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Transfer of LacZ Marker Gene to the Meniscus*

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Abstract

Background: **Lesions in the avascular two-thirds of the meniscus do not heal well and are of concern clinically. Various growth factors promote the synthesis of matrix by meniscal cells and thus have the potential to augment healing. However, their clinical application is severely hindered by problems with delivery. An attractive approach to overcoming such problems is to transfer genes that encode the growth factors in question to the site of the injury. As a prelude to this, we evaluated methods for delivering genes to the meniscus.**

Methods: **Gene transfer was evaluated *in vitro* and *in vivo* with a lacZ marker gene, which expresses the enzyme β-galactosidase. Two types of vectors were tested: an adenovirus and a retrovirus. Monolayers of lapine, canine, and human meniscal cells, as well as intact lapine and human menisci, were used for the *in vitro* studies. Lesions were created in the menisci of rabbits and dogs for the *in vivo* studies. Gene transfer to the sites of the experimental meniscal lesions *in vivo* was accomplished in two ways. In the lapine model, a suspension of adenovirus carrying the lacZ marker gene was mixed with whole blood and the clot was inserted into the lesion. In the canine model, retrovirally transduced allogenic meniscal cells carrying the lacZ marker gene were embedded in collagen gels and transferred to the defects. The animals were killed at various time-points, and gene expression was evaluated by histological examination of sections stained with 5-bromo-4-chloro-indolyl-β-D-galactose (X-gal), from which a blue chromagen is released in the presence of β-galactosidase.**

Results: **Monolayer cultures of lapine, canine, and human meniscal cells were susceptible to genetic transduction by both adenoviral and retroviral vectors. *In vitro* gene transfer to intact human and lapine menisci proved possible both by direct, adenoviral, delivery and indirect, retroviral, delivery. Gene expression persisted for at least twenty weeks under *in vitro* conditions. With regard to the *in vivo* studies, gene expression persisted within the clot and in some of the adjacent meniscal cells for at least three weeks in the lapine defect model. In the canine defect model, gene expression persisted within the transplanted, transduced meniscal cells for at least six weeks.**

Conclusions: **It is possible to transfer genes to sites of meniscal damage and to express them locally within the lesion for several weeks.**

Clinical Relevance: **Healing of the avascular portion of the meniscus may be improved by the transfer of genes encoding the appropriate growth factors. To our knowledge, the present report is the first to describe methods for transferring genes to the meniscus. When used in conjunction with the appropriate growth-factor genes, these techniques should help to provide the basis for potential alternative treatment options for meniscal lesions. Additional studies are needed to determine whether these techniques will lead to improved healing of meniscal defects *in vivo*.**

The menisci serve many important biomechanical functions, including load-bearing, shock absorption, and load transmission. They also contribute to the stability of the knee[14]. Because of a greater understanding of the importance of the menisci and many reports demonstrating an early onset of degenerative changes in the knee joint following meniscectomy[4,6,20], orthopaedic surgeons now aim to preserve as much functional meniscal tissue as possible when treating meniscal injuries.

Because the periphery of the meniscus receives a rich blood supply, tears in this location are reparable. In contrast, the inner two-thirds of the meniscus is relatively avascular. The results of meniscal repair in this zone are variable, although many techniques have been developed in attempts to promote healing in this region[2,7,15,24]. One approach is to use growth factors, which accelerate the healing process[12]. It has been demonstrated that meniscal fibrochondrocytes are capable of enhanced proliferation and matrix synthesis when exposed to growth factors that are normally present in wound hematomas[3,13,19,21,22]. Moreover, animal studies have shown that the addition of growth factors during conventional methods of meniscal repair improves healing[9].

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For this approach to be successful, a method is needed with which to deliver the growth factors locally to the lesion and to maintain the sustained release of the growth factors over time. Direct application of growth factors by themselves is not likely to be successful because of their short biological half-lives. The implantation of bioresorbable slow-release devices, while possible, has yet to be realized in a clinically useful manner.

Gene transfer provides a promising alternative strategy[5]. Delivery to the meniscus of a gene encoding the growth factor of interest may make it possible to achieve sustained local endogenous synthesis of the growth factor at the site of healing. There are many advantages to this approach, including the potential for regulating both the level and the duration of growth-factor production[5]. Endogenous synthesis also ensures that, unlike recombinant growth factors produced by prokaryotic cells, the growth factor has undergone appropriate posttranslation modification. The present study was designed to evaluate the feasibility of gene transfer to the meniscus.

