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OSTEOARTHRITIS and CARTILAGE

Transplantation of adenovirally transduced allogeneic chondrocytes into articular cartilage defects *in vivo*

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Summary

Gene transfer to chondrocytes followed by intra-articular transplantation may allow for functional modulation of chondrocyte biology and enhanced repair of damaged articular cartilage. We chose to examine the loss of chondrocytes transduced with a recombinant adenovirus containing the gene for *Escherichia coli* β-galactosidase (Ad.RSVntlacZ), followed by transplantation into deep and shallow articular cartilage defects using New Zealand White rabbits as an animal model. A type I collagen matrix was used as a carrier for the growth of the transduced chondrocytes and to retain the cells within the surgically created articular defects. Histochemical analysis of matrices recovered from the animals 1, 3 and 10 days after implantation showed the continued loss of lacZ positive chondrocytes. The number of cells recovered from the matrices was also compared with the initial innoculum of transduced cells present within the matrices at the time of implantation. The greatest loss of transduced cells was observed in the first 24 h after implantation. The numbers of transduced cells present within the matrices were relatively constant between 1 and 3 days postimplantation, but had progressively declined by 10 days postimplantation. These results suggest that transduction of chondrocytes followed by intra-articular transplantation in this rabbit model may enable us to examine the biological effects of focal transgenic overexpression of proteins involved in cartilage homeostasis and repair.

Key words: Gene therapy, Transplantation, Chondrocytes, Adenovirus.

Introduction

Transplantation of cultured chondrocytes into areas of cartilage injury has shown promise as a method to facilitate repair of deep articular defects [1]. The therapeutic application of this technology continues to be explored in a series of ongoing human trials [2]. However, many questions remain as to the cellular mechanisms that are responsible for growth of new tissue into the site of the treated defect. A more comprehensive understanding of the fate of transplanted cells and analysis of their functional biology would not only provide important scientific information but would also allow implementation of molecular methods that might alter function of the transplanted cells in order to facilitate appropriate repair/regeneration of damaged articular cartilage.

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Several groups have proposed somatic cell gene transfer as a method to alter the functional biology of chondrocytes that are used for intra-articular transplantation [3–5]. Somatic cell gene transfer is also an ideal method for molecular marking of cultured chondrocytes that can be used subsequently in transplantation studies. We have previously described *in vitro* experiments indicating that transduction of chondrocytes with recombinant adenoviruses might serve as a method to achieve focal delivery of biologically active proteins over sustained periods of time [3]. However, studies in animal models of cartilage injury are needed before the concept of transplanting transduced chondrocytes for modification of cartilage repair can be realized.

A major logistical problem preventing the widespread use of chondrocyte transplantation as a therapeutic modality involves the acquisition of articular cartilage for isolation of chondrocytes. The use of autologous cells for intra-articular transplantation although ideal from an immunological perspective, is often not a practical method

to obtain adequate numbers of explanted chondrocytes for application to large and/or multiple areas of cartilaginous injury. We hypothesized that the relatively poor antigen-presenting capacity of articular chondrocytes would enable us to test the feasibility of using allogeneic cells for transplantation into deep defects of articular cartilage *in vivo* [6, 7]. In addition, the use of adenovirally-transduced allogeneic cells represents an animal model of a worst-case scenario of immunological effects associated with *ex vivo* somatic cell gene transfer.

We hypothesized that transduction of chondrocytes with a marker transgene would provide a molecular and histochemical means to follow the fate of transplanted cells *in vivo*. In this study, we have attempted to model intra-articular chondrocyte transplantation in a way that would enable us to address the following questions. What is the fate of chondrocytes implanted into a defined deep defect? If the transplanted cells do not readily survive in the context of a deep defect, then what are the kinetics of cell loss? If the transplanted cells are destroyed, can we obtain information that would enable us to determine the mechanisms of cell death? We now present data indicating that limits to the successful transplantation of chondrocytes into deep defects primarily involve microenvironmental factors that effect cell loss within the initial 24 h after intra-articular transplantation.

