Transient Introduction of a Foreign Gene into Healing Rat Patellar Ligament

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Abstract

We investigated the in vivo introduction of a reporter gene into healing rat patellar ligaments using the hemagglutinating virus of Japan (HVJ)-liposome–mediated gene transfer method. The mid-portion of the medial half of the patellar ligament was cut transversely with a scalpel in 14-wk-old male Wistar rats. A HVJ-liposome suspension containing β-galactosidase (β-gal) cDNA was injected directly into the injured site and pooled in the fascial pocket covering the injured site 3 d postoperatively. Thereafter, β-gal–labeled cells were observed in the wound site accounting for 3% of the wound cells on the first day, 2% on the third, 7% on the seventh, 6% on the 14th, 2% on the 28th, and 0.2% on the 56th day after injection. The β-gal–labeled cells were initially localized in and adjacent to the wound site, but they were observed spreading into the ligament substance away from the wound on the seventh day after injection. On day 28, β-gal–labeled cells were observed throughout the length of the ligament substance. With double-labeling for marker antigens for monocyte/macrophage (ED-1) and for collagen I aminopropeptide (pN collagen I), it was revealed that fibroblastic (pN collagen I–positive) cells accounted for 63% and monocyte/macrophage lineage cells for 32% of the β-gal–labeled cells in the day 7 wound. On day 28, they formed 58 and 35% of the β-gal–labeled cells in the wound, respectively.

Thus, we succeeded in introducing the β-gal gene into healing rat patellar ligament. Moreover, labeling of the transfected cells made it possible to identify a biological event, namely that the cells in and around the wound site infiltrate into the uninjured ligament substance and come to populate the whole length of the ligament substance as repair progresses. These results suggest that ligament healing may involve not only the repair of the wound site itself but also extensive cellular infiltration of ligament substance adjacent to the wound. (*J. Clin. Invest.* 1996. 97:226–231.) Key words: gene transfer • ligament • wound healing • hemagglutinating virus of Japan • liposome

Introduction

Ligaments are dense fibrous tissue structures composed primarily of highly oriented and tightly packed collagen fibrils. Owing to such specialized matrix organization, ligaments possess large tensile strength and flexibility and are involved in stabilizing joints (1). At the same time, ligament injuries are one of the most common injuries to joints (2, 3), and significant joint instability due to loss of ligament function sometimes eliminates participation in competitive sports and may lead to degeneration of the affected joint (4). In this context, it is important to establish an optimal clinical treatment for ligament injuries.

The healing process of ligament tissue has been revealed to be analogous to that of other connective tissues, consisting of the inflammation, repair, and remodeling phases (5). However, in spite of advances in conservative and/or surgical treatment, it still takes longer for ligament to heal than for cutaneous tissue. In addition, repaired and remodeled injured ligament tissue is inferior to normal ligament tissue both biologically and biomechanically. Previous experimental studies demonstrated that ligament heals with the development of scar tissue. The healed tissue is therefore different from normal tissue in many aspects: elevated glycosaminoglycan content, decreased collagen content, different collagen constitution, abnormal collagen cross-linking, and inferior biomechanical properties (6, 7). Of these differences, the inferior recovery of biomechanical properties is clinically important. The tensile strength of injured skin reached ~90% of that of normal skin around 10 wk after injury (8), while the same value for injured medial collateral ligament of the knee was only 60% of normal ligament (6, 7). Taking into account that the major role of ligaments is to mechanically stabilize joints, such inferior mechanical properties of the healed ligament sometimes give rise to serious clinical concerns. Therefore, the establishment of a novel therapeutic approach for accelerating and improving ligament repair is needed.

Although the factors which improve the quality of scar tissue close to normal ligament tissue remain to be identified, a wealth of information from animal studies on cutaneous wound healing suggests the suitability of cytokine therapy for this purpose. Exogenous application of cytokines such as PDGF, TGF-β1, and basic fibroblast growth factor accelerates collagen synthesis, matrix organization, and the tensile strength of cutaneous wounds in vivo (9–14).

