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Biochemical and Biophysical Research Communications 317 (2004) 68–76
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Anti-tumor activity of chemokine is affected by both kinds of tumors and the activation state of the host's immune system: implications for chemokine-based cancer immunotherapy☆

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Received 23 February 2004

Abstract

In this study, we screened the anti-tumor activity of murine chemokines including CCL17, CCL19, CCL20, CCL21, CCL22, CCL27, XCL1, and CX3CL1 by inoculating murine B16BL6, CT26, or OV-HM tumor cells, all of which were transfected with chemokine-expressing fiber-mutant adenovirus vector, into immunocompetent mice. A tumor-suppressive effect was observed in mice inoculated with CCL19/B16BL6 and XCL1/B16BL6, and CCL22/OV-HM showed considerable retardation in tumor growth. In the cured mice inoculated with CCL22/OV-HM, a long-term specific immune protection against parental tumor was developed. However, we were unable to identify the chemokine that had a suppressive activity common to all three tumor models. Furthermore, an experiment using chemokine-transfected B16BL6 cells was also performed on mice sensitized with melanoma-associated antigen. A drastic enhancement of the frequency of complete rejection was observed in mice inoculated with CCL17-, CCL19-, CCL22-, and CCL27-transfected B16BL6. Altogether, our results suggest that the tumor-suppressive activity of chemokine-gene immunotherapy is greatly influenced by the kind of tumor and the activation state of the host's immune system.

Keywords: Adenovirus vector; Chemokine; Transfection; Anti-tumor activity; Gene immunotherapy

Chemokine consists of a superfamily of small (8–14 kDa), secreted basic proteins that regulate relevant leukocyte-migration and -invasion into tissue by interacting with their specific receptors, which belong to the superfamily of seven-transmembrane domain G-protein-coupled receptors [1,2]. The function of chemokine, which is capable of attracting specific immune cells, is demonstrated in inflammatory disease sites as well as normal lymphoid tissues [2]. Because of these properties, chemokine is considered as the intriguing molecule for cancer immunotherapy, which is based on the premise of

☆ *Abbreviations:* Ad, adenovirus vector; AdRGD, RGD fiber-mutant Ad; CTL, cytotoxic T lymphocyte; DC, dendritic cell; FBS, fetal bovine serum; MOI, multiplicity of infection; NK, natural killer; PBS, phosphate-buffered saline; $TCID_{50}$, tissue culture infectious $dose_{50}$.

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.03.013

the eradication of tumor cells as a consequence of interaction with immune cells that have migrated and accumulated in tumor tissues [3]. To date, more than 40 chemokines have been identified, and several chemokines have been demonstrated as candidates for cancer treatment for use either as sole agents or with an adjuvant [4–8].

We hypothesized that efficient in vitro transfection of chemokine gene into tumor cells could render the tumor sufficient chemokine expression in vivo for screening anti-tumor activity. The chemokine, secreted from inoculated tumor cells, would induce the accumulation of immune cells in the tumor tissue. Consequently, the interaction between the immune cells and the tumor cells should initiate and/or demonstrate the anti-tumor immune response. Among the various methods of gene transduction, recombinant adenovirus vector (Ad) can provide high-level transduction efficacy to a variety of cell types [9,10]. However, some tumor cells exhibit a resistance to Ad-mediated gene transduction due to a decline in the expression of a coxsackie-adenovirus receptor, a primary Ad-receptor, on their surface. We previously demonstrated that, compared with conventional Ad, the fiber-mutant Ad harboring the RGD sequence in the HI loop of the fiber knob (AdRGD) could more efficiently transduce foreign genes into several kinds of tumor cells due to their directivity to αv-integrin positive in the majority of tumors [11–13]. Therefore, chemokine-expressing AdRGD would be useful not only for screening the anti-tumor activity of chemokines by in vitro transfection, but also for developing in vivo cancer gene immunotherapy.

In the present study, we first confirmed the vector performance of eight AdRGDs encoding each mouse chemokine, CCL17, CCL19, CCL20, CCL21, CCL22, CCL27, XCL1, or CX3CL1. The anti-tumor activity of these chemokines was investigated in mice by inoculating three kinds of murine tumor cells, B16BL6 melanoma, CT26 colon carcinoma, and OV-HM ovarian carcinoma cells, transfected with each chemokine-expressing AdRGD. In addition, we examined the growth and rejection ratio of chemokine gene-transduced B16BL6 cells in mice sensitized with melanoma-associated antigen (gp100).

