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HVJ-Liposome-Mediated Gene Transfer to Healing Rat Meniscal Tissue*

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Abstract : The success rate of a repaired torn meniscus is not more than 70%. Gene transfer to healing soft tissues appears to be a feasible method for manipulating the healing process. In an attempt to evaluate the feasibility of gene transfer to meniscal tissue we tried to transfer a reporter gene (*Echerichia coli,* β-galactosidase gene) by using HVJ-liposome mediated gene transfer.

After performing complete radial tear on the medial meniscus of fifty 14-week-old male Wistar rats, HVJ-liposomes with DNA were injected into the femoral artery of 15 rats and into the knee joint of the other 15 rats. HVJ-liposomes without DNA were injected in the same way into 10 rats in each group as controls. Three rats from each experimental group and two rats from each control group were sacrificed 3, 7, 14, 28 and 56 days after the injections.

After X-gal staining the efficiency of transfection was estimated as 2.4% on day 3, 4.8% on day 7, 4.9% on day 14, 0.9% on day 28 and 0.3% on day 56 in the intra-arterially injected group. The same ratios for the intra-articularly injected group were 2.8%, 4.7%, 5.1%, 0.7% and 0.3% on post-injection days 3, 7, 14, 28 and 56 respectively. In all control sections of both groups the blue-stained cells were few at any point.

In conclusion we succeeded in introducing a reporter gene to healing rat meniscal tissue both by intra-arterial and intra-articular injection of HVJ-liposomes. We believe that particularly the intra-articular injection model appears to have the potential to be applicable in healing studies of intra-articular tissues within the knee joint.

Key Words: Gene therapy, hemagglutinating virus of Japan, meniscal healing, liposome

Introduction

The importance of preserving meniscal tissue has become increasingly apparent. Long-term follow-up studies showed that total and partial meniscectomies were the reason for degenerative changes within the knee joint (1-3). Meniscal suturing and more recently allograft meniscal transplantation and prosthetic meniscal replacement are recommended procedures for appropriate meniscal tears (4-7). However, the success of complete meniscal healing after meniscal sutures was not more than 50% (6). The use of exogenous fibrin clot was shown to significantly increase the healing potential of the meniscus but the rate of complete healing is still not more than 70% (7). There are not any long-term results of meniscal allograft transplantation and prosthetic meniscal replacement.

There is still a need for new procedures to enhance the healing potential of meniscal tissue. Manipulation of soft tissue healing has been a major focus of orthopedic research. Application of exogenous growth factors has been demonstrated to accelerate and modify skin wound healing in recent years (8-10). In meniscal tissue it was shown that the use of fibrin sealant and endothelial growth factor enhanced the neovascularization and formation of granulation tissue, which probably caused the greater healing level of the avascular portion of the meniscus (11). However, there have been no effective growth factor delivery methods developed for healing meniscal tissue. Gene transfer to the healing meniscus appears to be a feasible method for manipulating the healing process (9). The product of the transferred gene should accumulate at the injured site as long as the gene is expressed by the transfected cells.

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We previously demonstrated successful gene-transfer to healing rat patellar ligament by direct injection to the wound and by intra-arterial delivery of hemagglutinating virus of Japan (HVJ) conjugated liposomes (12-14). In this study we investigated the feasibility of an intra-arterial and intra-articular delivery of a reporter gene in HVJ-liposomes to healing rat meniscal tissue.

Materials and Methods

Construction of plasmid and preparation of HVJ-liposomes: *Escherichia coli* β-galactosidase (β-gal) cDNA driven chicken β-actin promoter and HVJ-liposomes were prepared as described previously (12,15). In short, 10 mg of dried lipid mixture (phosphatidylcholine, phosphatidylserine and cholesterol) and plasmid DNA, pre-incubated with high mobility group-1 (HMG-1) protein, were agitated and sonicated for preparation of unilamellar liposomes. Then the liposomes and ultraviolet inactivated HVJ were mixed to form HVJ-liposomes. Plasmid DNA and HMG-1 nuclear protein were capsulated into the liposomes with inactivated HVJ envelopes on their surfaces. Approximately 50 μg of plasmid DNA was entrapped in liposomes.

Surgical model and injection of HVJ liposomes: The rats were anesthetized by intraperitoneal injection of pentobarbital (50 mgr/kg). Complete radial tear was performed on fifty 14-week-old male Wistar rats, on the medial site of the medial meniscus of the right knee with a scalpel under a microscope. The rats were divided into 2 groups equally.

