

FIGURE 2. Cytokines and liver enzyme levels in serum after the systemic administration of Ad-L2 or AdK7-L2 into mice. Blood samples were collected by inferior vena cava at 3 h (A) or 24 and 48 h (B) after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP for A or 3.0×10^{10} VP for B). The livers were collected after 48 h following the injection (3.0×10^{10} VP) (C). A, IL-6 and IL-12 levels in the serum were measured by ELISA. B, AST levels in the serum were measured using a Transaminase-CII kit. C, Paraffin sections of the livers were prepared. Each section was stained with H&E. Data represent the means \pm SD of four mice.

Cell sorting of splenic cells

Splenic conventional DC, plasmacytoid DC, and B cells, which were CD11c⁺B220⁻, CD11c⁺B220⁺, and CD11c⁻B220⁺ cells, respectively, were sorted by FACS Aria (BD Biosciences). Total RNA samples were isolated from each cell by the reagent ISOGEN, and RT-PCR analysis was then performed as described above.

Results

This study was undertaken to elucidate the biological mechanism in the innate immune host responses toward i.v. administered Ad vector. The relationship between the innate immune response and liver toxicity by systemic administration of the Ad vectors was also examined.

Gene transduction and Ad vector accumulation in vivo

In this study we used the conventional Ad vector (Ad-L2) and a fiber-modified Ad vector containing a polylysine (K7) peptide (AdK7-L2), both of which express luciferase under the control of the CMV promoter. First, we examined luciferase production in the organ and the biodistribution of viral DNA after i.v. administration of AdK7-L2 (1.0×10^{10} VP) into mice compared with Ad-L2 (see Fig. 3). The vector dose of 1.0×10^{10} VP was selected because this dose did not induce any apparent toxicity (IL-6 and AST production) with either Ad-L2 or AdK7-L2. When a higher dose (3.0×10^{10} or 1.0×10^{11} VP) was used, only Ad-L2 and not AdK7-L2 showed toxicity (described later), which does not reflect an exact comparison of the transduction efficiency. The Ad type 5-based vector delivers the foreign gene predominantly in the liver after i.v. injection into mice (29, 30). Interestingly, AdK7-L2 mediated \sim 6-fold higher liver transduction

than Ad-L2 (Fig. 1A). In contrast, the luciferase production in the heart, lung, kidney, and spleen in response to AdK7-L2 was similar to that in response to Ad-L2. To examine the biodistribution of Ad-L2 and AdK7-L2 in mice, the amounts of Ad DNA in each organ 48 h after the injection of Ad vectors were measured with the TaqMan fluorogenic detection system. More AdK7-L2 DNA accumulated in the liver than Ad-L2 DNA (Fig. 1B), although the amounts of AdK7-L2 DNA in the heart, lung, kidney, and spleen were less than those of Ad-L2 DNA. In particular, the amounts of AdK7-L2 DNA in the spleen were \sim 56-fold less than those of Ad-L2 DNA. The data regarding luciferase production (Fig. 1A) and the amounts of Ad DNA in most organs (Fig. 1B) showed discrepancies. Luciferase production in the liver was >2 log order higher than that in other organs, while the amounts of Ad DNA in liver were not as striking among the organs compared with luciferase production. This difference is likely due to the difference in the amount of nonspecific viral uptake among the organs. Reduced spleen accumulation of AdK7-L2 DNA, compared with Ad-L2 DNA, was also observed at a dose of 1.0×10^{11} VP (data not shown).

Serum cytokines and AST levels

The systemic administration of Ad vectors results in the initiation of strong innate immune responses and inflammation in animals and humans (1), and this toxicity limits the utility of Ad vectors for gene therapy. To evaluate the innate immune response and liver toxicity of each Ad vector, we measured the levels of IL-6, IL-12, and AST in serum. Because IL-6 in the serum and hepatic toxicity

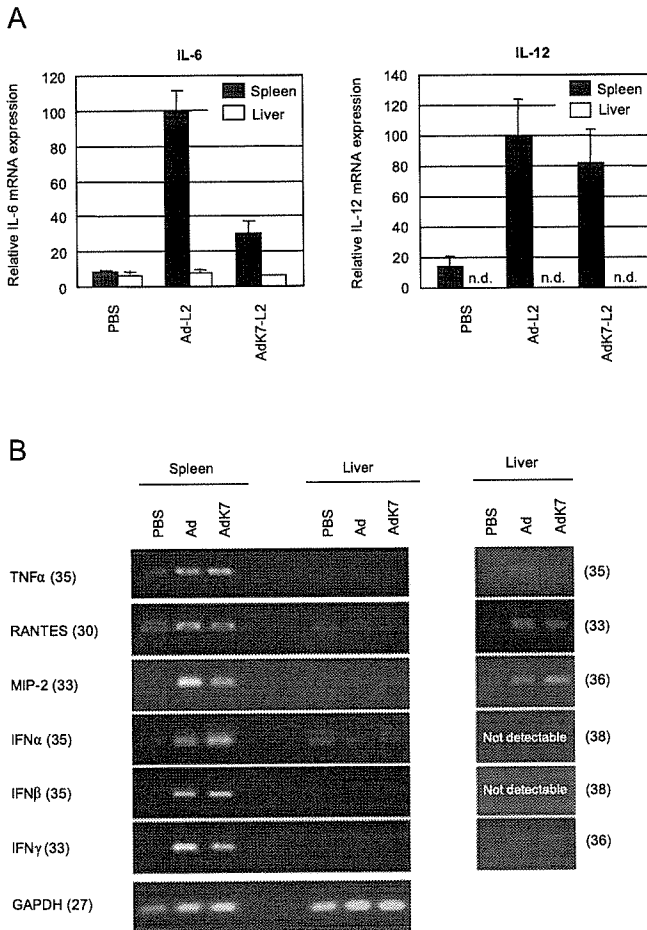


FIGURE 3. Cytokine, chemokine, and IFN mRNA levels in liver and spleen after the systemic administration of Ad-L2 or AdK7-L2 into mice. Total mRNA samples were isolated from liver and spleen at 3 h after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP). After the reverse transcriptase reaction, IL-6 and IL-12 cDNA were measured with the quantitative TaqMan PCR assay (A). The expression of TNF- α , RANTES, MIP-2, IFN- α , IFN- β , and IFN- γ was measured by semiquantitative RT-PCR assay (B). All data represent the means \pm SD of four mice. Cycle number is given in parentheses.

analysis was detected at a dose of $>1.0 \times 10^{11}$ or 3.0×10^{10} VP, respectively, these doses were used.