There are two main strategies for transferring genes to sites in the body[5]. With *in vivo*, or direct, gene delivery, the new genetic material is introduced directly into the patient. With *ex vivo*, or indirect, gene delivery, cells are removed, genetically manipulated outside the body, and then returned to the body. The cells used in *ex vivo* gene delivery do not necessarily have to be derived from the target organ.

Gene delivery is facilitated by the use of vectors, which enable the cellular uptake and expression of the transferred genes. A variety of viral and nonviral vectors are available for this purpose[17]. In the present study, we elected to evaluate vectors developed from an adenovirus and a retrovirus. Adenoviruses infect both dividing and nondividing cells very efficiently and may be used for both *in vivo* and *ex vivo* gene delivery. In contrast, retroviruses only transduce dividing cells and are predominantly used in *ex vivo* protocols. In both cases, the viruses in the present study were used to transfer a bacterial marker gene, lacZ, which expresses the enzyme β-galactosidase. Cells expressing this enzyme, which is not normally found in mammalian cells, can be stained blue by a simple histochemical procedure.

Materials and Methods

Viral Vectors

Two types of viral vectors were used in this study: adenovirus and retrovirus. The E1 and E3 regions of the genome were deleted in the adenoviral vector. The marker gene used in this study, lacZ, was cloned into the E1 region under the control of a human cytomegalovirus early promoter. The virus was prepared with standard methods, which yielded a suspension containing 10^9 plaque-forming units per milliliter.

The retroviral vector was based on the Moloney murine leukemia virus. It contained the lacZ gene under the control of the retroviral 5′ long terminal repeat and the neomycin phosphotransferase gene under the control of the simian virus 40 early promoter[16]. The latter gene renders cells resistant to the neomycin analog G418. This virus was generated at titers of approximately 10^5 infectious viral particles per milliliter from a producer line known as CRIP.

Meniscal Cell Culture

Menisci were retrieved from the knee joints of three-month-old New Zealand White rabbits that weighed approximately 2.5 kilograms, adult mongrel dogs that weighed twenty-five to thirty kilograms, and three patients who were fourteen, sixteen, and twenty-eight years old and had a medial meniscal tear. The removal of human menisci was approved by the local institutional review board. Cells were isolated from the menisci with the method described by Green[8]. Briefly, the menisci were cut into approximately three-millimeter-long pieces. The cells were released from the meniscal fragments by sequential digestion with 0.2 percent (weight per volume) trypsin for thirty minutes and 0.2 percent (weight per volume) collagenase for three hours at 37 degrees Celsius and were then seeded into twenty-five-square-centimeter tissue-culture flasks (T-25 tissue culture flask; VWR Scientific, Bridgeport, New Jersey) that contained four milliliters of Ham's F-12 medium (Gibco, Grand Island, New York) supplemented with 10 percent fetal bovine serum (Gibco), and 1 percent penicillin and streptomycin (weight per volume) (Gibco).

Cell Transduction in Vitro

Adenoviral transduction: When the cells were confluent, the medium was removed, and the cell monolayer was washed twice with four milliliters of Gey's balanced salt solution (Gibco). One milliliter of adenoviral vector suspension was added to give a ratio of 10^8 viral particles per million cells. The cells were incubated for three hours, with gentle shaking every thirty minutes. After the three hours of incubation, three milliliters of fresh medium was added and the cells were returned to the incubator.

Retroviral transduction: Confluent cultures were trypsinized and subcultured at a 1:3 split ratio. When the first-passage cultures were 60 percent confluent, the medium was removed, the cells were washed with Gey's balanced salt solution as described, and one milliliter of retroviral vector suspension was added in the presence of eight micrograms of polybrene (Sigma Chemical, St. Louis, Missouri) per milliliter. This provided a ratio of 5×10^6 viral particles per million cells. The cells were incubated for three hours, with gentle shaking every thirty minutes. After the three hours of incubation, three milliliters of fresh medium was added and the cells were returned to the incubator. When the cells were confluent, they were trypsinized and replated and 0.25 milligram of G418 (Sigma) per milliliter was added into the culture medium to select for transduced cells[11]. Cells that expressed the neomycin phosphotransferase gene contained in the retrovirus were resistant to the cytotoxic actions of G148. To detect lacZ gene expression by the transduced cells, the cultures were histochemically stained blue with the chromogenic substrate 5-bromo-4-chloro-indolyl-β-D-galactose (X-gal; Sigma), as described previously[16], at every passage. The percentage of $lacZ^+$ cells was determined by scoring cells as positive (blue) or negative (colorless) histologically. At least 100 cells were scored in each case.

