ICAM-2 Gene Therapy for Peritoneal Dissemination of ScirrhouS Gastric Carcinoma

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ABSTRACT
Purpose: Human scirrhous gastric carcinoma develops peritoneal dissemination with high frequency, and the prognosis of patients with peritoneal metastasis is poor. There have been few reports of an immunogene therapy for peritoneal dissemination. Intercellular adhesion molecule (ICAM)-2 is a second ligand of leukocyte function-associated antigen-1, which functions as a costimulatory molecule for effector cells. In the present study, we examined whether ICAM-2 transfection using adenovirus vector is effective gene therapy for peritoneal metastasis of gastric cancer.

Experimental Design: We constructed an adenovirus vector, AdICAM-2, that encodes the full-length human ICAM-2 gene under control of the cytomegalovirus promoter. This vector expresses high levels of ICAM-2 on the human gastric cancer cell line OCUM-2MD3, which has high peritoneal metastatic ability in nude mice. We investigated the antitumor effects of gene transfer of ICAM-2 using the adenovirus vector AdICAM-2 in vitro and in vivo.

Results: ICAM-2 expressed on OCUM-2MD3 cells by AdICAM-2 demonstrated significantly high adhesiveness to and cytotoxicity against peripheral blood mononuclear cells in vitro compared with the control adenovirus vector AdlacZ. Intratumoral injection of AdICAM-2 significantly inhibited the growth of s.c. tumor. Mice with peritoneal metastasis survived for a significantly longer time after AdICAM-2 injection, compared with injection of AdlacZ. Histopathological findings revealed that many natural killer cells infiltrated the peritoneal metastatic lesions after AdICAM-2 injection.

Conclusions: These findings suggest that transduction of ICAM-2 into cancer cells enhances the adhesion and activation of natural killer cells, resulting in a reduction of peritoneal metastasis. ICAM-2 transfection using adenovirus vector might be an effective form of gene therapy for peritoneal metastasis of gastric cancer.

INTRODUCTION
Intercellular adhesion molecule (ICAM)-1 is a member of the immunoglobulin supergene family of adhesion proteins that serves as the counter-receptor for leukocyte function-associated antigen (LFA)-1 (CD11a/CD18; Ref. 1). The ICAM-1/LFA-1 interaction is critical to the binding between effectors cells and cancer cells, and ICAM-1 is a costimulatory molecule for immune activation (2). Thus far, five ICAM families have been described. ICAM-2 is a glycoprotein belonging to the immunoglobulin superfamily (3). ICAM-2 is a second ligand for LFA-1. ICAM-2 has structural and functional homology to ICAM-1 (3). The extracellular domains of ICAM-1 consist of five C-like domains. ICAM-2 has two C-like domains resembling the NH2-terminal domains of ICAM-1. The first NH2-terminal domain of ICAM-2 is important to CD11a/CD18 binding (4). The ICAM-2/LFA-1 interaction may stimulate the migration and cytotoxicity of natural killer (NK) cells (5) and play other important biological roles (6).

Human scirrhous gastric carcinoma develops peritoneal metastasis with high frequency (7), and the prognosis of patients with peritoneal metastasis is poor (8, 9). Various types of therapy, including chemotherapy, hormonal therapy, hyperthermia therapy, and immunotherapy, have been tested for treatment of peritoneal metastasis of gastric carcinoma (10, 11); the effects of these therapies, however, have been found to be unsatisfactory. A new therapy following surgery or chemotherapy is necessary to improve the treatment strategy for scirrhous gastric carcinoma. We have reported previously that decreased expression of ICAM-1 in gastric cancer is associated with lymph node metastasis and poor prognosis (12). We have also reported (13) that transfection of the ICAM-1 gene inhibits lymph node metastasis, and we have reported (14) that gastric cancer cells overexpressing ICAM-1 have a tendency to cause a regression of peritoneal dissemination. It has been reported that NK cell activity in gastric cancer patients is decreased (15). In addition, serum transforming growth factor β is increased in patients with gastric carcinoma (16), resulting in a systemic immunosuppressive state (17). Immunogene therapy has been used for various types of cancers with effector cells, including macrophages, CTLs, and NK cells. However, there have been few reports of an immunogene therapy for peritoneal metastasis (18, 19). In the present study, we examined whether ICAM-2 transfection using adenovirus vector is an effective gene therapy for peritoneal metastasis of gastric cancer.
MATERIALS AND METHODS