Materials and Methods

ISOLATION AND CULTURE OF ARTICULAR CHONDROCYTES

Chondrocytes used in the study were isolated from pieces of articular cartilage separated from the underlying bone of cadaveric rabbits. The cartilage slices were minced finely and then digested sequentially with 0.2% testicular hyaluronidase (5 min at 37°C), 0.2% trypsin (30 min at 37°C), and 0.2% collagenase (1–2 h at 37°C). The resulting solutions were strained through sterile nylon mesh and centrifuged at 1200 ***g*** for 10 min. The cell pellets were washed twice with Ham's F-12 medium, resuspended, pooled and centrifuged at 1000 ***g*** for 10 min. The resulting pellet was suspended in F-12 media containing 10% FCS, 100 u/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B (complete F-12 media). This cell suspension was used to establish cell cultures in 25-cm^2 flasks (Falcon) at a density of $1–2\times10^6$ cells/flask. The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The chondrocytes used in this study were maintained as monolayer cultures for no more than two passages, in order to maintain the differentiated chondrocyte phenotype.

RECOMBINANT ADENOVIRUS

The adenoviral vector used in this study is based on a human adenoviral (serotype 5) genomic backbone deleted of sequences spanning E1A and E1B and a portion of E3 region. This impairs the ability of the virus to replicate or transform nonpermissive cells [8]. The transgene transcription was driven by the Rous sarcoma virus (RSV) LTR and a SV40 polyadenylation sequence cloned downstream of the reporter [9, 10]. The vector Ad.RSV*ntlacZ* contains a nuclear targeting (designated as *nt*) epitope linked to the 5' end of the *lacZ* gene, and as result, the cells transduced with this gene can be identified by the presence of blue nuclei after reaction with the chromogenic substrate X-gal.

TRANSDUCTION OF CHONDROCYTES

Primary rabbit chondrocyte cultures were trypsinized and split 1:4, and allowed to grow to 70% confluency in 75 cm^2 flasks. After the cells were 70% confluent, they were washed two times with serum-free Dulbecco's minimum essential medium (DMEM), infected with Ad.RSV*ntlacZ* (viral titers = 10^{10} plaque forming units (pfu)/ml; diluted 1:100 in serum free DMEM) at an approximate multiplicity of infection (MOI) of 10^3 pfu/cell, for 2 h at 37°C. Cells were then washed two times in fresh complete F-12 medium and allowed to grow for 24 h at 37°C. Mock infected cells were maintained in parallel.

PREPARATION OF CHONDROCYTE-COLLAGEN IMPLANTS

We used Helistate absorbent collagen type I hemostatic sponges (Intragra Life Sciences Co., Plainsboro, NJ, U.S.A.) as a carrier for growth of transduced chondrocytes and implantation to shallow and deep defects in the rabbit knee. Type I collagen implants have previously been shown to be a suitable carrier for transplantation of rabbit chondrocytes into articular defects [11]. Biomechanical, biochemical and histological analyses of chondrocyte containing matrices maintained in culture for 6 weeks indicate that the cells remain phenotypically stable, metabolically active, secrete glycosaminoglycans into the extracellular matrix and form an organized pericellular matrix. Using a 5 mm skin biopsy punch, discs were

obtained from a sheet of collagen sponge and sterilized using ethylene oxide before application of the chondrocytes. A disc was placed into each well of a 96-well plate, and covered with 100 µl complete F-12 medium containing 7×10^5 chondrocytes. Cells were allowed to adhere to the matrix for a period of 7–10 days before implantation *in vivo*.

ANIMALS

New Zealand White rabbits (3 kg in weight, pasteurella free, HRP, Inc., Kalamazoo, MI, U.S.A.) were individually housed in stainless steel caging in an AAALAC accredited facility. Rooms were environmentally controlled to provide a temperature between 61° and 71°F, a relative humidity of 35–65%, 100% fresh air at a rate of 10–12 room exchanges per hour, a light:dark cycle of 12:12 h. The rabbits were fed *ad libitum* a designated amount of chow (Purina High Fiber Rabbit Chow 5326, St. Louis, MO, U.S.A.). All procedures that used animals were conducted in compliance with federal laws and guidelines established by the Parke–Davis animal care and use committee.