In Vitro *Gene Transfer to Menisci*

Whole lapine menisci (four, five, or six per dish) and human meniscal fragments (two to five millimeters; eight, nine, or ten per dish) were placed in sixty-millimeter Petri dishes that contained five milliliters of the culture medium described earlier.

For direct gene delivery, 0.1 milliliter of the adenoviral vector suspension was injected into the menisci or meniscal fragments at four locations with use of a one-milliliter syringe and a 25-gauge needle. Four menisci or meniscal fragments were injected at each time-point (at one, two, eight, twelve, sixteen, and twenty weeks after the gene transfer).

For indirect gene delivery to the menisci or meniscal fragments, cultured meniscal cells were transduced with the retrovirus containing the lacZ and neomycin phosphotransferase genes and were selected

in G418 as described earlier. Monolayer cultures of cells were then trypsinized and centrifuged at 2000 revolutions per minute for ten minutes. The cells were collected and were suspended in 0.5 milliliter of Ham's F-12 medium with 10 percent fetal bovine serum and 1 percent penicillin and streptomycin to a final concentration of 10^6 cells per milliliter. One hundred microliters (10^5 cells) of the cell suspension was injected at multiple locations into the procured meniscal tissue with a one-milliliter syringe and a 25-gauge needle.

After the injections, the intact lapine and fragmented human menisci were placed in Petri dishes that contained the culture medium that lacked G418, and they were maintained in the incubator. The meniscal tissues were fixed sequentially, at one, two, four, eight, twelve, sixteen, and twenty weeks after the gene transfer, with 0.01-molar phosphate-buffered saline solution (Sigma) containing 4 percent paraformaldehyde (Fisher Scientific, Fair Lawn, New Jersey). The meniscal tissue was stained with X-gal to detect gene expression by the transduced cells. The stained menisci were then cut along the transverse plane through the site of the injection. Tissue specimens were dehydrated and embedded in paraffin wax. Paraffin sections were cut at a thickness of five micrometers and were affixed to glass slides. Deparaffinized and hydrated sections were stained with eosin as a counterstain for X-gal.

In Vivo *Gene Transfer to Operatively Created Meniscal Lesions*

Adenovirally Mediated ex Vivo *Gene Transfer to Lapine Meniscal Lesions*

Six New Zealand White rabbits, which weighed approximately 2.5 kilograms, were given anesthesia. Arthrotomies were performed on both knees under sterile conditions, and the medial menisci were exposed. A two-millimeter-long incision was made in the avascular area of the anterior part of the medial meniscus. A fibrin clot that had formed after 0.5 milliliter of whole blood was mixed with 0.3 milliliter of adenoviral suspension was transplanted into the incised meniscal lesion. A fibrin clot that did not have the viral vector was transplanted into the lesion in the contralateral knee to serve as a control. After the clot had been inserted, the capsule was closed carefully and the skin was closed with a subcuticular stitch. The animals were mobilized postoperatively and were given a standard diet. Two rabbits each were killed at one, two, and three weeks after the operation. The menisci were obtained, fixed with 0.01-molar phosphate-buffered saline solution that contained 4 percent paraformaldehyde, and stained with X-gal. The stained menisci were cut along the transverse plane through the tear and were embedded, sectioned, and stained as described.

TABLE I

LacZ Expression by Meniscal Cells After Retroviral Transduction *in Vitro*

Conditions*	Percentage of $LacZ^+$ Cells		
	Lapine Menisci	Canine Menisci	Human Menisci
48 hrs., unselected	20	18	10
1 wk., selected in G418	95	98	93
2 wks., selected in G418	98	95	98
4 wks., selected in G418	90	93	95
28 wks., selected in G418	93	98	92

*The time-periods refer to the interval after transduction.

Retrovirally Mediated ex Vivo *Gene Transfer to Canine Meniscal Lesions*

Canine meniscal cells were transduced with retrovirus that contained the lacZ and neomycin phosphotransferase genes and were selected in G418. Staining with X-gal before implantation into a defect demonstrated that nearly 100 percent of the cells were transduced. After confluence, they were trypsinized and centrifuged at 2000 revolutions per minute for ten minutes. The cell pellet was incorporated in a collagen gel suspension. To accomplish this, eight volumes of purified type-I collagen solution (Vitrogen 100; Celtrix, Santa Clara, California), one volume of ten-times-concentrated phosphate-buffered saline solution, and one volume of 0.1-molar sodium hydroxide were added, with gentle agitation, at 4 degrees Celsius, and the pH of the solution was adjusted to 7.2 ± 0.2. Finally, one volume of cell suspension was added to the neutralized, isotonic collagen mixture and was incubated in a culture dish at 37 degrees Celsius for twenty-four hours to gel the collagen (final concentration, 1.8×10^6 cells per milliliter; 0.21 percent collagen)[10,23].