Immunohistochemical Staining. ICAM-2 expression levels of cancer cells were compared between peritoneal metastasis and primary gastric carcinoma using an immunohistochemical method. Gastric cancer specimens were obtained from 15 patients, and both primary and metastatic lesions were sampled from the same patients. Anti-ICAM-2 antibody was supplied by Immuno- tech (Marseille, France). Immunohistochemical studies were performed by the avidin-biotin peroxidase complex method (DAKO, Carpinteria, CA). Tissue paraffin blocks were cut into 5-μm-thick sections. Sections were deparaffinized and then incubated at room temperature with anti-ICAM-2 antibodies (10 μg/ml) for 15 min. The sections were then incubated with biotinylated rabbit anti-mouse immunoglobulins in Tris-HCl buffer. For 15 min, sections were incubated with streptavidin-biotin-peroxidase complex, amplified reagent, and streptavidin peroxidase, followed by three washes with PBS. Finally, slides were reacted with diaminobenzidine tetrahydroxide for 10 min, counterstained with hematoxylin, and mounted. Normal mouse IgG was substituted for primary antibody as the negative control. ICAM-2 expression levels in primary carcinomas and metastatic peritoneal lesions in the same individual were compared. Staining was scored semiquantitatively. Scores were ranked as follows: weak, no immunoreactive tumor cells detectable or <10% of tumor cells positive with a weak staining intensity; moderate, 10–50% of tumor cells positive; and strong, >50% of tumor cells positive with strong staining intensity.

Cell Culture. The human scirrhous gastric cancer cell line OCUM-2MD3 was used (20). OCUM-2MD3 cells have a strong ability to metastasize into the peritoneum in nude mice (21). Human embryonic kidney cell line 293 was obtained from Damippon Pharmaceutical Company (Tokyo, Japan). OCUM-2MD3 cells and 293 cells were cultured in vitro at 37°C in DMEM with 10% fetal bovine serum, 100 μg/ml streptomycin and penicillin, and 0.5 mM sodium pyruvate.

Recombinant Adenovirus Vector. The replication-deficient adenovirus vectors used in this study were E1- and E3-deleted vectors based on the adenovirus serotype 5 genome. AdICAM-2 (adenovirus vector with ICAM-2 driven by the cytomegalovirus promoter) and AdlacZ (adenovirus vector with lacZ Escherichia coli driven by the cytomegalovirus promoter) were constructed using the Adeno X Expression Kit (Clontech, Palo Alto, CA). First, human ICAM-2 gene or E. coli lacZ gene was inserted into a shuttle vector. Next, the shuttle vector was transferred to the adenoviral genome by means of an in vitro ligation reaction. Direct sequencing checked the inserted ICAM-2 gene. Finally, the newly tailored recombinant adenoviral plasmid was packaged into infectious adenoviral particles by transfecting 293 cells. Recombinant adenoviruses were expanded in the 293 cells, and then the resultant viral solutions were stored at −80°C. The viral titer was determined by the 50% tissue culture infectious dose method (22–24).

Adenoviral Infection in vitro. To verify the infectious ability of the adenoviral vector, we performed infection tests at various multiplicities of infection (MOIs). Cancer cells were incubated until confluent in 6-well culture plates for 24 h, and the adenovirus vector was thawed in the plates and suspended in DMEM with 5% fetal bovine serum. The viral exposures were performed at MOIs of 0.1–100. After 120 min of incubation, fresh DMEM with 10% fetal bovine serum was added, and the plates were incubated at 37°C. To detect expression of the lacZ gene, cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, as described previously (25).