SURGICAL PROCEDURE

Animals were anesthetized with a single subcutaneous dose of 60 mg/kg Vetalar (Forte Dodge Laboratories Inc., Fort Dodge, IA, U.S.A.) and 6 mg/kg Rompun (Miles Inc., Shawnee Mission KA, U.S.A.). Supplemental inhalational anesthesia with AErrane (Aryquest, BOC Inc., Madison WI, U.S.A.) was used as needed. The hair around the knee was clipped and the skin was prepped for a sterile procedure. Using sterile technique, a lateral parapatellar arthrotomy was performed, and the patella was luxated medially. A steel drill bit (3 mm in diameter) and a dual torque low speed drill (Micro Mark) were used to create defects in the articular cartilage of the trochlear groove. Two types of defects were created. A deep defect (approximately 2 mm) penetrated the cartilage creating a well-circumscribed hole that exposed the cortical bone. Bleeding from the cortical bone was common. A shallow defect simply removed enough of the cartilage and bone to create an indentation deep enough to contain the implant. The collagen–chondrocyte implants were press-fitted into the defects. No additional materials or adhesives were applied to the matrices, the sponges were held in place within the defects by repositioning of the patella within the trochlear groove. One simple interrupted cruciate 3-0 Maxon suture (Davis & Geck Inc., American Cyanamid Co., Manati, Puerto Rico) was used to close the joint capsule. The skin was closed with 5-0 PDS II (Ethicon Inc., Johnson & Johnson Co., Somerville NJ, U.S.A.) sutures in a simple interrupted pattern.

TISSUE ANALYSES

Animals were euthanized 1, 3 or 10 days after implantation and tissue present within the articular defects was removed *en bloc*. Samples of defect tissue obtained at each time point were used for immediate isolation of adherent cells. Samples were rinsed briefly in phosphate-buffered saline (PBS) then digested 4 mg/ml collagenase (10 min at 37°C) then with 0.5% trypsin and 0.02% EDTA (30 min at 37°C), and cells were isolated by centrifugation. Cells were immediately fixed in 2% paraformaldehyde in 100 mM PIPES pH 6.9 for 30 min, washed for 30 min in PBS/2 mM $MgCl_2$ and reacted in 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ 0.01% sodium deoxycholate, 0.02% NP-40, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside in PBS (X-gal solution) for 1 h at 37°C [12]. Cells were then washed three times with PBS, post-fixed for 2 h in 2% paraformaldehyde/1% glutaraldehyde. The number of lacZ positive cells present in the samples was determined by identification of blue staining cells using light microscopy and a hemocytometer.

Parallel samples were either returned to sustained *in vitro* culture conditions for a period of 5 days, or were fixed and stained for lacZ expression, and frozen in OCT (Sigma). Serial transverse sections (7–9 µm) were obtained from frozen samples using a Leitz 1720 digital cryostat. Sections were counterstained with either hematoxylin and eosin, or Picrosirius red, using the procedure described by Dolber and Spach, then examined by light microscopy and representative photomicrographs were obtained (Vanox AHBS 3, Olympus Inc.) [13].

Results

The initial step in this study was to determine the growth and viability of adenovirally-transduced cells seeded onto the surface of type I collagen matrices *in vitro*. Chondrocytes within the matrices were compared directly with monolayer cultures maintained in parallel. Cells were seeded at a density of 1.6×10^5/implant, as well as 1.6×10^5/well (96 well plate). After 48 h growth the mean number of cells recovered from the implants was $10.1(\pm0.4)\times10^4$ and from the plastic plates

$13.4(\pm 0.7)\times10^4$ per well. The number of cells recovered after 9 days in culture were 13.7×10^4 and 15.5×10^4 from the matrices and plastic wells, respectively.

Adenovirally transduced cells were then seeded onto a set of matrices at a dose of 7.0×10^5 lacZ positive cells per disc. A subset of these matrices were maintained as *in vitro* cultures for a period of 10 days. Samples of matrix were examined after 1, 3 and 10 days of culture, and the total number of lacZ-expressing cells was determined. The mean number of lacZ-positive cells was $1.0\ (\pm0.2)\times10^5$ on day 1, $1.1\ (\pm0.5)\times10^5$ on day 3 and $0.5\ (\pm0.07)\times10^5$ on day 10. Although fewer lacZ-positive cells were recovered on day 10, these changes were not statistically significant.

