Anti-tumor Responses Induced by Chemokine CCL19 Transfected into an Ovarian Carcinoma Model via Fiber-Mutant Adenovirus Vector

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Considerable attention has recently been paid to the application of chemokines to cancer immunotherapy because of their chemotactic affinity for a variety of immune cells and because several chemokines are strongly angiostatic. In the present study, the recombinant adenovirus vectors encoding chemokine CCL19 or XCL1 in an E1 cassette (AdRGD-mCCL19 and AdRGD-mXCL1) were developed. The constructed fiber-mutant adenovirus vector, which contained the integrin-targeting Arg-Gly-Asp (RGD) sequence in the fiber knob, notably enhanced the transfection efficiency to OV-HM ovarian carcinoma cells compared to that induced by conventional adenovirus vector. The results of an in vitro chemotaxis assay for chemokine-encoding vector demonstrated that both AdRGD-mCCL19 and AdRGD-mXCL1 could induce the migration of cells expressing specific chemokine receptors. Of the two chemokine-encoding vectors evaluated in vivo, AdRGD-mCCL19 showed significant tumor-suppressive activity in B6C3F1 mice via transduction into OV-HM cells, whereas XCL1 did not exhibit any notable anti-tumor effects, suggesting that CCL19 may be a candidate for cancer immunotherapy.

Key words chemokine; CCL19; XCL1; recombinant adenovirus vector; anti-tumor effect; OV-HM cell

Chemokines attract a variety of immune cells and function at inflammatory disease sites as well as lymphoid tissue. Considering the eradication of tumor cells as a consequence of interaction with immune cells that have migrated and accumulated in tumor tissue, the usefulness of chemokines for cancer immunotherapy has received considerable attention. By now, more than 40 chemokines have been well characterized, but only a few have been identified as candidates for cancer therapy either independently or with an adjuvant. Tumor-suppressive activity of several chemokines has been observed after transduction into a variety of experimental tumors. Tumor cells that were transfected with the CC chemokine gene, CCL3, had reduced tumorgenicity and significantly increased infiltration of macrophages and neutrophils. Another CC chemokine, CCL22, was also strongly chemoattractive to dendritic cells, NK cells and T cells, which resulted in tumor regression in a murine lung carcinoma model due to its efficient induction of anti-tumor immunity. In the present study, we used the adenovirus vector, which exhibits very high gene transduction efficiency. Because a variety of tumor cells contain few Coxsackie adenovirus receptors (CAR), we used a recombinant adenovirus vector with a fiber mutation containing the Arg-Gly-Asp (RGD) sequence in the fiber knob. This fiber-mutant vector possesses higher transduction and anti-tumor activities compared to conventional adenovirus vectors when used in cytokine-gene therapy against melanoma. In the present study, ovarian carcinoma OV-HM cells were transfected with a chemokine-encoding recombinant vector, AdRGD-mCCL19 or AdRGD-mXCL1, and both the in vitro chemotactic activity and the in vivo tumor-suppressive response were investigated.

MATERIALS AND METHODS

Cell Lines and Animals OV-HM ovarian carcinoma cell line were kindly provided by Dr. Hiromi Fujiwara (School of Medicine, Osaka University, Japan) and were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS. A549 human lung carcinoma cells and human embryonic kidney (HEK) 293 cells were cultured in DMEM supplemented with 10% FBS. Murine pre-B lymphoma L1.2 cells and their stable transfectants, L1.2/mCCR7 and L1.2/mXCR cells, which expressing specific receptor for CCL19 and XCL1, respectively, were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS and 2-ME (50 μM, Life Technologies). All the cell lines were cultured at 37°C in a humidified atmosphere with 5% CO2. Female B6C3F1 mice were purchased from SLC Inc. (Hamamatsu, Japan) and used at 6—8 weeks of age. All of the experimental pro-
cures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