Materials and methods

Cell lines and animals. Human lung carcinoma A549 cells were purchased from ATCC (Manassas, VA, USA). Murine melanoma B16BL6 cells (H-2^b) and human embryonic kidney 293 cells were obtained from JCRB cell bank (Tokyo, Japan). Murine colon carcinoma CT26 cells (H-2^d) were kindly provided by Dr. Nicholas P. Restifo (National Cancer Institute, Bethesda, MD, USA). Murine ovarian carcinoma OV-HM cells (H-$2^{b/k}$) were kindly provided by Dr. Hiromi Fujiwara (School of Medicine, Osaka University, Osaka, Japan). A549 and 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. B16BL6 cells were cultured in a minimum essential medium supplemented with 7.5% FBS and antibiotics. CT26 and OV-HM cells were grown in an RPMI 1640 medium supplemented with 10% FBS and antibiotics. Murine pre-B lymphoma L1.2 cells and their stable transfectants of a specific chemokine receptor, L1.2/CCR4, L1.2/CCR6, L1.2/CCR7, L1.2/CCR10, L1.2/XCR1, and L1.2/CX3CR1 cells [14], were maintained in an RPMI 1640 medium supplemented with 10% FBS, 50 μM of 2-mercaptoethanol, and antibiotics. All the cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Female C57BL/6 (H-2^b), BALB/c (H-2^d), and B6C3F1 (H-$2^{b/k}$) mice, ages 7–8 weeks, were purchased from SLC (Hamamatsu, Japan). All of the animal experimental procedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

Vectors. Replication-deficient AdRGD was based on the adenovirus serotype 5 backbone with deletions of E1/E3 region. The RGD sequence for αv-integrin-targeting was inserted into the HI loop of the fiber knob using a two-step method as previously described [11]. Mouse cDNAs of CCL17, CCL19, CCL20, CCL21, CCL22, and XCL1 were obtained from pExCell-mCCL17, pT7T3D-Pac-mCCL19, pFastBac1-mCCL20, pT7T3D-Pac-mCCL21, pBluescript SK(+)-mCCL22, and pExCell-mXCL1, respectively. The expression cassette containing each mouse chemokine cDNA under the control of the cytomegalovirus promoter was inserted into E1-deletion site for constructing AdRGD-CCL17, -CCL19, -CCL20, -CCL21, -CCL22, and -XCL1, respectively, by an improved in vitro ligation method as previously described [15,16]. Mouse CCL27-expressing AdRGD (AdRGD-CCL27), mouse CX3CL1-expressing AdRGD (AdRGD-CX3CL1), gp100-expressing AdRGD (AdRGD-gp100), β-galactosidase-expressing AdRGD (AdRGD-LacZ), luciferase-expressing AdRGD (AdRGD-Luc), and AdRGD-Null, which is identical to the AdRGD vectors without the gene expression cassette, were previously constructed [11,17–19]. AdRGD-LacZ, -Luc, and -Null were used as negative control vectors in the present study. All recombinant AdRGDs were propagated in 293 cells, purified by two rounds of cesium chloride gradient ultracentrifugation, dialyzed, and stored at −80 °C. Titers (tissue culture infectious dose$_{50}$; TCID$_{50}$) of infective AdRGD particles were evaluated by the end-point dilution method using 293 cells.

RT-PCR analysis. A549 cells were transfected with each AdRGD at an MOI (multiplicity of infection; TCID$_{50}$/cell) of 50 for 2 h, and then the cells were washed twice with phosphate-buffered saline (PBS) and cultured for 24 h. The expression of mouse chemokine mRNA in these A549 cells was confirmed by an RT-PCR analysis as follows: total RNA was isolated from transduced A549 cells using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions, following which RT proceeded for 60 min at 42 °C in a 50-μl reaction mixture containing 5 μg total RNA treated with DNase I, 10 μl of 5× RT buffer, 5 mM $MgCl_2$, 1 mM dNTP mix, 1 μM random hexamers, 1 μM oligo(dT), and 100 U ReverTra Ace (Toyobo, Osaka, Japan). PCR amplification of each mouse chemokine and human β-actin transcripts was performed in 50 μl of a reaction mixture containing 1 μl of RT-material, 5 μl of 10× PCR buffer, 1.25 U *Taq* DNA polymerase (Toyobo), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.4 μM primers. The sequences of the specific primers used for PCR amplification and the expected PCR product sizes are defined in Table 1. After denaturation for 2 min at 95 °C, 30 (mouse chemokine) or 20 (human β-actin) cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C (human β-actin), 60 °C (mouse CCL17, CCL19, CCL20, CCL22, and CX3CL1), 62 °C (mouse CCL21 and XCL1), or 63 °C (mouse CCL27), and extension for 30 s at 72 °C were repeated and followed by completion for 4 min at 72 °C. The PCR product was electrophoresed on a 3% agarose gel, stained with ethidium bromide, and visualized under ultraviolet radiation. EZ Load (Bio-Rad, Tokyo, Japan) was used as a 100 bp molecular ruler.

In vitro chemotaxis assay. A549 cells were transfected with each AdRGD at an MOI of 50 for 2 h, and then the cells were washed twice with PBS and cultured in media containing 10% FBS. After 24 h

