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Suppression of cell migration in ovarian cancer cells mediated by PTEN overexpression

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Received April 8, 2003; Accepted May 30, 2003

Abstract. Peritoneal dissemination is the major progression pathway of ovarian cancer, and its control is important for improvement of the prognosis. *PTEN* is a tumor suppressor gene, and is known to inhibit cancer cell growth and migration. To investigate the possibility of gene therapy using PTEN for ovarian cancer, we introduced *PTEN* cDNA into an ovarian cancer cell line HRA carrying wild-type PTEN, and examined the effects *in vitro* and *in vivo*. Using *PTEN* cDNA cloned from a human liver cDNA library, a PTEN expression vector was constructed. This vector was introduced into HRA cells by the standard calcium phosphate precipitation method, and an HRA cell line overexpressing PTEN (HRA/PTEN) was established. On the cell migration test by *in vitro* scratch wound healing assay, the number of migrating cells was 6.3±0.9 cells/mm^2 in HRA/PTEN, which was significantly smaller than that in the control (39.7±3.2 cells/mm^2) ($p<0.01$). No significant differences were observed in the *in vitro* cell growth or *in vivo* tumor growth between HRA/PTEN and the control. The findings described above, show that enhanced expression of PTEN inhibits ovarian cancer cell migration, suggesting that gene therapy approaches using PTEN for control of peritoneal dissemination of ovarian cancer are possible.

Introduction

The incidence of ovarian cancer has been increasing in recent years, and it is currently the most frequent cause of death from gynecological neoplasia in western countries and Japan (1,2). Since the ovary is an intraperitoneal organ, early diagnosis of ovarian cancer is difficult in most cases, and more than half of the cases are found at the advanced stages III and IV accompanied by peritoneal dissemination and distant metastasis (2). To treat advanced ovarian cancer, surgical therapy and combination chemotherapy using a platinum analogue as the key drug are performed, but the long-term prognosis is still poor (3). Peritoneal dissemination is the most frequent progression pathway of ovarian cancer (4,5). Therefore, control of peritoneal dissemination is important for improvement of the prognosis of patients with advanced ovarian cancer.

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a tumor suppressor gene identified on 10q23 (6,7). In the gynecological field, abnormal *PTEN* was frequently observed in endometrial cancer, and its association with generation and progression of this disease has been drawing attention (8,9). Recently, PTEN has been reported to inhibit growth and migration of cancer cells (10-12). To investigate the possibility of gene therapy for ovarian cancer, we focused on the action of PTEN. In the present study, *PTEN* cDNA was introduced into an ovarian cancer cell line carrying wild-type *PTEN* to establish a PTEN-overexpressing cell line. The inhibitory effect of PTEN on the cancer cell growth and migration was investigated *in vitro* and *in vivo*.

Materials and methods

Cell culture. The human ovarian serous carcinoma cell line HRA (13) was provided by Professor Y. Kikuchi, and was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units penicillin, and 100 µg/ml of streptomycin at 37°C under 5% carbon dioxide.

PTEN sequence analysis in HRA. Total RNA of HRA cells was extracted using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany), and reverse-transcribed using Taq Man Reverse Transcription Reagents (Perkin Elmer, Applied Biosystems Division, Darmstadt, Germany). Using this cDNA as a template and the primers described previously (14), *PTEN* of HRA cells was amplified, and their nucleotide sequence was determined using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Applied Biosystems Division, Darmstadt, Germany)

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Key words: ovarian cancer, PTEN, cell migration, gene therapy, HRA cell line

with ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Applied Biosystems Division, Darmstadt, Germany).

Plasmid construction and transfection into HRA. The PTEN expressing vector pCMV-PTEN-IRES-bsr described previously (15) and the control vector pCMV-luciferase (LUC)-IRES-bsr (16) encoding LUC were transfected into HRA cells by the standard calcium phosphate precipitation method (17). In previous experiments, we confirmed that gene introduction using pCMV-LUC-IRES-bsr does not affect cell growth, migration, invasion, anticancer drug sensitivity, or radiosensitivity (15,18,19). The cells were selected with the concentration of 10 μg/ml blasticidin S hydrochloride (Funakoshi, Tokyo, Japan). Resistant clones were obtained after four weeks as HRA/PTEN and HRA/LUC. The cells were subsequently maintained in the presence of 10 μg/ml blasticidin S hydrochloride.

Western blotting. HRA, HRA/LUC and HRA/PTEN, $5x10^5$ cells each, were plated in 3.5 cm plastic dishes and cultured in 10% serum-supplemented DMEM. After 24 h, the cells were washed with phosphate-buffered saline(PBS) and were dissolved in 100 μl of lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1%NP-40, 100 IU/ml aprotinin and 1 mM phenylmethylsulfonylfluoride; PMSF]. Protein concentrations were measured using the Bio-Rad (Veenendaal, The Netherlands) protein assay kit. Western blotting was performed under standard procedures (20) utilizing Anti-PTEN polyclonal antibody (N-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the ECL Detection System (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Assessment of focal adhesion kinase (FAK) and AKT phosphorylation. FAK and AKT phosphorylation were assayed by Western blotting. Cell lysates were immunoprecipitated with an anti-FAK antibody (2A7; Upstate Biotechnology, Lake Placid, NY), or an anti-AKT antibody (New England Biolabs, Inc., Beverly, MA) for probing with each antibody. The membrane was then stripped, reprobed for phosphotyrosine (4G10; Upstate Biotechnology), or phospho-AKT (Ser473) (New England Biolabs), and visualized again by ECL.

In vitro cell growth kinetics. HRA, HRA/LUC and HRA/PTEN, $1x10^5$ cells each, were plated in 3.5 cm plastic dishes and cultured in 10% serum-supplemented DMEM. Every 24 h, the cells were dislodged using 0.05% trypsin-EDTA and counted by a hemocytometer.

In vitro scratch wound healing assay. Cell migration was measured by the *in vitro* scratch wound healing assay (21). Monolayer cells were scratched with a sterile pipette tip in 10 cm plastic dishes, and after 8 h of culture in 2% serum-supplemented DMEM, the cell migration was evaluated by counting cells that migrated from the wound edge.

In vivo tumor growth. Female BALB/c nude mice at 6-8 weeks of age (Japan Clea Laboratories, Tokyo, Japan) were used in the experiment. All of the animal experiments were performed under the guidelines of Jichi Medical School. HRA/LUC and HRA/PTEN, $5x10^6$ cells each, were subcutaneously transplanted on the backs of the mice. One week after transplantation, two dimensions of the tumors were measured using a caliper. The tumor volume was estimated by the equation: $[width]^2 \times [length] \times 1/2$.

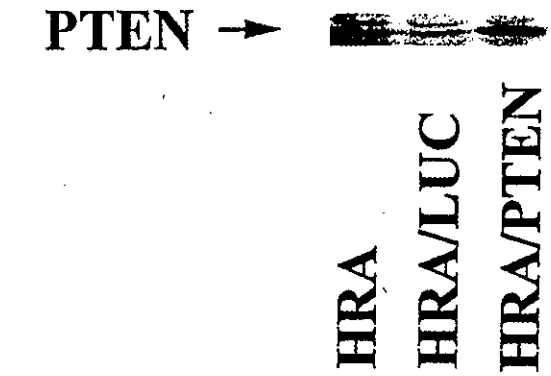

Figure 1. Detection of PTEN by Western blotting using anti-PTEN polyclonal antibody. PTEN expression was detected at the position of 55 kDa in HRA/PTEN, while only weak PTEN expression was detected in parent HRA or HRA/LUC.

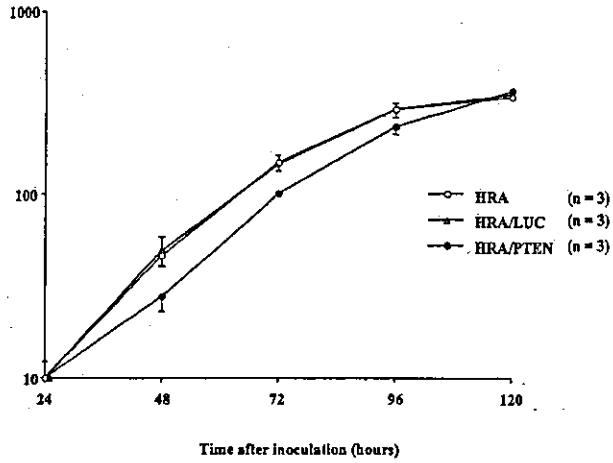

Figure 2. The cell growth of HRA, HRA/LUC and HRA/PTEN. There were no significant differences among the three groups.

Statistical analysis. Each experiment was performed three times. The significance of differences were analyzed by unpaired Student's t-test and analysis of variance. A p-value of <0.05 was defined as statistically significant.

Results

PTEN sequence analysis in HRA cells. Mutations in the PTEN gene were not found in HRA in any of nine coding exons (data not shown).

Expression of PTEN in HRA cells. The PTEN expression vector pCMV-PTEN-IRES-bsr, and the control vector pCMV-LUC-IRES-bsr were transfected into HRA cells. As shown in Fig. 1, PTEN expression was detected by Western blotting at the position corresponding to a molecular weight

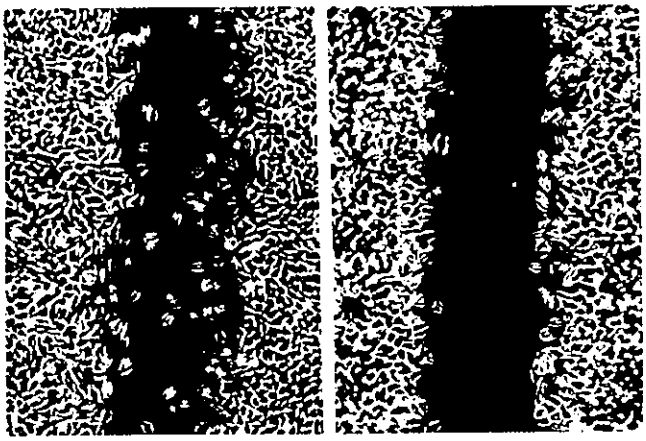

Figure 3. The cell migration of HRA/LUC (left) and HRA/PTEN (right) in the *in vitro* scratch wound healing assay. The cell number of HRA/PTEN migrating 8 h after scratching was 6.3±0.9 cells/mm², which was significantly smaller than those of HRA/LUC (39.7±3.2 cells/mm²) ($p<0.01$).