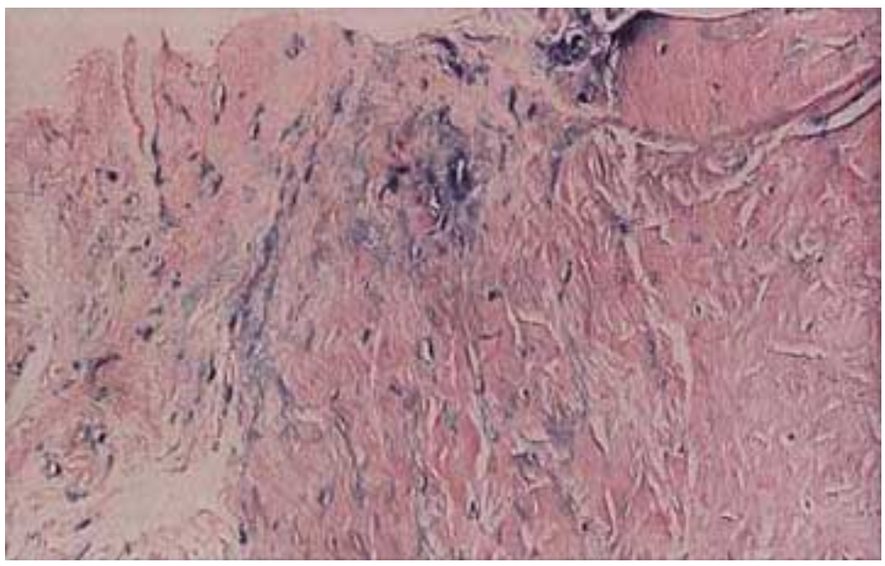

FIG. 1

Horizontal section of a human meniscus two weeks after an adenoviral vector containing the lacZ gene was injected directly into the meniscus *in vitro* (X-gal and eosin, × 200). High levels of gene expression are evident (blue areas).

Twelve adult mongrel dogs that weighed twenty-five to thirty kilograms were used. All of the dogs had a normal gait and a full range of motion of the limbs. General anesthesia was given and endotracheal intubation was used. Inhalation anesthesia was maintained with a mixture of isoflurane, nitrous oxide, and oxygen. The hindlimbs were shaved, prepared with iodine, and draped in a sterile fashion. A medial parapatellar skin incision was made through the subcutaneous tissue down to the joint capsule. The capsule was dissected to expose the medial collateral ligament. This ligament was detached at the femoral insertion and was reflected distally to expose the medial meniscus. A partial-thickness circular defect, two millimeters in diameter and three millimeters deep, was made three millimeters from the meniscosynovial junction in the middle third of the meniscus. The collagen gel with the transduced canine cells was implanted into the defect. A collagen gel without cells was implanted in the contralateral meniscus to serve as a control. After transplantation, the medial collateral ligament was reattached at the site of its original insertion with interosseous sutures. The capsule was closed carefully, and the skin was closed with a subcuticular stitch. The animals were mobilized postoperatively, given a standard diet, and allowed to walk outside the cages at one week after the operation. LacZ gene expression in the menisci was evaluated at one, two, four, and six weeks postoperatively. Three animals were killed at each time-period. The menisci were obtained, fixed with 0.01-molar phosphate-buffered saline solution that contained 4 percent paraformaldehyde, and stained with X-gal. The stained menisci were cut along the radial plane through the defect and were embedded, sectioned, and stained as described earlier.

Results

Our studies of transfer of the lacZ marker gene to the meniscus comprised three parts: *in vitro* transduction of cultured cells with adenoviral and retroviral vectors, direct and indirect *in vitro* delivery of the lacZ marker gene with adenovirus and retrovirus to procured menisci, and *ex vivo* delivery of the lacZ marker gene with adenovirus and retrovirus to operatively created meniscal lesions in the knee joints of rabbits and dogs.

TABLE II

LACZ EXPRESSION BY MENISCAL CELLS AFTER ADENOVIRAL TRANSDUCTION *IN VITRO*

Conditions*	Percentage of $LacZ^+$ Cells		
	Lapine Menisci	Canine Menisci	Human Menisci
48 hrs.	90	95	95
4 wks., passaged	12	15	18
20 wks., unpassaged	90	88	85

*The time-periods refer to the interval after transduction.

