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sFlt-1 Gene-transfected Fibroblasts: A Wound-specific Gene Therapy Inhibits Local Cancer Recurrence

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ABSTRACT

Local recurrence occurs frequently at the site of injury after surgical resection. On the other hand, fibroblasts have been shown to accumulate in the injured area to heal and remodel the damaged tissues. Therefore, fibroblasts are likely to be useful as wound-specific vectors for delivery of genes to sites of surgically injury. The present study was performed to investigate wound-specific migration of exogenously administered fibroblasts and efficacy of gene therapy using genetically engineered fibroblasts in an i.p. wound recurrence model in rats. We demonstrated that fibroblasts transfected with the GFP gene accumulated specifically around the site of injury immediately after i.p. injection. Then, fibroblasts transfected with an adenovirus designated as AdFex that encoded the soluble form of Flt-1 (sFlt-1), a vascular endothelial growth factor receptor, were administered i.p. to the rats to examine inhibition of tumor growth. At day16 after implantation, a significantly smaller tumor volume and less microvessel density in wound sites were observed in the AdFex/fibroblast-treated rats than in controls. Furthermore, this treatment also resulted in an improved survival rate. In conclusion, autologous fibroblasts show promise as a wound-specific vector for gene therapy, and administration of sFlt-1 gene-engineered fibroblasts contributed to local control of the tumor around the injured tissue.

INTRODUCTION

Although gene therapy against cancer is likely to become a useful method of treatment in the near future, there still remain a number of barriers to be overcome before this goal can be reached. One of the most important issues is the establishment of a cancer-specific system for gene delivery. Therefore, a great deal of effort has been made to develop specific vectors using the cancer-specific promoters (1–3). However, because of the weak transcription and expression efficiency of these promoters, no satisfactory strategy has yet been reported. In the present study, we directed our attention to the unique biological characteristics of fibroblasts and then attempted to apply these cells to cancer-targeting gene therapy. Fibroblasts are accumulated at the tissues of injury to heal and remodel the damaged tissues. Wound healing of the surgical resected areas also requires the accumulation of fibroblasts (4, 5). Local recurrence of cancer occurs frequently at and around the site of injury after surgical removal of the tumor (6–8). This type of recurrence is also observed at the port site in laparoscopic surgery (9, 10). Taken together, these observations suggested that fibroblasts may be applicable to cancer-targeting gene therapy for the treatment of local recurrence at the sites of injury, which can be called wound recurrence.

Moreover, fibroblasts have the advantage that they are easily isolated and cultured even from small biopsy and specimens from the recipients themselves can be expanded to large numbers in cell culture. In addition, they can be readily transfected with liposomal or viral vectors because of their rapid growth in vitro (11–13).

In regard to a strategy for killing the tumor, we focused on antiangiogenesis. Several studies have indicated that wound fluid has a high degree of angiogenic activity and could enhance the angiogenic switch of avascular, dormant microtumors (14, 15). Among the angiogenic molecules, VEGF2 is a potent participant in both wound healing and tumor growth. Brown et al. (16) showed that VEGF was up-regulated during wound repair, suggesting that it plays important roles in this process. We have demonstrated previously that VEGF induced strong tumorigenicity in the peritoneal cavity as well as in various organs (17, 18). Moreover, suppression of VEGF by competitive inhibition with the soluble form of Flt-1, which is a receptor for VEGF, contributed to the inhibition of tumor growth (19–21). These lines of evidence led us the hypothesis that halting VEGF-dependent angiogenesis by implantation of fibroblasts genetically modified to carry sFlt-1 could induce inhibition of tumor wound recurrence.

To evaluate this hypothesis, we first examined the wound-specific accumulation of fibroblasts by transfecting the cells with the GFP gene. Then, we developed an intraabdominal cancer recurrence model using a rat colon cancer cell line, RCN-9. In addition, we constructed an adenovirus designated as AdFex, which encoded the soluble form of Flt-1. Autologous FibroFex were implanted into the syngenic wound recurrence model. The evidence presented here provided a novel strategy for treatment of wound-related disease, particularly cancer recurrence.

MATERIALS AND METHODS

Cell Culture. Primary cultures of fibroblasts were prepared from the skin or retroperitoneum of 8-week-old male F344 rats as follows. The epithelium was extracted aseptically and washed with medium containing antibiotics then minced and spread across the flat surface of 10 cm2 tissue culture plates. Tissue chunks were kept in 1.5 ml of DMEM with 50% FCS (BioWhittaker) and P/S (penicillin 200 units/ml and streptomycin 100 μg/ml) for 2 days then in 7 ml of DMEM fortified with 30% FCS until the first passage. After the first passage, primary cells were cultured in DMEM with 10% FCS and P/S. Fibroblasts subcultured three or four times were used for transfection. A rat colon cancer cell line, RCN-9, was obtained from the Riken Inc. and maintained in RPMI 1640 supplemented with 10% FCS and P/S. All of the cultures were maintained at 37°C in a 5% CO2 atmosphere with 100% humidity.

