi-faceted attempt to heal articular cartilage. To achieve this we combined gene therapy approaches with cartilage transplantation. The cartilage transplantation allows for an increased population of chondrocytes that can contribute to the healing of injured articular cartilage. The introduction of gene therapy may be used to either diminish catabolic activity or increase anabolic activity of both native chondrocytes and transplanted chondrocytes. We believe that combining these two approaches enhances the potential to repair and resurface damaged articular cartilage.

**Methods**

**Cartilage Organ Culture and Cultures of Human Chondrocytes**

Cartilage is taken from various patients with osteoarthritis at the time of total knee arthroplasty. The slices were washed in Gey’s balanced salt solution and placed one slice per well in 24-well flat bottom tissue culture plates. Organ cultures are maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS) and the antibiotics penicillin, streptomycin, and ciprofloxacin.

Cartilage is minced finely in Gey’s balanced salt solution and the chondrocytes released from cartilage by sequential digestion at 37°C for 1 h with 2 mg/ml pronase (Boer Mann) followed by 1 mg/ml collagenase (Sigma) at 37°C overnight on a gyratory shaker. Isolated chondrocytes are centrifuged and washed three times in PBS (500 g, 5 min, 20°C). The cells are seeded at 2×10^5 cells/ml in DMEM containing 10% FCS and the antibiotics penicillin, streptomycin, and ciprofloxacin.

**Transplantation with Infected Chondrocytes**

Chondrocytes are seeded at 10^5 cells/well in six-well plates in DMEM containing 10% FCS and antibiotics. When cells are 70% confluent, they are washed twice with serum free DMEM and infected with AdlacZ [10] diluted in serum free DMEM. After 2 h at 37°C the cells are washed and cultured in complete medium overnight. The following day the chondrocytes are removed from the plate with trypsin, placed into complete medium and added slowly onto the articular surface of the cartilage organ cultures. One-half to one million cells are added to each 1 cm square piece of cartilage.

AdlacZ was provided by Dr Jeffrey M. Leiden at the University of Chicago [10]. AdlacZ is an E1 and E3-deleted replication defective adenoviral vector [10]. AdlacZ contains the β-galactosidase gene driven by the β-actin promoter and the cytomegalovirus enhancer [10].

**Transplantation with Chondrocytes Before Infection**

Uninfected chondrocytes are transplanted to cartilage organ culture (as above) and subsequently infected with AdlacZ diluted in serum free DMEM at a moi of 1000 pfu/cell. The infection of the transplanted chondrocytes was delayed for varying intervals of time as noted in the text. Two hours after infection the organ culture is washed and put into complete medium.

**Detection of β-Galactosidase Expression**

Chondrocytes or cartilage organ cultures were washed three times in PBS and fixed for 5 min at 4°C with 2% formaldehyde and 2% glutaraldehyde in PBS. Samples were washed in PBS and stained with 0.5 mg/ml bluo-gal in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgCl_2_. Samples were incubated in the staining solution for 14-24 h, and washed twice with PBS before being examined by light microscopy. Bluo-gal (halogenated indolyl-b-d-galactoside) is a histochemical substrate for β-galactosidase that produces a dark blue precipitate when hydrolyzed by β-galactosidase.
Cultures in Alginate Beads

Alginate (Keltone LVCR, Monsanto) is a negatively charged unsulfated copolymer of l-guluronan and d-mannuronan that polymerizes to form a gel in the presence of calcium ions or other multivalent counterions [11]. We prepare 1.2% alginate in 150 mM NaCl with agitation. The solution is heated to 50°C to reduce viscosity, filtered through a 0.45 μm filter to sterilize and cooled back to RT. The cells are encapsulated into alginate beads in 24-well plates at nine beads per well and 44,000 cells per bead. The chondrocytes are suspended in the sterile, filtered low viscosity alginate solution (1.2% in 150 mM NaCl), then slowly in a dropwise fashion through a 22 gauge needle dropped into excess 102 mM CaCl2 solution in 24-well plates. The beads are allowed to gel for 10 min. The beads are washed four times in 0.15 mM NaCl and once in DMEM medium and then placed in complete culture. Cells are released from alginate beads by suspending the beads in three volumes of 150 mM Na citrate and 0.15 mM NaCl at 37°C until the beads dissolve (about 15 min). Thereleased cells are centrifuged at 2000 g for 5 min.

