Transduction of Soluble Flt-1 Gene to Peritoneal Mesothelial Cells Can Effectively Suppress Peritoneal Metastasis of Gastric Cancer

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ABSTRACT

The prognosis of gastric cancer with peritoneal metastasis has not improved. Despite many promising studies, gene therapy has limited clinical application because of the lack of suitable vector systems to enable selective gene transduction to tumor cells. The aim of this study was to clarify whether gene therapy targeted to peritoneal mesothelial cells (PMCs) can inhibit peritoneal dissemination of gastric cancer. In vitro experiments showed that adenovirus expressing LacZ infected human omental tissue-derived PMCs more efficiently than human gastric cancer cell lines MKN1 and MKN45. When adenovirus expressing LacZ was injected into the peritoneal cavity of nude mice, the expression was detected in the peritoneum for at least 4 weeks. Furthermore, when adenovirus expressing soluble Flt-1 (Ad-sFlt-1) was i.p. administered in vivo, a high level of sFlt-1 protein could be detected in peritoneal lavage for 8 weeks. When MKN45 cells were i.p. inoculated 3 days after adenoviral vector injection, Ad-sFlt-1 markedly reduced the number of metastatic nodules larger than 1 mm in diameter on the peritoneal surface, and significantly prolonged the survival of nude mice without any significant side effects. Thus, peritoneal dissemination was significantly suppressed by a single i.p. injection of Ad-sFlt-1. Anti-angiogenic gene therapy targeted to PMCs could be a novel and practical strategy against peritoneal dissemination of gastric cancer, because it does not require tumor-specific gene transfer.

INTRODUCTION

The results of radical surgery with extended lymphadenectomy for advanced gastric cancers are unfavorable, and peritoneal recurrence is the most important factor determining the prognosis of such patients (1–6). Systemic chemotherapy is the main treatment against peritoneal metastasis, but an effective regimen to improve the gloomy prognosis has not been established (7). Because systemic chemotherapy appears to result in a marked dose reduction in the peritoneal cavity, various trials of i.p. chemotherapy have been performed, although the effect on the clinical outcome is still controversial (8, 9).

Recent studies on gene therapy have suggested the possibility of its clinical application for peritoneal metastasis in various cancers (10–16). Although many studies have suggested that gene therapy is a promising new treatment modality for disseminated cancer, the reason for the limited clinical application has been the inability to develop vector systems to enable selective gene transduction to tumor cells. To overcome this problem, modification of vector tropism (17, 18) and transcriptional targeting with tumor-specific promoters (19–21) have been attempted. However, a satisfactory method for tumor-specific gene delivery has not yet been developed.

In this study, we therefore changed the strategy and attempted to perform gene transfer to peritoneal mesothelial cells (PMCs). Because PMCs compose the most inner lining of the peritoneum, i.p. injected vector is supposed to be easily accessible to PMCs. Moreover, PMCs have the capacity to produce large amounts of cytokines such as interleukin (IL)-1, IL-6, (22), IL-8 (23), granulocyte colony-stimulating factor (24), and vascular endothelial growth factor (VEGF; Refs. 25 and 26) and thus may play various roles in peritoneal metastasis.

As the target gene, we selected anti-angiogenic genes, because their gene products are not cytolytic but induce tumor dormancy through modification of the circumstances of tumor development (27). A soluble form of VEGF receptor (sFlt-1) reduces the effects of VEGF by trapping VEGF with high affinity (28, 29). Previous studies have shown that gene transfer of sFlt-1 to tumor cells causes significant inhibition of tumor growth (30). Even in in vivo experiments using gastric cancer cells, i.p. injected sFlt-1 has been shown to suppress the development of peritoneal metastases (13, 30). However, in those studies, the sFlt-1 gene was considered to be transferred to cancer cells, and the effects of other host cells were neglected.

In this study, we investigated the transfection efficiency of PMCs by adenovirus vectors in vitro and monitored the transgene expression in the peritoneum induced by single i.p. injection of adenovirus vector into the peritoneal cavity of nude mice. Finally, we evaluated whether i.p. transfer of the sFlt-1 gene using adenovirus vector has potential as gene therapy against peritoneal dissemination of gastric cancer.

MATERIALS AND METHODS

Isolation and Culture of Human PMCs. Human PMCs were obtained from omental tissues of patients who underwent gastrectomy for gastric cancer with informed consent in our department. PMCs were isolated as described previously (20) and maintained in DMEM medium with 10% fetal bovine serum, penicillin/streptomycin, 2 ng/ml aFGF, and 5 μg/ml heparin in fibronectin-coated tissue culture flasks at 37°C in a humidified atmosphere of 5% CO2 in air. Cells of passage 3 cultures were used for experiments.