Transfection with GFP Gene. Fibroblasts were seeded in 10-cm tissue culture plates the day before transfection. For each plate, 36 μl of Fugene 6 was mixed with 600 μl of serum-free DMEM and left for 5 min. This mixture was added to 24 μg of reporter plasmid pEGFP-N1 (Clontech, CA) and incubated for 15 min. Then, 5.4 ml of medium was added, and the DNA/Fugene mixture was added to the plates without removing the medium from the cells. Cells were then harvested by trypsinization (0.25% trypsin/0.02% EDTA in PBS) and implanted into the animals.

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2 The abbreviations used are: VEGF, vascular endothelial growth factor; P/S, penicillin-streptomycin; GFP, green fluorescent protein; sFlt-1, soluble Flt-1; AdFex, adenovirus encoded with sFlt-1; AdGFP, adenovirus encoded with GFP; FibroFex fibroblasts transfected with AdFex; FibroGFP, fibroblasts transfected with AdGFP.
Retraperitoneal Wound Models and Accumulation of GFP Gene-transfected Fibroblasts. F344 rats (8-week-old males obtained from Charles River, Yokohama, Japan) were anesthetized with diethyl ether. A 1 cm × 1 cm intra-abdominal wound was made by resection of the right retraperitoneal epithelium. The wounded animals were divided into four groups comprised of five rats each as follows: (a) peritoneum-derived fibroblasts were i.p. administrated immediately after injury; (b) skin-derived fibroblasts were i.p. administered immediately after injury; (c) peritoneum-derived fibroblasts were i.p. administered 1 week after injury; and (d) 100 μg of naked plasmid pEGFP-N1 in 200 μl of PBS were i.p. injected immediately after injury. Aliquots of 1 × 10^{11} fibroblasts in 200 μl of PBS were used in each rat. Five days after administration, the rats were sacrificed and checked under a stereomicroscope (Olympus, Japan). Frozen sections were also examined to confirm the results.

Tumor Growth at the Site of Retraperitoneal Injury: Wound Recurrence Model. After 1-cm² right retraperitoneal resection, the abdominal wall was closed, and i.p. injection of 1 × 10^{7} RCN-9 cells in 1 ml of PBS was immediately performed.

Adenoviral Vectors. The replication-deficient adenovirus vectors used in this study were E1a-, partially E1b-, and partially E3- vectors based on human adenovirus type 5 (Riken, Inc., Tsukuba, Japan). Each vector contained an expression cassette using the CAG (chicken beta-actin promoter associated with cytomegalovirus enhancer) promoter/enhancer, an artificial splice sequence, followed by different transgenes and a poly(A) sequence. In AdFex, the cDNA encoded the first three immunoglobulin-like loops of sFlt-1, which were involved in high-affinity binding of VEGF (22) but lacked the transmembrane and intracellular domains. AdGFP contained the cDNA for GFP (Riken Inc.). All of the adenoviral vectors were propagated in 293 cells, purified by two rounds of cesium chloride density centrifugation, dialyzed, and stored at −70°C. The titer (expressed as plaque-forming units/milliliter) of each viral stock was determined by plaque assay with 293 cells. All of the vector preparations were demonstrated to be free of replication-competent adenoviruses. The transduction of AdGFP and AdFex into fibroblasts was performed at a multiplicity of infection of 50.

Western Blot Analysis for sFlt-1. Aliquots of 20 μg of protein were separated by 10% SDS-PAGE under reducing conditions and blotted onto polyvinylidene difluoride membranes (Immobilon-PVF, Millipore Corporation, Bedford, MA). First, they were incubated with rabbit antibody against NH₂ terminus of mouse sFlt-1 (kindly provided by Dr. Masabumi Shibuya, Tokyo University, Tokyo, Japan; Ref. 22), diluted 1:200 for 60 min at room temperature, and then with goat antibas IgG (H+L)-conjugated to horse-radish peroxidase (Zymed Laboratories, Inc., San Francisco, CA) diluted 1:1000 for 60 min at room temperature. Finally, the enhanced chemiluminescence system (Amersham) was used for detection. Protein concentration was determined using BCA® Protein Assay Reagents, Pierce (Rockford, IL). The amount of sFlt-1 was determined by area analysis of autoradiograms using an ARGUS-50, 3.3 (Hamamatsu, Japan).