IL-6 levels in response to AdK7-L2 were one-fourth of those with Ad-L2 (Fig. 2A). In contrast, there was no difference in serum IL-12 levels between Ad-L2 and AdK7-L2. Thus, IL-6 and IL-12 appear to be produced by a different mechanism. TNF- α in the serum after the injection of Ad-L2 or AdK7-L2 could not be detected (data not shown). Ad-L2 led to high levels of serum AST at 48 h after injection, while AdK7-L2 did not induce AST (Fig. 2B). At 24 h, neither Ad-L2 nor AdK7-L2 induced AST. In histological analysis, degranulation or denudation occurred in hepatocytes from Ad-L2, while AdK7-L2 did not induce hepatocyte toxicity (Fig. 2C). The results using AdK7-L2 were similar to those in the untreated mice (Fig. 2, B and C), suggesting that AdK7-L2 does not show any liver toxicity. These results suggest that AdK7-L2 shows less IL-6 production and almost no liver toxicity.

Cytokines mRNA levels in liver and spleen cells

Ad vectors induce the expression of various cytokines and chemokines in the innate immune responses by effector cells such as macrophages and DC (15, 17, 31–33). Liver and spleen are two

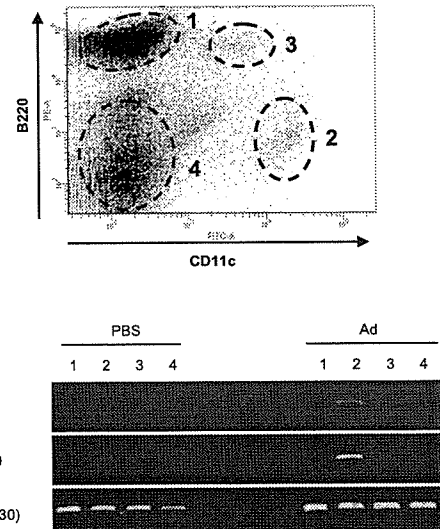


FIGURE 4. IL-6 and IL-12 mRNA levels in splenic CD11c-positive cells after the systemic administration of Ad-L2 into mice. Total mRNA samples were isolated from sorted splenic cells 3 h after i.v. administration of Ad-L2 (1.0×10^{11} VP). The expression levels of IL-6 and IL-12 mRNA were measured by RT-PCR assay. Lane 1, B cell ($B220^+CD11c^-$); lane 2, conventional DC ($B220^-CD11c^+$); lane 3, plasmacytoid DC ($B220^+CD11c^+$); lane 4, other cells ($B220^-CD11c^-$). Cycle number is given in parentheses.

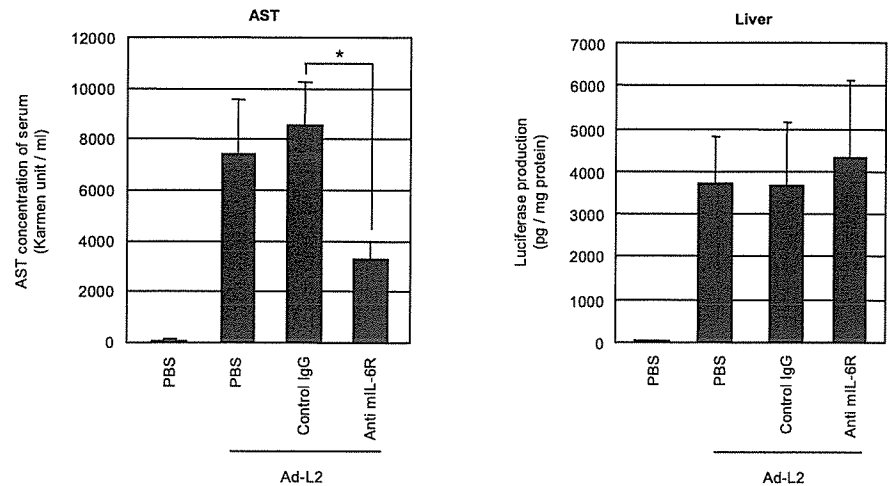
major organs responsible for the location of immune cells. We attempted to determine which organ (liver or spleen) produces cytokines, chemokines, and IFNs (IL-6, IL-12, TNF- α , RANTES, MIP-2, IFN- α , IFN- β , and IFN- γ) by quantitative real-time RT-PCR or semiquantitative RT-PCR analysis. IL-6 and IL-12 mRNA levels were not induced in the liver after i.v. administration of Ad vectors (Fig. 3A). This result was also checked by the result that specific IL-6 and IL-12 mRNA bands were not detected in the liver by RT-PCR analysis (data not shown). Expression of TNF- α , RANTES, MIP-2, IFN- α , IFN- β , and IFN- γ mRNA was also detected mainly in the spleen, not the liver (Fig. 3B). IL-6, MIP-2, and IFN- γ mRNA levels in the spleen in response to AdK7-L2 were lower than those in response to Ad-L2. In the liver, TNF- α , RANTES, MIP-2, and IFN- γ mRNA were detected by a high cycle number of PCR after Ad (Ad-L2 or AdK7-L2) injection, whereas IFN- α and IFN- β could be not detected (Fig. 3B).

We next identified the cell types responsible for the IL-6 and IL-12 expression in the spleen after i.v. administration of the Ad vector (Ad-L2). Spleen cells were sorted by FACS Aria based on the expression of CD11c and B220 in conventional DC ($CD11c^+B220^-$), plasmacytoid DC ($CD11c^+B220^+$), and B cells ($CD11c^-B220^+$ cells). IL-6 and IL-12 mRNA were mainly detected in the splenic conventional DC. Only a faint band of IL-12 mRNA was also detected in the splenic plasmacytoid DC ($CD11c^+B220^+$) (Fig. 4). These results suggest that splenic conventional DC are major effector cells of innate immune response (at least IL-6 and IL-12 production) against systemically administered Ad vectors.

Elimination of IL-6 signaling reduces liver toxicity

It has previously been shown that TNF- α is likely to be involved in host responses to Ad vectors in vitro and in vivo (34). Recently, Shayakhmetov et al. (35) have reported that IL-1 signaling, not TNF- α signaling, is involved in Ad vector-associated liver toxicity after i.v. administration. However, the mechanism of liver toxicity

FIGURE 5. Effects of serum IL-6 on serum AST levels and liver luciferase production after the systemic administration of Ad-L2 into mice. C57BL/6 mice were i.p. administered 100 μg per mouse of anti-IL-6R Ab (clone D7715A7), which was specific for blocking IL-6 signaling, or rabbit IgG as a control (clone; R3-34). Ad-L2 or AdK7-L2 (3.0×10^{10} VP) was i.v. injected into the mice 1.5 h later. Blood samples and liver tissue were collected 48 h after the injection of Ad-L2. The AST levels in the serum were measured using a Transaminase-CII kit. Luciferase production in the liver was measured by a luciferase assay system. All data represent the means \pm SD of three to four mice. *, $p < 0.01$.