Flow Cytometric Analysis. The OCUM-2MD3 cells were incubated with AdICAM-2 until they reached 70% confluence. The cells were washed with PBS containing 0.01% sodium azide and 0.1% BSA. The anti-ICAM-2 antibody (10 ng/μl; Immunotech) was added to each sample, and samples were incubated for 30 min. The samples were then incubated with 10 ng/μl FITC-conjugated goat antiserum IgG (Chemicon International Inc., Temecula, CA) for 30 min on ice. An aliquot of 1 × 10^4 cells was analyzed using FACScan.

Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs). PBMCs were prepared from fresh heparinized peripheral blood of healthy volunteers without atopic symptoms or a history of smoking. Mononuclear cells were isolated from fresh blood by Ficoll-Hypaque gradient centrifugation, as described previously (13). Isolated PBMCs were counted by a hemocytometer and diluted to a final concentration of 5 × 10^6 cells/ml in DMEM with 10% fetal bovine serum.

Adhesion Assay. Evaluation of the adhesion of PBMCs to cancer cells was performed as reported previously (26, 27). Briefly, cancer cells with AdICAM-2 and AdlacZ were cultured on 96-well plates until confluent. The adhesion assay was performed 48 h after coincubation with adenovirus vector. PBMCs (2 × 10^5) were added to each well in a final volume of 200 μl and coincubated with cancer cells for 60 min at 37°C. The wells were washed to remove nonadherent mononuclear cells. Cellular adhesion was quantified by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay. It was designed to measure the formazan product of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, using an MTP-120 microplate reader (Corona Electric Co.) to measure absorbance at 550 nm (28). Data are expressed as a percentage of adherent mononuclear cells [percentage of adhesion = (absorbance of experimental wells - absorbance of control wells)/absorbance of total PBMCs].

Cytotoxicity Assay. Cancer cells were cultured for 48 h with AdICAM-2 or AdlacZ on flat bottom 96-well plates. Cancer cells were then incubated with PBMCs at an E:T ratio of 5 or 10. After 6 or 12 h of incubation, 50 μl of supernatant were collected, and lysates were measured using the Cyto Tox 96 Assay Kit (Promega, Madison, WI), which measures lactate dehydrogenase released on cell lysis. Absorbance at 490 nm was measured using an MTP-120 microplate reader (Corona Electric Co.; Ref. 28). The percentage of specific cell lysis was calculated as follows: percentage of cytotoxicity = (A − B − C)/(D and E) × 100, where A = experimental lysis, B = effector spontaneous cell lysis, C = target spontaneous lysis, D = target maximum lysis, and E = target spontaneous lysis.

Effects of Anti-ICAM-2 Neutralizing Antibody on Adhesiveness to and Cytotoxicity against PBMCs. Cancer cells transduced with AdICAM-2 or AdlacZ were treated with neutralizing antibody against ICAM-2 for 1 h. Final solutions contained anti-ICAM-2 antibody concentrations of 25 μg/ml. The adhesion and cytotoxicity assays were then performed as described above. Mouse IgG1 antibody (DAKO) was used as a standard for comparison.
Animal Models and Evaluation of Gene Transfer in vivo. Xenografts were established by injecting $5 \times 10^6$ OCUM-2MD3 cells into the flanks of female athymic BALB/c nude mice at 4–6 weeks of age (Oriental Kobo, Tokyo, Japan). From 9 days to 29 days after injection, AdlacZ or AdICAM-2 (2.4 $\times 10^6$ plaque-forming units/mouse) was injected into each tumor every 2 days. Injections were performed 11 times in total. Tumor size was measured with calipers every 2 days until the 35th day. Tumor area was calculated using the following formula: area = length (mm) $\times$ width (mm). Peritoneal metastatic models were established by inoculating $5 \times 10^6$ OCUM-2MD3 cells into the peritoneal cavities of mice. AdlacZ and AdICAM-2 (2.4 $\times 10^6$ plaque-forming units/mouse) were injected into the peritoneal cavities of mice every 2 days from the 9th day after i.p. injection (11 times in total). To enhance the effects of ICAM-2 transduction, the first day of adenoviral injection was brought forward 5 days. The survival time of mice was observed until all mice had died. At the 20th day after inoculation, some mice were sacrificed and examined macroscopically to determine the distribution of disseminated metastasis. All experiments with nude mice were performed regarding their welfare in accordance with the guidelines approved by the United Kingdom Coordinating Committee on Cancer Research. For histopathological examination, tissue paraffin blocks were cut into 5-μm-thick sections and stained with H&E or anti-NK1.1 antibody (Southern Biotechnology Associates, Birmingham, AL) by the avidin-biotin peroxidase complex method.