In Vitro *Gene Transfer to Monolayer Cultures*

The tranduction of lapine, canine, and human meniscal cells was first accomplished with a retrovirus capable of expressing the lacZ and neomycin phosphotransferase genes simultaneously.

After the lapine, canine, and human meniscal cells were transduced with this retrovirus, expression of the lacZ gene was seen initially in 20, 18, and 10 percent of cells, respectively (Table I). Selection of transduced cells in G418 increased the percentage of transduced cells to nearly 100 (Table I). Transgene expression after transduction with the retrovirus and selection in G418 was stable and lasted twenty-eight weeks *in vitro*, at which time the experiment was terminated. During this twenty-eight-week period, the cells were taken through approximately fifteen passages, without reduction in the percentage of cells expressing lacZ. These experiments were repeated five times with the same results.

Lapine, canine, and human meniscal cells were also transduced with an adenovirus carrying the lacZ gene. At a multiplicity of infection of fifty, nearly 100 percent of the cells of each species expressed the lacZ gene after

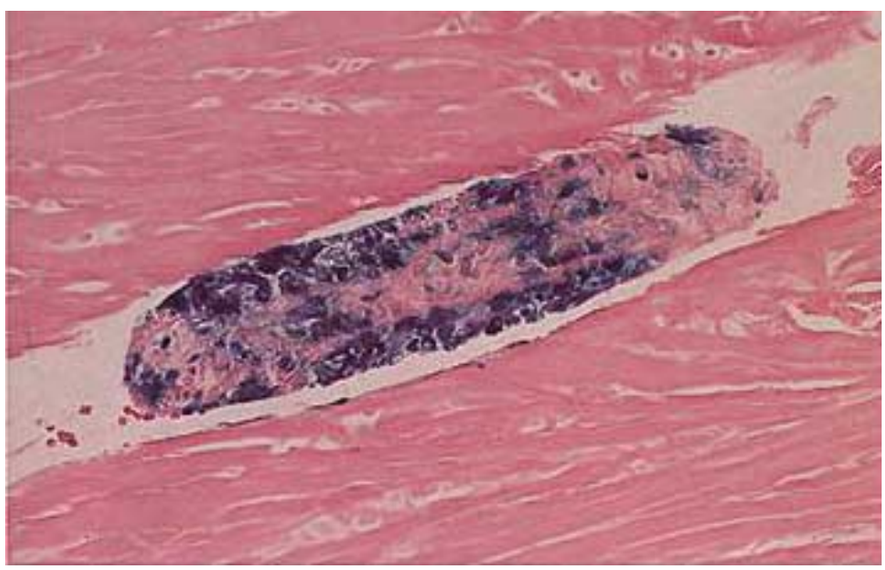

FIG. 2

Horizontal section of a lapine meniscus three weeks after an adenoviral vector containing the lacZ gene was incorporated into a fibrin clot and then implanted into a meniscal tear (X-gal and eosin, × 200). Cells within the fibrin clot have expressed the transgene (blue areas).

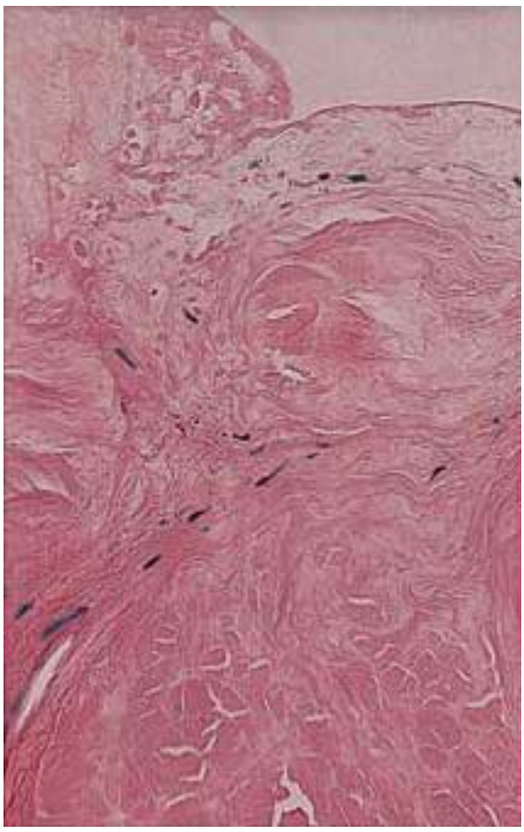

FIG. 3-A

Figs. 3-A through 3-E: Radial sections of canine menisci after retrovirally mediated *ex vivo* gene transfer to an operatively created defect (and control specimens) (X-gal and eosin).