after i.v. Ad administration is poorly understood. In the present study, although AdK7-L2 mediated higher luciferase expression and a higher accumulation of viral DNA in the liver than Ad-L2, it remains unclear why AdK7-L2 showed almost background levels of liver toxicity while Ad-L2 showed high toxicity. As reported previously, inflammatory cytokines, chemokines, and IFNs could be the mediators responsible for liver toxicity (2). IL-6 levels in the serum were the most strikingly different between AdK7-L2 and Ad-L2. Furthermore, IL-6 stimulated acute phase protein (serum amyloid A, fibrinogen, α_1 -anti-trypsin, and α_1 -acid glycoprotein) in rat and human hepatocytes (36, 37). Therefore, we next examined the effects of serum IL-6 on liver toxicity (Fig. 5). To do this, we used an anti-IL-6R Ab that inhibits the signal through the IL-6 receptor. The IL-6 receptor system consists of two functional molecules, an 80-kDa ligand-binding chain (IL-6R) and a 130-kDa nonligand-binding but signal-transducing chain (gp130). The anti-IL-6R Ab blocks the binding of IL-6 to the IL-6R (38, 39). The anti-IL-6R Ab or the control Ab was i.p. injected 1.5 h before the injection of Ad-L2. The AST levels in the serum and luciferase production in the liver were determined 48 h later. Administration of anti-IL-6R Ab significantly (~ 2 -fold) reduced Ad vector-mediated AST levels in the serum compared with PBS or the control Ab (Fig. 5A). Importantly, anti-IL-6R Ab injection did not interfere with luciferase production in the liver (Fig. 5B). These results suggest that IL-6 signaling is involved in liver toxicity after i.v. administration of an Ad vector.

Discussion

In this study we found that the fiber-modified Ad vector containing the K7 peptide, which has high affinity with heparin sulfate, shows much lower serum IL-6 and liver toxicity than the conventional Ad vector. This improved characteristic is likely involved with the reduced biodistribution of the vector to the spleen compared with that of the conventional Ad vector. RT-PCR analysis showed that the spleen, not the liver, is the major site of cytokine, chemokine, and IFN (IL-6, IL-12, TNF- α , RANTES, MIP-2, IFN- α , IFN- β , and IFN- γ) production and that splenic conventional DC are the major effector cells of the innate immune response (at least IL-6 and IL-12 production) after i.v. administration of Ad vectors. We also showed that IL-6 signaling is involved in part with liver toxicity in response to Ad vectors. Importantly, this fiber-modified Ad vector containing the K7 peptide maintained higher transduction efficiency in all the organs examined, and the liver transduction was higher than that of the conventional Ad vector. Although there have been some reports that modified Ad vectors such as the pe-

glylated Ad vector (18–21), the Ad vector containing the Ad type 35 fiber shaft and knob (40), and the triple mutant Ad vector with ablation of CAR, α_v integrin, and HSG binding (22) show decreased innate immune response and liver toxicity, these types of vector lose their transduction activity in vivo. To our knowledge, this is the first report of an Ad vector that maintains high transduction efficiency in vivo with reduced toxicity.

The fiber-modified Ad vector containing the K7 peptide has been developed to overcome the limitations imposed by the CAR dependence of Ad infection. Expanded and efficient gene transfer has been reported based on the use of mutant fiber proteins containing a stretch of lysine residues (23–25). However, there has been no report on the difference in gene transfer activity and toxicity in vivo between the conventional Ad vector and the fiber-modified Ad vector containing the K7 peptide. We have demonstrated that the fiber-modified Ad vector containing the K7 peptide mediates ~ 6 -fold higher mouse liver transduction in response to i.v. administration than the conventional Ad vector (Fig. 1A). The amounts of fiber-modified Ad vector DNA in the liver after i.v. administration were also 5-fold higher than those with the conventional Ad vector (Fig. 1B). It has been reported that the interaction between the Ad type 5 fiber and the HSG of a hepatocyte is involved in the accumulation in the mouse liver and the cynomolgus monkey liver of systemically administered Ad vectors (41, 42). This fiber-modified Ad vector might mediate more efficient gene transduction through a much higher affinity for HSG. In contrast, the amounts of fiber-modified Ad vector DNA in the spleen after i.v. administration were 56-fold lower than those of the conventional Ad vector (Fig. 1B). Biodistribution of viral DNA reflects the total of receptor-mediated uptake and nonspecific uptake. Luciferase production in the cells mainly reflects receptor-mediated uptake. We previously reported that most Ad DNAs are taken up in the liver nonparenchymal cells, not parenchymal cells, after i.v. administration (22). In this study, the conventional Ad vector would also be taken up in the macrophages and DC by nonspecific uptake, resulting in significantly higher Ad DNA and lower luciferase production in the spleen. In contrast, the fiber-modified Ad vector would be taken up more in the liver via receptor-mediated uptake and nonspecific uptake, resulting in significantly lower Ad DNA in the other organs, especially the spleen. Even though the amount of AdK7-L2 uptake in the spleen, heart, lung, and kidney was less than that of Ad-L2 uptake, the amount of receptor-mediated uptake in these organs would be similar between Ad-L2 and AdK7-L2, suggesting that these vectors showed similar levels of luciferase production in the organs other than the liver.

The initiation of inflammatory innate immune responses occurs after the systemic administration of Ad vectors to animals and humans, and this toxicity limits the utility of Ad vectors for gene therapy. Increased cytokine/chemokine production after the injection of Ad vectors has been reported to be due to the introduction of input Ad vectors to Kupffer cells in the liver and DC (15, 17, 43–46). Detailed analysis of the organs responsible for the expression of cytokines, chemokines, and IFNs by RT-PCR suggests that their production can mainly be attributed to spleen cells (especially splenic conventional DC), not liver cells (Figs. 3 and 4), which is consistent with the recent report of Bart et al. (47). Therefore, interference with spleen distribution of the Ad vector should provide a useful method for safer gene therapy.

TLRs, which are crucial to the recognition of pathogen-associated molecular patterns, are expressed on various types of immune cells including macrophages, DC, B cells, splenic types of T cells, and even on nonimmune cells such as fibroblasts and epithelial cells (48). For example, HSV and CMV (dsDNA virus) activate inflammatory cytokines and type I IFN secretion by the stimulation of TLR9 (49–53). The innate immune receptor to the Ad has not yet been identified. It has not even been determined whether TLRs are involved in Ad-mediated innate immune response *in vivo*, although it has been reported that TLR signals are not involved in the DC maturation induced by the Ad vector (46). As shown in Fig. 3B, cytokine production against the Ad vector occurred mainly in conventional DC. It is noted that the TLR9-mediated innate immunity responses to DNA virus are cell type-specific and limited to plasmacytoid DC (50). The unidentified sensor receptor(s) for double-stranded Ad DNA or Ad capsid protein in conventional DC might play a critical role in the expression of inflammatory cytokines/chemokines and type I IFN. Although we have previously reported that large amounts of conventional Ad vector accumulate in nonparenchymal cells, including Kupffer cells and liver sinusoidal (endothelial) cells (22, 54), the expression of mRNA of cytokines, chemokines, and IFNs in the liver was weak after administration of the Ad vector (Fig. 3B). A lack of putative sensor receptor(s) against Ad or the inability of sensor receptor(s) to recognize Ad due to the specific cellular disposition of Ad in Kupffer cells might result in a reduced production of cytokines/chemokines/IFNs in the liver.