The kinetics of cell growth within the matrix approximated those observed in monolayer cultures of articular chondrocytes. Collagen matrices seeded with the transduced cells were also analyzed histologically after 24 h [Fig. 1(a)]. The transduced (lacZ expressing) chondrocytes were

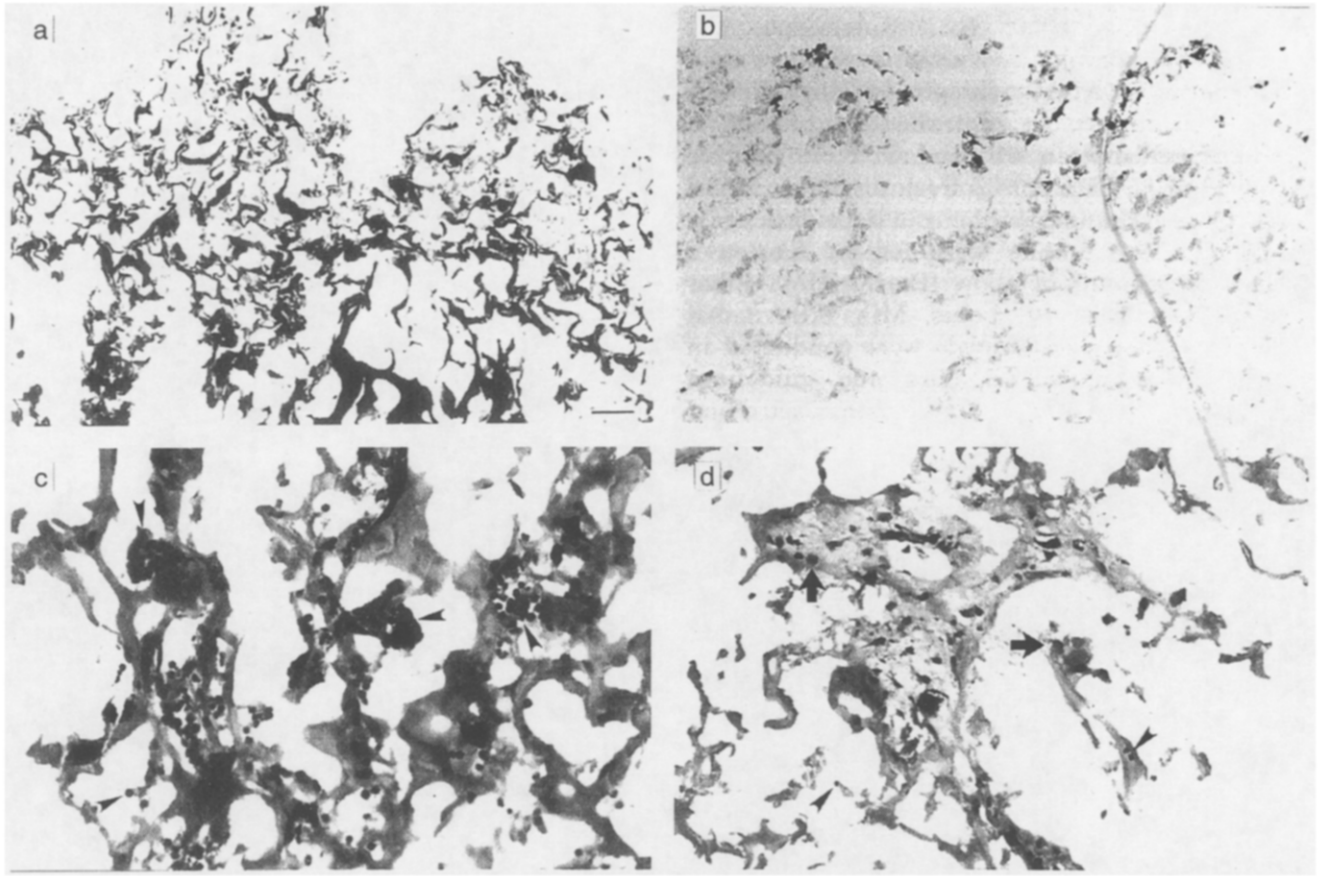

FIG. 1. Implantation of collagen sponges containing adenovirally-transduced chondrocytes into defined articular defects allows for persistence of transduced cells *in vivo*. (a) Transverse sectional photomicrograph of collagen sponge seeded *in vitro* with Ad.RSVntlacZ-transduced chondrocytes before implantation *in vivo*. Twenty-four hours after application of chondrocytes to the matrix samples were fixed, reacted with X-gal solution, snap frozen and cryosectioned. Sections (7 μm) were counterstained with picrosirius red stain to identify collagen fibers. Note that large numbers of lacZ positive cells with blue staining nuclei (transduced chondrocytes) can be seen along the surface of the collagen matrix but that few cells can be observed at a depth of greater than approximately 0.1 mm. Bar = 100 μm. Magnification = 13.2×. (b) Transverse sectional protomicrograph of collagen matrix seeded *in vitro* with Ad.RSvntlacZ-transduced chondrocytes, recovered 24 h after implantation *in vivo*. Chondrocytes expressing lacZ (blue nuclei) can be observed near the implant border but are substantially decreased in the total number of cells compared with the pre-implantation samples (a). These sections were not counterstained. Magnification = 33×. (c) Transverse sectional photomicrograph of collagen sponge seeded *in vitro* with Ad.RSVntlacZ-transduced chondrocytes recovered 72 h after implantation *in vivo*. Sections were reacted with X-gal and counterstained with hematoxylin and eosin. LacZ-positive chondrocytes continue to be observed in a location proximal to the collagen fibers (pink) of the matrix. A large number of red blood cells (arrowheads) are also present throughout the matrix. Magnification = 132×. (d) Transverse sectional photomicrograph of collagen sponge seeded *in vitro* with Ad.RSVntlacZ-transduced chondrocytes recovered 10 days after implantation *in vivo*. Sections were reacted with X-gal and counterstained with hematoxylin and eosin. Compared with samples obtained 72 h after implantation, chondrocytes remain concentrated along the implant border. Red blood cells (arrowheads) present within the matrix are decreased in number compared to days 1 and 3. A few mononuclear cells (arrows) are scattered throughout the section. Magnification = 66×.