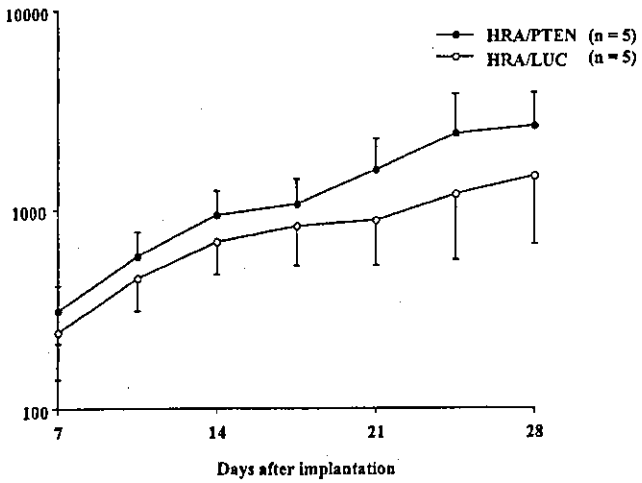

Figure 4. The tumor growth of HRA/LUC and HRA/PTEN. Tumor cells ($5x10^6$ each), were subcutaneously injected on the back of mice. There was no difference in the growth between the two groups. The tumor volumes were estimated following the equation of [width]² x [length] x 1/2 (mm³).

of 55 kDa in HRA/PTEN, while only weak PTEN expression was detected in parent HRA or HRA/LUC.

In vitro cell growth kinetics. The growth curves of HRA, HRA/ LUC and HRA/PTEN are shown in Fig. 2, no significant difference was observed between the three groups. Therefore, enhanced expression of the PTEN did not affect the cell growth *in vitro*.

In vitro scratch wound healing assay. The effects of PTEN expression on cell migration *in vitro* are shown in Fig. 3. The number of HRA/PTEN migrated to the scratched area was 6.3±0.9 cells/mm², which was significantly smaller than those

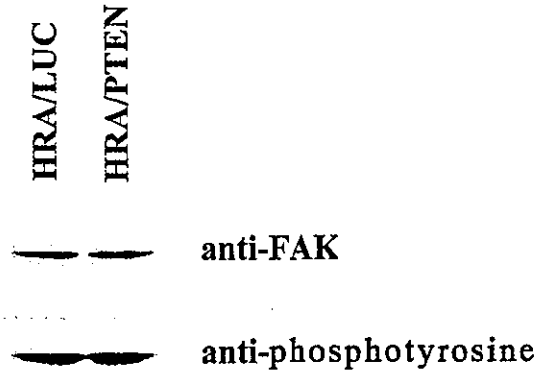

Figure 5. FAK tyrosine phosphorylation in HRA/LUC and HRA/PTEN. Lysates were immunoprecipitated with FAK and immunoblotted with anti-FAK or anti-phosphotyrosine. There was no difference in FAK tyrosine phosphorylation between the two groups.

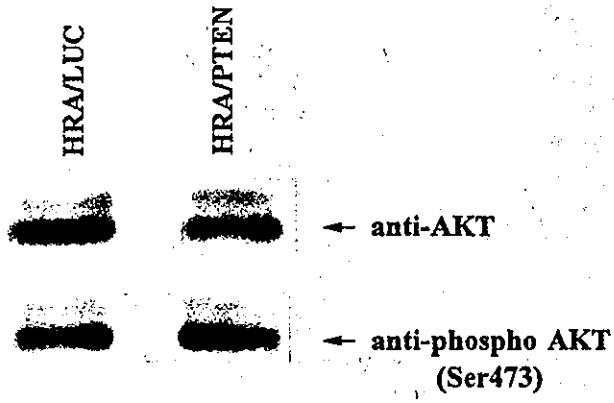

Figure 6. AKT phosphorylation in HRA/LUC and HRA/PTEN. Lysates were immunoprecipitated with AKT and immunoblotted with anti-AKT or anti-phospho-AKT. There was no difference in AKT phosphorylation between the two groups.

of HRA/LUC (39.7±3.2 cells/mm²) ($p<0.01$). Therefore, the enhanced expression of PTEN inhibited the cell migration.

In vivo tumor growth. Based on the *in vitro* experiments, we tested the effect of PTEN expression on tumor growth *in vivo*. As shown in Fig. 4, there was no difference in the growth between the two groups. Therefore, the enhanced expression of PTEN did not affect the tumor growth.

FAK tyrosine phosphorylation. FAK tyrosine phosphorylation in HRA/LUC and HRA/PTEN was assessed by immunoblotting with anti-phosphotyrosine. As shown in Fig. 5, there was no difference in tyrosine phosphorylation between the two groups. Therefore, the enhanced expression of PTEN did not affect FAK tyrosine phosphorylation in HRA.

AKT phosphorylation. AKT phosphorylation in HRA/LUC and HRA/PTEN was assessed by immunoblotting with anti-phospho-AKT. As shown in Fig. 6, the level of AKT phosphorylation was not significantly different between HRA/LUC and HRA/PTEN. Therefore, the enhanced expression of PTEN did not affect the level of AKT phosphorylation in HRA.

Discussion

In this study, we introduced a PTEN-expression vector into an ovarian cancer cell line HRA carrying wild-type *PTEN* to establish a cell line overexpressing PTEN (HRA/PTEN), and investigated the effects of overexpressed PTEN. No significant differences were observed in the *in vitro* cell growth or *in vivo* tumor growth between HRA/PTEN and the control cells (HRA/LUC), confirming that overexpression of PTEN does not affect the ovarian cancer cell growth. In contrast, the number of migrating cells was significantly smaller in HRA/PTEN than in the control *in vitro* scratch wound healing assay, demonstrating that overexpression of PTEN inhibits ovarian cancer cell migration. There have been several reports of the inhibitory effect of PTEN on cancer cell migration (12,21,22). However, in these studies, *PTEN* was introduced into *PTEN*-mutated or deleted cell lines. It has been reported that mutations in the *PTEN* gene were not found in any of the 50 primary ovarian cancers or 11 immortalized ovarian cancer cell lines (23). From this point of view, it is not clear whether these experimental models correspond to clinical ovarian cancer. Since the HRA cell line used in this study carries wild-type *PTEN*, it is appropriate as a general model of clinical ovarian cancer.

Recently, we introduced HGF/NK4, a hepatocyte growth factor (HGF) antagonist, into an ovarian cancer cell line, and showed that HGF/NK4 overexpression inhibits ovarian cancer cell migration *in vitro* and peritoneal dissemination *in vivo* (18). Thus, it is considered that cell migration is one of the critical factors that directs peritoneal dissemination, and that the inhibition of cell migration can control peritoneal dissemination. However, in this study, the overexpression of PTEN significantly inhibited cell migration but did not suppress peritoneal dissemination in an *in vivo* study (data not shown). In order to achieve therapeutic effects, either a more potent expression of PTEN, or other co-factors might be required, and a precise mechanism of action should be understood.

Although the mechanism of PTEN by which cell migration is inhibited is not known, the following possibilities can be raised. The mechanism via FAK, which is one of the specific substrates of PTEN (12,21). Tamura *et al* showed, using a PTEN-mutated glioblastoma cell line, that PTEN-dephosphorylated FAK, which resulted in inhibition of cell migration (12,21). The second possibility is inhibition of cell migration by an AKT-mediated mechanism. AKT is one of the substrates for PTEN, and is dephosphorylated by PTEN (24). Kwon *et al* have reported that AKT inhibits the binding of Rac1 to GTP (25). Rac1-GTP binding is closely involved in cell migration (26). These observations suggest that PTEN overexpression inhibits ovarian cancer cell migration by an AKT-Rac1-mediated mechanism. However, in this study, no dephosphorylation of FAK or AKT was observed after introduction of the *PTEN* gene, excluding the possibility that inhibition of cell migration by overexpression of PTEN in HRA cells is mediated by FAK or AKT. PTEN has both lipid phosphatase activity and protein phosphatase activity (27), whereas the G129E mutant of PTEN has only protein phosphatase activity (27). Therefore, establishing transfectants with the G129E mutant to examine cell migration-inhibitory activity in the transfectants may elucidate part of the mechanism.

In this study, overexpression of PTEN did not influence cancer cell proliferation, while Minaguchi *et al* have reported that it suppresses the proliferation of ovarian cancer cells (14). The reasons for this difference are as follows. First, the different methods of gene transfer may have produced different results. We introduced the PTEN-expressing vector by the calcium phosphate precipitation method, while Minaguchi *et al* used the PTEN-expressing adenovirus vector. In general, gene transfer with the adenovirus vector has a higher transfer efficacy than the calcium phosphate precipitation method, leading to high gene expression. PTEN transfer had little effect on the proliferation of 3 of the 9 ovarian cancer cell lines used in their study. They ascribed it to a difference in the efficacy of gene transfer. To influence the proliferation of ovarian cancer cells, a high transfer efficacy, that is, a very high expression may be needed. Secondly, amplification of the phosphatidylinositol 3'-kinase (PIK3) gene may be involved. In ovarian cancers, amplification of the PIK3 gene is frequently observed (28). PIK3 phosphorylates phosphatidylinositol 3,4,5-triphosphate (PIP3) and promotes cell proliferation (29,30). PIP3 is also a PTEN-specific substrate (30), and overexpression of PTEN causes dephosphorylation of PIP3, presumably suppressing cell proliferation. Probably because no PIK3 gene amplification was present in the HRA cell line used in this study, no cell proliferation-inhibitory effect was noted. It is possible that the cell proliferation-inhibitory activity of PTEN is observed only in ovarian cancers with amplified PIK3 gene.

Using the p53 gene, many fundamental studies (31) and several clinical trials (32,33). have been conducted to develop gene therapy with a tumor suppressor gene. Many clinical trials have been conducted in patients with p53 mutations. However, since mutant p53 is thought to impair the function of wild-type p53 (34) the effect of therapy for patients with tumors carrying a mutant p53 gene would be limited. In contrast, a stronger effect may be expected from gene therapy with the *PTEN* gene, because mutant *PTEN* rarely occurs in ovarian cancers. An attempt to produce antitumor activity through overexpression of a mutation-free gene in the patient being treated may provide a gene therapy strategy with a tumor suppressor gene.

In summary, the results in this basic study *in vitro* showed that overexpression of PTEN inhibits ovarian cancer cell migration, and suggest the future possibility of gene therapy for ovarian cancer through the inhibition of peritoneal dissemination by PTEN.