**Construction of Adenovirus Vectors** The replication-deficient adenovirus vectors containing a fiber mutation, which were used in this study, were developed based on the adenovirus type 5 backbone with deletions of the E1 and E3 regions. The RGD sequence was inserted into the HI loop of the fiber knob using a two-step method developed by Mizuguchi et al. Murine chemokine genes derived from pT73-D-Pac-mCCL19 and pExCell-mXCL1 were used as sources of cDNA. Recombinant adenovirus vectors with the RGD fiber mutation, AdRGD-mCCL19 and AdRGD-mXCL1, carrying the chemokine cDNA under the control of the cytomegalovirus (CMV) promoter, were constructed by an improved *in vitro* ligation method described previously. The luciferase expressing adenovirus vectors with the RGD fiber mutation (AdRGD-luc), serving as a negative control, is identical to the AdRGD-mCCL19 and AdRGD-mXCL1 vectors and contains the luciferase gene in the expression cassette (Fig. 1). Conventional adenovirus vector expressing luciferase (Ad-Luc) was also developed by Mizuguchi et al. The adenovirus vectors were propagated in 293 cells and purified by cesium chloride gradient ultracentrifugation. Virus particle (VP) was accomplished spectrophotometrically. The titer (tissue culture infectious dose) of AdRGD-luc was determined by plaque-forming assay using 293 cells.

**Gene Expression by AdRGD-Luc or Conventional Ad-Luc in OV-HM Ovarian Carcinoma Cells** In 96-well plates, the OV-HM cells were treated with Ad-Luc or AdRGD-Luc at 1250, 2500, 5000, and 10000 viral particles/cell for 1.5 h, respectively. Cells were washed with PBS and cultured for an additional 48 h. Subsequently, the cells were washed, collected, and their luciferase activity was measured using the Luciferase Assay System (Promega, U.S.A.) and Microlumat Plus LB96 (Perkin Elmer, U.S.A.) after the cells were lysed with the Luciferase Cell Culture Lysis (Promega, U.S.A.) according to the manufacturer’s instruction.

**In Vitro Chemotaxis Assay** The AdRGD-Luc and indicated AdRGD-chemokine were transfected into A549 cells for 2 h at a multiplicity of infection (MOI) of 50, and the cells were washed twice with PBS and cultured in media containing 10% FBS. The cells were subsequently washed after 24 h cultivation, and incubated with an assay medium (phenol red-free RPMI 1640 containing 0.5% bovine serum albumin and 20 μM HEPES, pH 7.4) for another 24 h. The resulting conditioned medium was collected, and its chemotactant activity was measured by an *in vitro* chemotaxis assay across a polycarbonate membrane with 5-μm pores (Chemoatixcell-24; Kurabo, Osaka, Japan) using L1.2 transfectants expressing the specific receptor for chemokines. The culture supernatants of intact A549 cells, AdRGD-Luc-transfected A549 cells, and chemokine gene-transduced A549 cells were prepared. These samples and recombinant chemokines dissolved in the assay medium were added to a 24-well culture plate. Cells expressing specific receptors for CCL19 (L1.2/CCR7) or XCL1 (L1.2/XCR1) were suspended in the assay medium (1 × 10^6 cells) and placed in a Chemotaxicell-24 installed on each well. Likewise, parental L1.2 cells for these transfectants were prepared and added to the Chemotaxicell-24. Cell migration was allowed for 2 h at 37 °C in a 5% CO₂ atmosphere. The cells that migrated to the lower well were lysed and quantitated using a PicoGreen dsDNA quantitation reagent (Invitrogen, Tokyo, Japan). The data are expressed as mean±S.E. of the triplicate results and the migration activity was expressed in terms of the percentage of the input cells. Recombinant chemokines (mouse: mCCL19 and mXCL1) corresponding to each specific receptor (CCR7 and XCR1) were purchased from DakoCytomation (Kyoto, Japan) and used as a positive control.