Another interesting finding is that the fiber-modified Ad vector containing the K7 peptide showed almost background levels of AST activity, which reflects liver toxicity (Fig. 2B). Histological analysis supported this finding (Fig. 2C). Because the K7-modified Ad vector showed higher transgene activity and a higher accumulation of viral DNA into the liver (Fig. 1), the transduction and distribution of the vector into the liver did not participate in liver toxicity. The cytokines/chemokines play a major causative role in liver damage associated with systemic Ad infusion as well as in the induction of an antiviral immune response (2). Ad-induced cytokines/chemokines recruit immune effector cells (neutrophils, monocyte/macrophages, and NK cells) to Ad-transduced cells (mainly liver), resulting in acute hepatic toxicity. Shayakhmetov et al. (35) have reported that hepatocytes and Kupffer cells trigger IL-1 transcription in liver tissue after *i.v.* administration of Ad vectors and that interference of IL-1-signaling reduces liver toxicity. We speculated that IL-6 could be the main mediator for hepatic toxicity because IL-6 is one of the main cytokines in the early stages of inflammation, IL-6 production by the fiber-modified Ad vector was much reduced (approximately a quarter) compared with that by the conventional Ad vector, and all of the cytokines/chemokines/IFNs we examined (including IL-6) were mainly produced by the spleen, not the liver. Treatment of the anti-IL-6R Ab decreased liver toxicity (Fig. 5), suggesting that IL-6 plays at least

some role in liver toxicity induced by systemic injection of the Ad vector. Because the AST levels were only partially reduced by the treatment with the anti-IL-6R Ab, another mechanism such as IL-1 signaling, rapid Kupffer cell death (55, 56), activation of the liver endothelium (55), or other factors might be involved in the liver toxicity. Nevertheless, it is attractive that the K7-modified Ad vector did not show liver toxicity despite the higher transduction efficiency and higher accumulation of the vector into the liver (probably Kupffer cells).

Our present study provides new insight into the cellular biological mechanism related to the innate immune response and liver toxicity against the systemically administered Ad vector. Modification of vector tropism should contribute to safe gene therapy procedures.

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Disclosures

The authors have no financial conflict of interest.

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ORIGINAL ARTICLE

Cotransduction of CCL27 gene can improve the efficacy and safety of IL-12 gene therapy for cancer

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Interleukin-12 (IL-12) is a potent antitumoral cytokine, but high doses are toxic. Herein, we demonstrate that combinational transduction of IL-12 and CC-chemokine ligand-27 (CCL27) genes into pre-existing murine OV-HM ovarian carcinoma and Meth-A fibrosarcoma, by using RGD fiber-mutant adenoviral vectors, could induce tumor regression and relieve systemic side effects more effectively than either treatment alone. The antitumor activity of the IL-12 and CCL27 combination treatment was T-cell-dependent, and development of long-term specific immunity was confirmed in rechallenge experiments. Immunohistochemical analysis of tumors transduced with CCL27 gene alone or cotransduced with IL-12 and CCL27 genes showed significant increases in numbers of infiltrating CD3⁺ T cells, which included both CD4⁺ and CD8⁺ cells. Additionally, cotransduction with IL-12

and CCL27 genes could more efficiently activate tumor-infiltrating immune cells than transduction with CCL27 alone, as determined by the frequency of perforin-positive cells and expression levels of IFN- γ . Furthermore, mice treated with the IL-12 and CCL27 combination compared with those treated with IL-12 alone showed milder pathological changes, for example, lymphocyte infiltration and extramedullary hematopoiesis, in lung, liver and spleen. Our data provide evidence that combinational *in vivo* transduction with IL-12 and CCL27 genes is a promising approach for the development of cancer immunogene therapy that can simultaneously recruit and activate tumor-infiltrating immune cells.

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Keywords: IL-12; CCL27; cancer immunogene therapy; adenovirus; tumor-infiltrating immune cell

Introduction

Multifunctional cytokine interleukin-12 (IL-12) is a 70 kDa (p70) heterodimeric protein in which the 40 kDa (p40) and 35 kDa (p35) subunits are connected by one disulfide bond.^{1,2} IL-12 is mainly secreted by antigen-presenting cells including dendritic cells (DCs) and macrophages and is known for its antitumor properties, effected through the induction of specific cellular immune responses, such as the enhancement of proliferative and cytotoxic activities of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs),^{3,4} the production of interferon- γ (IFN- γ) from activated cells,^{4–6} and the promotion of helper T type 1 (Th1) cell differentiation from Th0 cells.^{4,7,8} IFN- γ is involved in IL-12-mediated tumor regression,⁹ and IL-12 also exhibits an antiangiogenic effect that can account for some antitumor activity.¹⁰ Although recombinant IL-12 administration

at high doses is effective for cancer treatment, major drawbacks of this therapy are the serious adverse effects associated with high systemic peak IL-12 concentrations.^{11,12} Moreover, even in gene therapy aiming for persistent local IL-12 expression by using intratumoral injection of a vector carrying the IL-12 gene, careful evaluation of the vector dosage is required to restrict IL-12 leakage from the tumor tissue to the systemic blood circulation.¹³ Therefore, in order to realize clinical applications of IL-12-based cancer therapy, we need to explore innovative approaches that can elicit more effective tumor immunity at IL-12 doses lower than the maximum tolerance dosage.

On the basis of the relative benefits and risks of IL-12 for cancer treatment, we hypothesized that if small amounts of IL-12 can efficiently and persistently act on a large number of immune cells only within the local tumor tissue, such IL-12-based cancer immunotherapy may suppress adverse effects while maintaining excellent tumor immunity inducibility. However, it is known that infiltration of immune cells is generally hard to achieve in tumor tissue, and that several tumor models, such as Meth-A and MCH-1A1 tumors, are resistant to treatment with systemically administered IL-12 specifically due to poor tumor infiltration of lymphocytes.^{14,15} In addition, because conventional cancer immunotherapy research, involving the development of various vaccine strategies, has mainly focused on efficient

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induction and activation of the host's immune response to tumors, the methodology that can adequately recruit immune cells into tumor tissue is not yet established. In order to better control trafficking and biodistribution of immune cells in cancer immunotherapy, we and several other groups recently proposed novel strategies based on the application of chemokine-chemokine receptor coupling.¹⁶⁻²³

Chemokines, which are small (8–14 kDa), basic, secreted proteins, regulate leukocytic migration and infiltration of local sites through cooperation with various cell adhesion molecules.²⁴ To date, more than 50 chemokines have been identified, and they compose a superfamily that contains four subgroups: C-chemokine, CC-chemokine, CXC-chemokine and CX3C-chemokine.²⁵ Within this superfamily, immune chemokines, which primarily target lymphocytes and DCs, would be useful molecules to improve the efficacy of cancer immunotherapy by augmenting tumor-infiltrating immune cells. We previously reported that tumorigenicity of the murine ovarian carcinoma cell line, OV-HM cells, was attenuated through enhanced tumor-infiltration by immune cells including NK and T cells, effected via *in vitro* transfection with CC-chemokine ligand-27 (CCL27) using RGD-fiber mutant adenoviral vector (AdRGD).²¹ Although 70% of mice inoculated with CCL27-transfected OV-HM cells achieved complete rejection, these mice exhibited only limited long-term immunity in OV-HM-rechallenged experiments. Our results suggested that the tumor-infiltrating immune cells attracted by CCL27 gene transduction should be activated in a tumor-specific manner in order to induce more effective tumor immunity. In addition, because the *ex vivo* approach is not applicable for clinical use in patients with established malignant disease, we need to analyze both antitumor efficacy and activation state of tumor-infiltrating immune cells on direct *in vivo* transduction models using AdRGD-CCL27 plus other immunomodulators. Hence, a combinational therapy using IL-12- and CCL27-expressing vectors at low doses, for example, could potentially provide enhanced antitumor activity and long-term immune responses through the effective recruitment and activation of tumor-infiltrating immune cells, without the serious adverse effects of IL-12 leakage into the systemic circulation.