Fig. 3-A: Two weeks after gene transfer, there was a large number of $lacZ^+$ cells (stained blue) in the defect (× 200).

transduction with this virus (Table II). Gene expression after adenoviral transduction was stable for all species for twenty weeks in confluent, nondividing cultures, but, unlike expression after retroviral transduction, it was not stable on passage (Table II).

In Vitro *Gene Transfer to Menisci*

Direct Gene Transfer

Direct retroviral transfer was not attempted because retroviral vectors only transduce dividing cells.

Whole lapine menisci and fragments of human menisci were used to assess the feasibility of direct viral gene transfer with the adenoviral vector carrying the lacZ gene. *In vitro* injection of this vector directly into lapine menisci led to the transduction of cells adjacent to the track of the needle but not elsewhere, suggesting that the dense, meniscal matrix impeded diffusion of the virus. Human meniscal cells were also transduced after direct injection of adenoviral vectors (Fig. 1). High levels of gene expression persisted in both lapine and human menisci for twenty weeks *in vitro*, at which time the experiment was discontinued. In general, injection of the adenovirus vector carrying the lacZ gene into human menisci transduced cells within a wider radius than did injection into lapine menisci.

Indirect Gene Transfer

LacZ gene expression was detected within lapine and human menisci by X-gal-staining at all time-points during the twenty weeks after the injection of allogenic cells transduced with the retrovirus carrying the lacZ and neomycin phosphotransferase genes.

In Vivo *Gene Transfer to Operatively Created Meniscal Lesions*

Ex Vivo *Gene Transfer with a Fibrin Clot*

Histochemical analysis of retrieved lapine menisci confirmed that the virus had succeeded in transferring the lacZ gene to cells within the clot and also to certain meniscal cells lining the lesion. Expression of the lacZ gene persisted in these tissues for three weeks (Fig. 2), at which time the experiment was discontinued.

Ex Vivo *Transfer of Transduced Meniscal Cells in Collagen Gel*

Transplanted cells were detected by X-gal-staining of the tissue in the defect and the meniscosynovial junction near the defect at one, two, four, and six weeks

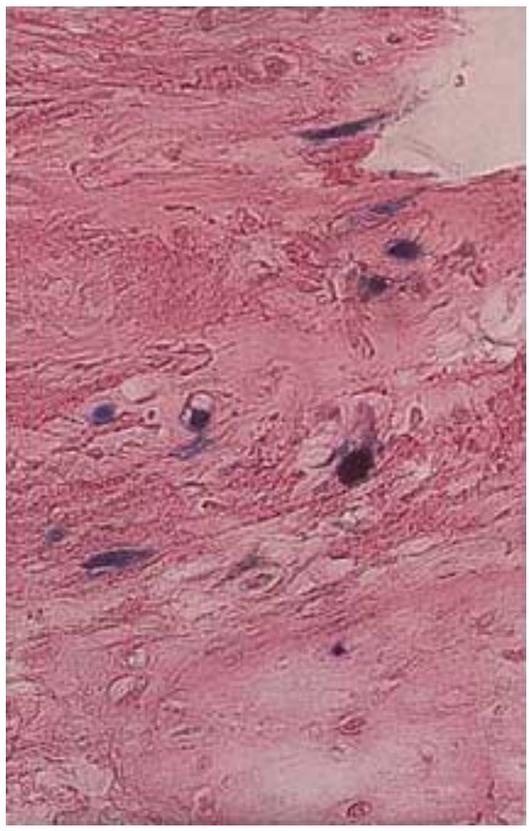

FIG. 3-B

Four weeks after gene transfer, there were $lacZ^+$ cells (stained blue) embedded in a dense fibrous tissue in the defect (× 400).