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[CANCER RESEARCH 63, 5091–5094, August 15, 2003]

Interleukin-10-mediated Inhibition of Angiogenesis and Tumor Growth in Mice Bearing VEGF-producing Ovarian Cancer[1]

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ABSTRACT

Interleukin-10 (IL-10) is an immunosuppressive cytokine produced by T lymphocytes and drawing attention as an inhibitor of tumor angiogenesis. In this study, we investigated antiangiogenic and tumor suppressive effects of IL-10 in ovarian cancer cells. mIL-10-expressing plasmid was transferred into two ovarian cancer cell lines, SHIN-3 [vascular endothelial growth factor (VEGF) producing] and KOC-2S (non-VEGF producing). After selection, mIL-10-expressing cells were obtained as SHIN-3/mIL-10 and KOC-2S/mIL-10. No significant differences were observed in *in vitro* growth properties between mIL-10-expressing cells and control (luciferase expressing) cells in either KOC-2S or SHIN-3. The angiogenic activities of mIL-10-expressing cells were measured by dorsal air sac assay, which detected the number of newly formed blood vessels within a chamber *in vivo*. In addition, tumor formation was evaluated by s.c. tumor transplantation, and survival was monitored after i.p. injection of ovarian cancer cells into BALB/c nude mice. Both *in vivo* angiogenic activity and tumor growth were significantly inhibited in SHIN-3/mIL-10 cells compared with the control. Moreover, peritoneal dissemination was inhibited, and the survival period was significantly prolonged (mean survival days > 90 *versus* 36). In contrast, in the case of KOC-2S cells, no significant differences were observed in any of the parameters tested. These results indicate that IL-10 has suppressive effects on angiogenesis, tumor growth, and peritoneal dissemination of VEGF-producing ovarian cancer cells. Although the mechanisms of the antiangiogenic effect of IL-10 are still unclear, the potential usefulness of IL-10-mediated gene therapy of ovarian cancer was suggested.

INTRODUCTION

Ovarian cancer has been on the increase in recent years and is currently a leading cause of death from gynecologic malignancy (1). Ovarian cancer is relatively asymptomatic and first detected as an advanced disease with ascites and peritoneal dissemination in more than half of patients (2). Although the progress of anticancer agents, such as platinum analogues and paclitaxel, has improved therapeutic response in advanced ovarian cancer, the long-term prognosis remains unsatisfactory (3). In the case of ovarian cancer, peritoneal dissemination is the most common form of progression and recurrence (2), and both the size of disseminated tumors and amount of ascitic fluid are known to correlate inversely with the prognosis (4). Therefore, the control of peritoneal dissemination is crucial to improve the prognosis.

The growth and spread of malignant neoplasms largely depend on angiogenesis (5, 6). Angiogenesis in ovarian cancer also plays a major role in the growth of disseminated tumors (7). Thus, inhibition of angiogenesis may not only suppress peritoneal dissemination and growth of the disseminated lesions but also improve the prognosis for advanced ovarian cancer.

In addition to being known as an immunosuppressive cytokine, IL-10[3] has recently been reported to have angiogenesis inhibitory activity (8, 9). We focused on this action of IL-10 and investigated the inhibition of peritoneal dissemination through angiogenesis inhibition by IL-10 both *in vitro* and *in vivo* using an IL-10-transduced human ovarian cancer cell line.

MATERIALS AND METHODS

Cell Lines and Plasmids. The human ovarian serous adenocarcinoma cell lines SHIN-3 (10) provided by Dr. Y. Kiyozuka (Hyogo College of Medicine, Japan), and KOC-2S (11) provided by Dr. A. Kataoka (Kurume University, School of Medicine, Japan) were maintained as reported. Cloned mIL-10 cDNA was inserted into the *Bam*HI site of the plasmid pCMV-IRES-bsr (12). Then either the plasmid pCMV-mIL-10-IRES-bsr or the control plasmid pCMV-LUC-IRES-bsr (12) encoding LUC were transferred into SHIN-3 and KOC-2S cells by the standard calcium phosphate precipitation method (13). The cells were selected in the presence of 10 μg/ml blasticidin S hydrochloride (Funakoshi, Tokyo, Japan). Resistant clones were obtained after 4 weeks and named as SHIN-3/mIL-10, SHIN-3/LUC, KOC-2S/mIL-10, and KOC-2S/LUC, respectively. The cells were subsequently maintained in the presence of 10 μg/ml blasticidin S hydrochloride.

Confirmation of mIL-10 Secretion and VEGF Quantitation. Conditioned media from 5×10^6 of cells in 10 ml of serum-free DMEM:F12 medium (Life Technologies, Inc., Grand Island, NY) in 10-cm dishes were collected at 24 h. Western blotting was performed under standard procedures (14) using anti-mIL10 monoclonal antibody (Genzyme, Cambridge, MA). VEGF concentrations of cultured supernatants were measured with a Quantikine Human VEGF ELISA kit (R&D Systems) according to the manufacturer's instructions.

***In Vitro* Cell Growth Kinetics and *in Vivo* Tumor Growth.** Both mIL-10-expressing and control cells were plated in 3-cm dishes at 5×10^4 cells/dish and cultured subsequently in 10% serum-supplemented DMEM/F12 medium. Every 24 h, one set of the cells was dislodged using 0.05% trypsin-EDTA and counted by hemocytometer. To investigate the effect of mIL-10 expression on tumor growth, 5×10^6 cells were s.c. transplanted on the back of the female BALB/c nude mice (Japan Clea Laboratories, Tokyo, Japan) at 4 weeks of age. Six days after transplantation, two dimensions of the tumors were measured using a caliper every 3 days. The tumor volume was estimated with the equation $[\text{width}]^2 \times [\text{length}] \times 1/2$. All of the animal experiments were performed under the guidelines of Jichi Medical School.

Dorsal Air Sac Assay. The angiogenic activities of mIL-10-expressing cells and control cells were measured by dorsal air sac assay as described previously (15). Briefly, diffusion chambers (Millipore Co., Bedford, MA) were filled with each of the suspension of cells (3×10^6) in 150 μl of PBS. The cell-containing chamber was implanted into the preformed s.c. air sac in the dorsum of an anesthetized female BALB/c nude mouse. On day 5, the implanted chambers were removed from treated mice. The angiogenic response was assessed by determining the number of newly formed vessels >3 mm in length within the area marked by a black ring. As described previously (15), the blood vessels formed by angiogenic factors released from tumor cells were morphologically distinct from the pre-existing background vessels.

Received 11/7/02; revised 4/14/03; accepted 6/5/03.

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[1] Supported in part by grants from the Ministry of Health, Labor and Welfare of Japan; grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan; and a grant-in-aid of the Japan Medical Association.

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[3] The abbreviations used are: IL-10, interleukin-10; CMV, cytomegalovirus; IRES, internal ribosome entry site; VEGF, vascular endothelial growth factor; *bsr*, blasticidin S resistance gene; mIL-10, murine interleukin-10; LUC, luciferase.

***In Vivo* Ascites Accumulation, Peritoneal Dissemination, and Survival Rate.** BALB/c nude mice, maintained under a pathogen-free environment, were inoculated i.p. with either mIL-10-expressing cells or control cells at 5×10^6 cells/body. Two and 3 weeks after the i.p. injection, the mice were sacrificed; 1 ml of PBS was injected i.p., and the ascites fluid was recovered totally. Peritoneal dissemination was evaluated by counting the number of tumor nodes on the surface of the small intestine. Survival of the mice was monitored twice daily. The survival rate was calculated by the Kaplan-Meier method.

Statistical Analysis. All experiments were independently repeated twice or more. The significance of differences were analyzed by unpaired Student's *t* test. The survival rates were analyzed by the generalized Wilcoxon and Log-rank tests. A value of $P < 0.05$ was defined as statistically significant.

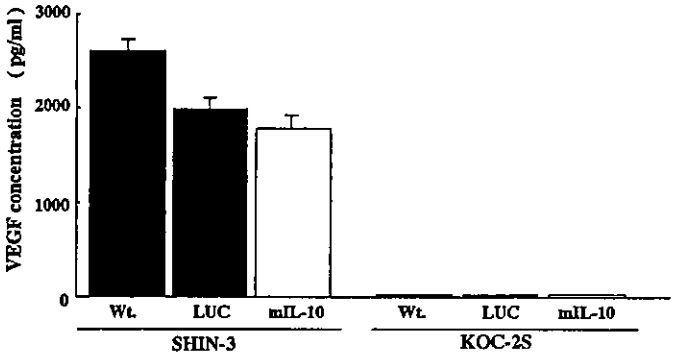

Fig. 1. Quantitation of VEGF in culture supernatant. mIL-10-expressing cells and control cells were plated in wells (5×10^6 cells/well) and cultured in serum-free medium for 24 h. SHIN-3 cells produced VEGF in contrast to KOC-2S cells. There were no significant differences between transduced and wild-type cells. The data represent the mean ± SD based on triplicate experiments.

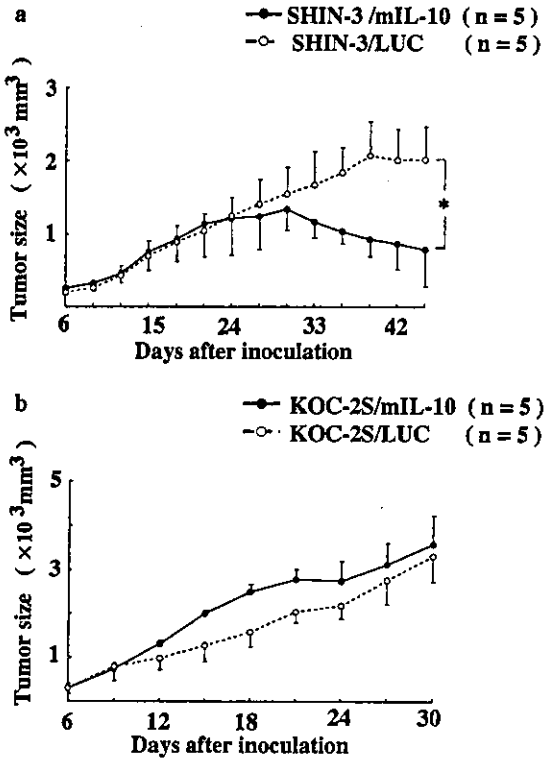

Fig. 2. *In vivo* tumor growth of mIL-10-expressing cells. Tumor cells were s.c. injected into the back of mice and measured every 3 days. In *a*, the tumor size of SHIN-3/mIL-10 (●) at 45 days after inoculation was 0.8 ± 0.5 × 10^3 mm^3, which was significantly smaller than SHIN-3/LUC (○; 2 ± 0.4 × 10^3 mm^3; *, $P < 0.05$). In *b*, the tumor growth curves of KOC-2S/mIL-10 (●) and KOC-2S/LUC (○) did not show significant differences. The tumor volumes were calculated based on the equation [width]^2 × [length] × 1/2 (mm^3). The data represent the mean ± SD.