Thus, in the present study using the established murine OV-HM tumor (responsive to IL-12) and Meth-A tumor (unresponsive to IL-12) models, we compared intratumoral injections with AdRGD-IL12 alone, AdRGD-CCL27 alone, and the combination of AdRGD-IL12 and AdRGD-CCL27, in terms of tumor suppressive effects, frequency and activation state of tumor-infiltrating immune cells, as well as systemic adverse effects.

Results

Synergistic antitumor effects and long-term specific immune response induced by the combinational intratumoral injection of AdRGD-IL12 and AdRGD-CCL27

In order to evaluate the antitumoral effects of direct *in vivo* gene transduction in established OV-HM and Meth-A tumors, we investigated the changes in tumor

size and the survival ratios of mice tested after intratumoral injection with various combinations of AdRGDs. We utilized a total vector dosage of 2×10^7 PFU (plaque-forming unit) in the therapeutic experiments based on our observations that any group, including mice injected intratumorally with AdRGD-IL12 alone, did not show severe adverse effects, such as body weight reduction and sudden death, at dosages lower than this level (data not shown).

As shown in Figure 1a, OV-HM tumors injected with luciferase-expressing AdRGD (AdRGD-Luc; control vector) showed delayed growth compared with that of vehicle-injected tumors, and the growth of OV-HM tumors injected with AdRGD-CCL27 alone was nearly equal to that of AdRGD-Luc-injected tumors. These results suggested that OV-HM tumors were easily injured by direct *in vivo* transduction with AdRGDs, and that their growth was not affected by only CCL27 gene transduction, at least in this therapeutic model. On the other hand, intratumoral injection of AdRGD-IL12 alone induced obvious suppressive effects on OV-HM tumor growth, and even achieved complete regression in 4 of 7 mice (Figure 1b). Although the survival ratio in mice treated with AdRGD-IL12 alone improved with increased vector dosage, there were also higher numbers of mice exhibiting body weight reduction (data not shown). Therefore, we examined whether the combination of AdRGD-IL12 and AdRGD-CCL27 could induce any synergistic effects for inhibition of OV-HM tumor growth without any concomitant side effects. Intratumoral co-injection of AdRGD-IL12 and AdRGD-CCL27 in a total vector dose ratio of 1:1 showed better antitumor effects than injection with AdRGD-IL12 alone, whereas treatment with AdRGD-IL12 and AdRGD-CCL27 in a ratio of 1:9 was ineffective compared with injection with AdRGD-CCL27 alone. Importantly, all mice treated with co-injection of AdRGD-IL12 and AdRGD-CCL27 in a vector dose ratio of 9:1 achieved complete regression of the primary tumor and survived more than 3 months after treatment without any severe adverse effects, such as body weight reduction and sudden death. In the established Meth-A tumor model, intratumoral injection of AdRGD-Luc alone or AdRGD-CCL27 alone did not affect tumor growth and survival ratio as compared with phosphate-buffered saline (PBS)-administration (Figure 1c and d). Although Meth-A tumors injected with AdRGD-IL12 alone showed marked regression, the combination of AdRGD-IL12 and AdRGD-CCL27 in a vector ratio of 9:1 produced very strong antitumor effects, similar to those seen in the OV-HM tumor model. Furthermore, most of the mice which achieved complete regression of primary tumors by the combination of AdRGD-IL12 and AdRGD-CCL27 in a ratio of 9:1 were also protected against rechallenge with the same tumor at 3 or 6 months after treatment (Table 1). In contrast, rechallenge of different tumor (B16BL6 or CT26) of which haplotype matched to recipient induced palpable tumor formation in all primary tumor-regressed mice within 2 weeks, indicating that the systemic long-term immunity generated in mice receiving the AdRGD-IL12 and AdRGD-CCL27 combinational therapy was specific for primary tumors (OV-HM or Meth-A).

Taken together, these results clearly revealed that the combination of low doses AdRGD-CCL27 could synergistically enhance the efficacy of IL-12 gene therapy for