observed to be present (adherent) almost exclusively along the surface of the matrices.

The remaining matrices were implanted into cartilage defects within rabbit knees and allowed to grow *in vivo* for the same duration as the *in vitro* cultures. The number of lacZ-positive cells recovered after transplantation of matrices *in vivo* were $5(\pm 0.09)\times 10^3$ at 1 day, $3.0(\pm 0.09)\times 10^3$ at 3 days and $0.2(\pm 0.03)\times 10^4$ at 10 days. The survival of transgene expressing cells *in vivo* at the histologic level is also shown in Fig. 1. At the light microscopic level, samples obtained 24 h [Fig. 1(b)] and 72 h [Fig. 1(c)] after implantation were indistinguishable. In all samples examined, lacZ-expressing cells continued to be found along the surface of the implanted matrix but were greatly reduced in number compared with pre-implantation matrix [Fig. 1(a)]. By 10 days after implantation [Fig. 1(c)], lacZ-expressing cells continued to be observed within the recovered matrices. No lacZ-expressing cells were observed within the deeper portions of the matrices, and no areas of necrotic cells were identified at any of the time points examined.

The kinetics of transduced chondrocyte survival *in vivo* are shown in Fig. 2. The greatest loss of viable lacZ-positive cells occurred within 24 h after implantation of matrix into the deep defects [Fig. 2(a)], with a mean cell loss approaching 90%. Additional cell losses observed from days 2–10 were not statistically significant. Parallel controls, consisting of matrix seeded with equivalent numbers of lacZ-expressing cells, were maintained in tissue culture conditions for a period of 3 days, and showed no significant (percentage based) loss of lacZ-positive cells. This suggests that the loss of lacZ-positive cells observed *in vivo* is not a consequence of transduction with a recombinant adenovirus, but is a function of the implantation procedure itself and or the immediate micro-environmental conditions proximal to the implanted matrix.