Statistical Analysis. Differences between the control and experimental groups were analyzed using the Student’s $t$ test. $P$ values of <0.05 were considered statistically significant. Survival evaluation was carried out using Kaplan-Meier analysis.

RESULTS

Comparison of ICAM-2 Expression between Peritoneal Metastatic Lesions and Primary Tumor in the Same Individual. Figure 1A provides a comparison of ICAM-2 expression between the primary tumor and peritoneal dissemination in the same 13 individuals. The primary tumors showed strong, moderate, and weak staining in cases 3, 9, and 1, respectively. In contrast, the peritoneal dissemination showed strong, moderate, and weak staining in cases 0, 5, and 8, respectively. Although the entire primary lesion showed more than a moderately strong stain of ICAM-2, most of the peritoneal metastatic lesions demonstrated weaker staining. Figure 1B shows a primary lesion positive for ICAM-2 and a peritoneal metastatic lesion negative for ICAM-2 from the same patient.

Ability of Adenoviral Vector to Transfer the ICAM-2 Gene. As shown in Fig. 2, ICAM-2 expression on the cell surface was confirmed by FACSscan 48 h after infection with AdICAM-2. The basal expression level of ICAM-2 in OCUM-2MD3 was 0.7% in the control. The percentages of positive cells were 3%, 15%, 51%, and 75% at MOIs of 0.1, 5, 10, and 100, respectively (Fig. 2A). No β-galactosidase activity was found in parent cells. OCUM-2MD3 cells infected with AdICAM-2 at a MOI of 100 demonstrated that maximal expression of ICAM-2 occurred on the 2nd day (Fig. 2B). To determine whether transduction of ICAM-2 altered the proliferation of OCUM-
2MD3 cells, a cell proliferation assay was performed. The doubling time of OCUM-2MD3 cells infected with AdICAM-2 was 12.3 h, and that of uninfected cells was 11.7 h.

Effects of Transduction on the Adhesion and Cytotoxicity of PBMCs. We examined the effects of ICAM-2 transduction on adhesion and cytotoxicity 48 h after adenoviral infection at a MOI of 100. The percentage of adhesion of PBMCs with cancer cells was 85 ± 14% for AdICAM-2-transduced cells, compared with 47 ± 10% for AdlacZ-transduced cells. There was a significant increase in the adhesion of PBMCs to AdICAM-2-transduced cells (Fig. 3A). Figure 3B shows the results of a PBMC-mediated cytotoxicity experiment including NK activity. Lysis of cancer cells was recognized in 52 ± 6% and 94 ± 6% of AdICAM-2-transduced cells 6 and 12 h later, respectively, at an E:T ratio of 10:1. In contrast, values for cancer cell lysis resulting from AdlacZ transduction were 26 ± 9% and 42 ± 15% at 6 and 12 h, respectively. The differences in cytotoxicity between AdICAM-2 transduction and AdlacZ transduction were statistically significant (P < 0.01). The cell lysis of AdICAM-2-transduced cells was significantly higher at an E:T ratio of 10:1 than at an E:T ratio of 5:1 (Fig. 3B).

Effects of Anti-ICAM-2 Neutralizing Antibody on the Adhesiveness to and Cytotoxicity against PBMCs. Adhesiveness was significantly (P < 0.01) decreased by coadministration of anti-ICAM-2 antibody to OCUM-2MD3 cells after AdICAM-2 transduction, whereas AdlacZ-transduced cells were not affected by anti-ICAM-2 (Fig. 4A). The cell lysis of AdICAM-2-transduced cells was significantly (P < 0.01) decreased by the neutralizing antibody against ICAM-2, compared with that of AdlacZ-transduced cells (Fig. 4B).