Collection of Wound Fluid and a Migration Assay. Before and 1, 3, 24, 72, and 168 h after retraperitoneal wounding in 10 rats, 3 ml of PBS used to wash each wound was collected and designated as wound fluid 0, 1, 3, 24, 72, and 168 h, respectively. The migration ability of fibroblasts was assayed using a Transwell chamber (Corning Inc., Corning, NY). A 4 × 10^6/ml suspension of single cells in conditioned medium was applied to the upper chamber, and medium containing 10% wound fluid was applied to the lower chamber. After incubation for 20 h, cells on the upper side of the membrane were removed. The number of cells that had passed through the membrane to the lower side was counted under a microscope after staining with Diff-Quik (International Reagent Corp., Kobe, Japan).

Detection of Neovascularization with the Dorsal Air Sac Method. The dorsal air sac method was performed as described previously (23). FibroFex or AdGFP were washed three times with PBS and suspended in PBS at a concentration of 2 × 10^6 cells/0.2 ml. A Millipore chamber (diameter, 10 mm; filter pore size, 0.45 μm) was filled with 0.2 ml of the cells and implanted s.c. into the dorsal side of the rats. On day 5 after implantation, the rats were anesthetized and fixed in the prone position. A wide, rectangular incision was made in the skin on the dorsal side, and the skin was carefully ablated. To locate the chamber-contacting region, a ring (Millipore) of the same shape as the chamber was placed onto the s.c. tissues adjacent to the chamber region, and the area was photographed. The s.c. tissues were then fixed and made into paraffin sections for microvessel staining.

Microvessel Staining and Evaluation. As an indicator of angiogenesis, microvessels were counted in immunohistochemically stained sections under light microscopy. Antifactor VIII polyclonal antibody (Dako Polyclonal; Dako Corporation, Carpenteria, CA) was used after the immunoperoxidase procedure (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA; Ref. 24). After screening the areas with intense neovascularization at a low magnification (×40 or ×100), microvessels were counted in a ×200 field (0.739 mm²/field). In all of the samples, the mean value for the number of microvessel was calculated from five different ×200 fields.

Statistical Analysis. The statistical analyses were performed with SPSS, Release 6.0 (NANKODO Co. Ltd., Chicago, IL). Analysis of the significance of difference among multiple groups (chemoattractant activity, tumor volume, and MVD in the wound recurrence model after treatment) was performed by ANOVA. The survival curves were plotted according to the Kaplan-Meier method, and the statistical differences were analyzed by using the generalized log-rank test. P < 0.05 was considered to indicate statistical significant.

RESULTS AND DISCUSSION

Tumor Growth at the Site of Retraperitoneal Injury: Wound Recurrence Model. Spreading of RCN-9 cells in the abdominal cavity of syngenic rats with retroperitoneal injury resulted in a significantly greater degree of tumor growth in the wound site on the 14th postoperative day than in the noninjured site (tumor volume in the wound site: 6.93 ± 1.29 cm³; total tumor volume in noninjured site: 1.21 ± 0.50 cm³; n = 5; P < 0.001).

GFP Gene-transfected Autologous Fibroblasts Accumulate Specifically in the Wound after Immediate Implantation. Green fluorescence was observed in the right retraperitoneal wound after immediate postoperative implantation of GFP gene-transfected autologous fibroblasts (Fig. 1). Under higher magnification (×100), we identified morphologically that the fluorescence was derived from fibroblasts as confirmed on frozen sections. Fibroblasts derived from both the peritoneum and skin migrated exclusively to the wound after implantation. Such specific accumulation was also observed in the wound recurrence model. Faint fluorescence was observed at the paramedian incision site. On the other hand, when the fibroblasts were administered i.p. 7 days after retroperitoneal injury, no specific fluorescence was observed in the retroperitoneal space. Injection into the wound of either naked pEGFP-N1 cDNA or AdGFP resulted in no specific fluorescence.

By in vitro migration assay, we demonstrated larger amounts of chemotactic growth factors for fibroblasts in wound fluids than in controls (Fig. 2). The wound fluid taken at 1 h showed strongest chemotactic activity. It was then down-regulated, and on day 7 when the wound healed, it backed to the same level of the peritoneal washing buffer (PBS) without injury.

sFlt-1 Gene-transfected Fibroblasts Inhibit Tumor Growth around the Injured Site. AdFex or AdGFP was transferred into fibroblasts derived from rat skin in primary culture. These cells were designated as FibroFex or FibroGFP. The transfection efficiency is 70−80%. The expression of sFlt-1 was detected in the 24-h serum-free culture medium of FibroFex by Western blotting using an antibody to the NH₂ terminus of Flt-1. FibroFex exhibited potent antiangiogenic activity in the dorsal air sac method (Fig. 3). It was also confirmed by microvessel staining (FibroFex: 32.17 ± 10.91/200× field; FibroGFP: 57.83 ± 12.17/200× field; n = 5; P < 0.001).