The viability of the lacZ-expressing cells identified by light microscopy in the 10-day samples was established by recovery of matrices *en bloc* after 10 days *in vivo*, followed by transfer to cell culture [Fig. 2(b)]. Cells were isolated from the explanted matrices after 5 days of growth *in vitro* and stained for lacZ expression. The number of lacZ cells isolated from the matrices after 10 days *in vivo* (Fig. 2 Column a), compared with the number isolated from matrices returned to cell culture for an additional 5 days of growth *in vitro* (Fig. 2 Column b) were not significantly different ($P=0.05$). These results confirm that the transduced allogeneic chondrocytes continue to remain

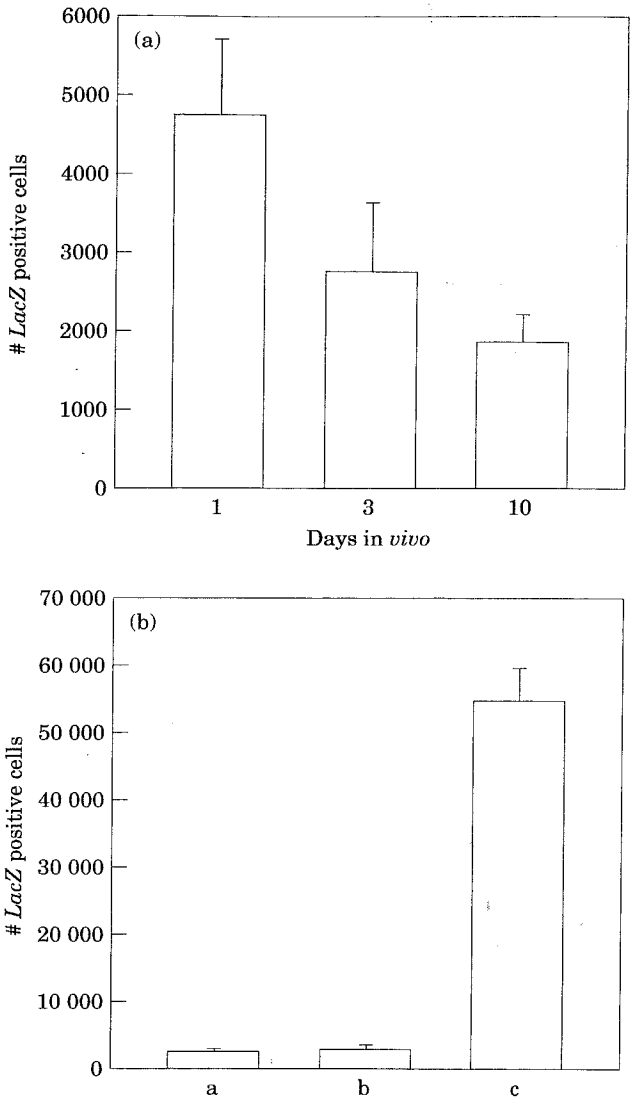

FIG. 2. Recovery of viable transduced chondrocytes from collagen matrices after implantation into deep articular cartilage defects *in vivo*. (a) Collagen matrices were seeded *in vitro* with Ad.RSVntlacZ-transduced chondrocytes (mean $=7\times 10^5$ lacZ positive cells/matrix) and implanted into deep articular defects. Tissue present within the defect was recovered 1, 3 or 10 days after implantation, digested with collagenase and trypsin, and cells were isolated by centrifugation. The number of cells expressing lacZ was determined by staining with X-gal and counting blue cells with a hemocytometer. Six animals were examined at each time point. Column heights represent mean number of lacZ-positive cells per matrix ± standard error. (b) Cells were isolated from the collagen matrices after 10 days of growth *in vivo* and the number of lacZ expressing cells was determined (Column a). Parallel samples of cells recovered from matrices after 10 days of growth *in vivo* were returned to monolayer culture *in vitro* for an additional 5 days before quantification of lacZ positive cells (Column b). Controls consisting of seeded matrices were maintained *in vitro* for a period of 10 days before recovery and quantification of adherent lacZ positive cells (Column c). Column heights represent mean number of lacZ-positive cells per matrix ± standard error. Column a, $N=4$. Columns b and c, $N=3$.

viable for at least 10 days after implantation into deep articular defects *in vivo*. Viable lacZ-expressing cells present within the matrices after 10 days *in vivo* represent 3.8% of the initial innoculum, as determined by comparison with parallel controls maintained for the entire 10 day period *in vitro* (Fig. 2 Column c). In order to determine whether physical contact of the transduced cells with bone marrow cells and/or immune effector cells present in subchondral bone were responsible for the *in vivo* loss of cells from the matrix, we repeated the transplantation experiments using a series of shallow (1 mm) articular defects. The results (Fig. 3) indicate that there is no apparent difference between deep and shallow defects with regard to the kinetics of cell survival or the total percentage of transduced cells that remain within the matrix after the initial implantation procedure at 1 or 3 days.