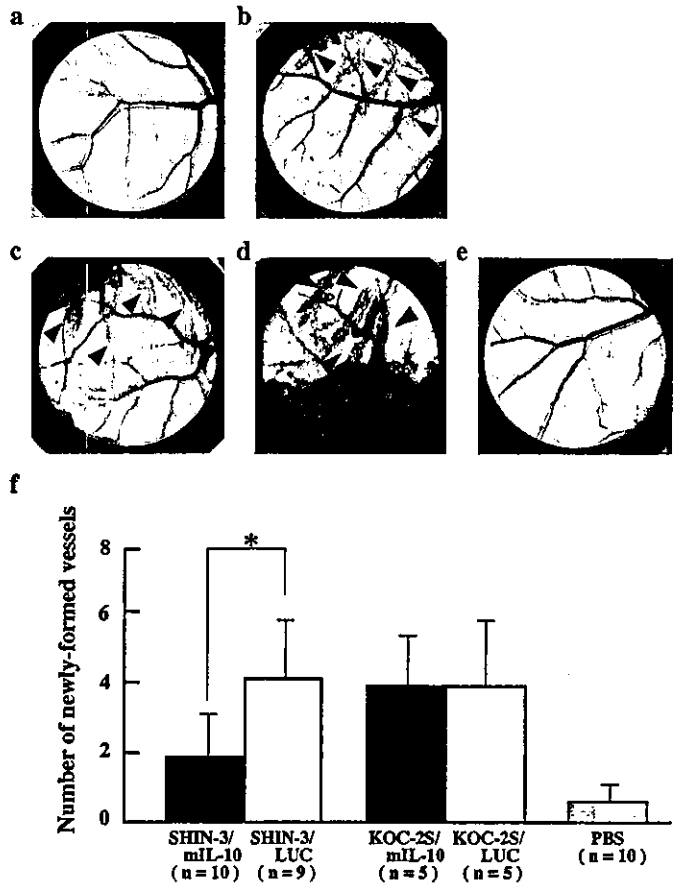

Fig. 3. Angiogenic response induced by tumor cells. Chambers containing SHIN-3/mIL-10 (*a*) and KOC-2S/mIL-10 (*c*) were implanted s.c. into mice. Chambers containing SHIN-3/LUC (*b*) and KOC-2S/LUC (*d*) were implanted as control group. Chambers containing only PBS (*e*) were implanted as vehicle-treated group. Newly formed vessels induced by SHIN-3 cells were mostly abolished by mIL-10 expression (*a*). *Arrowheads* point to newly formed vessels. Photographs were taken at day 5. The angiogenic response was assessed by counting the number of newly formed vessels longer than 3 mm(f). The numbers of newly formed vessels are significantly reduced in the case of SHIN-3/mIL-10 (1.9 ± 1.2) compared with the case of SHIN-3/LUC (4.22 ± 1.72). KOC-2/mIL-10 and KOC-2S/LUC did not show significant differences. Each bar represents the mean ± SD. *, $P < 0.05$.

RESULTS

Expression of mIL-10 and VEGF Concentration in Culture Supernatant. Either the mIL-10-expressing plasmid vector pCMV-mIL-10-IRES-bsr or the control vector (pCMV-LUC-IRES-bsr) was transferred into the SHIN-3 and KOC-2S cell lines, and the clones were developed. The presence of mIL-10 in the culture supernatant of mIL-10-transfected cells was confirmed by Western blotting. In contrast, no mIL-10 could be detected from culture supernatants of control cells (data not shown). Fig. 1 shows VEGF concentrations in culture supernatants from SHIN-3 and KOC-2S cells. The wild-type SHIN-3 cells were VEGF hypersecretory (2607 ± 124 pg/ml), whereas the wild-type KOC-2S cells were not (20 ± 4 pg/ml). There were no significant differences in the level of VEGF secretion between the mIL-10-transfected SHIN-3 or KOC-2S cell line and the corresponding control, showing that mIL-10 expression did not influence the VEGF secretion by tumor cells *in vitro*.

***In Vitro* Cell Growth Kinetics.** These mIL-10-transduced cells and the control cells were microscopically indistinguishable and had similar growth rates *in vitro*. No differences were noted in either the SHIN-3 or KOC-2S cell line. Therefore, the expression of the mIL-10 gene did not influence the tumor cell growth *in vitro* (data not shown).

***In Vivo* Tumor Growth.** On the basis of the results of *in vitro* experiments, we investigated the effect of mIL-10 expression on tumor growth *in vivo*. As shown in Fig. 2*a*, SHIN-3/mIL-10 tumors tend to shrink from day 30 after inoculation, significantly smaller than the control on day 45 [$(0.8 \pm 0.5) \times 10^3$ mm^3 *versus* $(2 \pm 0.4) \times 10^3$ mm^3, $P < 0.05$]. In contrast, KOC-2S/mIL-10 tumors did not show a difference from the control (Fig. 2*b*). These results indicate that mIL-10 expression suppressed SHIN-3 tumor growth.

Dorsal Air Sac Assay. We examined the effect of mIL-10 expression on angiogenesis by dorsal air sac assay. The number of newly formed vessels in chambers filled with PBS alone (negative control) was 0.6 ± 0.52, and little or no angiogenic response occurred after experimental manipulation or during the subsequent healing process (Fig. 3*e*). The number of newly formed vessels in SHIN-3/LUC tumors separated by a semipermeable filter (positive control) was 4.22 ± 1.72 (Fig. 3*b*). However, as shown in Figs. 3, *a* and *f*, the number of newly formed vessels in SHIN-3/mIL-10 tumors was significantly decreased (1.9 ± 1.2, $P < 0.05$) compared with that in SHIN-3/LUC tumors. These results indicate that, in SHIN-3 cells, mIL-10 expression suppresses the SHIN-3-induced angiogenesis. In contrast, mIL-10 expression did not significantly influence angiogenesis in KOC-2S cells *in vivo* (Figs. 3, *c*, *d*, and *f*).

***In Vivo* Ascites Accumulation, Peritoneal Dissemination, and Survival Kinetics.** To investigate the effect of persistent mIL-10 expression on peritoneal dissemination and ascites accumulation, we injected either mIL-10-transduced cells or control cells into nude mice i.p. The mean volume of ascitic fluid in the SHIN-3/mIL-10-injected group was significantly smaller than that in the control group (0.73 ± 0.15 ml *versus* 2.8 ± 0.4 ml, $P < 0.05$; Fig. 4*a*). As the peritoneal dissemination, gross tumor nodes on small intestines in the SHIN-3/mIL-10-injected group were significantly decreased (6.7 ± 2.5 *versus* 96 ± 14.5, $P < 0.05$; Fig. 4*b*). In contrast, there were no significant differences in the mean volume of ascitic fluid or number of peritoneal disseminations between the KOC-2S/mIL-10-injected group and positive control group (Fig. 4, *a* and *b*).

In the SHIN-3/LUC-injected group, marked ascites accumulation was observed around day 20 after i.p. injection, and all mice died within 48 days after injection. In contrast, in the SHIN-3/mIL-10-injected group, the ascites accumulation was suppressed, and a significantly longer survival was observed ($P < 0.05$; Fig. 4*c*). On the other hand, there were no significant differences in the survival duration between the KOC-2S/mIL-10-injected group and control group (Fig. 4*d*). These results indicate that, specifically in SHIN-3 cells, persistent mIL-10 expression suppresses ascites production and peritoneal dissemination and prolongs mouse survival.

DISCUSSION

In this study, mIL-10 expression in a VEGF-hypersecretory cell line resulted in suppression of *in vivo* tumor growth, peritoneal dissemination, ascites accumulation, and prolonged survival in a mouse model of peritoneal dissemination. Presumably, the mechanism of the difference with these results was related to the angiogenesis-inhibitory activity of IL-10, because angiogenic activity was significantly suppressed in mIL-10-overexpressing SHIN-3/mIL-10 cells. In addition, the finding that mIL-10 expression had little effect on the VEGF-hyposecretory cell line KOC-2S suggests that the angiogenesis inhibitory activity of mIL-10 is mediated by VEGF. Investigating the mechanism of the VEGF-mediated angiogenesis inhibitory activity of mIL-10 in an experiment with mIL-10 knockout mice, Silvestre *et al.* (8) reported that IL-10 inhibited angio-

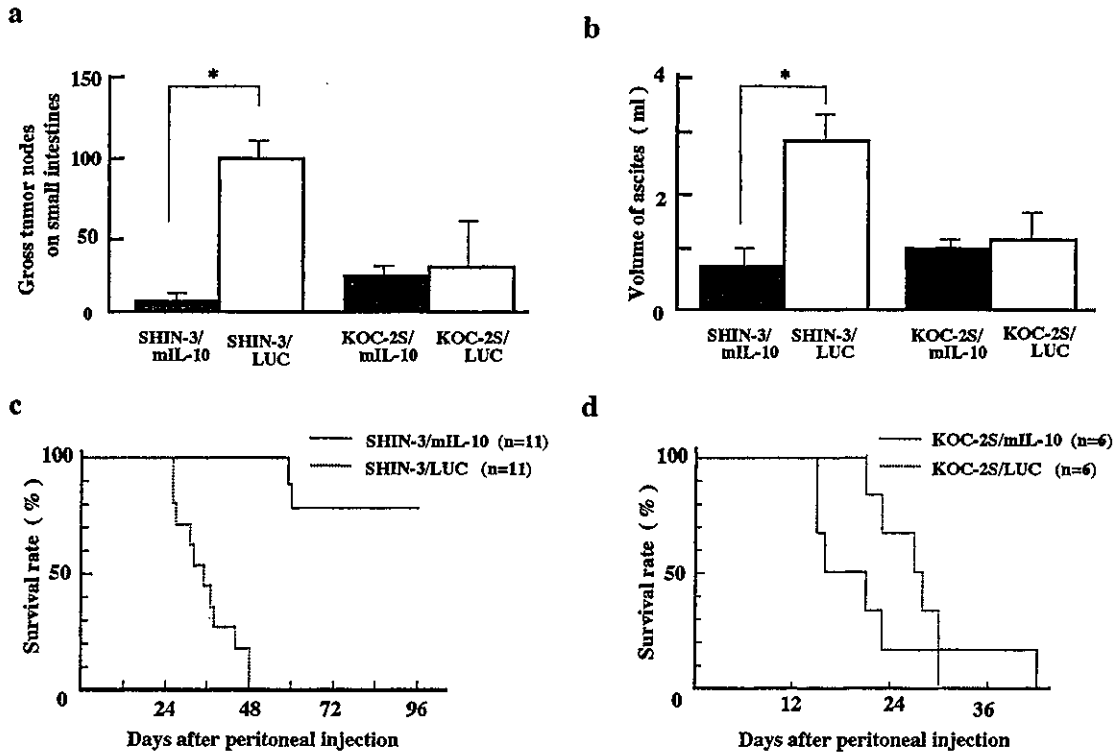

Fig. 4. Peritoneal dissemination, ascites accumulation, and survival kinetics of mIL-10-expressing cells. Tumor cells, 5×10^6 each, were i.p. injected into mice. In *a*, peritoneal dissemination at 3 weeks after injection was significantly less extensive in SHIN-3/mIL-10 (6.7 ± 2.5) than SHIN-3/LUC (96 ± 14.5). In *b*, ascites accumulation 3 weeks after injection was significantly reduced in SHIN-3/mIL-10 (0.73 ± 0.15 ml) compared with SHIN-3/LUC group (2.8 ± 0.4 ml). In *c*, the survival of mice injected with SHIN-3/mIL-10 was significantly longer than that of mice injected with SHIN-3/LUC ($P < 0.01$, by generalized Wilcoxon and Log-rank tests). No significant differences were observed in peritoneal dissemination, ascites accumulation, and survival kinetics between KOC-2S/mIL-10 and KOC-2S/LUC at 2 weeks after injection (*a*, *b*, and *d*). The data represent the mean $\pm$ SD based on triplicate experiments. *, $P < 0.05$.

genesis by down-regulating VEGF production. In an experiment with a human umbilical endothelial cell line, Cervenak *et al.* (9) found that IL-10 inhibits angiogenesis by competitively inhibiting VEGF. These observations, including our present study, suggest that mIL-10 inhibits angiogenesis through VEGF. However, the mechanism by which mIL-10 inhibits VEGF is yet to be elucidated and awaits further study.