Tumor Growth in Nude Mice. We examined the effects of ICAM-2 transduction on the growth of xenografted tumors. Xenografts in nude mice were recognized as tumors at 7 days after inoculation of OCUM-2MD3 cells. The xenografts were treated by intratumoral injection of AdICAM-2. To produce a treatment model, adenoviruses were injected into xenografts 9
days after inoculation of OCUM-2MD3 cells. Tumor growth of xenografts was significantly suppressed after AdICAM-2 injection compared with AdlacZ injection (Fig. 5). No mouse died, and no remarkable change in skin was induced by adenoviral injection.

**Inhibitory Effects of ICAM-2 Transduction on Peritoneal Metastasis of Scirrhous Gastric Cancer.** Intraperitoneal inoculation of OCUM-2MD3 cells yielded many metastatic nodules in the peritoneal cavity and resulted in death within 3 weeks after i.p. inoculation in all mice. AdlacZ-treated mice also developed peritoneal metastasis. However, mice treated with AdICAM-2 had fewer metastatic nodules than those treated with AdlacZ at 20 days after i.p. injection. The survival time of AdICAM-2-treated mice was significantly prolonged compared with that of AdlacZ-treated mice (log-rank \( P < 0.05 \); Fig. 6). Although the median survival time of AdlacZ-treated mice was 18.5 days, that of AdICAM-2-treated mice from day 9 was 22.5 days. The median survival time for mice with earlier injection of AdICAM-2 (day 4) was 5 days longer than that for mice with the later injection (day 9).

**Histopathological Findings.** To investigate whether gene delivery into cancer cells was successful after i.p. adenoviral injections, 5-bromo-4-chloro-3-indolyl-\( \beta \)-galactopyranoside staining of cancer cells was performed as described in “Materials and Methods.” \( \beta \)-Galactosidase expression was recognized in cancer cells of peritoneal metastatic nodules after i.p. injections of AdlacZ (Fig. 7B), especially at the tips of the metastatic nodules. Cancer cells in the ascitic field of the peritoneal cavity also expressed \( \beta \)-galactosidase (Fig. 7A). \( \beta \)-Galactosidase expression was also recognized in the cancer cells of xenografted tumors surrounding the adenoviral injection at 48 h after AdlacZ injection (data not shown). Mononuclear cells infiltrated the peritoneal metastatic cancer cells after i.p. injection of AdICAM-2 (Fig. 7C). To investigate whether the effector cells included NK cells, immunohistochemical analysis with anti-NK1.1 antibody was performed. In the region of intratumoral AdICAM-2 injection, mononuclear cells were recognized to be NK1.1-positive cells (Fig. 7D).

**DISCUSSION**

Peritoneal metastasis is an important factor in the prognosis of patients with gastric cancer. For patients with peritoneal metastasis who do not undergo resection, the median survival time is 6.7 months (29). Takeuchi et al. (15) have reported that patients with low NK activity show a significantly poorer survival rate. Ishigami et al. (30) have reported that serosal infiltration correlates with low levels of intratumoral NK cell infiltration, suggesting that peritoneal dissemination might be closely associated with insufficient immune responses to cancer cells. ICAM-2 is a second ligand for LFA-1 (CD11a/CD18),...
which is expressed on leukocytes. The interaction between CD11a/CD18 and ICAM-2 plays a role in mediating many leukocyte functions, including immunoglobulin production and the cytotoxicity of T cells and NK cells. Kotovuori et al. (31) have reported that a peptide from the ICAM-2 domain is a more efficient activator of leukocyte adhesion than ICAM-1 and ICAM-3. In the present study, ICAM-2 expression levels of cancer cells were examined in peritoneal metastasis and primary gastric carcinoma using an immunohistochemical method. The primary lesions showed more than moderately strong ICAM-2 staining, whereas most of the peritoneal metastatic lesions demonstrated weak ICAM-2 staining. These data indicate that the expression levels of ICAM-2 in peritoneal metastases tend to be decreased compared with those of the primary lesions. We hypothesized that ICAM-2 expression on cancer cell surfaces might elicit an antimetastasis effect. We then examined the effects of ICAM-2 gene therapy using an adenoviral vector for peritoneal metastasis.