Cell Lines. Two human gastric cancer cell lines, MKN1 and MKN45, were obtained from Riken Cell Bank (Tsukuba, Japan) and maintained in DMEM medium with 10% fetal bovine serum and penicillin/streptomycin at 37°C in a humified atmosphere of 5% CO2 in air.

Preparation of Adenoviral Vectors. Replication-deficient recombinant adenovirus vectors expressing the human soluble VEGF receptor (Ad-sFlt-1) or a control vector expressing β-galactosidase (Ad LacZ) were described previously (31). The titers of recombinant adenovirus were determined by plaque assay in 293 cells, and the titer was expressed in plaque-forming units (pfu). Recombinant adenoviruses were kept at −80°C until use.

In Vitro Transfection Efficiency of PMCs with Adenoviral Vector. MKN1, MKN45, and PMCs were infected with Ad LacZ at multiplicities of infection (MOI) ranging from 0.1 to 1 for 1 h. The cultures were then fed with medium with 10% fetal bovine serum, penicillin/streptomycin, 2 ng/ml aFGF, and 5 μg/ml heparin in fibronectin-coated tissue culture flasks at 37°C in a humidified atmosphere of 5% CO2 in air.

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in the cultures was determined as the percentage of blue cells relative to total cells counted in five randomly chosen microscopic fields.

**β-Galactosidase Expression in Murine Peritoneum Induced by i.p. Injection of Ad-LacZ.** Five-week-old female BALB/c nude mice (Nihon CLEA, Tokyo, Japan) were used. The mice were maintained under specific-pathogen-free conditions according to the Guidelines for Animal Experiments of Tokyo University. Mice received i.p. injections of Ad-LacZ at a dose of 1.4 \times 10^9 pfu/4 ml. After 1, 2, 3, and 4 weeks, mice were sacrificed by cervical dislocation, and β-galactosidase activity was determined by *in situ* X-gal staining. The mice received i.p. injections of the above-described fixative for 15 min. Then, the peritoneal cavity was rinsed six times with PBS to remove the fixative before infusing 10 ml of staining solution. After a 4-h incubation, the staining solution was removed, and the peritoneal cavity was opened for visual inspection and photography.

**Soluble Flt-1 Expression in Vivo.** Five-week-old female BALB/c nude mice received i.p. injections of Ad-sFlt-1 at a dose of 1.4 \times 10^9 pfu/4 ml. After 1–8 weeks, mice were sacrificed, 1 ml of PBS was injected i.p., and the peritoneal fluid was fully recovered. The cell-free lavage was obtained by centrifugation at 15,000 rpm for 10 min at 4°C and stored at −80°C. Analysis of sFlt-1 in the peritoneal lavage was performed using standard ELISA methodology (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Treatment with LipoTransduction of Soluble Flt-1 Gene against Peritoneal Metastasis of MKN45 Cells.** Five-week-old female BALB/c nude mice were allocated to three groups (PBS, LacZ, and sFlt-1). First, each mouse received i.p. injection of 4 ml PBS, 1.0 \times 10^9 pfu/4 ml Ad-LacZ, or 1.0 \times 10^9 pfu/4 ml Ad-sFlt-1. Three days later, a single cell suspension of 3 \times 10^6 MKN45 cells resuspended in 1 ml of PBS was injected into the peritoneal cavity of each mouse with a 23-gauge syringe. On day 24, all mice were sacrificed, and the number of macroscopic nodules on the peritoneal surface was counted. Using the same methods, the survival of mice was evaluated in each group up to day 120. All of the *in vivo* experimental protocols were approved by the animal care committee of Tokyo University.

**Statistical Analysis.** Transfection efficiency and number of metastatic nodules were analyzed by ANOVA, and survival was analyzed by the Kaplan-Meier method. Differences with *P* < 0.05 were considered significant.

**RESULTS**

**In Vitro Transfection Efficiency to PMCs with Adenovector.** We first evaluated the transfection efficiency of Ad-LacZ to PMCs as well as MKN1 and MKN45 *in vitro*. Ad-LacZ was added to these cell cultures, and gene expression was evaluated by X-gal staining 3 days later (Fig. 1). The infection rate of MKN1 was 10.5 ± 2.3% (mean ± SD) and 63.8 ± 13.4% at 0.1 and 1 MOI, respectively. No significant infection of MKN45 was observed at 0.1 MOI, and 9.7 ± 1.8% of MKN45 were infected at 1 MOI. In comparison, more than one-half of PMCs (57.9 ± 0.6%) were infected at 0.1 MOI, and almost all PMCs were infected at 1 MOI. Thus, human omental tissue-derived PMCs showed markedly higher transfection efficiency with Ad-LacZ than two human gastric cancer cell lines (*P* < 0.05, at 0.1 and 1 MOI). This suggested that i.p. administered Ad-LacZ may infect PMCs more efficiently than cancer cells.