In the first group, three days after the injury HVJ-liposomes suspensions including 25 μg of plasmid DNA were injected into the right femoral artery of 15 Wistar rats using a 29G needle, after flushing with 1 ml of saline physiologic solution, under a microscope. The femoral artery and vein were clamped and solution was pooled for about 5 minutes in the lower extremity. For the control study, HVJ-liposomes without DNA were injected into the right femoral artery of 10 Wistar rats by the same method.

In the second group, three days after the injury, HVJ-liposomes suspensions including 25 μg of plasmid DNA were injected into the right knee of 15 Wistar rats. For the control study, HVJ-liposomes without DNA were injected into the right knee of 10 Wistar rats by the same method.

The animals were allowed normal cage activity and were sacrificed 3, 7, 14, 28, 56 days after the injection. Three positive and two control solution injected rats were sacrificed on each of these days from each intra-arterially and intra-articularly injected group.

Detection of β-gal expression: Immediately after sacrifice the whole medial meniscus of the right knee was excised and fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4). After fixation cryosections (6 μm thick) were prepared. The sections were stained with 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal) (16). The staining was performed overnight, at 37° (pH 7.4).

Evaluation of efficiency of transfection: To evaluate the efficiency of transfection X-gal stained sections were further subjected to hematoxylin nuclear staining for cell counting. Efficiency was calculated as the ratio of β-gal expressing cells to 1000 randomly counted cells (in 10 different fields) by light microscope in the wound of each rat.

Characterization of β-gal expressing cells: To determine approximately whether β-gal expressing cells are fibroblastic or monocyte/macrophage linage cells, cryosections of the meniscal tissue on day 14 were subjected to double immunofluorescence staining for type I collagen aminopropeptide and monocyte/macrophage antigen. Polyclonal antibody to bovine aminopropeptide of collagen type I (pN collagen I) (provided by Dr. Rupert Timpl, Max Planck Institude, Munich, Germany) and monoclonal antibody to rat monocyte/macrophage (ED-1) (Serotec Ltd., Oxford, United Kingdom) were used and sections were examined with a fluorescent microscope equipped with light microscope optics (Axiophoto, Carl Zeiss GmbH, Th¸ringen, Germany) as described previously (12). The data were obtained from three separate experiments for evaluation of 1000 cells in five different fields.

The protocol of this study was approved by the institutional animal care committee of Osaka University.

Results

In both groups β-gal expressing positively blue-stained cells were predominant around the wound. There were only very limited positively blue-stained cells seen on meniscal tissue other than the wound area (Fig. 1D). These blue stained meniscal cells were located near the

wound. In the intra-arterially injected group there were more blue-stained cells around the blood vessels on the menisco-capsular junction and around blood vessels in wound than in the intra-articularly injected group (Fig. 1A). In the intra-articularly injected group there were more blue-stained cells near the rim of the wound than in the intra-arterially injected group (Fig 1C).

The efficiency of transfection was estimated to be 2.4% ± 0.178% on day 3, 4.8% ± 0.214% on day 7, 4.9% ± 0.277% on day 14, 0.9% ± 0.076% on day 28 and 0.3% ± 0.044% on day 56 in the intra-arterially injected group (Fig. 2). The same ratios for the intra-articularly injected group were 2.8% ± 0.212%, 4.7% ± 0.261%, 5.1% ± 0.289%, 0.7% ± 0.060% and 0.3% ± 0.028% on post injection days 3, 7, 14, 28 and 56 respectively (Fig. 2).

In all control sections of both groups the blue-stained cells were few at any point (Fig. 1B and E). The proportions of blue stained cells in the intra-arterially injected control group were 0.18% ± 0.040%, 0.12% ± 0.035%, 0.14% ± 0.030%, 0.11% ± 0.020% and 0.13% ± 0.020% on post-injection days 3, 7, 14, 28 and 56 respectively. The proportions of blue stained cells for the intra-articularly injected control group for the same post-injection days were 0.16% ± 0.045%, 0.14% ± 0.033%, 0.15% ± 0.038%, 0.19% ± 0.040% and 0.09% ± 0.022%.