Table 1
Primer sequences used for PCR amplification

Gene	Primer sequence (5′ → 3′)	Product size (bp)
Mouse CCL17	(F) TGC TTC TGG GGA CTT TTC TG (R) CCT TGG GTT TTT CAC CAA TC	242
Mouse CCL19	(F) GAA AGC CTT CCG CTA CCT TC (R) TGC TGT TGC CTT TGT TCT TG	164
Mouse CCL20	(F) CGA CTG TTG CCT CTC GTA CA (R) CAC CCA GTT CTG CTT TGG AT	157
Mouse CCL21	(F) CTG AGC CTC CTT AGC CTG GT (R) TCC TCT TGA GGG CTG TGT CT	381
Mouse CCL22	(F) TAT GGT GCC AAT GTG GAA GA (R) GCA GGA TTT TGA GGT CCA GA	102
Mouse CCL27	(F) CTC CCG CTG TTA CTG TTG CT (R) AGT TTT GCT GTT GGG GGT TT	331
Mouse XCL1	(F) ATG GGT TGT GGA AGG TGT G (R) GGG AAC AGT TTC AGC CAT GT	250
Mouse CX3CL1	(F) GCA GTG ACC GGA TCA TCT CT (R) GGC ACC AGG ACG TAT GAG TT	701
Human β-actin	(F) CCT TCC TGG GCA TGG AGT CCT G (R) GGA GCA ATG ATC TTG ATC TTC	202

cultivation, cells were washed and incubated with an assay medium (phenol red-free RPMI 1640 containing 0.5% bovine serum albumin and 20 mM Hepes, pH 7.4) for another 24 h. The resulting conditioned medium was collected, and its chemoattractant activity was measured by an in vitro chemotaxis assay across a polycarbonate membrane with 5-μm pores (Chemotaxicell-24; Kurabo, Osaka, Japan) using L1.2 transfectants expressing the specific receptor for chemokines. Recombinant chemokines corresponding to each specific receptor (mouse: CCL19, CCL20, CCL22, CCL27, XCL1, and CX3CL1) were purchased from DakoCytomation (Kyoto, Japan) and used as a positive control for cell migration. Migration was allowed for 2 h at 37 °C in a 5% CO_2 atmosphere. The migrated cells were lysed and quantitated using a PicoGreen dsDNA quantitation reagent (Invitrogen, Tokyo, Japan), and the migration activity was expressed in term of the percentage of the input cells calculated by the following formula: (% of input cells) = (the number of migrated cells)/(the number of cells placed in Chemotaxicell-24; 1×10^6 cells) $\times$ 100.

Evaluation of growth of chemokine gene-transduced tumor cells in immunocompetent mice. B16BL6, CT26, and OV-HM cells were transfected with each AdRGD at an MOI of 400, 50, and 10, respectively. After 24 h cultivation, the cells were harvested and washed three times with PBS, and then 2×10^5 transduced B16BL6 cells, 2×10^5 transduced CT26 cells, and 1×10^6 transduced OV-HM cells were intradermally inoculated into the flank of C57BL/6 mice, BALB/c mice, and B6C3F1 mice, respectively. The major and minor axes of the tumor were measured using microcalipers, and the tumor volume was calculated by the following formula: (tumor volume; mm^3) = (major axis; mm) $\times$ (minor axis; mm)^2 $\times$ 0.5236 [20]. The mice were euthanized when one of the two measurements was greater than 15 mm. On day 60 after tumor inoculation, the tumor-free mice were judged as individuals that could achieve complete rejection. In some cases, the mice that could completely reject a primary tumor were rechallenged by intradermal injection into the flank with 1×10^6 parental or irrelevant tumor cells without chemokine gene-transduction at 3 months after the initial challenge.

Evaluation of growth and rejection ratio of chemokine gene-transduced B16BL6 cells in mice sensitized with melanoma-associated antigen. The immunization of mice with melanoma-associated antigen was performed by the administration of dendritic cells (DCs) transduced with the gp100 gene. The isolation, cultivation, and gene transduction procedures for C57BL/6 mouse bone marrow-derived DCs conformed to the methods previously described [21]. DCs transfected with Ad-RGD-gp100 at an MOI of 50 for 2 h were intradermally injected into the right flank of C57BL/6 mice at 5×10^5 cells/50 μl. At 1 week after the vaccination, 2×10^5 intact or transduced B16BL6 cells were inoculated into the left flank of the mice. The tumor growth and complete rejection were assessed as described above.

Results

Expression of chemokine mRNA and protein in cells transfected with AdRGD

In order to verify the vector performance of mouse chemokine gene-carried AdRGDs, we first examined

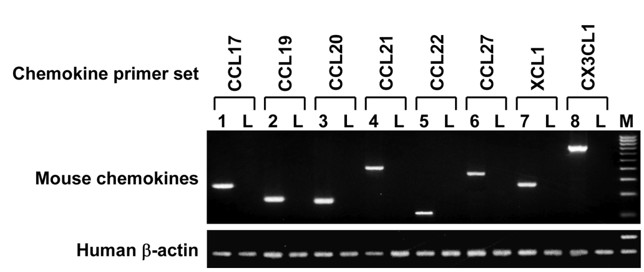

Fig. 1. RT-PCR analysis of chemokine mRNA expression in A549 cells transfected with each chemokine gene-carried AdRGD. PCR for mouse chemokine and human β-actin transcripts was performed on the same RT samples using each specific primer set (summarized in Table 1) to ensure the quality of the procedure. Lane L is negative control using AdRGD-LacZ-transfected A549 (LacZ/A549) cell-derived RT material. Lanes 1–8 represent CCL17/A549, CCL19/A549, CCL20/A549, CCL21/A549, CCL22/A549, CCL27/A549, XCL1/A549, and CX3CL1/A549, respectively. Lane M is a 100 bp molecular ruler.