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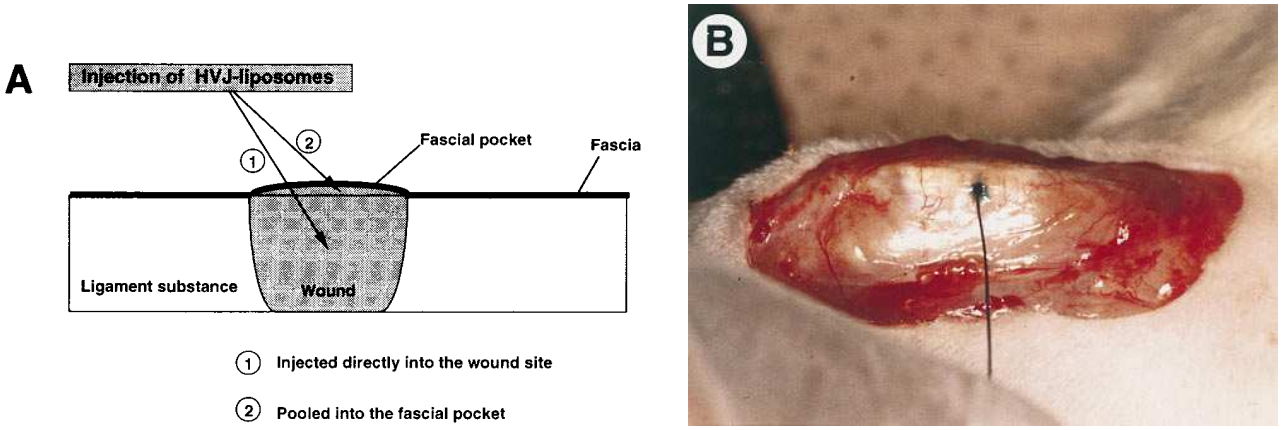

Figure 1. (*A*) A diagram showing the injection of the HVJ-liposomes into the healing ligament. They were directly injected into the wound as well as pooled into the fascial pocket. (*B*) The location of methylene blue dye at the time of injection of 10 μl into the wound and fascial pocket. The use of a fine 33-gauge needle ensured that the injected dye would surely bathe the wound site. The dye is also entrapped in the fascial pocket with minimum leakage.

Ligaments are subcutaneously located, so it is difficult to accurately identify the injured sites from outside the skin. Since repetitive surgical incisions for direct visualization of the wound sites are too invasive, and repetitive injections of specific molecules are not feasible for ligament healing models, it is important to establish a method to chronically express target molecules within the ligament wound. For this purpose, an in vivo gene transfer appears to be one of the best of several strategies for cytokine delivery. With this, the product of the transferred gene will accumulate at the injured site as long as the gene is expressed by the transfected cells. Recently, we have established a highly efficient method for in vivo gene transfer that involved the entrapment of DNA and nonhistone chromosomal protein within liposomes and the use of hemagglutinating virus of Japan (HVJ; Sendai virus)[1] to both enhance the fusion of liposomes to cell membranes (15) and to accelerate the transportation of DNA to the nucleus (16). Using this technique, we have succeeded in introducing foreign genes into several organs or tissues (17–21). In the present study, we used this technique for the introduction of a reporter gene into healing rat patellar ligaments.

Methods

Construction of plasmid. pAct-c-myb (a gift from Dr. S. Ishii, Institute of Physical and Chemical Research, Tsukuba, Japan), which contained the 5′-promoter region (370 bp) and the first intron (900 bp) of the chicken β-actin gene (22), was restricted with XhoI/BamHI and cloned into the SalI/BamHI site of pUC19. This plasmid (pUC-Act-c-myb) was restricted with NcoI/XbaI to remove c-myb and ligated with SalI linker (8-mer) (Takara Ltd., Kyoto, Japan). The *Escherichia coli* β-galactosidase (β-gal) gene (3.1 kb), isolated from pMC1871 (23) by restriction with SalI, was cloned into this site.