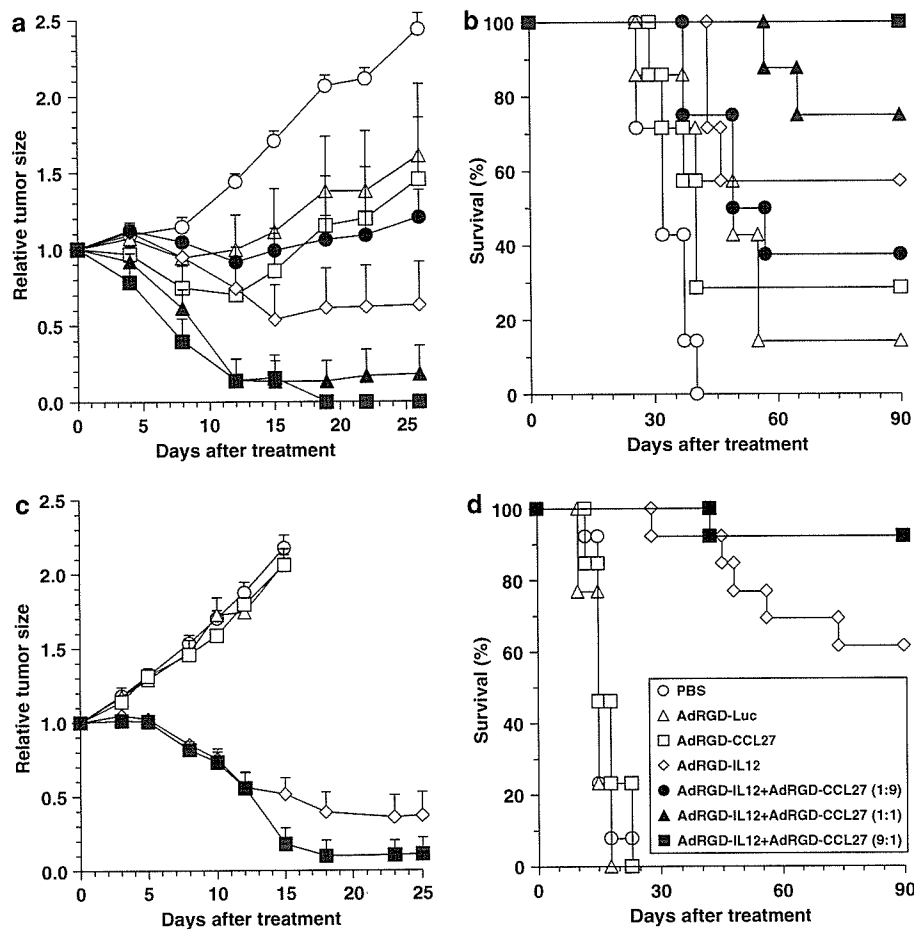


Figure 1 Antitumor efficacy of intratumorally injected AdRGD-IL12 plus AdRGD-CCL27 in OV-HM or Meth-A tumor model. B6C3F1 mice (a and b) or BALB/c mice (c and d) were intradermally inoculated with 10^6 OV-HM cells or 2×10^6 Meth-A cells, respectively, into the flanks. The tumors (7–9 mm in diameter) were injected with either AdRGD-Luc alone, AdRGD-IL12 alone, AdRGD-CCL27 alone, or the combination of AdRGD-IL12 and AdRGD-CCL27 in a ratio of 1:9, 1:1 or 9:1 at the same dose totaling 2×10^7 PFU. PBS was injected into the tumors as a control. (a and c) The sizes of growing tumors were measured twice or three times a week using microcalipers. The data were expressed as a relative tumor size to the initial (day 0) tumor size. Each point represents the mean \pm s.e. of results from seven or eight mice. (b and d) Data represent the number of mice for which tumors were smaller than 20 mm, expressed as a percentage of the total mice tested in each group.

OV-HM and Meth-A tumors, and that the antitumor responses were both specific and long lasting.

Subset of immune cells responsible for antitumor efficacy of the combination of AdRGD-IL12 and AdRGD-CCL27

Next, in order to elucidate whether T-cell functions were involved in the induction of the observed antitumor effects, we carried out therapeutic protocols with intratumoral co-injection of AdRGD-IL12 and AdRGD-CCL27 in a ratio of 9:1 totaling 2×10^7 PFU against established OV-HM and Meth-A tumors in BALB/c nude mice (data not shown). The AdRGD-IL12 and AdRGD-CCL27 combinational treatment was not able to restrain growth of OV-HM tumors established in nude mice. Although growth of Meth-A tumors was slightly delayed in nude mice treated with the AdRGD-IL12 and AdRGD-CCL27 combination, there were no individuals demonstrating tumor regression, unlike the experiments with immunocompetent mice. These results confirmed that tumor regression by the intratumoral co-injection with AdRGD-IL12 and AdRGD-CCL27

was mainly based on a T-cell-dependent immune response.

In addition, we performed *in vivo* depletion analysis using specific antibodies against CD4, CD8 and asialoGM1 in the OV-HM and Meth-A tumor model. As shown in Figure 2a, anti-OV-HM tumor effects of the combination of AdRGD-IL12 and AdRGD-CCL27 were attenuated by depletion of CD8⁺ T cells, CD4⁺ T cells and NK cells in B6C3F1 mice, with CD8-depletion being the most effective at abrogating the therapeutic effects. Moreover, OV-HM tumors in mice with depletion of both CD4⁺ and CD8⁺ T cells, in spite of treatment with AdRGD-IL12 and AdRGD-CCL27, showed more enhanced growth than those in mice with PBS-administration. On the other hand, growth suppressive effects of AdRGD-IL12 and AdRGD-CCL27 combinational therapy against Meth-A tumors were not affected by CD4⁺ T cell depletion, whereas the depletion of CD8⁺ T cells, NK cells and both CD4⁺ and CD8⁺ T cells attenuated antitumor effects, similar to the pattern shown in OV-HM tumor model (Figure 2b). Therefore, we concluded that CD8⁺ CTLs, which were activated by the helper function of CD4⁺ T cells, were the predominant effector

Table 1 Induction of long-term specific immunity in mice that could achieve complete regression of the primary tumor by intratumoral injection with the combination of AdRGD-IL12 and AdRGD-CCL27

Strain	Groups	Rechallenging cells	Tumor rejected mice/tested mice		
			Exp. 1 ^a	Exp. 2 ^a	Exp. 3 ^b
B6C3F1	Intact mice	OV-HM ^c	0/6	0/6	0/6
	OV-HM-regressed mice	OV-HM ^c	4/5	5/5	5/5
	OV-HM-regressed mice	B16BL6 ^d	0/3	0/5	0/5
BALB/c	Intact mice	Meth-A ^e	0/10	—	—
	Meth-A-regressed mice	Meth-A ^e	12/12	—	—
	Meth-A-regressed mice	CT26 ^f	0/5	—	—

^aTumor challenge was performed 3 months after treatment with the combination of AdRGD-IL12 and AdRGD-CCL27 in a ratio of 9:1 at a total of 2×10^7 PFU.

^bTumor challenge was performed 6 months after treatment with the combination of AdRGD-IL12 and AdRGD-CCL27 in a ratio of 9:1 at total of 2×10^7 PFU.

^cOV-HM cells were inoculated at 10^6 cells/mouse.

^dB16BL6 cells were inoculated at 3×10^5 cells/mouse.

^eMeth A cells were inoculated at 10^6 cells/mouse.

^fCT26 cells were inoculated at 3×10^5 cells/mouse.

cells in our therapeutic experiments, although the cytotoxic activity of NK cells also partly contributed to the enhanced antitumor effects seen with intratumoral co-injection of AdRGD-IL12 and AdRGD-CCL27.

The subset and activation state of infiltrating T cells in OV-HM and Meth-A tumors co-injected with AdRGD-IL12 and AdRGD-CCL27

We attempted to identify, by immunohistochemical analysis, the subset of infiltrating T cells in OV-HM and Meth-A tumors 6 days after injection of the combination of AdRGD-IL12 and AdRGD-CCL27 in a ratio of 9:1 totaling 2×10^7 PFU. In comparison with PBS-injected tumors, tumors injected with AdRGD-Luc exhibited nearly equal numbers of infiltrating CD3⁺ T cells in both tumor models (Figure 4a and b), indicating that intratumoral administration of AdRGD at 2×10^7 PFU did not influence T-cell accumulation. Notably, a large number of CD3⁺ T cells were detected in tumors co-injected with AdRGD-IL12 and AdRGD-CCL27 as well as in tumors injected with AdRGD-CCL27 alone (Figures 3a and b, and 4a and b). OV-HM tumors injected with AdRGD-IL12 alone showed a significantly smaller number of infiltrating CD3⁺ T cells than those injected with AdRGD-CCL27 alone or AdRGD-IL12 plus AdRGD-CCL27 (Figure 4a). In the Meth-A tumor model, although no differences in CD3⁺ T-cell accumulation were observed between tumors injected with AdRGD-CCL27 alone and AdRGD-IL12 alone, the frequency of CD3⁺ T cells in tumors treated with the combination of AdRGD-IL12 and AdRGD-CCL27 was slightly higher than that in tumors injected with each AdRGD alone (Figure 4b). In addition, subset analysis of tumor-infiltrating T cells revealed that co-injection of AdRGD-IL12 and AdRGD-CCL27 promoted infiltration into tumor tissue of both CD4⁺ and CD8⁺ subsets, composed of Th cells and CTLs respectively (Figures 3c–f and 4c–f). Injection of AdRGD-CCL27 alone induced a large number of infiltrating CD4⁺ cells and a relatively small number of infiltrating CD8⁺ cells in the OV-HM tumor model (Figure 4c and e). In contrast, the CCL27-induced