Alcian Blue Staining

The cultures are fixed for 48 h in 2.5% glutaraldehyde and simultaneously stained with alcian blue 8GX using 0.05% alcian blue in 0.4 M M gCl2, 25 mM Sodium acetate (pH 5.6) [12]. Excess stain is removed by washing in 3% acetic acid; then 25 and 50% ethanol with 3% acetic acid and finally 70% ethanol. The cultures are stored in 70% ethanol.

Fm DiI Labeling of Chondrocytes

Chondrocytes are removed from culture with trypsin and washed first with medium and then three times with phosphate-buffered saline. A stock solution of the Fm DiI (Molecular Probes; 50 μg DiI in 50 μl ethanol) is diluted 1:100 in phosphate-buffered saline. The cells are incubated with the diluted Fm DiI dye for 5 min at 37°C followed by 15 min at 4°C. The cells are centrifuged to remove excess dye before they are resuspended in phosphate-buffered saline.

Results

We have established human cartilage organ culture and cultures of human chondrocytes allowing us to assess chondrocyte transplantation to cartilage and gene transfer to chondrocytes as a prelude to producing genetically modified chondrocytes in vivo. The chondrocytes take on a fibroblast-like appearance in monolayer culture. This loss of phenotype is reversible as they readopt a rounded morphology typical of chondrocytes and produce proteoglycan when placed in a matrix such as alginate beads [11, 13]. Fig. 1 shows rounded human chondrocytes cultured in beads for 20 days before being released from the beads and stained with alcian blue to detect proteoglycan synthesis.

Transplantation with Infected Chondrocytes

We have shown high efficiency of infection of monolayers of human chondrocytes with the adenoviral vector, AdlacZ that codes for β-galactosidase. By staining with blue gal we see expression of the β-galactosidase gene by close to 100% of the chondrocytes. The predominantly nuclear staining of cells growing in monolayer culture is shown in Fig. 2. Uninfected chondrocytes do not give any background staining (data not shown).

Human chondrocytes were infected with AdlacZ before being transplanted to organ culture. Previous studies showed that transplanted chondrocytes adhered to cartilage in organ culture [14] or to focal articular cartilage defects in vivo in rabbits [15, 16] and continued to express transfected genes up to the 10 [15] or 28 [16] days tested. In this study we stained the tissue for β-galactosidase activity at varying time points after transplant to determine if the transplanted chondrocytes survived and the β-galactosidase gene introduced with the adenoviral vector, AdlacZ, continued to be expressed. The chondrocytes continued to express β-galactosidase for the duration of the trial 14 days after transplanting cells infected with AdlacZ (Table I). We have also shown that human chondrocytes will bind to cartilage in organ culture using fluorescently-labeled cells. We labeled chondrocytes with the membrane dye Fm DiI (Molecular Probes), and allowed the chondrocytes to adhere to cartilage. With this approach we have shown that the chondrocytes adhere to the cartilage for at least 28 days (Table I).

Transplanting Human Chondrocytes Before Infection with AdlacZ

Human chondrocytes were infected with AdlacZ after being transplanted to organ culture.
Human chondrocytes were cultured in alginate beads for 20 days. The cells were released using sodium citrate to chelate the calcium ions and to dissolve the beads. The clustered cells were subsequently stained with alcian blue to detect proteoglycan.

Fig. 2. Human chondrocytes were infected with the adenoviral vector, AdlacZ, that codes for β-galactosidase. The cultures were subsequently stained with blue gal we see expression of the β-galactosidase gene. The staining is predominantly nuclear.

Fig. 3(a)–(c) show expression 6, 20 and 27 days after infection. Transplant was 2 days before infection in each case. Direct infection with AdlacZ of cartilage with no prior repopulation with cultured chondrocytes results in very few blue foci compared to other trials where the cartilage was resurfaced [Fig. 3(d)]. This is likely a consequence of the extracellular matrix of cartilage that would exclude penetration of the virus to endogenous chondrocytes. It is necessary first to repopulate the surface of the cartilage with infected chondrocytes or to infect the chondrocytes subsequent to repopulating the surface of the cartilage.

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<td>Preinjected or prestained* chondrocytes</td>
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<td>Infection at variable times following transplant</td>
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(a) Chondrocytes were infected with adenovirus AdlacZ (†) or stained with the fluorescent dye Fm DiI (*) prior to transplantation and analyzed for β-galactosidase activity or fluorescence on cartilage at variable times after transplant.