**Expression of LacZ Gene in Murine Peritoneum Induced by i.p. Injection of Ad-LacZ.** We next examined β-galactosidase expression in murine peritoneum after i.p. administration of Ad-LacZ. As shown in Fig. 2, 7 days after injection, almost the entire peritoneum,
including the parietal peritoneum, visceral peritoneum, diaphragmatic peritonuem, and liver surface, was strongly and homogeneously stained. Although the staining intensity gradually decreased, parts of the mesentery still showed significant X-gal staining even 4 weeks after injection. This suggests that genes transduced to PMCs can continuously function for at least 4 weeks.

**Soluble Flt-1 Expression in Murine Peritoneum.** We confirmed the level of sFlt-1 protein in the peritoneal cavity by ELISA method after an i.p. injection of $1.0 \times 10^9$ pfu/4 ml of Ad-sFlt-1. The level of sFlt-1 in the peritoneal lavage of nude mice remained high for 5 weeks and gradually declined thereafter. However, sFlt-1 was still detectable at 8 weeks after gene transduction (Fig. 3). In a control study, sFlt-1 was not detected in the peritoneal lavage of mice infected with Ad-LacZ at any time point (data not shown). No significant ascites or other abnormality was observed in any mouse infected with Ad-sFlt-1 up to 8 weeks.

**Treatment with Soluble Flt-1 Gene Transduction for Peritoneal Dissemination of MKN45.** Finally, we examined whether an i.p. injection of Ad-sFlt-1 resulted in the inhibition of peritoneal dissemination. Mice received i.p. injections of PBS, Ad-LacZ, or Ad-sFlt-1, and 3 days later, MKN45 cells were i.p. inoculated. The macroscopic nodules of peritoneal dissemination were then counted 3 weeks after tumor inoculation. Metastatic nodules were observed on the peritoneal surface, especially on the vicinity of stomach and intestine, whereas few nodules were detected on the surface of parietal peritoneum and i.p. organs (Fig. 4A).

The total number of metastatic nodules was $190 \pm 69.5$ and $202 \pm 69.5$ in the PBS and LacZ groups, respectively. Mice that received injections of Ad-sFlt-1 showed comparatively fewer nodules ($142 \pm 83.3$), although the difference was not statistically significant (Fig. 4B). However, the size of metastatic nodules on the peritoneum was significantly different (Fig. 4C). When we counted the large metastatic nodules that exceeded 1.0 mm in diameter, the PBS and LacZ groups showed $22.0 \pm 12.0$ and $18.4 \pm 12.0$ large nodules,
whereas the sFlt-1 group showed only 1.3 ± 1.8 (P < 0.05). The survival of these mice is shown in Fig. 4D. Consistent with the finding of macroscopic peritoneal metastasis, the outcome showed a significant difference among the three groups. On day 120, almost all of the mice in the PBS and LacZ groups were dead, whereas half of the mice in the sFlt-1 group were still alive. Moreover, median survival was significantly prolonged in the sFlt-1 group (P < 0.05 versus PBS and LacZ groups). This indicates that sFlt-1 gene transfer to the murine peritoneum can effectively suppress the growth of peritoneal metastases and improve the prognosis in this experimental condition.

DISCUSSION

VEGF is a potent growth factor for endothelial cells, and many studies have demonstrated a positive correlation between VEGF expression and malignant potential in various cancers (32–36). Moreover, tumor suppression has been achieved in animal experiments by neutralization of VEGF or by blocking the VEGF receptors (37, 38), indicating that VEGF plays critical roles in tumor angiogenesis. In fact, gene therapy targeted to VEGF and its receptors has been reported to be a feasible and effective strategy for cancer treatment (39–42). The important thing is that anti-angiogenic gene therapy does not require a direct and selective transduction of target genes into cancer cells, but rather transduction around the tumor to create an antiangiogenic environment.

In the current study, we therefore attempted transfection of the sFlt-1 gene to PMCs, because they line all of the internal organs in the peritoneal cavity, and cancer cells detached from the primary site interact with PMCs in the first stage of peritoneal dissemination. Our results clearly showed that PMCs were efficiently transduced with the adenovirus vector both in vitro and in vivo. In vivo experiments also showed that sFlt-1 protein could be detected in the peritoneal lavage for several weeks after a single i.p. injection of Ad-sFlt-1. We similarly examined the sFlt-1 level in the peritoneal lavage after i.p. injection of 10 ng of recombinant sFlt-1 protein. However, a significant level of sFlt-1 could not be detected even 7 days after injection (data not shown), which was in marked contrast to i.p. injection of Ad-sFlt-1. These results indicate that adenoviral transduction of the sFlt-1 gene to PMCs by a single i.p. injection can continuously provide a sufficient level of sFlt-1 protein at the peritoneal surface for at least several weeks.

