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

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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 vivo 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 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 BamHI site of the plasmid pCMV-IRE S-bsr (12). Then either the plasmid pCMV-mIL-10-IRE S-bsr or the control plasmid pCMV-LUC-IRE S-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 × 10^6 cells/dish and cultured subsequently in 10% serum-supplemented DMEM:F12 medium. Each cell line was seeded in the presence of 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 [width]^2 × [length] × 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 15 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.

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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 \times 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.

RESULTS

Expression of mIL-10 and VEGF Concentration in Culture Supernatant. Either the mIL-10-expressing plasmid vector pCMV-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-2S/mIL-10 and KOC-2S/LUC did not show significant differences. Each bar represents the mean ± SD. * \( P < 0.05 \).

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. 2a, SHIN-3/mIL-10 tumors tend to shrink from day 30 after inoculation, significantly smaller than the control on day 45 [(0.8 ± 0.5) × 10^3 mm^3 versus (2 ± 0.4) × 10^3 mm^3, *P* < 0.05]. In contrast, KOC-2S/mIL-10 tumors did not show a difference from the control (Fig. 2b). 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. 3a). The number of newly formed vessels in SHIN-3/LUC tumors separated by a semipermeable filter (positive control) was 4.22 ± 1.72 (Fig. 3b). However, as shown in Figs. 3a 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. 3c, 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 decreased (1.9 ± 1.2, *P* < 0.05) compared with that in the control group (0.73 ± 0.15 ml versus 2.8 ± 0.4 ml, *P* < 0.05; Fig. 4a). 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. 4b). 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. 4c). 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. 4d). 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-

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**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 ± SD based on triplicate experiments. *P* < 0.05.
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REFERENCES


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