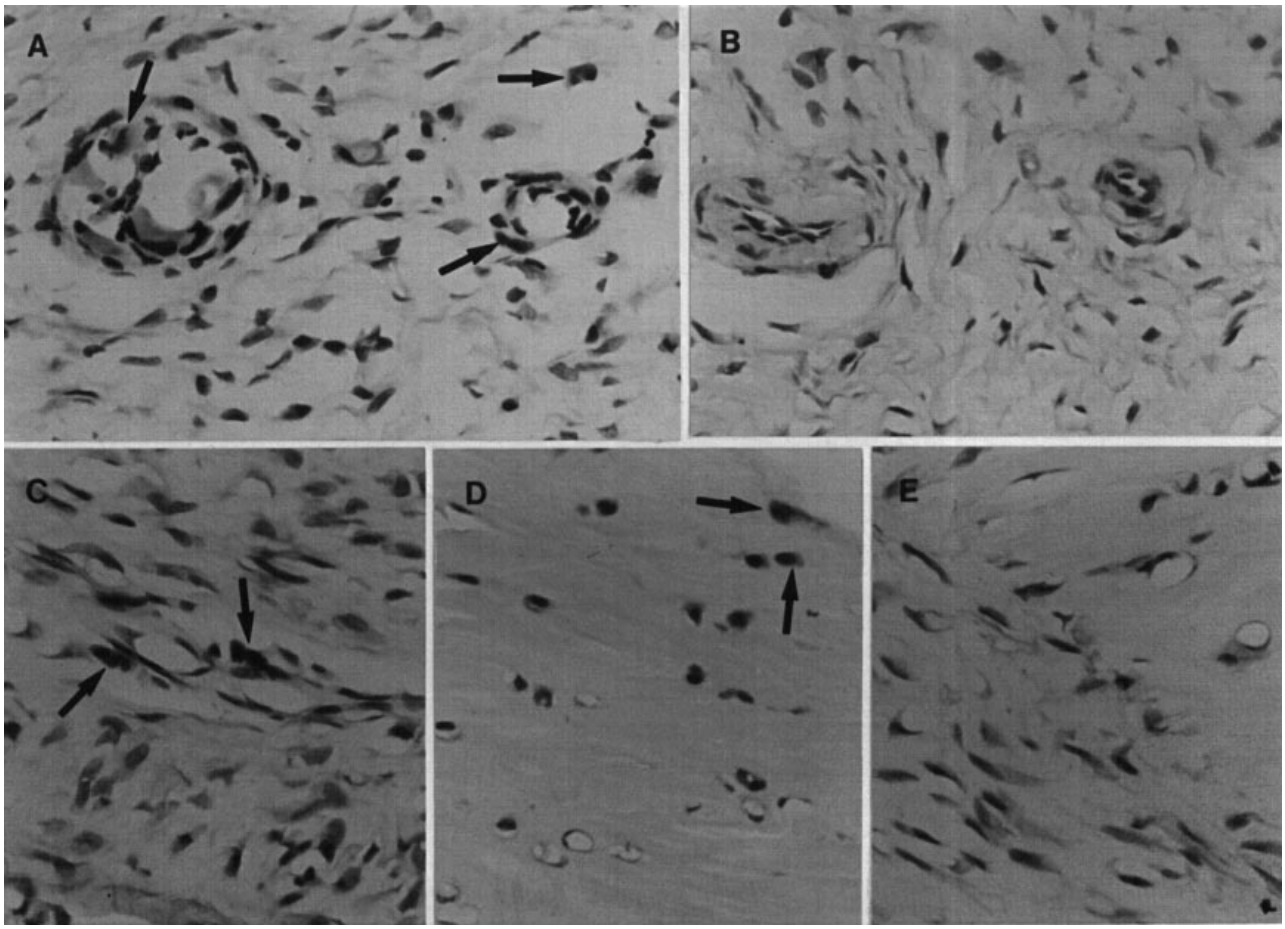

Fig. 1. Photomicrography of the X-gal and haematoxylin stained healing rat meniscal tissue. Positively blue stained cells around blood vessels on day 7 sections of intra-arterially injected study group (A) (x 400), and day 7 control section for intra-arterial injection group (B) (x400). A day 14 section, blue stained cells in the wound area (C) (x400) and in the meniscus tissue (D) (x400) near the joint space in intra-articularly injected study group. Control section for intra-articular injection on day 14 (E) (x400). (Arrows show positively blue-stained cells.)