Preparation of HVJ-liposomes. HVJ-liposomes were prepared as described previously (16, 18). Briefly, 10 mg of dried lipid mixture (phosphatidylcholine, phosphatidylserine, and cholesterol) was hydrated in balanced salt solution (140 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.5) containing plasmid DNA and high mobility group-1 nuclear protein. The mixture was agitated and sonicated for preparation of unilamellar liposomes. The liposomes were then incubated with HVJ, which had been inactivated with ultraviolet irradiation, to allow formation of HVJ-liposomes. Thus, plasmid DNA and high mobility group-1 nuclear protein are capsulated into the liposomes with inactivated HVJ spikes on their surfaces.

Surgical model and injection of HVJ-liposomes. The medial half of the patellar ligaments of 18, 14-wk-old male Wistar rat was cut transversely at the center portion with a scalpel. HVJ-liposomes containing ~2–5 μg of β-gal DNA in a total volume of 10–15 μl were injected directly into the wound site and were also pooled into the fascial pocket (the pocket of the periligamentous tissue) 5 d after the injury by using a 33-gauge needle (Hamilton Co., Reno, NV) (Fig. 1 *A*). The methylene blue dye injection experiment was used to confirm that the injected solution bathed the wound site of the ligament (Fig. 1 *B*). During the first 24 h, we checked that the dye did not spread > 1 mm into the adjacent ligament substance. For the control study, HVJ-liposomes instilled in balanced salt solution were injected into the injured region.

The animals were allowed to return to normal cage activity and were killed at 1, 3, 7, 14, 28, and 56 d after injection. Three rats were killed on each of these days.

Detection of β-gal expression. Immediately after killing, the whole length of the patellar ligament was excised and was fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4). After fixation, cryosections (6 μm thick) were prepared. The cryosections were stained with 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-Gal) for identification of individual cells expressing β-gal (24). The staining was performed overnight at 30°C.

Evaluation of the efficiency of transfection. To evaluate the efficiency of transfection, β-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 1,500 randomly counted cells (by light microscopy in five different fields) in the wound of the dead rats. The data were obtained from three separate experiments.

Characterization of β-gal–expressing cells. To determine approximately whether β-gal–expressing cells are fibroblastic or monocyte/macrophage lineage cells, cryosections of the ligament on days 7 and 28 after injection were subjected to double-immunofluorescence staining for type I collagen aminopropeptide and monocyte/macrophage antigen. The cryosections were incubated with polyclonal antibody to bovine aminopropeptide of collagen type I (pN collagen I) (provided by Dr. Rupert Timpl, Max Planck Institute, Münich, Ger-

1. *Abbreviations used in this paper:* β-gal, β-galactosidase; ED-1, monoclonal antibody to rat monocyte/macrophage; HVJ, hemagglutinating virus of Japan; pN collagen I, aminopropeptide of collagen type I.