mRNA expression in transfected cells by an RT-PCR analysis (Fig. 1). In this experiment, human lung carcinoma A549 cells were used instead of murine tumor cells to eliminate the influence of the expression of endogenous mouse chemokine. A549 cells transfected with AdRGD-CCL17, -CCL19, -CCL20, -CCL21, -CCL22, -CCL27, -XCL1, or -CX3CL1 expressed corresponding mouse chemokine mRNA, whereas no PCR products derived from the transcripts of the mouse chemokine gene were detected in AdRGD-LacZ-transfected A549 cells. Next, using in vitro chemotaxis assay, we investigated whether A549 cells transfected with each chemokine gene-carried AdRGD could secrete chemokine protein as a biologically active form into culture supernatants. As shown in Fig. 2, the culture supernatants of each chemokine gene-transduced A549 cell could induce greater migration of cells expressing the corresponding chemokine receptor than those of the intact A549 cells or the AdRGD-Luc-transfected A549 (Luc/A549) cells. The migration of parental L1.2 cells for chemokine receptor-transfectants was not observed in recombinant chemokine-added wells, and they were

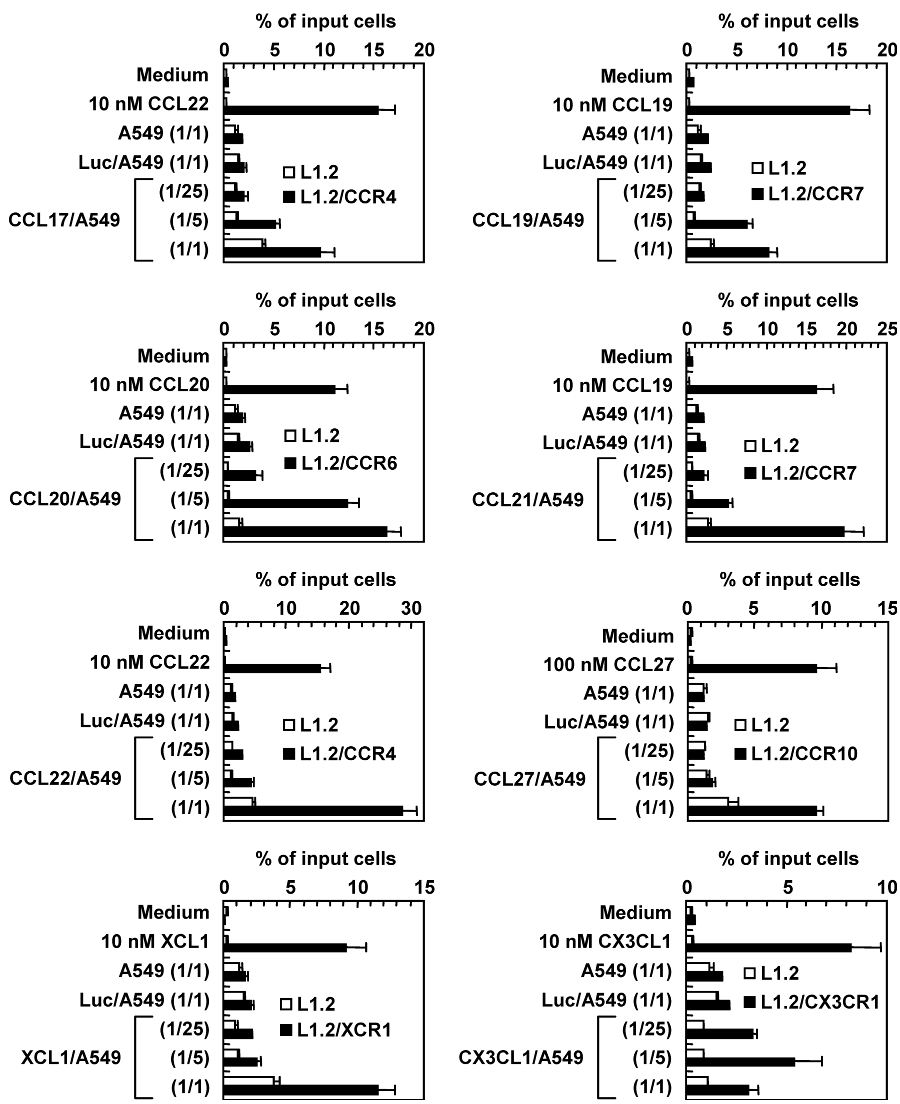

Fig. 2. Chemoattractant activity of culture supernatants of A549 cells transfected with each chemokine gene-carried AdRGD against the stable specific chemokine receptor-expressing cells. The culture supernatants of intact A549 cells, AdRGD-Luc-transfected A549 (Luc/A549) cells, and chemokine gene-transduced A549 cells were prepared and diluted with an assay medium. The fractional values with parentheses in each panel express the dilution factor. These samples and recombinant chemokines dissolved with the assay medium were added to a 24-well culture plate. Cells expressing specific receptors for CCL17 and CCL22 (L1.2/CCR4), CCL20 (L1.2/CCR6), CCL19 and CCL21 (L1.2/CCR7), CCL27 (L1.2/CCR10), XCL1 (L1.2/XCR1), or CX3CL1 (L1.2/CX3CR1) were suspended with the assay medium and placed in a Chemotaxicell-24 installed on each well at 1×10^6 cells. Likewise, parental L1.2 cells for these transfectants were prepared and added to Chemotaxicell-24. Cell migration was allowed for 2 h at 37 °C in a 5% CO_2 atmosphere. The cells that migrated to the lower well were lysed and quantitated using a PicoGreen dsDNA quantitation reagent. The data are expressed as means ± SE of the triplicate results.