(b) Chondrocytes were infected with adenovirus AdlacZ 2 days after transplantation and analyzed for β-galactosidase activity at variable times after transplant.

(c) The adenovirus, AdlacZ was used to infect endogenous chondrocytes in cartilage without prior transplant of chondrocytes from tissue culture.

(d) Chondrocytes were infected with adenovirus AdlacZ at variable times after transplantation and subsequently analyzed for β-galactosidase activity.
Fig. 3. Human chondrocytes were infected with AdlacZ 2 days after being transplanted to organ culture. The tissue was stained for β-galactosidase activity at (a) 6, (b) 20 and (c) 27 days after infection. As a control cartilage was infected with AdlacZ with no prior repopulation with cultured chondrocytes and the tissue was stained for β-galactosidase activity (d).

days after transplant and β-galactosidase activity determined at 45 days (Fig. 4). The ability to infect chondrocytes after transplant raises the possibility of repeated infections of surface chondrocytes as an alternative to repeated injections of chondrocytes into the joint space.

Fig. 4. Human chondrocytes were transplanted to cartilage in organ culture and infected with AdlacZ 35 days later. The β-galactosidase activity was determined 10 days after infection.

Discussion

Our goal is to deliver chondroprotective genes directly to the chondrocytes in cartilage. In this way we hope to re-establish the equilibrium between matrix synthesis and breakdown. This should both repair damaged cartilage and extend the life of hypermetabolic chondrocytes attempting to maintain the extracellular matrix. As a prelude to gene therapy trials in patients with osteoarthritis we have explored the persistence of binding of transplanted chondrocytes to cartilage in vitro and the expression of the β-galactosidase gene introduced into the chondrocytes before and after transplant of the chondrocytes to cartilage.

Establishment of cartilage organ culture from human cartilage allows for assessment of chondrocyte transplantation to cartilage and gene transfer to chondrocytes as a prelude to producing genetically modified chondrocytes in vivo. We have shown that human chondrocytes will bind to cartilage in organ culture using fluorescently-labeled cells. We labeled chondrocytes with the membrane dye Fm Dil (Molecular Probes), and allowed the chondrocytes
to adhere to cartilage. We have shown with this analysis that the chondrocytes adhere to the cartilage for at least 28 days. We have shown that these chondrocytes remain viable on the surface of the cartilage as they continue to express the β-galactosidase gene. We initially infect the chondrocytes with adenovirus coding for the β-galactosidase gene. Transplantation of the infected chondrocytes to cartilage results in the continued stable expression of the β-galactosidase gene out to 14 days tested to date. Chondrocytes infected with AdlacZ 2 days after being transplanted to organ culture continued to express β-galactosidase 29 days after transplant and 27 days after infection of chondrocytes following transplant [Fig. 3(c)]. Chondrocytes that can adopt a fibroblast-like appearance in culture readopt a round and cuboidal shape typical of chondrocytes when attached to cartilage [17]. The transplanted chondrocytes will synthesize new matrix and integrate into the cartilage [14].

We have also transplanted unmodified chondrocytes to cartilage and shown that the transplanted chondrocytes can be efficiently infected well after the transplant (Fig. 4). We can infect the chondrocytes as late as 35 days after transplant suggesting that repeated infections instead of repeated chondrocyte transplants may be feasible. Direct infection of chondrocytes in cartilage without prior transplantation gives many fewer infected cells. Our results may indicate that the matrix synthesized by these cells over the short term is not sufficient to block infection by adenovirus through steric hindrance. It is also possible that the transplanted cells are more easily infected than endogenous chondrocytes as a result of a different profile of cell surface receptors. In either case it is necessary first to repopulate the surface of the cartilage with infected chondrocytes or to infect the chondrocytes after repopulating the surface of the cartilage.

We are attempting to combine gene therapy approaches with cartilage transplantation. The introduction of new gene products has the potential to both decrease catabolic and increase anabolic activity of transplanted chondrocytes and chondrocytes native to the cartilage. The cartilage transplantation itself provides an increased population of chondrocytes that can contribute to healing injured articular cartilage. The combination of these methods should enhance efforts to resurface and repair degenerated articular cartilage.

Acknowledgments

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References