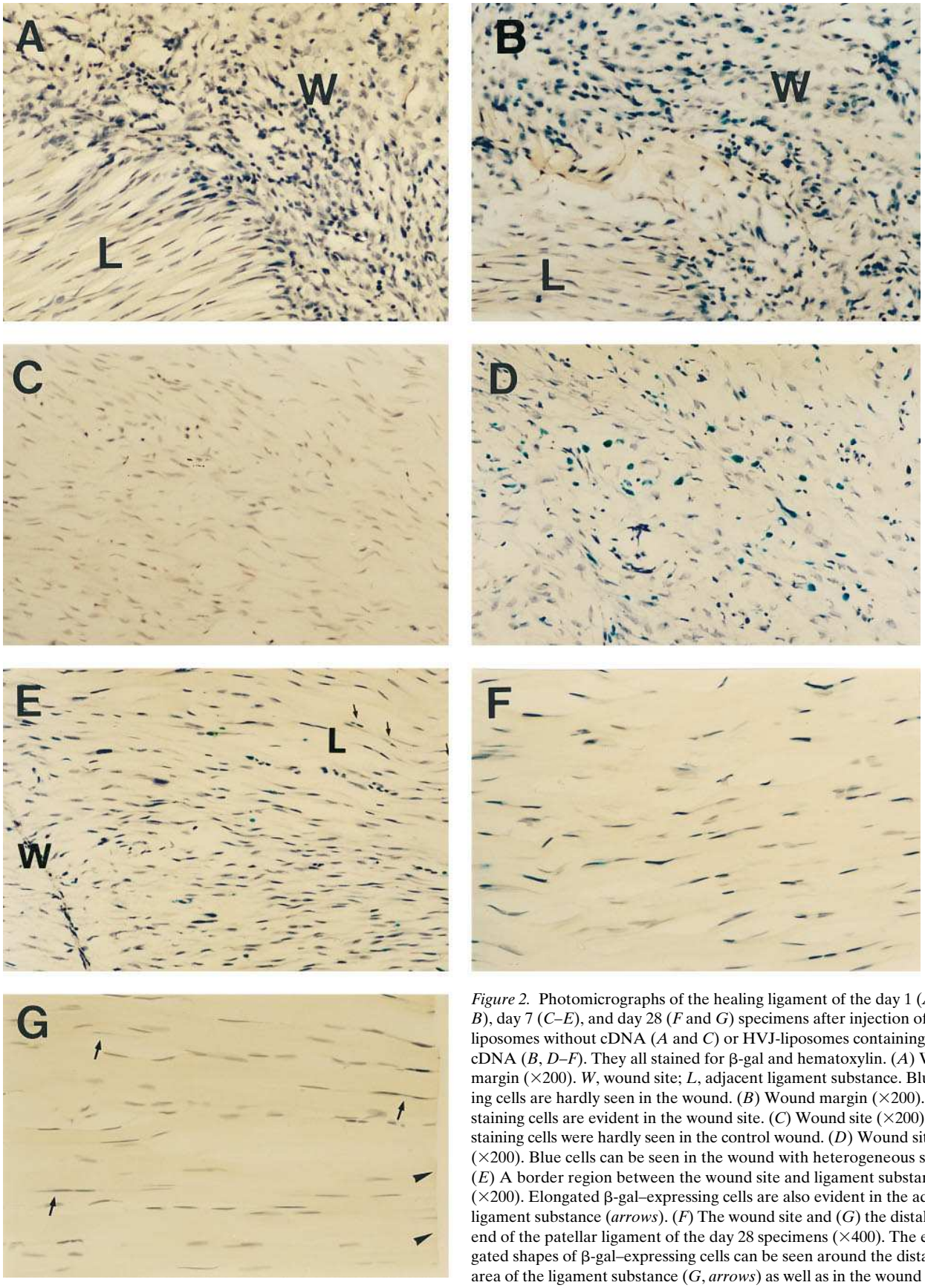

Figure 2. Photomicrographs of the healing ligament of the day 1 (*A* and *B*), day 7 (*C–E*), and day 28 (*F* and *G*) specimens after injection of HVJ-liposomes without cDNA (*A* and *C*) or HVJ-liposomes containing β-gal cDNA (*B*, *D–F*). They all stained for β-gal and hematoxylin. (*A*) Wound margin (×200). *W*, wound site; *L*, adjacent ligament substance. Blue staining cells are hardly seen in the wound. (*B*) Wound margin (×200). Blue staining cells are evident in the wound site. (*C*) Wound site (×200). Blue staining cells were hardly seen in the control wound. (*D*) Wound site (×200). Blue cells can be seen in the wound with heterogeneous shapes. (*E*) A border region between the wound site and ligament substance (×200). Elongated β-gal–expressing cells are also evident in the adjacent ligament substance (*arrows*). (*F*) The wound site and (*G*) the distal (tibial) end of the patellar ligament of the day 28 specimens (×400). The elongated shapes of β-gal–expressing cells can be seen around the distal end area of the ligament substance (*G*, *arrows*) as well as in the wound site (*F*). Arrowheads in *G* indicate the distal end of the patellar ligament.