The cells were then administered into the wound recurrence models immediately after injuring the retroperitoneum. When sacrificed on 16th, a significantly smaller tumor volume was observed in the wound site in rats received FibroFex than in the controls. Less neovascularization was observed in the FibroFex-treated rats than in
Fig. 1. The specific accumulation of fibroblasts in the retroperitoneal wound. A, retroperitoneal wound 5 days after implantation of GFP-expressing fibroblasts, stereomicroscope, daylight, 20×. N, normal tissue; W, wound tissue. B, same site as A under fluorescent stereomicroscope, 20×. C, part of B, stereo fluorescent microscope, 100×. D, frozen section of normal peritoneum, 200×. E, frozen section of peritoneal wound after GFP-expressing fibroblasts implantation. F, wound recurrence site, 1 day after implantation of FibroGFP and RCN-9, stereomicroscope, daylight, 50×. N, normal tissue; W, wound tissue. G, same site as F under fluorescent stereomicroscope, 50×.
the controls (Fig. 4). The sFlt-1 expression was detected only in the wound recurrence site by Western blotting in day 7. In the disseminated tumors, no sFlt-1 expression was detected. All of the visible disseminated tumors were collected, and the total volume in each group showed no significant difference (FibroFex: 1.30 ± 0.31 cm³; FibroGFP: 1.57 ± 0.35 cm³; PBS: 1.21 ± 0.50 cm³; \( P = 0.398 \)). These evidences demonstrated that the administration of FibroFex changed the local environment within the wound site where angiogenesis was significantly inhibited. Furthermore, an improvement of survival rate was also observed in animals treated with FibroFex (Fig. 5; \( P = 0.045 \)).

The main mechanism of the i.p. recurrence of the cancers in the abdominal organs is the regrowth of microscopically residual cancer cells around the surgically resected areas. This type of recurrence is commonly very difficult to remove by reoperation. Therefore, it is necessary to establish a novel strategy to prevent such i.p. recurrence after surgical resection. In the present study, we confirmed that primary cultured gene-transfected fibroblasts, derived from either peritoneum or skin, accumulated specifically in retroperitoneal wound, whereas administration of naked cDNA or adenovirus did not result in such specific accumulation. Although this was a preliminary study, we expect that fibroblast will be useful as vectors for targeting wound-related diseases. That is, using genetically engineered fibroblasts we will be able to modify the disadvantageous microenvironment for tumor growth around the wound. When wound recurrence is suspected, genetically manipulated fibroblasts secreting a substance unfavorable for the proliferation of cancer cells may contribute to suppression of cancer growth. Moreover, the observation that fibroblasts originating from either orthotopic or heterotopic anatomical sites accumulated to the injured tissue implied that fibroblasts taken from skin of the patient would be available for this treatment, obviating the need for immunosuppression.

Considering the potential role of angiogenic factors, especially VEGF, on tumor growth, sFlt-1 is a potent candidate for transfection into fibroblasts, thereby leading to the prevention of tumor recurrence around the surgically resected area. The VEGF expression in the wound fluid of normal rat appeared as fast as 1 h. After a peak at 24 h, it was down-regulated and disappeared on day 7 (data not shown). We believe 7 day is the predominant period for angiogenesis in wound healing of rats. The transient expression of adenovirus is efficient in this study. Because fibroblasts administrated i.p. were preferentially accumulated in the retroperitoneal wound, systemic angiogenesis and healing of the abdominal wall were not affected.

The present study also showed that injured tissue secreted chemoattractants for migration of fibroblasts as early as 1 h after injury, production of which decreased as wound repair progressed. In addition, changes in the pattern of chemoattractant activity in the wound fluid suggested that early after injury is an appropriate time to administer the fibroblasts.

Thus, exogenously inoculated fibroblasts exhibited wound-specific accumulation, and genetically engineered fibroblasts can facilitate specific gene delivery to the surgical wound, which is an area of
high-risk of cancer recurrence. In conclusion, fibroblasts transfected with sFlt-1 using an adenoviral vector inhibited tumor growth at the site of surgical injury. This method of fibroblast-mediated gene therapy is a promising novel strategy for preventing and controlling wound-related recurrence of cancer after surgery.

REFERENCES


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