Although this study found that IL-10 inhibited the growth and peritoneal dissemination of ovarian cancer, the application of IL-10 in clinical practice will require a prolonged, persistent expression. Therefore, it is desirable to transfer the IL-10 gene into a certain tissue and establish a gene therapy system. Because IL-10 is a secretory protein, it is not necessary to directly transfer the gene into cancer cells; transfer into cells such as skeletal muscle or peritoneal cells and its expression may turn the cells into "a factory" from which IL-10 is secreted into circulation to exert its effect. We consider that AAV vectors (9), which allow efficient gene transfer into a variety of cells, are suitable for implementing this strategy. We are in the process of testing its efficacy.

There may be further advantage in the use of IL-10 to treat cancer patients. In addition to angiogenesis inhibitory activity, IL-10 has been reported to have the effect of improving cancerous cachexia through suppression of the production of tumor necrosis factor-α and other cytokines (16, 17); therefore, IL-10 holds promise for improving the patient's QOL not only through its direct effect on tumors but also through the improvement of cancerous cachexia.

In summary, this study suggests that IL-10 inhibits the growth and peritoneal dissemination of ovarian cancer through the inhibition of angiogenesis, leading to improved survival. It also suggests the possibility of a novel gene therapy method using IL-10 and targeted at inhibition of peritoneal dissemination.

ACKNOWLEDGMENTS

We thank Avigen, Inc. (Alameda, CA) for the AAV vector production system.

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Journal of General Virology (2003), 84, 2127–2132 DOI 10.1099/vir.0.19193-0

Short Communication

Positive and negative effects of adeno-associated virus Rep on AAVS1-targeted integration

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Adeno-associated virus type 2 integrates preferentially into the AAVS1 locus on chromosome 19 of the human genome. It was reported previously that transfection with two plasmids, one for Rep and the other carrying a transgene flanked by inverted terminal repeats (ITRs), enables preferential integration of the latter into AAVS1. Aiming at increasing the frequency of AAVS1-specific integration, the Rep- to transgene-plasmid ratio necessary to achieve a higher frequency of site-specific integration was examined. 293 cells were co-transfected with the Rep78 plasmid and an ITR-flanked Neo gene at different ratios. G418-resistant clones were selected randomly. Extensive Southern blot analysis showed an optimum range of Rep78 expression. In that range, approximately 20% of clones harboured the Neo gene at AAVS1. Excess Rep expression, however, resulted in 'abortive' integration of the Neo gene, a rearrangement of AAVS1 without transgene integration. Rep78 appeared to cause abortive integration more extensively than Rep68. Deleterious effects of the Rep protein on the AAVS1 locus should be considered to develop an improved AAVS1-targeted system.

Received 3 March 2003
Accepted 1 April 2003

Retrovirus vectors are used widely for gene therapy applications. However, the random integration of retrovirus vector sequences may cause insertional mutagenesis, and the accidental activation of proto-oncogenes cannot be prevented. Adeno-associated virus type 2 (AAV) is a non-pathogenic parvovirus being considered as a gene transfer vehicle (Berns & Giraud, 1996; Kotin, 1994; Muzyczka, 1992). The AAV genome, a linear single-stranded DNA of 4·7 kb long, integrates preferentially into a defined locus in the human genome, AAVS1, on chromosome 19 (19q13·3-qter) (Kotin *et al.*, 1990, 1992; Samulski *et al.*, 1991). AAV can provide a potentially ideal gene delivery system for site-specific integration.

Each end of the AAV genome consists of inverted terminal repeats (ITRs), which are required in *cis* for AAVS1-specific integration. The AAV *rep* gene encodes four overlapping non-structural proteins, Rep78, Rep68, Rep52 and Rep40, while the *cap* gene encodes structural Cap proteins. The unspliced and spliced transcripts from the p5 promoter encode Rep78 and Rep68. Either Rep78 or Rep68 plays a key role in AAVS1-specific integration, binding ITRs (Im & Muzyczka, 1989) and AAVS1 (Weitzman *et al.*, 1994) via tandem repeats of the GAGC tetramer (McCarty *et al.*, 1994). The mechanism of AAVS1-specific integration of AAV has not been elucidated fully. However, a model whereby integration proceeds via a circular intermediate of the AAV genome by a deletion-substitution mechanism has been proposed (Dyall & Berns, 1998; Linden *et al.*, 1996).

A structural difference between Rep78 and Rep68 is that Rep78 possesses a zinc finger-like motif at its carboxyl terminus. Both Rep proteins share essentially the same functions: strand-specific DNA binding (Im & Muzyczka, 1989), site-specific nicking and ATP-dependent helicase activity (Im & Muzyczka, 1990). Either Rep protein alone is sufficient for replication of the AAV genome (Hölscher *et al.*, 1994) and for AAVS1-specific integration (Surosky *et al.*, 1997). The multifunctional Rep proteins inhibit cellular transformation by heterologous genes (Labow *et al.*, 1987; Yang *et al.*, 1992) and suppress heterologous promoters, including the c-*fos*, c-*myc*, H-*ras* and LTR of human immunodeficiency virus type 1 (HIV-1) (Hermonat, 1991, 1994; Oelze *et al.*, 1994). The Rep proteins also modulate cell cycle-regulating proteins (Hermanns *et al.*, 1997). These results indicate that overexpression of Rep proteins has negative effects on cells and is, on occasion, lethal to cells.

AAV vectors lacking the *rep* gene fail to integrate into AAVS1, showing apparent random integration into the host chromosomal DNA (Kearns *et al.*, 1996). A non-viral plasmid-based system capable of integrating a transgene specifically into AAVS1 has been described; this was achieved by transferring the transgene flanked by the ITRs with transient expression of Rep78 or Rep68 (Balagué *et al.*, 1997; Pieroni *et al.*, 1998; Shelling & Smith, 1994; Surosky *et al.*, 1997; Tsunoda *et al.*, 2000). Thus, this system is safer than integrating retrovirus and AAV vectors randomly. A strategy utilizing two plasmids, one harbouring the transgene cassette between the ITR sequences and the other for Rep expression, allows only the transgene plasmid to integrate into the AAVS1 locus (Surosky *et al.*, 1997). This method successfully introduced the transgene into AAVS1 in haematopoietic K562 cells (Kogure *et al.*, 2001).

The frequency of AAVS1-specific integration by the plasmid-based methods has differed among studies. Shelling & Smith (1994) reported that 9 of 12 cell clones (75 %) obtained by transfecting HeLa or 293 cells with an AAV vector plasmid on which the Neo gene was placed under the control of the p40 promoter, the original promoter for Cap proteins, had rearranged AAVS1 and mentioned that approximately 50 % of the rearranged bands also hybridized to an AAV probe. Another strategy using one plasmid on which both a Rep cassette and an ITR-flanked transgene cassette were placed has targeted the transgene to AAVS1 in 6 of 21 (29 %) 293 cell clones (Balagué *et al.*, 1997). Similar methods applied to other cell lines, HeLa and Huh-7 cells, have been able to insert the transgene to AAVS1 in up to 20 % of clones (Lamartina *et al.*, 1998; Pieroni *et al.*, 1998). All the studies mentioned here used a one plasmid system and the p5 promoter for Rep expression.

Aiming at increasing the frequency of AAVS1-directed integration, we first examined whether AAVS1-specific integration depended on the levels of Rep protein expressed in cells. To control the expression of the cytotoxic Rep proteins, we chose to vary the amount of Rep plasmid DNA. 293 cells were transfected using the calcium phosphate precipitation method with 2, 0·4, 0·2, 0·04, 0·02 or 0 μg pCMVR78, which expresses Rep78 under the control of the CMV promoter (Surosky *et al.*, 1997), and 2 μg pWNeo (Rep:Neo ratio of 1, 0·2, 0·1, 0·02, 0·01 or 0). pWNeo bears a Neo gene under the control of the CMV promoter between the ITRs. To monitor the amount of plasmid DNA incorporated, extrachromosomal DNA was analysed by Southern blot with a plasmid backbone probe (Fig. 1a). As the amount of Rep plasmid decreased, signal intensities corresponding to pCMVR78 decreased gradually, whereas those corresponding to pWNeo changed little, indicating that the amount of plasmid DNA incorporated into the cells correlated with that used for transfection. Western analysis of the transfected 293 cells confirmed that the expression level of the Rep protein was a function of the amount of pCMVR78 (Fig. 1b).