many) and monoclonal antibody to rat monocyte/macrophage (ED-1) (Serotec Ltd., Oxford, United Kingdom) diluted in Tris-buffered saline (20 mM Tris-HCl, 0.9% NaCl, pH 7.6) overnight also at room temperature, after incubation with FITC-conjugated goat anti–rabbit IgG and rhodamine-conjugated goat anti–mouse IgG for 3 h at room temperature. The cross-reactivity of the antibody against pN collagen I to rat tissue was confirmed by immunoblotting (data not shown). The monoclonal antibody ED-1 recognizes mainly cytoplasmic and some surface antigens on most monocytes and macrophages in rat and appears to be related to phagocytosis (25). After extensive washing, labeled sections were further subjected to β-gal staining and, in addition, hematoxylin counter staining. The sections were examined with a fluorescent microscope equipped with light microscope optics (Axiophoto, Carl Zeiss GmbH, Thüringen, Germany). A comparison of the staining patterns of pN collagen I, monocyte/macrophage antigen, β-gal, and hematoxylin was used to determine whether β-gal–expressing cells were fibroblastic cells or monocyte/macrophage cells. The data were obtained from three separate experiments for evaluation of 1,500 cells in five different fields.

Results

On day 1 after injection, the wound space was filled with histologically hypercellular granulation-like tissue (Fig. 2, *A* and *B*, *W*). In the control experiments, blue staining was histochemically observed at only a negligible number of cells in the wound (Fig. 2 *A*, *W*). On the other hand, blue staining was observed in the cytoplasm of a larger number of the cells in the β-gal gene-transferred wound site. Most of them were ovoid and looked undifferentiated (Fig. 2 *B*, *W*). 7 d after injection, cell shapes in the wound became more heterogeneous. Blue staining was scant in the control wound (Fig. 2 *C*) but was observed in the cells with heterogeneous shapes, from ovoid to elongated, in the β-gal gene-transferred wound (Fig. 2 *D*). The clear difference in the appearance of blue staining between the control wounds and the β-gal gene-transferred wound on days 1 and 7 indicated that the majority of blue staining cells in the β-gal gene-transferred wound were transfected (β-gal–expressing) cells, and that nonspecific staining was negligible.

It is noteworthy that on day 7 the β-gal–expressing cells, which had been initially localized in and adjacent to the wound site, were observed as far away from the wound as approximately one-fourth the length of the ligament (Fig. 2 *E*, *arrows*). Thereafter, β-gal–expressing cells were seen to spread further into the ligament substance. 28 d after injection, β-gal–expressing cells in the wound site were mostly elongated (Fig. 2 *F*). At this time, β-gal–expressing cells were also observed throughout the length of the ligament substance (Fig. 2 *G*, *arrows*).

Efficiency of transfection in the wound site was 3% on day 1, 2% on day 3, 7% on day 7, 6% on day 14, 2% on day 28, and 0.2% on day 56 after injection (Fig. 3). Thus, β-gal gene products were shown to be present in the wound site ~1–2 mo after injection.

To roughly identify the cell types of the β-gal–expressing cells in the gene-transferred wound, β-gal–stained sections on days 7 and 28 after injection 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. 4, *A–C*,*), and monocyte/macrophage lineage cells showing ED-1–positive and pN collagen I–negative cells (Fig. 4, *D– F*, *arrow*). According to this classification, the fibroblastic cells accounted for 89% and monocyte/macrophage lineage

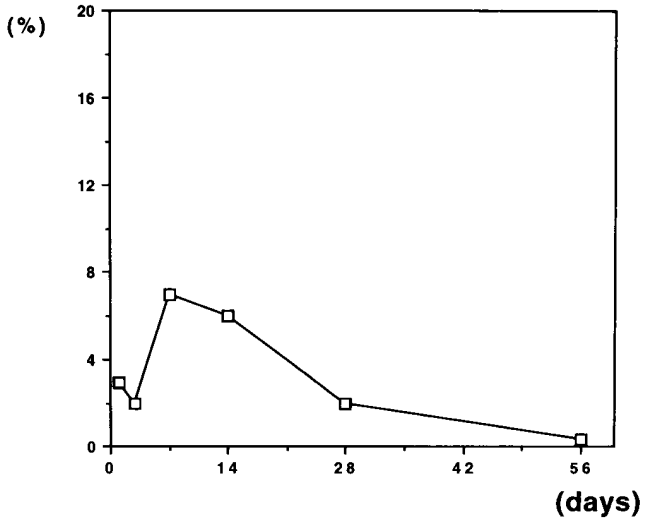

Figure 3. Time course changes in the efficiency of postinjection β-gal gene expression in the wound site; ordinate, percentage of β-gal–labeled cells in the wound; abscissa, time (days).

cells for 5% of all wound cells on day 7. Furthermore, the fibroblastic cells accounted for 63% and monocyte/macrophage cells for up to 32% of the β-gal–expressing cells in the wound. On day 28, the fibroblastic cells constituted 88% of all wound cells, and monocyte/macrophage lineage cells formed 5%, while the two types of cells accounted for 58 and 35%, respectively, of the β-gal–expressing cells in the wound.