**Evaluation of Growth of OV-HM Cells Transfected with Chemokine-Encoding Adenovirus Vectors in Immunocompetent Mice** To evaluate the gene transfection efficiency of the fiber-mutant adenovirus vector developed for this study, OV-HM cells were transfected with conventional adenovirus vector or fiber-mutant adenovirus vector at indicated particles/cell and luciferase activity was measured. The results shown in Fig. 2 demonstrated that luciferase gene expression induced by fiber-mutant vector was much higher than that induced by conventional adenovirus vector. For example, at 10000 VP/cell transfection, 16-fold greater gene expression was obtained in response to fiber-mutant vector than to Ad-Luc. This demonstrated that the insertion of the RGD peptide into the viral fiber enhanced transfection efficiency to OV-HM cells via the adenovirus vector.

**Expression of Murine CCL19 and XCL1 by Transfection with Chemokine-Encoding Adenovirus Vector** To

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**RESULTS**

**OV-HM Tumor Cells Transfected with Fiber-Mutant Adenovirus Vector Induced Higher Gene Expression Than That Induced by Conventional Vector** To evaluate the gene transfection efficiency of the fiber-mutant adenovirus vector developed for this study, OV-HM cells were transfected with conventional adenovirus vector or fiber-mutant adenovirus vector at indicated particles/cell and luciferase activity was measured. The results shown in Fig. 2 demonstrated that luciferase gene expression induced by fiber-mutant vector was much higher than that induced by conventional adenovirus vector. For example, at 10000 VP/cell transfection, 16-fold greater gene expression was obtained in response to fiber-mutant vector than to Ad-Luc. This demonstrated that the insertion of the RGD peptide into the viral fiber enhanced transfection efficiency to OV-HM cells via the adenovirus vector.

**Expression of Murine CCL19 and XCL1 by Transfection with Chemokine-Encoding Adenovirus Vector** To
After incubation, cells were collected and luciferase activity was measured. Data are presented as mean ± S.E. of relative light units (RLU)/well determined from three experiments.

### Table 1. Specific Chemoattractant Activity in Vitro Induced by Transfection of AdRGD-mCCL19 or AdRGD-mXCL1 into A549 Cells

<table>
<thead>
<tr>
<th></th>
<th>L1.2 % of input cells (mean ± S.E.)</th>
<th>L1.2/XCR1 % of input cells (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>0.2 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>10 nM mCCL1</td>
<td>0.3 ± 0.0</td>
<td>9.2 ± 0.8</td>
</tr>
<tr>
<td>A549</td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Luc/A549</td>
<td>1.5 ± 0.0</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>mXCL1/A549</td>
<td>3.7 ± 0.3</td>
<td>11.6 ± 0.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>L1.2 % of input cells (mean ± S.E.)</th>
<th>L1.2/CCR7 % of input cells (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>0.2 ± 0.0</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>10 nM mCCL1</td>
<td>0.2 ± 0.0</td>
<td>16.3 ± 1.2</td>
</tr>
<tr>
<td>A549</td>
<td>1.2 ± 0.1</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Luc/A549</td>
<td>1.5 ± 0.0</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>mCCL19/A549</td>
<td>2.5 ± 0.1</td>
<td>8.2 ± 0.5</td>
</tr>
</tbody>
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Chemotaxis assay was performed using L1.2 cells expressing specific receptors for CCL19 (L1.2/CCR7) or XCL1 (L1.2/XCR1).