infiltrating T cells were biased in favor of the CD8⁺ subset rather than the CD4⁺ subset in the Meth-A tumor model (Figure 4d and f). Although the cause of the resulting differences in CCL27-induced effects between both tumor types was unclear, these results suggest that the balance of immune cell subsets attracted by chemokines might be affected by different tumor tissue micro-environments. Collectively, intratumoral co-injection of AdRGD-IL12 and AdRGD-CCL27 more effectively augmented infiltration of both CD4⁺ and CD8⁺ T cell subsets in OV-HM and Meth-A tumor parenchyma than injection of AdRGD-IL12 alone.

Subsequently, we examined the activation states of tumor-infiltrating T cells by immunohistochemical analysis for perforin, which is the major cytotoxic molecule in activated CTLs. As shown in Figures 3g, 5a and b, few perforin-positive cells were detected in OV-HM or Meth-A tumors injected with AdRGD-Luc as well as in PBS-administered tumors. Similarly, the perforin-positive cells were hardly detectable in tumor-infiltrating T cells attracted by injection of AdRGD-CCL27 alone (Figure 5a and b), indicating that CCL27 did not significantly attract activated effector cells possessing potent tumor cell-killing activity. On the other hand, tumors injected with AdRGD-IL12 alone exhibited significantly higher numbers of perforin-positive cells than those injected with AdRGD-CCL27 alone. Importantly, compared with the AdRGD-IL12-treated group, tumor-infiltrating cells in a sensitized/activated state were more effectively augmented by the AdRGD-IL12 and AdRGD-CCL27 combination treatment (Figures 3h, 5a and b). In addition, reverse transcription-polymerase chain reaction (RT-PCR) analysis using isolated total RNA from OV-HM tumors subjected to the various treatments, showed that PCR products derived from transcripts of IFN- γ , which are the secreted cytokines from IL-12-activated cells including Th1 cells and CTLs, were detected at high levels only in tumors injected with AdRGD-IL12 alone or AdRGD-IL12 plus AdRGD-CCL27 (Figure 5c). Furthermore, CD4⁺ or CD8⁺ T cells isolated from Meth-A tumors with the co-injection of AdRGD-IL12 and AdRGD-CCL27 contained more IFN- γ -producing cells than those

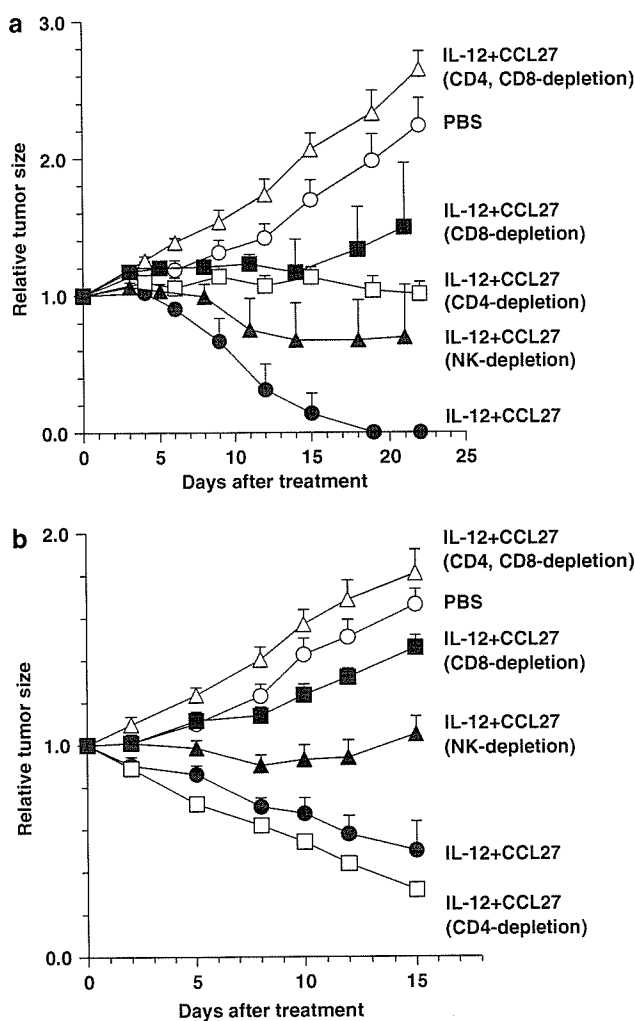


Figure 2 Determination of immune subsets responsible for the antitumor efficacy induced by the IL-12/CCL27 combination. On day -7, B6C3F1 mice (a) or BALB/c mice (b) were intradermally inoculated with 10^6 OV-HM cells or 2×10^6 Meth-A cells, respectively, into the flanks. For depletion of CD4⁺ T cells, CD8⁺ T cells or NK cells in the mice, GK1.5 ascites (anti-CD4), 53-6.72 ascites (anti-CD8) or anti-asialoGM1 antisera were intraperitoneally injected on days -3, -2, -1, 0, 5, 10 and 15. On day 0, OV-HM tumors received the IL-12/CCL27 combination intratumoral injection in a ratio of 9:1 totaling 2×10^7 PFU. The data were expressed as a relative tumor size to the initial (day 0) tumor size. Each point represents the mean \pm s.e. of results from five to seven mice.

from tumors with other treatments in flow cytometric analysis (Figure 5d). These findings support the observation that tumors co-injected with AdRGD-IL12 and AdRGD-CCL27 could recruit activated T cells in higher frequency compared with tumors treated with AdRGD-IL12 alone or AdRGD-CCL27 alone.