Throughout the experiment, no evidence of any abnormal inflammatory, cytotoxic, or immunological reaction was detected in the patellar ligament or adjacent tissues.

Discussion

The HVJ-liposome method is a unique gene transfer method, characterized by safe and high efficiency of transfection with short incubation time, which avoids the risks of pathogenesis or cytotoxicity (16, 21, 26). However, similar to other gene transfer methods using liposomes, only transient gene expression can be expected from our method, because the transferred gene is rarely integrated into the host genome (15). This transient gene expression seems nevertheless to be advantageous for studies of wound healing and remodeling, because such processes are not long-lasting events in which permanent gene expression may carry the risk of inducing pathological conditions. Results of this study show that we have succeeded in using this characteristic vector for direct in vivo introduction of a foreign gene into a healing ligament. Although the efficiency of transfection in this study was modest, amounting to <10%, the duration of 4–8 wk was comparable to that for previous in vivo transfection studies using this method for transfer into other organs or tissues (17–19, and Kaneda, Y., unpublished data).

Fibroblasts and monocyte/macrophage lineage cells were proved to be the major targets of transfection, the source of foreign gene products in this model, until at least 28 d after injection. The high transfection rate to monocytes/macrophages was remarkable. Although monocyte/macrophage cells constituted at most 5% of the cells in the wound site, they accounted for about one-third of the transfected cells there. Double staining for β-gal and ED-1 epitope showed that the activity of en-