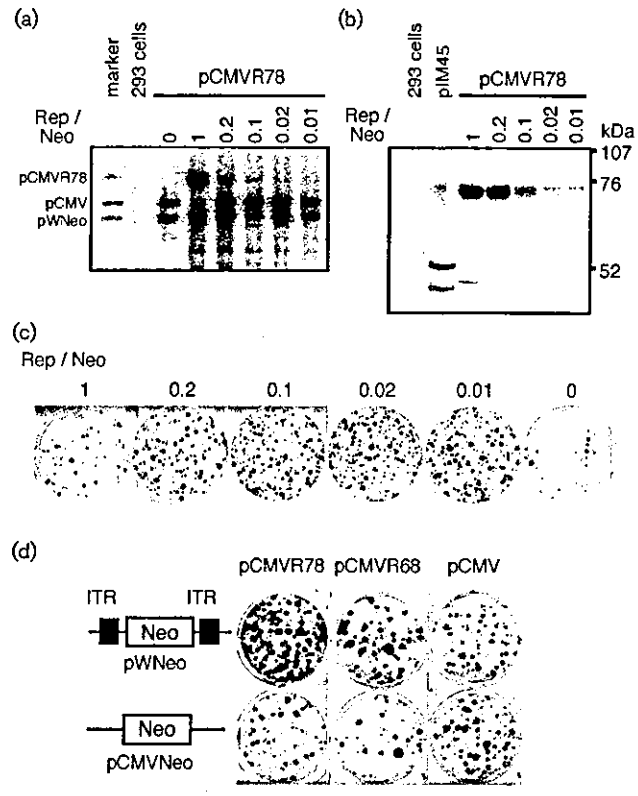

Fig. 1. (a) Quantification of plasmid DNAs incorporated into 293 cells. 293 cells (2×10^5 cells per well) in 6-well plates were transfected with 2 μg pWNeo and various amounts of pCMVR78 (0, 2, 0·4, 0·2, 0·04 or 0·02 μg) at a Rep to Neo plasmid ratio of 0, 1, 0·2, 0·1, 0·02 or 0·01 by the calcium phosphate precipitation method. The amount of plasmid DNA transfected per well was made up to a total of 4 μg with a plasmid devoid of a Rep cassette (pCMV). Following transfection, extrachromosomal DNA was isolated and treated with *Bam*HI. *Bam*HI digestion generates a 5·9, 3·4 or 4·0 kb band, derived from pCMVR78, pWNeo or pCMV, respectively, that hybridizes to a plasmid backbone probe. (b) Expression of Rep78 in 293 cells transfected with various amounts of pCMVR78. The Rep to Neo ratio is indicated above each lane. pIM45 harbours the AAV *rep* and *cap* genes (McCarty *et al.*, 1991). Anti-Rep antibody 294.4 (a gift from J. Kleinschmidt) was used. (c) Comparison of the number of G418-resistant colonies obtained using various amounts of pCMVR78. Following transfection with pCMVR78 and pWNeo at different Rep to Neo ratios (1, 0·2, 0·1, 0·02, 0·01 or 0 μg), a 1/500 fraction of transfected cells was replated onto a 10 cm dish in triplicate and cultured for 10 days in the presence of G418. (d) Representative comparison of the number of G418-resistant colonies generated using pCMVR78 or pCMVR68. After transfection with 0·8 μg pCMVR78, pCMVR68 or pCMV and 3·2 μg pWNeo or pCMVNeo, a 1/500 fraction of transfected cells was replated onto 6-well plates in triplicate and cultured for 10 days in the presence of G418. When either Rep plasmid was co-transfected with pWNeo, a larger number of colonies was formed.

Fig. 1(c) compares G418-resistant colonies grown after transfection with pCMVR78. The number of colonies increased significantly when pCMVR78 was added to the transfection solution. The number of colonies observed did not differ significantly at the Rep to Neo ratios of 0·2–0·01. On transfection at the Rep to Neo ratio of 1, however, the number of colonies decreased, probably due to the strong cytotoxicity of Rep78. To estimate the frequency of integration of the Neo gene to AAVS1, we extensively analysed clones by Southern blot. From each group, 14–20 clones were expanded and their genomic DNA was digested with *Hin*dIII or *Eco*RV, enzymes that do not cleave plasmid pWNeo or the proximal portion of AAVS1 where integration of the AAV genome occurs predominantly (Giraud *et al.*, 1994; Kotin *et al.*, 1992). The presence of co-migrating bands that hybridized to both AAVS1 and Neo probes on both *Hin*dIII- and *Eco*RV-blots was a criterion to conclude that the Neo gene was integrated into AAVS1. Table 1 summarizes the result of Southern blot analysis of the 293 cell clones. When the pCMVR78 to pWNeo ratio was 1 or 0·2, approximately 95% of clones showed rearrangement of AAVS1. The frequency of rearrangement of AAVS1 decreased gradually as the amount of pCMVR78 was reduced. Unexpectedly, integration of the Neo gene into AAVS1 was observed only in 1 of 16 clones (6%) at the Rep to Neo ratio of 1. In contrast, transfection at the Rep to Neo ratio of 0·2–0·02 produced approximately 20% of clones that delivered the Neo gene to AAVS1. These results indicated that a high-level expression of the Rep proteins increased the frequency of AAVS1 rearrangement and rather decreased the frequency of AAVS1-specific integration of the transgene.

In the second experiment, we compared Rep78 with Rep68 by transfecting 0·8 μg pCMVR78, pCMVR68 or pCMV along with 3·2 μg pWNeo or pCMVNeo (Rep to Neo ratio of 0·25). pCMVR68 expresses Rep68 alone (Surosky *et al.*, 1997) and pCMVNeo is the same as pWNeo except for the absence of ITRs. Fig. 1(d) is a representative comparison of G418-resistant colony formation. The results of the Southern blot analysis of clones selected randomly are summarized in Table 1. Rep78 generated rearrangement of AAVS1 in approximately 90% of clones and 24% of clones had the Neo gene at AAVS1. The frequency of rearrangement of AAVS1 is 65% with the use of pCMVR68 and 40% of clones integrated the Neo gene to AAVS1. This result suggested that Rep78 appeared to cause more 'abortive' integration of the Neo gene, rearrangement of AAVS1 without integration of transgene, although the difference between Rep78 and Rep68 was not statistically significant.

Fig. 2 shows Southern blot analysis of representative clones with the Neo gene at AAVS1. Fig. 2(a, b) is the *Hin*dIII- or *Eco*RV-digest probed with an AAVS1-specific probe (upper panel) or a Neo probe (lower panel). Each clone presented here has an upshifted band(s) other than a basal band (arrow). Common bands that hybridized to both AAVS1 and Neo probes are indicated by arrowheads. Fluorescent *in situ* hybridization (FISH) analysis confirmed the integration of the Neo gene into chromosome 19 in 11 of 12 clones. A representative chromosomal analysis is shown in Fig. 2(c). The 293 cells used in the present study have four copies of chromosome 19 labelled with Cy-3-conjugated chromosome 19-specific probe (arrowheads). The left panel shows a metaphase spread of clone C6/6. Fluorescein Neo signals are localized to chromosome 19 and another unidentified site (arrows). In the right panel showing analysis of clone C6/18, one chromosome 19 harbours the Neo signals at its terminal portion.

'Abortive' integration into AAVS1, rearrangement of AAVS1 without foreign gene insertion, has been described in 293 or HeLa cells (Balagué *et al.*, 1997; Shelling & Smith,

Table 1. Summary of Southern blot analysis

	Experiment 1									Experiment 2			
Rep plasmid	pCMVR78									pCMVR78		pCMVR68	
Rep to Neo ratio	1		0·2		0·1		0·02		0·01	0·25		0·25	
No. of clones analysed	16		20		19		14		14	17		20	
Enzyme used	*Hin*dIII	*Eco*RV	*Hin*dIII	*Eco*RV	*Hin*dIII	*Eco*RV	*Hin*dIII	*Eco*RV	*Hin*dIII	*Hin*dIII	*Eco*RV	*Hin*dIII	*Eco*RV
Rearranged AAVS1 band*	14	14	19	18	14	8	9	7	6	13	15	12	13
Common band†	4	6	7	4	8	5	4	4	0	9	4	8	8
AAVS1 rearrangement (%)‡	15 (94)		19 (95)		14 (74)		9 (64)		6 (43)	15 (88)		13 (65)	
Neo at AAVS1(%)§	1 (6)		4 (20)		4 (21)		3 (21)		0 (0)	4 (24)		8 (40)	
Neo signal on chromosome 19‖										3		8	

*Number of clones with rearrangement of AAVS1.

†Number of clones with common bands hybridizing to both AAVS1 and Neo probes.

‡Number of clones with rearrangement of AAVS1 on either the *Hin*dIII or the *Eco*RV blot.

§Number of clones with common bands on both *Hin*dIII and *Eco*RV blots.

‖Number of clones with the Neo signal on chromosome 19.

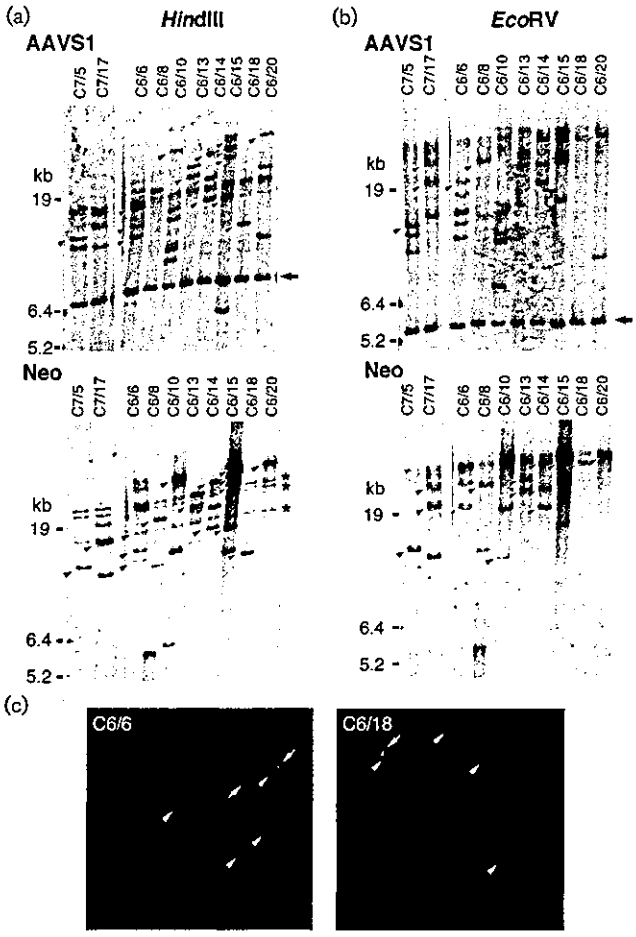

Fig. 2. Southern blot analysis of clones with the Neo gene at AAVS1. *Hin*dIII- or *Eco*RV-digested genomic DNA was hybridized initially with a ^{32}P-labelled AAVS1-specific probe [the 3·0 kb *Acc*I fragment from pRVK (a gift from K.I. Berns)]. After stripping the probe, membranes were rehybridized with a Neo-specific probe (the 2·0 kb *Not*I fragment derived from pWNeo). The presence of co-migrating bands that hybridized to both AAVS1 and Neo probes on both *Hin*dIII and *Eco*RV blots was a criterion to conclude that the Neo gene was integrated into AAVS1. (a) *Hin*dIII-digest probed with an AAVS1-specific probe (upper panel) or with a Neo probe (lower panel). (b) Blot of genomic DNA digested with *Eco*RV isolated from the same clones. Each clone presented here has an upshifted band(s) other than a basal 6·5 kb band (arrow). Common bands that hybridized to both AAVS1 and Neo probes are indicated by arrowheads. Asterisks indicate non-specific bands that cross-hybridized to the Neo probe used. Clones C7/5 and C/17 were derived from transfection with pCMVR78. Clones C6/6 to C6/20 were obtained using pCMVR68. (c) Representative FISH of clones shown to harbour the Neo gene at AAVS1 by Southern blot analysis. Note that the 293 cells used in the present study have four copies of chromosome 19 (arrowheads). Signals detected by a Neo probe (arrows) are indicated.