We found that the ICAM-2 gene can be transduced into gastric cancer cells, resulting in a high expression of ICAM-2 on cancer cells in vitro. Transfection of adenovirus with the ICAM-2 gene did not influence the proliferative ability of cancer cells. ICAM-2 expression was high 2 days after transfection and then decreased with time; this transient expression of ICAM-2 might have been due to the rapid proliferation of OCUM-2MD3 cells. Zheng et al. (32) have reported that cancer cells can escape from preexisting CTLs either by a loss of tumor antigen or by down-regulation of costimulatory molecules. It has been reported that ICAM-2 enhances antigen presentation and provides costimulatory signals to T cells or NK cells through LFA-1 ligation (33). In addition, the migratory capacity of NK cells is enhanced by the ICAM-2/CD11a/CD18 pathway (34). Our results indicate that ICAM-2 on cancer cell surfaces exhibits higher adhesiveness to PBMCs containing NK cells and higher cytotoxicity against by PBMCs than the control. The adhesiveness was decreased by coadministration of anti-ICAM-2 neutralizing antibody to OCUM-2MD3 cells after AdICAM-2 transduction. Cytotoxicity was also decreased by the neutralizing antibody against ICAM-2. These findings suggest that ICAM-2 expression on cancer cells is responsible for increases in adhesion and cytotoxicity and that the increased ICAM-2 expression on cancer cells reinforces the cytotoxic activity of immune cells such as NK cells.

We then examined the effects of adenovirus-mediated ICAM-2 gene transfer on peritoneal metastasis of gastric carcinoma. It was found that i.p. inoculation of adenoviral vector can introduce a gene into the cancer cells of peritoneally disseminated tumors. Mice with peritoneal metastasis survived for a longer time after AdICAM-2 injection. In addition, AdICAM-2-treated mice had fewer bloody ascites and fewer metastatic nodules in the peritoneal cavity. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining after i.p. injection of AdlacZ revealed adenoviral transfection into cancer cells in the peritoneal cavity. Immunohistochemical findings revealed that NK cells had infiltrated the peritoneal metastatic lesions after AdICAM-2 injection. NK cells in nude mice play an important role in transplanted tumor growth in nude mice (35). Carroll et al. (36) have reported that i.p. administration of adenovirus vector of Ad-CMV-p53 results in NK cell activation and recruitment to the peritoneal cavity. As a target for NK cells, ICAM-2 is concentrated into the biologically active cell surface region by cytoskeletal reorganization in cancer cells (37). In our study, NK cells may have played an important role as antitumor effectors. These results show that transduction of ICAM-2 onto cancer cells enhances the adhesion and activation of NK cells, resulting in a reduction of peritoneal metastasis. Adenovirus ICAM-2 is not selective for tumor cells and can be transduced to all types of cells, including normal cells and tumor cells that are exposed in the peritoneal cavity. However, in cases of peritoneal metastasis, the peritoneal cavity is covered with many cancer cells and few normal cells (38). Therefore, in peritoneal dissemination, most adenovirus ICAM-2 is transduced into tumor cells, not into normal cells.

There have been few reports of immunogene therapy for peritoneal dissemination of gastric cancer cells (39). It has been reported, however, that antitumor immune responses using adenoviral gene delivery are useful for cancer therapy (40, 41). This report is the first to indicate that peritoneal dissemination is decreased after i.p. adenoviral delivery of ICAM-2 in vivo as immunogene therapy. Although only NK cells are effector cells in nude mice, more efficiency might be expected in humans.
because humans have not only NK cells but also CTLs. LFA-1/ICAM-2 costimulation of T cells induces secretion of the T-helper 1 cytokine (42). As such, transduction of ICAM-2 into cancer cells with a recombinant adenoviral vector should be a potent strategy for tumor immunogene therapy.

REFERENCES


Transfection Suppresses Peritoneal Metastasis


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