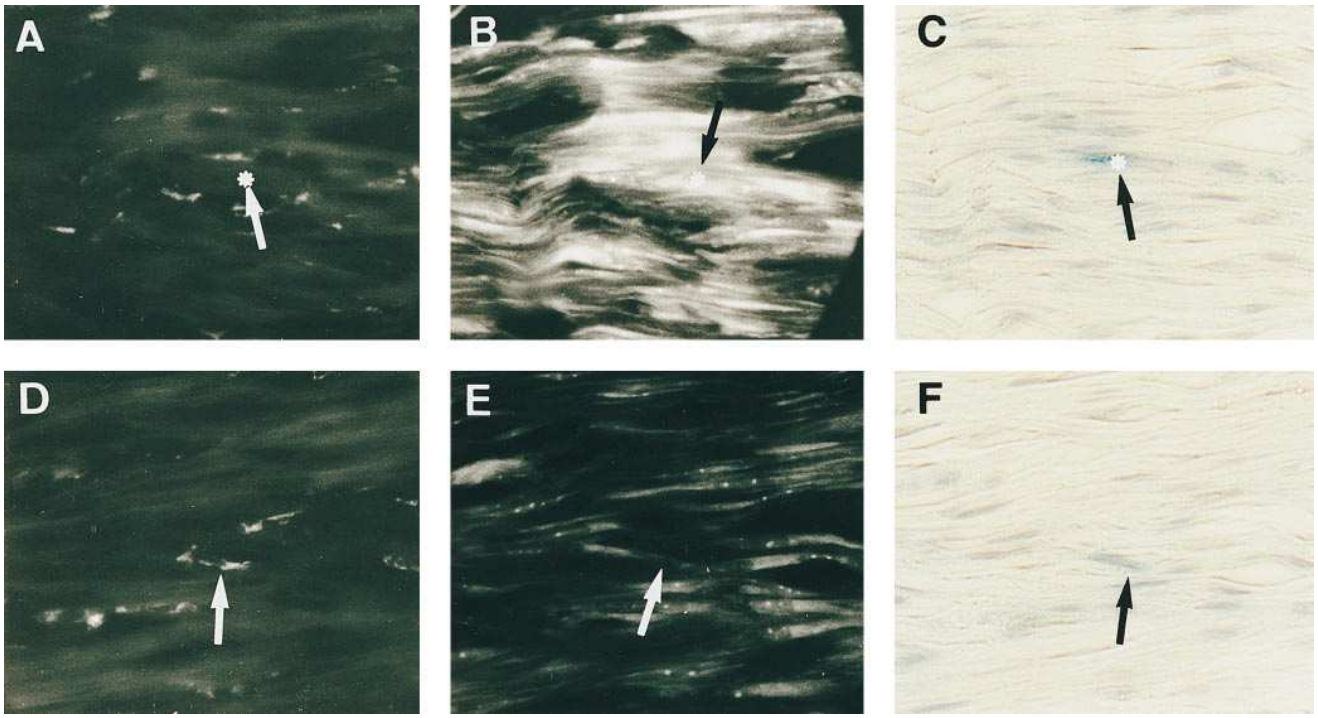

Figure 4. Fourth labeling for ED-1 (*A* and *D*), pN collagen I (*B* and *E*), and β-gal and hematoxylin (*C* and *F*) of the day 7 wound site specimens (×630). Upper columns show an example of β-gal–positive fibroblastic cells, which is negative for ED-1 (*A*, *), positive for pN collagen I (*B*, *), and positive for β-gal (*C*, *). *D–F* show examples of typical β-gal–positive monocyte/macrophage cells, that is, positive for ED-1 (*D*, *arrow*), negative for pN collagen I (*E*, *arrow*) and positive for β-gal (*F*, *arrow*).

dogenous β-gal in monocyte/macrophage cells was negligible (data not shown). Thus, the transfection rate for monocyte/macrophage cells was demonstrated to be about seven times higher than that for fibroblasts. A previous wound healing study provided evidence of active phagocytosis by macrophage cells in the wound (27). Thus, the encapsulated contents of liposomes may be introduced into the cytoplasm of monocyte/macrophage cells by phagocytosis, in addition to the cell fusion mechanisms peculiar to HVJ-liposomes.

However, the result of this gene transfer study also revealed a biological event involved in ligament healing. We observed that the β-gal–labeled cells spread into the uninjured ligament substance away from the wound, and that they finally populated the entire length of the ligament substance as healing progressed. Taking into account that the injected methylene blue dye only slightly penetrated and/or spread into the adjacent ligament substance from the injection site 1 d after injection (see Methods), and furthermore, that the HVJ-liposomes are not stable for >24 h (Kaneda, Y., unpublished data), we interpreted the spreading of β-gal–labeled cells into the ligament substance as representing the migration of the initially transfected cells at the injection site. In support of our interpretation, a previous study reported the presence of "a wave of cells" that extended out with time to the uninjured parts of the ligament from the wound site after injury in the rabbit medial collateral ligament of the knee (28). We observed that the β-gal–expressing cells existing in the ligament substance were mostly fibroblastic cells and monocyte/macrophage lineage cells by using triple staining for β-gal, ED-1, and pN collagen I as shown in Fig. 4 (data not shown). Therefore, it is suggested that wound fibroblasts and monocyte/macrophage cells take part in not only the repair of the wound site but also in remodeling of extensive ligament substance adjacent to the wound. Thus, the results of our present study may provide new insights into the connective tissue healing concept that tissue repair involves the remodeling of comprehensive tissue adjacent to the wound site.

In conclusion, we succeeded in introducing a reporter gene into the healing patellar ligament during the early processes of repair. This model should be potentially beneficial for therapeutic procedures or study of the ligament's biology. Our preliminary study showed that introduction of the PDGF-BB gene into this model leads to enhanced collagen deposition in and adjacent to the wound site as long as 4 wk after transfection (Nakamura, N., S. Horibe, and T. Natsuume, unpublished data). Thus, the introduction of key molecules for wound healing, including several cytokines, into this model should lead to a better understanding of the in vivo biological effects of these molecules on wound healing processes and will also provide some therapeutic insights into ligament healing and/or remodeling.

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