1994; Surosky *et al.*, 1997). A similar disruption of AAVS1 has been detected in cell lines latently infected with wild-type AAV (Kotin *et al.*, 1990). This phenomenon may be explained in three ways. First, the integrated transgene or AAV genome is disrupted during or after an integration event such that Southern blot analysis cannot detect it. The instability of the integrated AAV genome over passages in a latently infected cell line was described (Cheung *et al.*, 1980). An additional rearrangement can occur in the rearranged AAVS1 region (Shelling & Smith, 1994). Second, recombination between the AAVS1 region and other sites may cause rearrangement of AAVS1 without integration of the transgene at AAVS1. Third, the Rep protein may excise the integrated plasmid DNA or AAV genome, resulting in the loss of the preintegrated sequences.

The 293 cells used in the present study have four copies of chromosome 19. Southern blot analysis showed that some clones had more than three upshifted bands besides a basal band. We used a relatively large probe (3·0 kb) for detecting AAVS1 bands. It is possible that Rep-mediated disruption of the AAVS1 region can produce the multiple bands hybridizing to the AAVS1 probe. Another explanation is as follows: at 24 h post-transfection, we replated transfected cells to isolate clones derived from single cells. At this time-point, the Rep protein was still being expressed in cells and an additional integration event might occur in some cells after cell division.

Lamartina *et al.* (1998) reported no apparent difference between Rep78 and Rep68 in the ability to deliver foreign DNA to AAVS1 in HeLa cells. Several studies have reported the functional differences between Rep78 and Rep68. Rep68 is more efficient in processing dimers to monomer duplex DNA and possesses a stronger nicking activity (Ni *et al.*, 1994, 1998), while the helicase activity of Rep78 is stronger (Wollscheid *et al.*, 1997). The differential effects of Rep78 and Rep68 on the p5, p19 and p40 promoters were described (Weger *et al.*, 1997). In addition, Rep78 inhibits CREB-dependent transcription by interacting with protein kinase (Chiorini *et al.*, 1998; Di Pasquale & Stacey, 1998). None of these findings explains why Rep78 appears to cause more abortive integration. Rep68 may be more suitable for the AAVS1-targeted integration system. To confirm the usefulness of Rep68 in the AAVS1-targeted integration system, further analysis of a larger population of cell clones would be required. Also, the exact functions of the Rep protein in AAVS1-specific integration should be elucidated.

The results presented here have important implications for developing an AAVS1-directed integration system as well as for understanding the mechanism of AAVS1-specific integration by the Rep proteins.

ACKNOWLEDGEMENTS

We thank K. I. Berns for pRVK and J. A. Kleinschmidt for the anti-Rep antibody 294.4. This work was supported in part by grants from the Ministry of Health and Welfare of Japan, Grants-in-Aid for

Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and Special Coordination Funds for Promoting Science and Technology of the Science and Technology Agency of the Japanese Government.

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Int. J. Cancer: 106, 324–326 (2003)

Publication of the International Union Against Cancer

OVEREXPRESSION OF THYMIDYLATE SYNTHASE MEDIATES DESENSITIZATION FOR 5-FLUOROURACIL OF TUMOR CELLS

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The prognosis of cancers of various organs overexpressing thymidylate synthase (TS) has been reported to be poor. It has been suggested that the poor prognosis is partly due to a low sensitivity of TS-overexpressing tumors to TS-targeting 5-fluorouracil (5-FU). To investigate the relationship between TS expression and sensitivity to 5-FU, we used the TS-overexpressing cervical cancer cell line SKG-II/TS and SKG-I/TS that had been established by TS gene transfer. The 50% growth inhibitory concentration (IC_{50}) of 5-FU for SKG-II/TS was 24 ± 6.0 μM, which was 6 times as high as that for the control (4.0 ± 1.1 μM), showing significantly decreased sensitivity to 5-FU ($p < 0.01$). The IC_{50} of 5-FU for SKG-I/TS was 90 ± 15 μM, which was over 2 times as high as that for the control (40 ± 0.6 μM), showing significantly decreased sensitivity to 5-FU ($p < 0.05$). Thus, TS-overexpressing tumors have decreased sensitivity to 5-FU, which may be one of the factors that determine the prognosis of these tumors.

Key words: *thymidylate synthase; cervix neoplasms; 5-fluorouracil; gene transfer; SKG-II; SKG-I*

Thymidylate synthase (TS; EC 2.1.1,45) is the key regulatory enzyme that catalyzes the methylation of deoxyuridine monophosphate in the *de novo* synthesis pathway of pyrimidine. Clinically, the relationship between TS expression and the degree of malignancy of tumors of various organs including colorectal cancer has attracted attention. TS-overexpressing tumors have been reported to be generally poor.[1–8] Although the mechanism leading to the poor prognosis is unknown, exaggerated cell proliferation in TS-overexpressing tumors,[9,10] rapid invasive growth and metastatic activity of tumors[7] and decreased sensitivity chiefly of colorectal cancers to 5-fluorouracil (5-FU)[1,5] have been implicated. However, there is no direct evidence that TS overexpression decreases the sensitivity to 5-FU. In our study, we investigated whether TS overexpression decreases the sensitivity to 5-FU in a TS-overexpressing cervical cancer cell line established by TS gene transfer.

MATERIAL AND METHODS

TS-overexpressing human uterine cervical carcinoma transfectants

TS-overexpressing human uterine cervical carcinoma transfectant SKG-II/TS and control transfectant SKG-II/Luciferase (LUC) were created as described previously.[11]

SKG-I, the human uterine cervical carcinoma cell line, was a kind gift from Professor Nozawa, and was maintained as described.[12] A TS-expressing vector described previously[11] was transfected into SKG-I cells by the standard calcium phosphate precipitation method.[13] The cells were selected in the presence of 10 mg/mL blasticidin S hydrochloride. Resistant cells were obtained after 4 weeks, and SKG-I/TS was obtained. The level of TS in SKG-I/TS was measured by the fluorodeoxyuridine monophosphate (FdUMP)-TS binding assay according to the method of Spears *et al.*,[14] using [6-^{3}H]FdUMP as substrate. Protein content was determined using the Bio-Rad (Veenendaal, The Netherlands) protein assay kit. The TS activity measured as the number of FdUMP-binding sites was calculated per mg protein.

Colorimetric assay

The sensitivity of the cells to 5-FU was investigated by colorimetric assay using a Cell Proliferation Kit II (XTT)(Boehringer Mannheim GmbH Biochemica, Mannheim, Germany). The cells were exposed to 5-FU (Kyowa Hakko, Tokyo, Japan) at concentrations of 0.2–200 μ*M* for 72 hr. The viable cell count measured by colorimetric assay was presented as a percent ratio to the count of the control untreated with 5-FU. A dose-response curve was prepared and the 50% growth inhibitory concentrations (IC_{50}) was obtained.

Statistical analysis

Significance of difference was analyzed by unpaired student's *t*-test. A p value < 0.05 was regarded as significant.

RESULTS

First, we investigated the TS activity in SKG-I and SKG-I/TS by FdUMP-TS binding assay. The TS activity of SKG-I/TS was 0.6±0.03 pmol/mg protein, which was significantly higher than those of SKG-I (0.3±0.04 pmol/mg protein)($p < 0.01$).

Next, we investigated the effect of TS expression on 5-FU-sensitivity *in vitro*. The IC_{50} of 5-FU for SKG-II/TS was 24 ± 6.0 μ*M*, which was 6 times as high as that for SKG-II/LUC (4.0 ± 1.1 μ*M*), showing significantly decreased sensitivity to 5-FU ($p < 0.01$, Fig. 1). The IC_{50} of 5-FU for SKG-I/TS was 90 ± 15 μ*M*, which was over 2 times as high as that for SKG-I (40 ± 0.60 μ*M*), showing significantly decreased sensitivity to 5-FU ($p < 0.05$, Fig. 2). Therefore, the expression of the TS resulted in reduced 5-FU-sensitivity.

DISCUSSION

Studies have shown a relationship between TS expression in tumors and sensitivity to 5-FU. In an *in vitro* study using gastrointestinal cancer cell lines, Habara *et al.* found a significant correlation between TS activity and sensitivity to 5-FU.[15] Wang *et al.* examined TS gene expression in five 5-FU-resistant colorectal cancer cell lines using complementary DNA (cDNA) microarrays, and reported that these cell lines showed a higher TS gene expression than the 5-FU-sensitive parent cell lines.[16] These *in vitro* studies suggest that TS expression in tumors determines the sensitivity to 5-FU. In our study, we examined the effect of TS

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Received 1 July 2002; Revised 6 February 2003; Accepted 15 March 2003

DOI 10.1002/ijc.11221
Published online 16 May 2003 in Wiley InterScience (www.interscience.wiley.com).

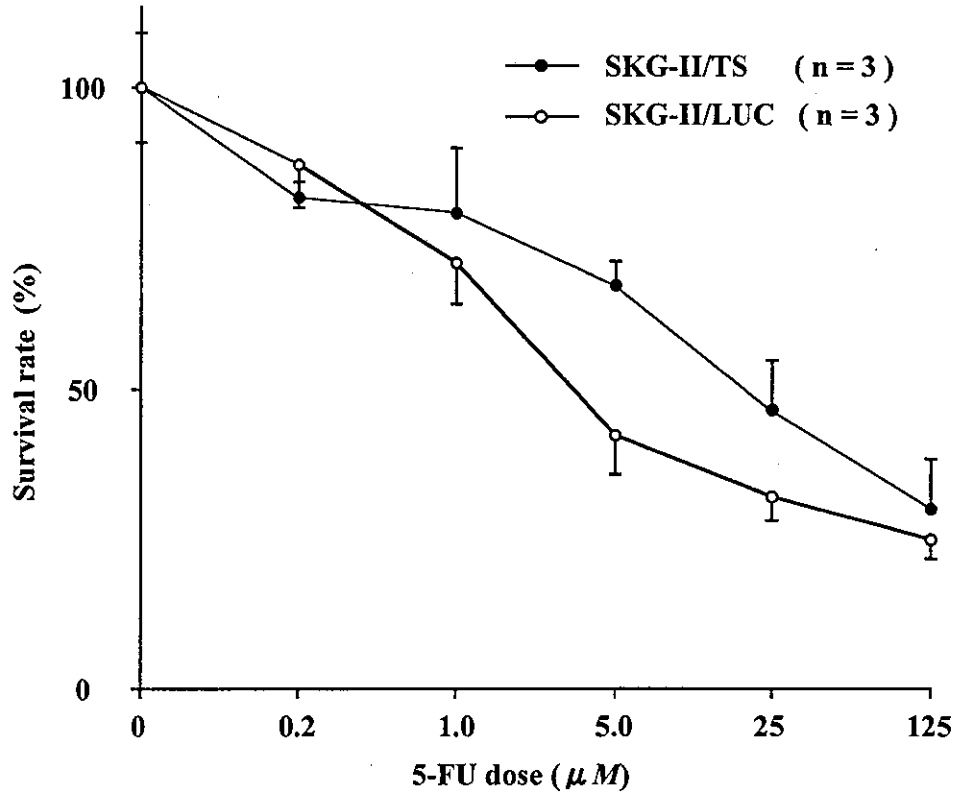

FIGURE 1 – Dose-response curves of SKG-II transfectants exposed to 5-FU. The 50% growth inhibitory concentration (IC_{50}) of 5-FU for SKG-II/TS was 24 ± 6.0 μ*M*, which was 6 times as high as that for SKG-II/LUC (4.0 ± 1.1 μ*M*), showing significantly decreased sensitivity to 5-FU ($p < 0.01$). Bars, SD.