Discussion

Intra-articular transplantation of explanted chondrocytes for the repair of cartilaginous injury is a promising area of clinical research. A major scientific question associated with this clinical technology concerns the fate of cells introduced into the microenvironment of a deep defect. A corollary to this is can genetically modified cells be successfully introduced into deep articular defects, and can these cells continue to express transgenic proteins for defined periods of time? We have determined that in the context of an acellular matrix seeded with genetically modified cells *in vitro*, the cells can persist within the microenvironment of a deep defect, and can continue to express transgenic protein for at least 10 days.

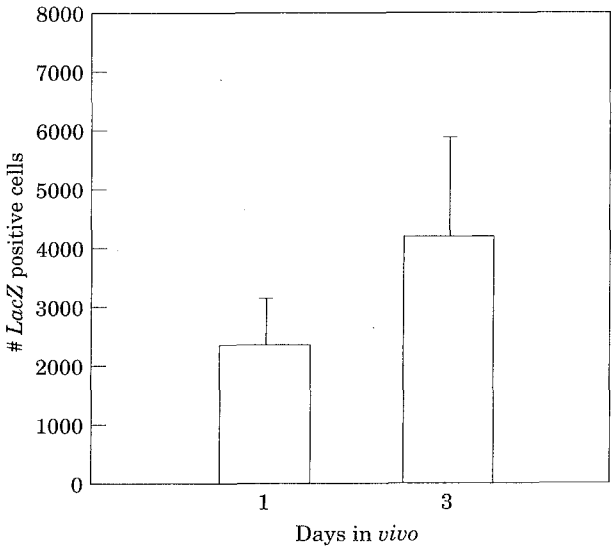

FIG. 3. Recovery of viable transduced chondrocytes from collagen matrices after implantation into shallow articular cartilage defects *in vivo*. Collagen matrices were seeded *in vitro* with Ad.RSVntlacZ-transduced chondrocytes (mean = 5.0×10^5 cells/matrix) and implanted into shallow articular defects (diameter = 3 mm). Tissue present within the defect was recovered 1 and 3 days after implantation, digested with collagenase and trypsin, and cells were isolated by centrifugation. The number of cells expressing lacZ was determined by staining with X-gal and counting blue cells with a hemocytometer. Three animals were examined at each time point. Column heights represent mean number of lacZ-positive cells per matrix ± standard error.

Realistic limits to the application of intra-articular transplantation of chondrocytes in humans are based on identification of a cost-effective source of chondrocytes for transplantation. In this study, we used cadaveric animals as a source of allogeneic chondrocytes and modeled a worst-case scenario with regards to the immunological effects that might be associated with this cellular transplantation procedure. These experiments show that transduced allogeneic cells survive for at least 10 days after intra-articular transplantation, and continue to express transgenic protein for the duration of their survival *in vivo*.

Previous studies have used a periosteal cap to retain transplanted cells within the confines of the full-thickness defect. As an alternative to the use of a periosteal cap, we examined the use of a type I collagen sponge seeded with transduced chondrocytes as a cellular implant that can form fit to the dimensions of shallow or deep articular defects. Chondrocytes grown within these matrices are metabolically active, phenotypically stable, and express extracellular structural proteins characteristic of articular cartilage [11]. The present findings suggest that type I collagen matrices are suitable for use as implantable devices for retention of genetically-modified chondrocytes proximal to deep and shallow articular defects. These matrices also provide a suitable environment for continued viability of transduced cells *in vivo*.

We hypothesized that the use of these matrices would increase the potential for cell survival compared with introduction of recently-trypsinized cells into a microenvironment filled with serum, bone marrow components and differentiated white blood cells. This approach is also technically much simpler and less invasive than the use of the periosteal cap. In addition, the matrix is biodegradable and is gradually replaced by new extracellular matrix proteins secreted by the transplanted cells. Unfortunately, compared with matrices seeded with transduced chondrocytes and maintained *in vitro*, approximately 90%

of the cells adherent to the matrices were lost after 24 h *in vivo*.