maintained at low levels against the culture supernatants of intact A549, Luc/A549, and chemokine gene-transduced A549 cells. These results clearly demonstrated that all AdRGDs encoding each chemokine gene could deliver the concerned gene to target cells, and that transfected cells could secrete the chemokine protein which maintained original chemoattractant activity.

In vivo anti-tumor effect by transfection with chemokine-expressing AdRGD

B16BL6 and CT26 cells were each transfected with eight kinds of chemokine-expressing AdRGDs and AdRGD-Luc, as a control vector, at an MOI of 400 and 50, respectively. OV-HM cells were transfected with

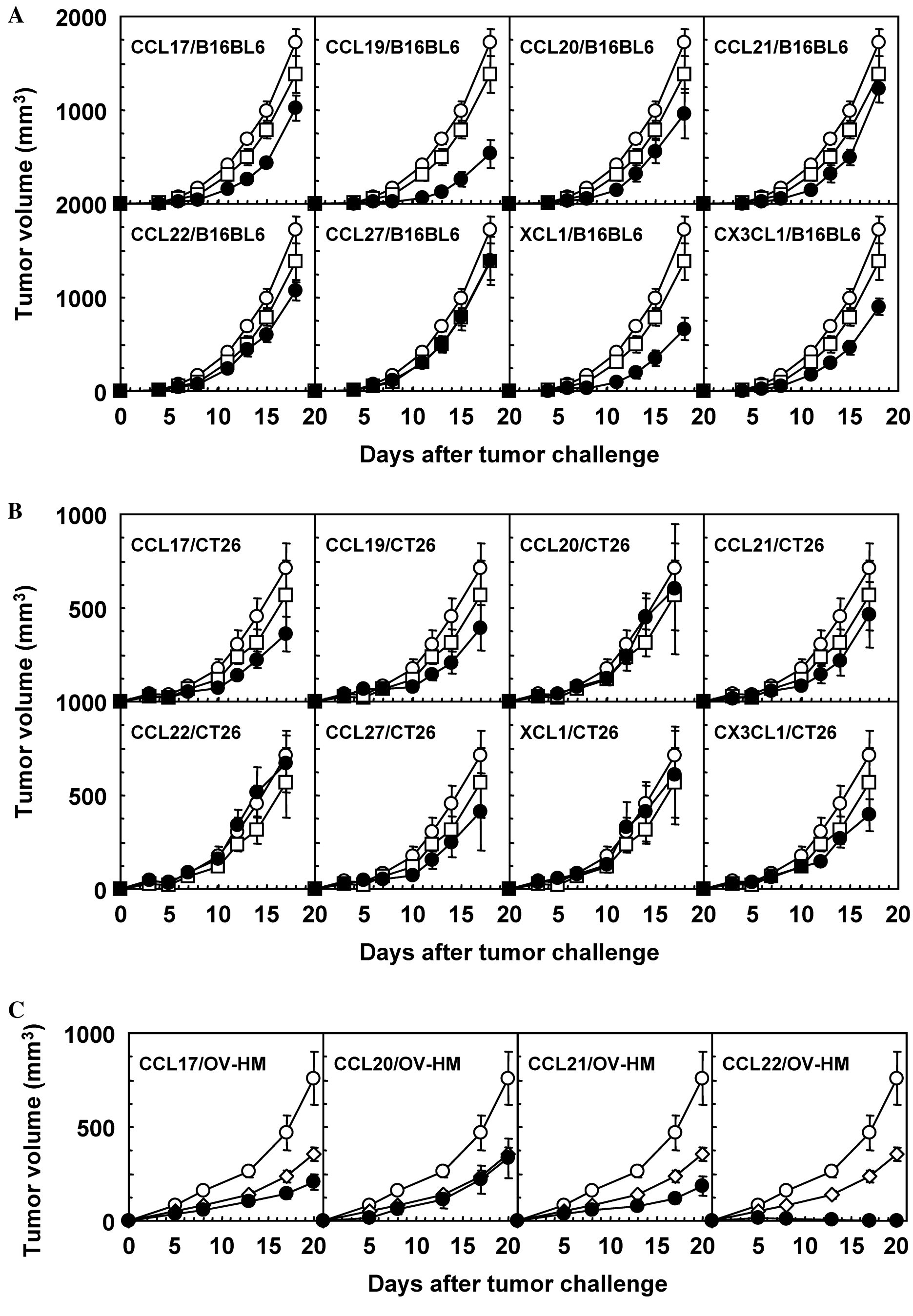

Fig. 3. In vivo growth of three kinds of murine tumor cells transduced with the chemokine gene. B16BL6 cells (A), CT26 cells (B), and OV-HM cells (C) were transfected with each chemokine-expressing AdRGD at an MOI of 400, 50, and 10, respectively, for 24 h. C57BL/6 mice, BALB/c mice, and B6C3F1 mice were intradermally injected in the flank with 2×10^5 transduced B16BL6 cells, 2×10^5 transduced CT26 cells, and 1×10^6 transduced OV-HM cells (●), respectively. Similarly, mice were inoculated with three kinds of intact tumor cells (○), AdRGD-Luc-transfected B16BL6 cells or CT26 cells (□), or AdRGD-Null-transfected OV-HM cells (◇), as control groups. The tumor volume was calculated after measuring the major and minor axes of the tumor at indicated points. Each point represents the mean ± SE of 6–10 mice. The data are representative of two independent experiments.

AdRGD-CCL17, -CCL20, -CCL21, -CCL22, or control AdRGD-Null at an MOI of 10. These transduced tumor cells were intradermally inoculated into H-2 haplotype-matched mice, and tumor growth was compared with that of intact tumors. As shown in Fig. 3, the tumorigenicity of B16BL6 and CT26 cells was hardly affected by transfection with the control vector, whereas OV-HM cells transfected with AdRGD-Null exhibited a slight delay of tumor growth as compared with intact OV-HM cells. Among 20 combinations of chemokine and tumor cells, an obvious tumor-suppressive effect was recognized in mice inoculated with CCL19/B16BL6, XCL1/B16BL6, or CCL22/OV-HM cells. In contrast, the in vivo growth of CCL27/B16BL6, CCL20/CT26, CCL22/CT26, XCL1/CT26, and CCL20/OV-HM cells was the same as that of the control vector-transfected cells, and only a slight delay of tumor growth was observed in five B16BL6 groups (CCL17, CCL20, CCL21, CCL22, and CX3CL1), five CT26 groups (CCL17, CCL19, CCL21, CCL27, and CX3CL1), and two OV-HM groups (CCL17 and CCL21). Importantly, CCL22/OV-HM cells not only demonstrated considerable retardation in tumor growth but were also completely rejected in 9 of 10 mice. In the rechallenge experiment, these cured mice were intradermally injected with 1×10^6 parental OV-HM cells or irrelevant B16BL6 cells at 3 months after the initial challenge. Five of six mice rechallenged with OV-HM cells remained tumor-free for more than 2 months, whereas rechallenging with B16BL6 cells perfectly developed palpable tumors in three additional mice within 2 weeks (data not shown). These results indicate the generation of long-term specific immunity against OV-HM tumor in mice that could once reject CCL22/OV-HM cells.