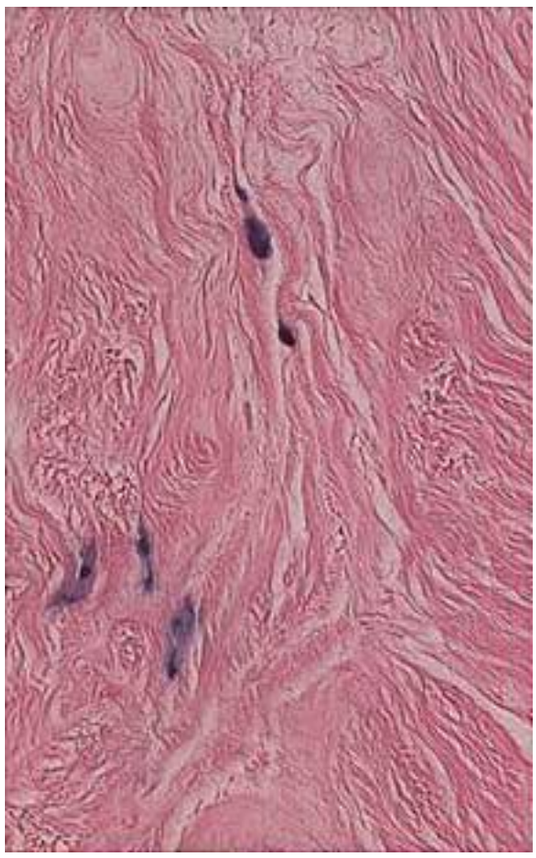

FIG. 3-C

Six weeks after gene transfer, there were lacZ+ cells (stained blue) in the defect (× 400).

after implantation (Figs. 3-A, 3-B, and 3-C). The cells detected in the fibrous tissues appeared fibroblastic, whereas cells that resembled fibrochondrocytes could be identified in the body of the meniscus (Fig. 3-A). Transduced allografted cells were detected in lesions six weeks after the operation (Fig. 3-C). Control defects in which no cells had been implanted showed no staining at any time postoperatively (Figs. 3-D and 3-E). The operatively created defects were filled with fibrous tissue that contained fibroblastic cells and inflammatory cells.

Discussion

The results of the present study confirm that, although a meniscal defect becomes filled with fibrous tissue containing fibroblasts by six weeks postoperatively, this tissue is histologically different from normal meniscal tissue. This finding agrees with that of Arnoczky et al.[2], who showed that a similarly sized, full-thickness defect in a canine meniscus filled with a fibrin clot was still morphologically different from the adjacent meniscal tissue at three months.

Port et al.[15] evaluated healing biomechanically after fibrin clots and cultured autogenous marrow cells were placed in operatively created, full-thickness meniscal lesions in goats. They found no enhancement in healing at four months. Their results demonstrated that the fibrin clot, even though it contains chemotactic and mitogenic factors as well as additional cellular components, could not induce complete healing of caprine meniscal lesions. This finding suggests that a single application of the growth factors present in fibrin clots may not promote healing, a circumstance that could reflect the necessity for growth factors to be present within the lesion for an extended period of time. However, with existing technology, high concentrations of growth factors cannot be delivered to sites of meniscal damage in a sustained fashion. In an effort to solve this problem, two of us (P. D. R. and C. H. E.) are developing gene-transfer techniques[5] that could be used to promote the sustained, localized production of growth factors within the meniscal lesion in order to improve healing.

The present study confirms that gene transfer to meniscal cells is feasible in lapine, canine, and human menisci. With use of the lacZ gene as a marker, we showed that both adenovirally and retrovirally mediated

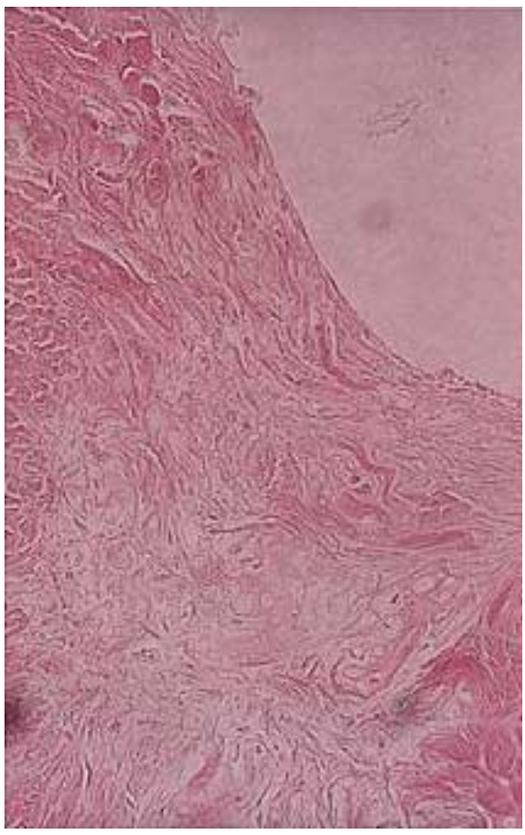

FIG. 3-D

A control specimen in which collagen gel without cells had been implanted (× 200). Two weeks postoperatively, the defect was filled with inflammatory cells and fibroblasts in a sparse fibrous tissue. No background staining was seen after treatment with X-gal, and thus no lacZ+ cells were present.