Murphy et al. (43) have shown that human mesothelial cells transduced with human growth hormone adenovector can secrete human growth hormone protein for 6 weeks in vitro. In the same study, they confirmed that human growth hormone-transduced mesothelial cells can be identified on the peritoneal surface for at least 19 days when injected into the peritoneal cavity in nude mice. Their results are mostly consistent with our data and suggest that gene expression in PMCs is an ideal approach against peritoneal metastasis from the viewpoint of drug delivery.

In our results, the development of large metastatic nodules was markedly suppressed in the sFlt-1 group, although the total number of nodules in the peritoneal cavity was not decreased significantly. This appears to be reasonable because the growth of solid tumors greater than 1–2 mm³ is critically dependent on angiogenesis (44). In these experiments, we inoculated tumor cells 3 days after i.p. transduction of the sFlt-1 gene to clarify whether the gene expression in PMCs, but not in tumor cells, can affect tumor growth. Thus, the suppression of peritoneal metastasis is considered to be attributable to host-derived sFlt-1, although it cannot be ruled out that the residual adenovirus directly infected the inoculated cancer cells. However, the transduction of PMCs is more efficient than that of MKN45 cells in vitro, and we could not detect any residual fluid in the abdominal cavity at the time of tumor inoculation. Moreover, even if the cancer cells were infected with residual adenovirus, the period of gene expression is thought to be shorter in cancer cells than in PMCs because of the rapid cell cycle. In fact, dissemination nodules 24 days after i.p. administration of Ad-LacZ did not show significant X-gal staining (data not shown). From these findings, we can conclude that the antitumor effect in our experiments was dependent on sFlt-1 protein secreted from PMCs.

We did not evaluate the therapeutic effects of i.p. injection of Ad-sFlt-1 after tumor inoculation in the peritoneal cavity. However, Mahasreshti et al. (30) have reported that i.p. administration of adenovector containing the sFlt-1 gene at 1 and 14 days after ovarian cancer cell inoculation in the peritoneal cavity can significantly improve the outcome. Mori et al. (13) have also reported that repetitive i.p. injections of HVJ cationic liposomes encapsulating a plasmid expressing sFlt-1 significantly reduced the peritoneal dissemination of MKN45 cells. In the present study, the sFlt-1 gene was transduced 3 days before i.p. injection of tumor cells in nude mice. From the results of these studies, it can be speculated that the same treatments can also suppress the growth of MKN45 even in our experimental system. Importantly, in those two studies, it is uncertain whether sFlt-1 protein was derived from cancer cells or other host cells, although cationic liposomes have been shown to fuse preferentially with cancer cells. In fact, Engler et al. (45) have shown that when Ad-LacZ was injected into the abdominal cavity of nude mice bearing PC3 prostate cancer in the peritoneal cavity, transgene expression was higher in the peritoneal wall than in tumor tissue. Their data together with our results suggest the possibility that the sFlt-1 gene was also transduced to PMCs and might have a positive contribution in tumor suppression, even in the experiments of those two studies.

Takayama et al. (31) have reported that i.m. administration of the sFlt-1-expressing adenovirus in nude mice induced a significant level of sFlt-1 in plasma for at least 3 weeks and inhibited s.c. tumor growth. In our experiments, however, we could detect sFlt-1 in the peritoneal lavage but not in the plasma of mice (data not shown). Therefore, i.p. administration of an adenovector-carried gene is expected to exhibit local antitumor effects in the abdominal cavity but does not appear to exhibit additional effects on distant organs. This may result in a lack of unfavorable side effects and thus produce the therapeutic benefit. On the other hand, Setoguchi et al. (46) have reported that i.p. administration of an adenovirus vector containing human erythropoietin resulted in a high level of human erythropoietin in serum for at least 24 days in rabbits. The reason for the discrepancy between their results and ours is not clear, but it may be attributable to the differences in the gene expression system or the degradation process of gene products between sFlt-1 and human erythropoietin.

In conclusion, anti-angiogenic gene therapy targeted to PMCs could effectively suppress the development of peritoneal metastasis and prolong survival without any side effects in a mouse xenograft model. This method might be more practical for clinical application, because it does not require frequent administration or the development of tumor-specific gene transfer system. The peritoneum can be a novel target of gene therapy against peritoneal dissemination of gastric cancer. Genetic modification of the “soil” may effectively control the growth of “seeded” cancer cells in the peritoneal cavity.

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