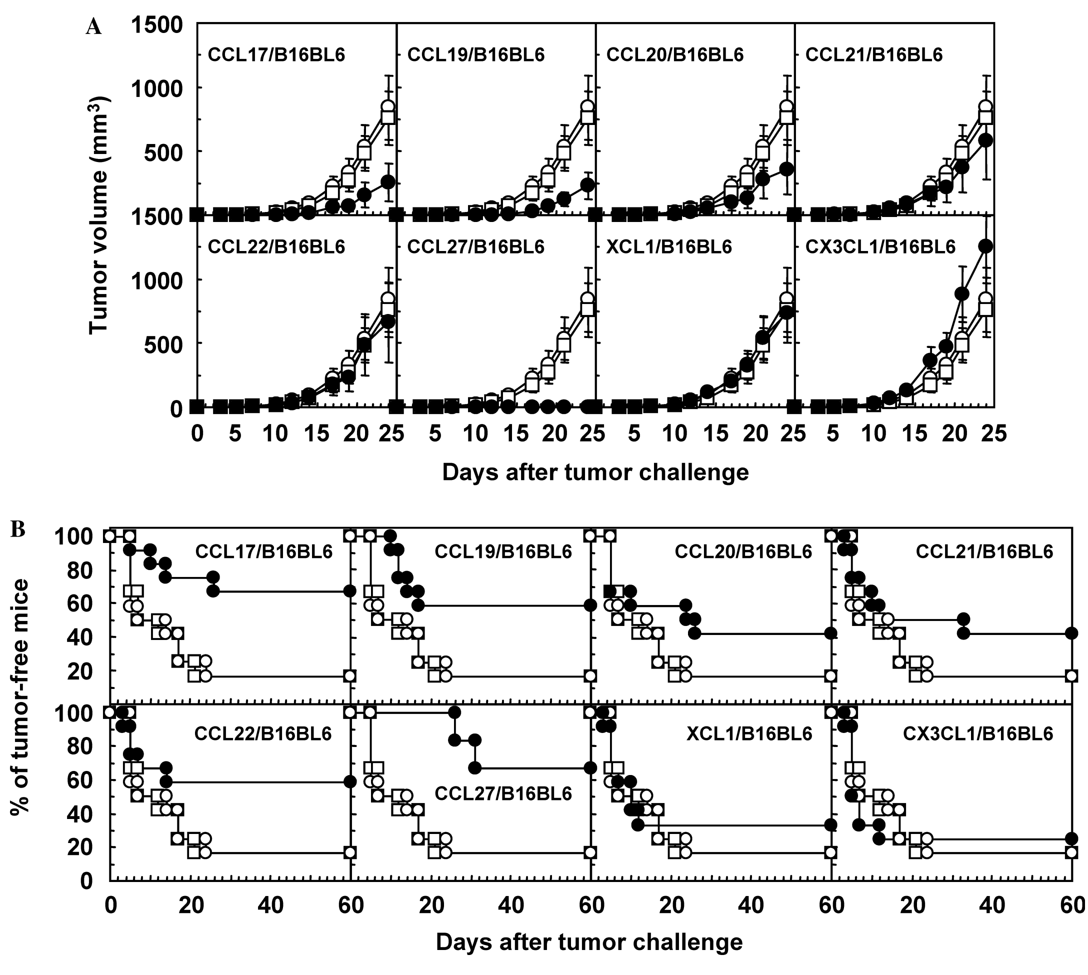

Fig. 4. Growth and rejection ratio of B16BL6 cells transduced with the chemokine gene in mice primed with melanoma-associated antigen. C57BL/6 mouse bone marrow-derived DCs were transfected with AdRGD-gp100 at an MOI of 50 for 2 h and they were intradermally injected into the right flank of syngeneic mice at 5×10^5 cells. At 1 week after the vaccination, these mice were intradermally inoculated in the left flank with 2×10^5 B16BL6 cells transfected with each chemokine-expressing AdRGD at an MOI of 400 for 24 h (●). Likewise, intact B16BL6 cells (○) or AdRGD-Luc-transfected B16BL6 cells (□) were inoculated in the gp100-primed mice, which were used as control groups. (A) The tumor volume was assessed three times per week. Each point represents the mean ± SE of results obtained from 12 mice. (B) Data are expressed in terms of the percentage of mice without visible tumor against the total mice tested in each group.

Growth and rejection ratio of chemokine gene-transfected B16BL6 cells in gp100-primed mice

For the purpose of examining the influence of chemokine against tumor growth in hosts specifically sensitized with tumor-associated antigen, B16BL6 cells transfected with chemokine-expressing AdRGD were inoculated into mice that were vaccinated with DCs presenting gp100, one of the identified melanoma-associated antigens. As shown in Fig. 4A, CCL17-, CCL19-, or CCL27-transfection was very effective for tumor growth suppression in gp100-primed mice, whereas AdRGD-Luc-transfected B16BL6 cells did not show any difference in tumor growth as compared with intact cells. A remarkable enhancement was observed in the complete rejection ratio at 2 months after tumor inoculation in the CCL22-transfected group as well as in the CCL17, CCL19, and CCL27 groups (Fig. 4B). Also, transfection with AdRGD-CCL20 or -CCL21 moderately improved the rejection ratio of B16BL6 cells in gp100-primed mice. XCL1 did not show a notable difference in both the growth and the rejection ratio of B16BL6 cells as compared with the control groups, and CX3CL1-transfected cells showed a tendency to promote tumor growth as compared with the intact B16BL6 cells.

Discussion

The application of chemokines to cancer immunotherapy has recently attracted great attention, because of their chemoattractant activity for a variety of immune cells as well as the angiostatic activity of some chemokines such as CXCL9 and CXCL10. In addition, it has been known that some tumor cells express a lower level of chemokines than normal cells [22]. Therefore, we may obtain novel cancer gene