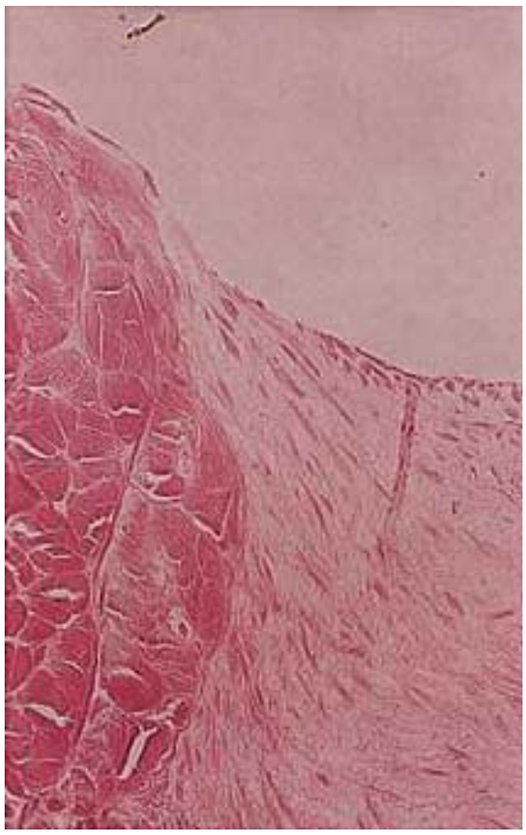

FIG. 3-E

A control specimen in which collagen gel without cells had been implanted (× 200). Six weeks postoperatively, the defects were filled with fibrous tissue that contained fibroblastic cells, and thus the material filling the defect was histologically different from normal meniscal tissue.

gene transfer can maintain gene expression for at least twenty weeks *in vitro*.

Because the *in vitro* data suggested that *ex vivo* gene delivery to a meniscus is more efficient than *in vivo* gene delivery, the former strategy was employed for gene transfer to operatively created meniscal lesions in experimental animals. Since adenovirus infects nondividing cells, it was possible to utilize this vector in a fibrin-clot technique. The adenoviral vector suspension and the fibrin clot were mixed before implantation into the meniscal lesion. Although few meniscal cells were transduced by this method, cells trapped within the clot expressed the transgene very efficiently and could serve as a local source of growth-factor synthesis. In this sense, the identity of the transduced cells within the clot is not critical, provided that they transduce and express the transgene efficiently. An advantage of the fibrin-clot method is that fibrin clots are already used in operative meniscal repairs, and thus they lend themselves to early use as an adjunct to gene transfer in humans. Moreover, unlike the method involving use of retroviruses, this method does not require cell culture. Adenovirally mediated gene delivery combined with the fibrin-clot technique was successful for delivery of the lacZ gene to the meniscal lesion, with gene expression persisting within the clot for the entire three-week period of the experiment.

Retroviruses do not transduce nondividing cells, such as those found in fibrin clots, but they proved to be effective mediators of gene transfer to meniscal cells under *in vitro* conditions in which the cells divide. Moreover, because retroviruses insert their genetic material stably into the chromosomal DNA of the cells that they infect, there is no dilution of the transferred genes during cell division and it is possible to select for cells expressing the genes of interest. For *ex vivo* gene delivery with retroviruses, cells were transduced, selected in G418, and embedded in collagen gels before transplantation and insertion into the meniscal defect. This technique was efficient not only in preventing the cells from migrating from the meniscal lesion but also in maintaining the morphology of the transferred meniscal cells. The collagen gel used in this study contained 95 to 98 percent type-I collagen, with the remainder being type-III collagen. Therefore, this collagen-and-cell mixture could be a suitable scaffold for maintaining the integrity of meniscal tissue after transplantation to the lesion. Transgene expression persisted for six weeks after allotransplantation of the retrovirally transduced cells into partial-thickness meniscal defects.

The three to six-week duration of gene expression in the meniscal defects noted in the present study may be long enough to enhance healing if a therapeutic gene is utilized. Meniscal cells are known to respond to exogenously supplied growth factors[3,9,21], and articular chondrocytes have been shown to respond to transfer of a transforming-growth-factor gene by increasing matrix synthesis[1,18]. This permits cautious optimism regarding the possibility that these techniques could form the basis of a gene-transfer approach to the repair of meniscal lesions.

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