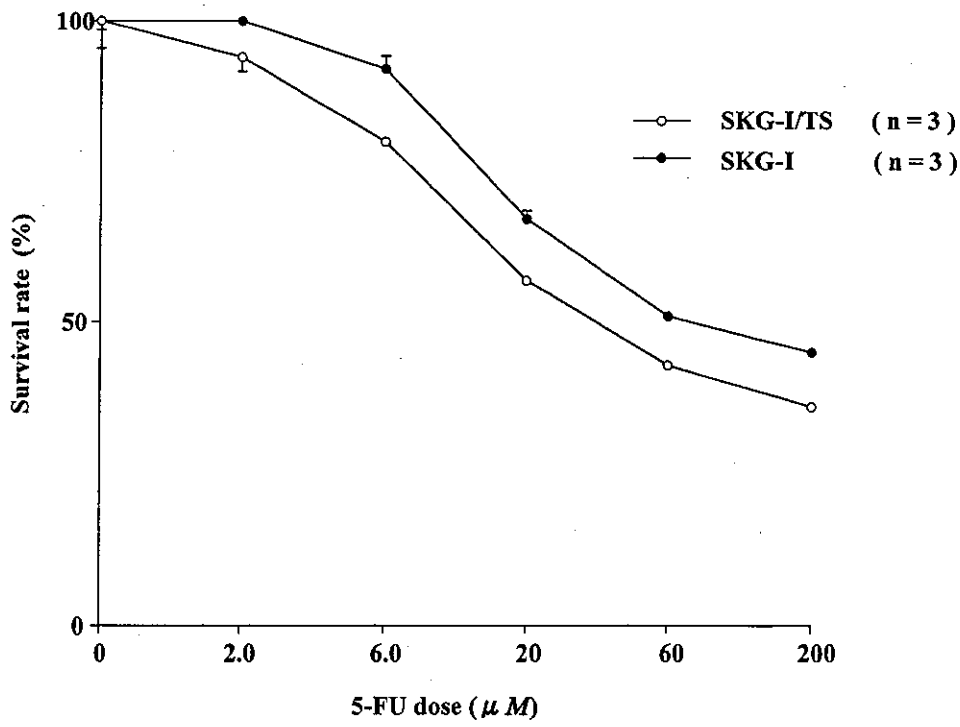

FIGURE 2 – Dose-response curves of SKG-I and SKG-I/TS exposed to 5-FU. The 50% growth inhibitory concentration (IC_{50}) of 5-FU for SKG-I/TS was 90 ± 15 μ*M*, which was over 2 times as high as that for SKG-I (40 ± 0.60 μ*M*), showing significantly decreased sensitivity to 5-FU ($p < 0.05$). Bars, SD.

expression on 5-FU sensitivity in TS-overexpressing cell lines established by TS gene transfer, and showed that TS expression was associated with decreased sensitivity to 5-FU. These findings suggest that the poor prognosis of TS-overexpressing tumors is partly due to their decreased sensitivity to 5-FU. The mainstay of treatment for cervical cancer is surgery and radiotherapy, and therapy with 5-FU or other anticancer agents is rarely the treatment of choice. Therefore, it is difficult to speculate from the 5-FU sensitivity that TS expression determines the prognosis of cervical cancer. However, based on clinical analysis of patients with cer-

vical cancer treated by radiotherapy, we reported that TS-overexpressing patients had a poorer prognosis than patients with low TS expression.[17] In addition, we found in an *in vitro* study that TS-overexpressing cervical cancer cells were less sensitive to radiation.[11] Recently, chemoradiation for advanced cervical cancer, which combines radiation and chemotherapy with 5-FU and cisplatin, has attracted attention.[18,19] Thus, analysis of TS expression in cervical cancers may predict the efficacy of chemoradiation.

The TS overexpression may decrease the sensitivity to 5-FU by the following mechanism. 5-FU is an anticancer agent targeting TS, which is converted *in vivo* to FdUMP by thymidine kinase, and this reportedly binds to TS to inhibit it, thereby exerting a cell-killing effect.[20,21] Thus, a larger dose of 5-FU is required to exert its effect on TS-overexpressing tumors, which means a decreased sensitivity to 5-FU.

In addition to decreased sensitivity to 5-FU and radiation, tumor invasion or distant metastasis has recently been implicated in the poor prognosis of TS-overexpressing tumors: immunostaining of rectal cancer specimens from clinical patients has shown that TS expression is an independent prognostic factor for local recurrence, distant metastasis, disease-free and overall survival.[22] TS may be involved in tumor invasion and metastasis. Therefore, the investigation of invasive and metastatic activity and angiogenesis using our TS transfectants should be performed in the future.

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Reduction of CTLL-2 cytotoxicity by induction of apoptosis with a Fas-estrogen receptor chimera

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(Received March 27, 2003/Accepted April 30, 2003)

Allogeneic bone marrow transplantation and donor lymphocyte infusion are powerful treatments for chemotherapy-resistant leukemia. Tumor eradication is attributed to a graft-versus-leukemia reaction by the donor-derived cytotoxic T lymphocytes (CTLs), but the same cell population may cause severe graft-versus-host disease. One strategy to suppress harmful CTL activity is to incorporate a suicide gene into the donor lymphocytes prior to infusion, and to destroy these cells when they aggressively attack nonmalignant host tissues. In this study, we investigated the feasibility of using a Fas-estrogen receptor fusion protein (MfasER) to control T cell-mediated cytotoxicity, based on our previous finding that the chimera transmits a Fas-mediated death signal through activation by estrogen binding. A murine CTL line CTLL-2 was transfected with a vector encoding MfasER, and the growth, viability and cytotoxic activity of the transfected cells (CTLL/MfasER) were analyzed. The expression of apoptosis-related proteins such as Fas ligand and perforin was also investigated. In the absence of estrogen, CTLL/MfasER showed similar growth to parental CTLL-2, and the killing activity was preserved. Addition of 10^{-7} *M* estrogen induced a rapid apoptosis of CTLL/MfasER, and the cytotoxicity was severely impaired. A decrease of Fas ligand and perforin in the estrogen-treated CTLL/MfasER was seen in an immunoblot analysis. These functional and biochemical analyses showed that the estrogen-inducible apoptosis in MfasER-expressing CTLs rapidly terminated their target cell killing. The feasibility of using the MfasER-estrogen system to control graft-versus-host disease was demonstrated. (Cancer Sci 2003; 94: 639–643)

Today, allogeneic cell therapies such as bone marrow transplantation (BMT) and donor lymphocyte infusion (DLI) are indispensable means of treating hematological malignancies and achieve a life-long cure in many patients. The advantage of such therapies over conventional chemotherapy is at least twofold. First, an intensified, myeloablative dose of chemoradiotherapy can be given during the pre-transplant period. Second, immunocompetent allogeneic donor T lymphocytes eradicate residual malignant cells. This combat reaction by the donor T cells is called a graft-versus-leukemia (GVL) effect.[1] However, GVL may be closely associated with graft-versus-host disease (GVHD), a serious complication following BMT and DLI. Currently, the dominant antigens on leukemia cells driving the GVL response are unknown, and efforts to discriminate T cell subsets responsible for GVL and GVHD have been mostly unsuccessful.

One way to avoid this dilemma is to incorporate a suicide gene into the donor lymphocytes and eliminate them when severe GVHD occurs. So far, the thymidine kinase gene from herpes simplex virus type 1 (HSVtk) has been most extensively studied for this purpose.[2] This strategy is based on the fact that the viral enzyme converts barely toxic prodrugs such as ganciclovir (GCV) into highly toxic intermediates. Although the toxic metabolites should kill only the manipulated cells expressing HSVtk, this suicide system has some limitations. Conceivable problems are as follows. (1) The rate of cell killing is relatively slow, while a rapid eradication of responsible CTLs is necessary to terminate severe GVHD. (2) Nondividing cells are refractory. The HSVtk/GCV system depends on DNA synthesis to exert cytotoxicity, but activated effector cells are not necessarily dividing at that moment. Indeed, this system was not fully functional in a patient with chronic GVHD, where responsible CTLs might be slowly cycling.[2] (3) GCV may cause nonspecific bone marrow suppression. Patients undergoing DLI are usually in a myelosuppressive state, and GCV treatment may further suppress their hematopoietic activity to cause severe marrow failure. (4) Anti-cytomegalovirus treatment may hamper GVL by the HSVtk-transduced T cells. Cytomegalovirus infection is common in immunocompromised patients, where GCV is one of the few therapeutic options. GCV administration will eradicate HSVtk-transduced cells, including the donor-derived lymphocytes contributing to GVL and graft-versus-infection. (5) HSVtk is a foreign protein to the hosts and may evoke immunological reactions against the transduced cells.[3]

To overcome these limitations, we have employed a novel suicide system using Fas and the ligand-binding domain (LBD) of a nuclear receptor.[4,5] When fused with heterologous proteins, an LBD can work as a molecular switch to control the fusion protein, and a variety of effector proteins have been converted to function in a ligand-dependent manner.[6] In fact, an apoptosis-inducing system was designed with Fas and the LBD of estrogen receptor (ER). Fas, also called CD95 or APO-1, belongs to the tumor necrosis factor receptor superfamily and regulates apoptosis upon binding to Fas ligand (FasL) or crosslinking antibodies.[7] In a previous study, the transmembrane and cytoplasmic domains of Fas (Mfas) were fused with ER, and expression of the chimeric molecule (MfasER) led to estrogen-inducible apoptosis in L929 cells both *in vitro* and *in vivo*.[4] The estrogen-induced apoptosis was rapid and extensive whether the challenged cells were proliferating or resting, unlike the HSVtk/GCV system, which was ineffective in killing nonproliferating cells.[5] We expected that the MfasER/estrogen system would also be effective in eliminating the cells involved in GVHD, because Fas-mediated apoptosis is physiologically vital in activated T lymphocytes. In addition, immunological reactions are less likely to be elicited against the fusion protein than against HSVtk, because MfasER is composed of endogenous proteins. In the present study, we investigated the feasibility of using MfasER to control T cell-mediated cytotoxicity. A murine T cell line CTLL-2,[8] with many characteristics of normal CTLs, was used as an effector to evaluate the efficacy of MfasER in controlling cytotoxic activity.

Materials and Methods

Plasmids. An MfasER expression vector was constructed as follows. MfasER cDNA was derived from pEF-BOS/MfasER (a gift from Dr. A. Kakizuka, Kyoto University, Kyoto).[4] An in-

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