immunotherapy capable of demonstrating an excellent therapeutic effect, if a specific chemokine is adequately expressed at a local tumor site by gene transduction. The tumor-suppressive activity of several chemokines was observed in actuality in various experimental tumor models using the in vitro transfection method [8,23–27]. We also previously demonstrated that a CC family chemokine, CCL27, could suppress OV-HM tumor growth via transfection into the tumor cells due to the local recruitment of T cells and natural killer (NK) cell, whereas the transfection of CX3CL1 did not show a significant effect [19]. However, there are few reports comparing the antitumor activity of a specific chemokine between distinct tumor models.

Thus, we screened the potential anti-tumor activity of CCL17, CCL19, CCL20, CCL21, CCL22, CCL27, XCL1, and CX3CL1 in three murine tumor models by in vitro transfection. In order to efficiently transduce the chemokine gene into tumor cells, we constructed the AdRGDs carrying an expression cassette containing each murine chemokine cDNA by an improved in vitro ligation method. AdRGD can enhance gene transduction efficiency against a variety of tumor cells as compared with conventional Ad because of the expression of the RGD sequence, the αv-integrin-targeting peptide, at the HI-loop in their fiber knob [11–13]. Moreover, the improved in vitro ligation method enables speedy construction of a series of AdRGDs for screening by easy insertion of the expression cassette for the concerned gene into E1-deletion site [15,16]. With respect to the RT-PCR analysis and in vitro chemotaxis assay, transfection using our eight AdRGDs encoding the chemokine gene allowed tumor cells to express each corresponding chemokine mRNA and secrete a specific chemokine protein in a biologically active form (Figs. 1 and 2). Murine B16BL6 melanoma, murine CT26 colon carcinoma, and murine OV-HM ovarian carcinoma cells were transfected with chemokine-expressing AdRGDs at the MOI, which was suitable for adequately introducing a reporter gene into each tumor cell in preliminary examinations. To address the possibility of growth suppression depending on the cytotoxicity by AdRGD itself or secreted chemokine, we evaluated the viability of tumor cells transfected with each AdRGD at 48 h after transfection by MTT assay. The in vitro growth of the transfected cells was essentially identical to that of the intact cells with the exception of the OV-HM cells transduced with AdRGD-CCL19 or -XCL1 (data not shown). Therefore, CCL19 and XCL1 were excluded from the in vivo experiment using OV-HM cells.