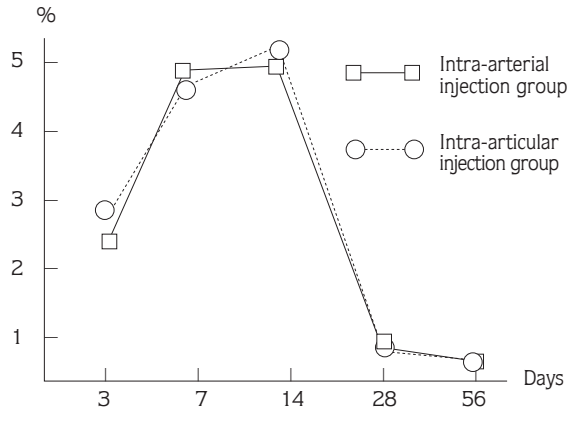

Fig. 2. The efficiency of transfection of healing meniscal wound. Ordinate, percentage of β-gal labeled cells, abscissa, time (days).

To identify the cell types of the β-gal expressing cells in the gene transferred wound, β-gal stained sections on day 14 were also subjected to double labeling for pN collagen I and ED-1. On the basis of the results, the β-gal expressing cells were classified into two types: fibroblastic cells showing ED-1 negative and pN collagen I positive cells (Fig. 3A, B, C), and monocyte/macrophage lineage cells showing ED-1 positive and pN collagen I negative cells (Fig. 3D, E, F). According to this classification the fibroblastic cells accounted for 85% and monocyte/macrophage linage cells for 8% of all wound cells on day 14. The fibroblastic cells were counted as 51% and monocyte/macrophage cells were counted as 43% of the β-gal expressing cells in the wound. The ratio for fibroblastic and monocyte/macrophage lineage cells was not different for intra-arterially and intra-articularly injected groups. The above mentioned ratios were average ratios of both groups.

We did not observe any cytotoxic effect of the procedure on the cells of the wound or meniscal tissue by light microscopy.

Discussion

Gene transfer is a new form of therapeutic intervention at a molecular level with applications in many areas of medical treatment. It can be used to correct a genetic defect and it can also be used to deliver a very specific, biologically active agent at a particular place with the possibility of permanent, transient or inducible expression (17). In a previous study by our group it was shown that the introduction of a platelet-derived growth factor gene into healing rat patellar ligament has some biological effects (12). In this study, in vivo gene transfer to healing meniscal tissue is reported for the first time. Gene transfer to meniscal tissue has the potential of application to transfer some growth factor genes to the target tissue for modifying the quality and speed of the healing process.

The efficiency of gene transfer was highest on days 7 and 14 in both groups while it was near 0 on days 28 and 56. This shows a transient gene transfer to healing meniscal tissue like our previous studies in which transient gene transfer to healing rat patellar ligament was observed (12,14). When it is considered that the healing process in soft tissues lasts for a limited period, the transient gene transfer in these tissues is advantageous. In this study it was observed that most of the gene-transferred cells were in the wound area rather than meniscal tissue. To modify a wound healing by gene transfer the main target must be the wound area. We think that extracellular matrix composition and vascularity were the main reasons for the low gene transfer efficiency in this fibrocartilage tissue.

The efficiency of gene transfer by HVJ-liposomes did not differ between the intra-arterially and intra-articularly injected groups. One major concern about gene transfer by intra-arterial delivery is the possibility of transfecting cells in other vital organs. After the release of vascular clamping, the HVJ-liposomes solution is circulated systemically. HVJ-liposomes are mainly trapped by the liver and spleen when they are circulated systemically (R. Morishita, unpublished data). In our previous study we examined the X-gal staining of liver and kidney tissue after intra-arterial delivery of HVJ liposomes and observed no specific staining in these tissues (14). However, we believe that further investigations will be required to evaluate the systemic effects of intra-arterial delivery. The risk of systemic complications is very low in intra-articular injection as the knee is a closed cavity. Furthermore, multiple intra-articular injections to the knee to increase the efficiency of gene transfer can be easily performed. It seems that gene transfer to the meniscus and other intra-articular tissues of the knee by intra-articular injection of HVJ-liposomes has more advantages than intra-arterial delivery.