**DISCUSSION**

Cytokines or chemokines encoded by a viral vector are currently regarded as intriguing options for cancer gene immunotherapy. Adenovirus vector, which shows high gene transduction efficiency and which can infect both dividing and non-dividing cells, is widely used as a carrier for gene therapy. It has been reported that the initial process of adenovirus infection involves at least two sequential steps. The first step is the attachment of the virus to the cell surface, which occurs by binding of the fiber knob to the Coxsackie virus and Adenovirus Receptor (CAR).26,27 Following this, in the second step, the interaction between the RGD motif of the penton base with αv integrins, the secondary host-cell receptors, facilitates internalization through receptor-mediated endocytosis.28,29 In other words, if the host cell surface lacks CAR, efficient gene transfer using a conventional adenovirus vector is difficult. Unfortunately, some malignant cells, including ovarian carcinoma, exhibit a resistance to adenovirus-mediated gene transduction due to low CAR expression on their surface. To overcome the low gene expression levels in CAR negative cells by adenovirus vectors, we constructed a fiber-mutant Ad vector with an integrin-targeting RGD peptide by a simple in vitro method.20 The results of gene transfection in vitro (Fig. 2) demonstrated that OV-HM transfected using AdRGD-Luc carrying the luciferase gene significantly induced gene expression compared to that induced by the conventional Ad-Luc, suggesting that the recombinant adenovirus vector is a better option for cancer gene therapy.

We also inserted the murine chemokine cDNA of the CC family chemokine, CCL19, and C family chemokine, XCL1, into the E1 cassette of this fiber-mutant adenovirus vector, and AdRGD-mCCL19 and AdRGD-mXCL1 were developed. The expression of chemokine mRNA was reported pre-
Previously,30) A chemotaxis assay of chemokine-encoding vectors was conducted in vitro to evaluate the biological activity of these vectors. The results demonstrated that the produced protein in the culture supernatants of cells infected with these vectors could efficiently cause migration of the specific receptor-expressing cells (Table 1).

The C family chemokine, XCL1, has been widely used for cancer immunotherapy, but in general, XCL1 by itself did not induce notable anti-tumor effects, even though it is a chemoattractant for both T cells and NK cells.31) The CC chemokine, CCL19, reportedly induces T cell and dendritic cell migration and exhibits tumor-suppressive effects in several mouse malignant cell models.32,33) Hillinger et al. reported that intratumoral injection of recombinant CCL19 led to significant systemic reduction in tumor volumes. CCL19-treated mice exhibited remarkably increased infiltration of T cells and dendritic cells at the tumor sites. These cell infiltrates were accompanied by increases in several cytokines and chemokines such as IFN-γ, CXCL9, CXCL10, GM-CSF, and IL-12.34) We have also shown that CCL19 and XCL1 elicited anti-tumor response, to some extent, through transfection into B16BL6 melanoma cells. But our study, which used eight chemokines to evaluate the anti-tumor effects in three tumor cell types, suggests that the tumor-suppressive activity of chemokine gene immunotherapy is very complicated and is greatly influenced by the type of tumor and activation state of the host's immune system.30) Moreover, as we previously reported,30) transfection with the chemokine CCL27 induced tumor-suppressive effects, whereas another chemokine, CX3CL1, did not show any notable anti-tumor activity. However, both of these chemokines induced the accumulation of T cells as well as NK cells at the tumor site. Our results indicated that the distribution of immune cells that have migrated to the tumor and the angiogenic or angiostatic activity may play an important role in the anti-tumor response.

Several groups have reported much stronger anti-tumor activity when using chemokines as adjuvants with other agents.35—39) In the present study, CCL19 could not induce complete tumor regression, but merely inhibited its growth. On the other hand, remarkable anti-tumor activity could be obtained when XCL1 was combined with cytokines or transfected into dendritic cells.40,41) A recent report showed that combination of both XCL1 and CXCL10 can enhance the efficiency of adoptive T cell therapy for EG7 tumor cells via accumulation of effector T cells in tumor tissue.42) Many factors are likely to influence the tumor-suppressive effects of chemokines, but the relatively weak anti-tumor activity and long-term immuno-protective effects of chemokines may be mainly related to the activation level of migrating immune cells. In other words, not only the accumulation but also the activation of immune cells migrating into tumors is important in cancer immunotherapy using chemokines. Therefore, combination therapy using both chemokines and cytokines will increase the anti-tumor effects and improve cancer immunotherapy.

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REFERENCES