Our data indicates that the major loss of transduced chondrocytes from the matrix (approximately 90%) occurs within the first 24 h after implantation. Subsequent rates of loss betwen 1–10 days were minimal. This kinetic profile indicating relatively rapid cell loss within the initial 24 h would argue against a mechanism for cell death involving cell-mediated cytotoxicity [major histocompatibility complex (MHC) II-restricted cytotoxic T cells] or pathways involving humoral immune responses. It also suggests that similar rate of transplanted cell death may occur after the introduction of autologous chondrocytes into deep defects covered by a periosteal cap. The data presented by Kang *et al.* [14] are consistent with our observations, and also argue against host immunity as the primary mechanism responsible for loss of transplanted cells *in vivo* over the first three days. Clearly the identification of a mononuclear cell infiltrate within the matrix 10 days after implantation [Fig. 1(d)], is consistent with a host immune response directed against allogeneic cells and/or transduced cells expressing low levels of adenoviral proteins or *E. coli* β-galactosidase.

An alternative explanation for the rapid loss of cells would be that transplanted cells migrated out of the matrix and onto the surface of the subchondral bone or deeper layers of articular cartilage exposed during the surgical creation of the defects. Chondrocytes maintained in monolayer culture do acquire a fibroblastic phenotype, however, there is no reported evidence of dedifferentiated chonrocytes exhibiting a propensity for functional migration between substrata (e.g., collagen matrix to subchondral bone).

Another alternative explanation for loss of transduced cells would be inaccessibility to nutrients and/or oxygen at levels necessary to maintain cell viability. The hypothesis would be that transduced cells present in the interior of the matrix at the time of transplantation, or cells that migrated into the interior of the collagen matrix after *in vivo* transplantation would be in a microenvironment characterized by relatively slow (poor) exchange of gases and small molecules and susceptible to death. Our *in vitro* method for seeding the collagen matrix allows for adherence and growth of cells on the exterior surface of the collagen matrix. Our lacZ gene marking studies have shown no evidence to suggest that cells can migrate into the interior of the collagen matrix (Fig. 1). We also did not see evidence of necrotic cells present within the interior of matrices recovered 24 or 72 h after *in vivo* implantation. The fact that relatively-stable numbers of cells remain within the matrix between 24 and 72 h would also suggest that ischemic necrosis is not the major factor associated with the rapid rate of cell loss observed *in vivo*.

It is also possible that cells continue to exist within the matrix, or within the physical confines of the surgical defect, but that extinction of transgene expression renders the cells invisible to histochemical staining and identification by light microscopy. The vector systems used a retroviral long terminal repeat (LTR) to drive transgene expression. Although transcriptional silencing of LTR promoters in transduced cells has been reported to occur after transplantation of cells *in vivo*, it is difficult to invoke promoter inactivation that occurs within period of 24 h as a mechanism for loss of lacZ-positive cells [15–17]. In addition the half life of transgenic lacZ would also argue in favor of cell death as the mechanism responsible for the loss of X-gal-positive cells. The fact that we could readily recover transduced cells from the matrix, and that these cells continued to express lacZ *in vitro* (both immediately and after sustained monolayer culture), argues strongly against the hypothesis that loss of transgene expression can explain the rapid loss of lacZ positive cells that we observed *in vivo*. Also the kinetics of cell loss *in vivo* appear to be independent of the vector system used for cellular transduction.

We favor the hypothesis that mechanical forces transmitted through (impact loading) and/or along the edges (shear) of the matrix directly effect survival of transplanted cells. Although no necrotic cells were identified in histologic sections of the matrices recovered from the experimental animals, it is unclear at this time whether the mechanism(s) of chondrocyte death involve necrosis vs apoptosis, and whether these two pathways can be distinguished using this *in vivo* model [18–20]. If cell loss is indeed primarily due to the effects of mechanical forces acting on the transplanted cells, then the use of perioperative joint immobilization may enhance cell viability *in vivo*.

The continued use of genetically-modified chondrocytes for further *in vivo* studies may enable us to answer many of the outstanding questions that have arisen regarding the functional biology of chondrocytes implanted into articular defects. The use of chondrocytes transduced to overexpress biologically-active proteins may enable us to modify the functional biology of articular tissue repair along defined pathways of growth and differentiation, and will promote the further

development of this technology as a therapeutic modality.

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