Although a slight delay in tumor growth was observed in most of the combinations of tumor cells and chemokines, only CCL19/B16BL6, XCL1/B16BL6, and CCL22/OV-HM cells demonstrated a notable tumor-suppressive activity in immunocompetent mice as compared with the control vector-transfected cells (Fig. 3). In particular, CCL22-transfection was highly efficacious for the repression of OV-HM tumor growth, since complete rejection was observed in 9 of 10 mice. Furthermore, five of six cured mice could resist rechallenge with parental OV-HM cells, indicating the generation of a long-term tumor-specific immunity by rejection of CCL22/OV-HM cells. CCL22 exhibits a strong chemoattractant activity for a variety of immune cells including T cells, NK cells, and DCs. Guo et al. [28] also reported that the intratumoral injection of conventional Ad encoding human CCL22 resulted in a marked tumor regression in a murine 3LL lung carcinoma model with significant cytotoxic T lymphocyte (CTL) activity. However, CCL22-transfection did not show an anti-tumor effect in both B16BL6 and CT26 cells, and the chemokine that could demonstrate an obvious suppressive effect common to tumor cells of all three kinds was not found even if the results of CCL27/OV-HM and

CX3CL1/OV-HM cells, which were examined in our previous work [19], were included. In addition, some chemokines such as CCL17, CCL20, CCL21, and CX3CL1 failed to induce a notable suppressive effect against all three kinds of tumors although their chemoattractant activity for immune cells was reported. These complicated phenomena suggest that the anti-tumor effect via chemokine expression might be affected by several factors, for example, (1) the immunogenicity of the tumor cells, (2) the quantity and population ratio of the immune cells accumulated in tumor tissue, and (3) the activation state and deviation of the immune system in host.

We considered that not only the accumulation but also the activation of immune cells in tumor tissue is very important in cancer immunotherapy using chemokines, because several approaches that combined chemokines with cytokines or costimulatory molecules resulted in the synergic enhancement of anti-tumor activity as compared with the application of chemokine alone [29–32]. DCs, unique antigen-presenting cells capable of priming and stimulating naive T cells, not only play a critical role in establishing antigen-specific adaptive immune responses but also regulate the innate immune system [33–35]. Because of these properties, DCs loaded with tumor-associated antigen are ideal for generating a primary immune response against cancer as "nature's adjuvant" [33,36]. We previously reported that the vaccination of DCs transfected with gene coding gp100, one of the melanoma-associated antigens, by AdRGD could induce anti-B16BL6 tumor immunity based on increasing cytotoxic activities of NK cells and gp100-specific CTLs [21]. When chemokine-transfected B16BL6 cells were inoculated into mice vaccinated with gp100-expressing DCs, CCL17, CCL19, CCL22, and CCL27 could promote resistance to tumor formation (Fig. 4). Upon comparing the outcomes in Figs. 3A and 4, CCL19 demonstrated B16BL6 tumor-suppressive activity in both intact and gp100-primed mice, whereas the enhancement of the anti-tumor effect by CCL17, CCL22, or CCL27 was observed only in gp100-primed mice. Surprisingly, the anti-tumor activity of XCL1 detected in intact mice was lost in gp100-primed mice, and the CX3CL1/B16BL6 tumor grew more rapidly than the control tumor in gp100-primed mice. We speculated that the weak anti-B16BL6 tumor activity of XCL1 or CX3CL1 was masked by vaccine efficacy of gp100-expressing DCs, and that the angiogenic activity of CX3CL1 [37] might be emphasized in a tumor-specifically sensitized host.

Collectively, our data suggested that the tumor-suppressive activity of chemokine was greatly influenced by the kind of tumors and the activation state of the immune cells, and that a search for an effective chemokine for cancer immunotherapy should be performed in an experimental model that can reflect clinical status, including the immunogenicity of tumors, the state of the host's immune system, and the combination of other treatments, as much as possible.

Acknowledgments

We are grateful to Dr. Nicholas P. Restifo (National Cancer Institute, Bethesda, MD, USA) for providing the CT26 cells, and to Dr. Hiromi Fujiwara (School of Medicine, Osaka University, Osaka, Japan) for providing the OV-HM cells. This study was supported in part by the Research on Health Sciences focusing on Drug Innovation from The Japan Health Sciences Foundation; by grants from the Bioventure Development Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan; by the Science Research Promotion Fund of the Japan Private School Promotion Foundation; by Grants-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and by grants from the Ministry of Health and Welfare in Japan.

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