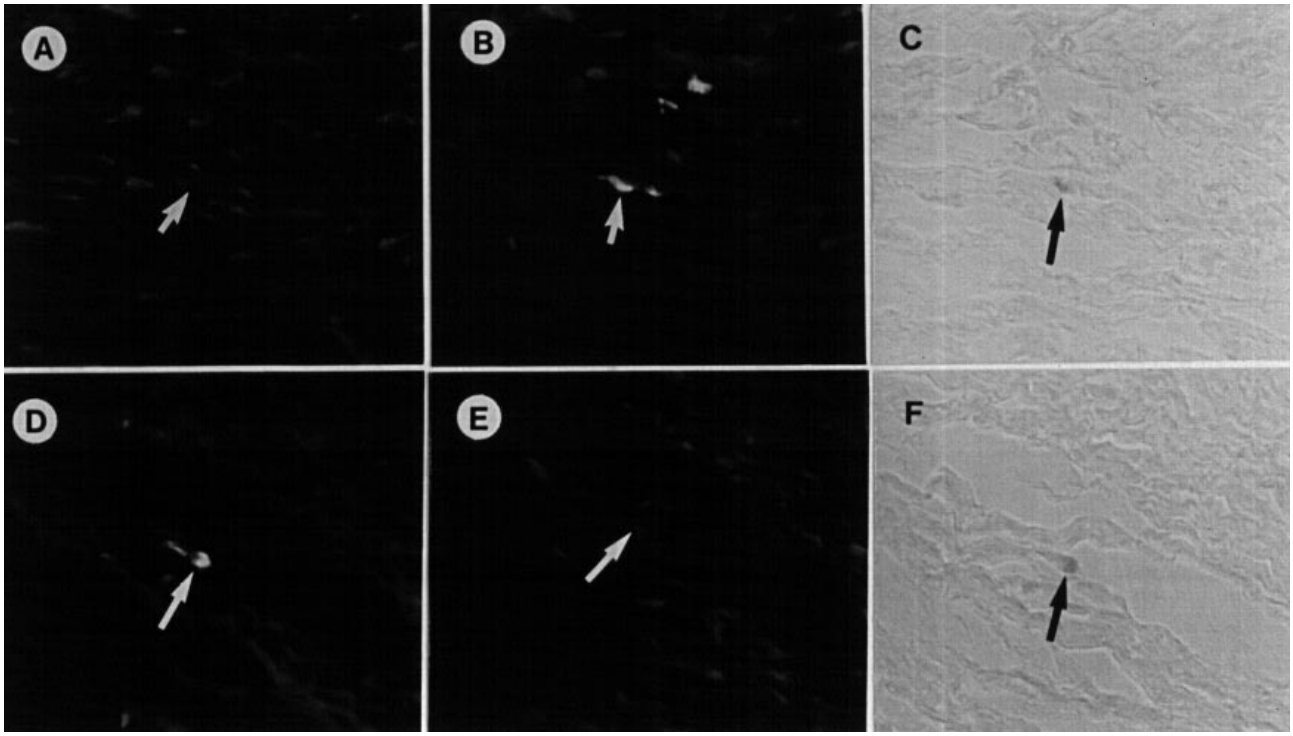

Fig. 3. Labeling for ED-1 (A and D), pN collagen I (B and E) hematoxylin (C and F) on day 14 sections (x600). A, B and C shows an example of a fibroblastic cells, which is negative for ED-1 (A), positive for pN collagen (B) and positive for X-gal (C). A monocyte/macrophage lineage cell which is positive for ED-1 (D), negative for pN collagen (E) and positive for X-gal (F).

Although monocyte/macrophage lineage cells constituted 8% of all the cells in the wound site, they accounted for about half of the transfected cells there. The high transfection rate to monocyte/macrophages was remarkable. The transfection rate of monocyte/macrophage lineage cells was nine times higher than that of fibroblastic cells. Similar findings were also observed in gene transfer to healing rat patellar ligament by direct injection (12). The phagocytosis of the encapsulated contents of liposomes by monocyte/macrophages in addition to the cell fusion mechanism may be the reason for this high rate of transfection.

In conclusion we succeeded in introducing a reporter gene to healing rat meniscal tissue both by intra-arterial and intra-articular injection of HVJ-liposomes. We believe that particularly the intra-articular injection model appears to have the potential to be applicable in healing studies of intra-articular tissues within the knee joint. To increase the efficiency of transfection and tissue specific gene transfer are the future directions of our study.

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