Taken together, these data from immunohistochemical, RT-PCR and flow cytometric analyses demonstrate that the simultaneous production of IL-12 and CCL27 in tumor tissues could work synergistically, via the chemoattractant activity of CCL27 and the immunostimulating ability of IL-12, to expand the numbers of infiltrating T cells in an effector phase. Also, the frequencies of both perforin-positive cells and IFN- γ -producing T cells in tumor parenchyma correlated closely with differences in the antitumor effects (Figures 1 and 5), strongly suggest-

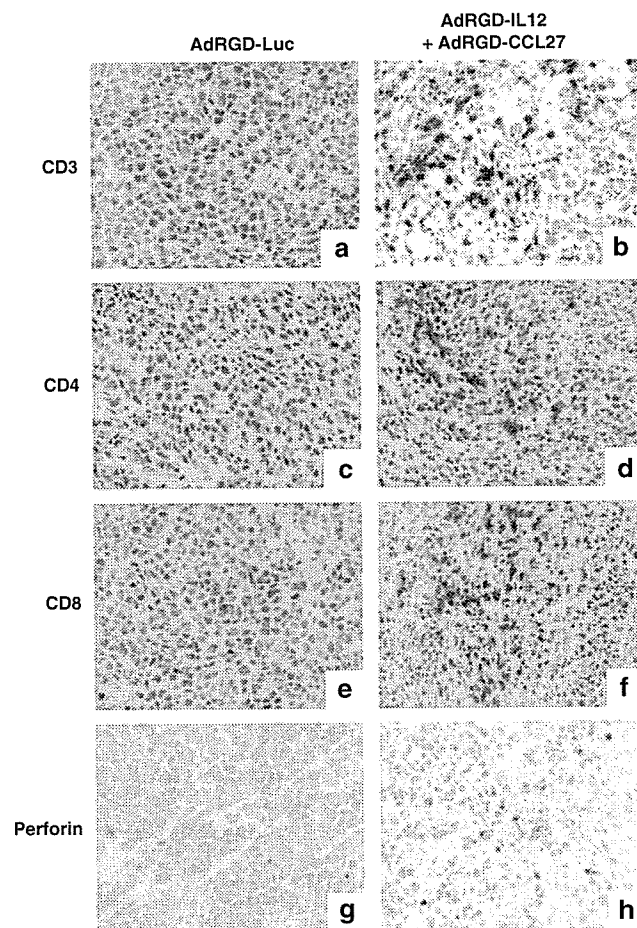


Figure 3 Images of infiltrating T cells in OV-HM tumors injected with the IL-12/CCL27 combination. OV-HM cells were intradermally inoculated into the flanks of B6C3F1 mice at 10^6 cells/mouse. The tumors (7-9 mm in diameter) were injected with AdRGD-Luc alone (a, c, e and g) or AdRGD-IL12 plus AdRGD-CCL27 in a ratio of 9:1 (b, d, f and h) at the same dose totaling 2×10^7 PFU. On day 6 after the intratumoral injections, immunohistochemical staining against CD3 (a and b), CD4 (c and d), CD8 (e and f) and perforin (g and h) was performed using frozen tumor sections. Original magnifications are $\times 400$.

ing that efficient accumulation of activated tumor-specific CTLs at a local tumor site is a key factor for establishment of efficacious cancer immunotherapy.

Reduction of adverse effects caused by IL-12 in the combinational therapy using AdRGD-IL12 and AdRGD-CCL27

In our therapeutic experiments, severe adverse effects including body weight reduction and sudden death were not observed in any treated mice. However, mice achieving complete regression of primary Meth-A tumor by treatment with AdRGD-IL12 alone showed some histopathological changes in the lung, liver and spleen. In all lungs collected from mice treated with AdRGD-IL12 alone, rare to moderate levels of lymphocyte infiltration into the perivascular lymph sinus were observed (Figure 6a and g), and hemorrhagic lesions were also often found. Also, microgranuloma, interstitial lymphocyte infiltration and extramedullary hematopoi-

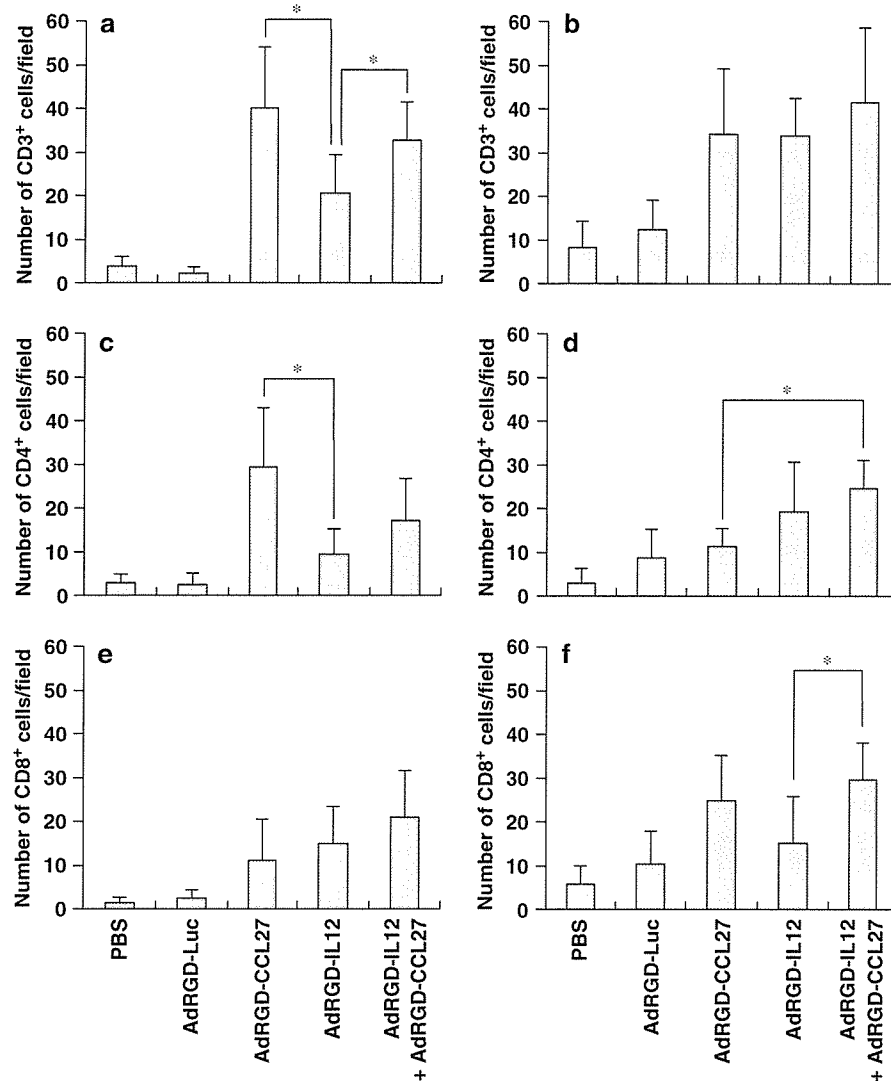


Figure 4 Quantification of tumor-infiltrating T-cell subsets in OV-HM and Meth-A tumors injected with the IL-12/CCL27 combination. OV-HM cells (a, c and e) or Meth-A cells (b, d and f) were intradermally inoculated into the flanks of syngeneic mice at 10^6 or 2×10^6 cells/mouse, respectively. The tumors (7–9 mm in diameter) were injected with AdRGD-Luc alone, AdRGD-IL12 alone, AdRGD-CCL27 alone, or the AdRGD-IL12 and AdRGD-CCL27 combination in a ratio of 9:1 at the same dose totaling 2×10^7 PFU. PBS was injected into the tumors as a control. On day 6 after the intratumoral injections, immunohistochemical staining against CD3, CD4 and CD8 was performed using frozen tumor sections. These immunohistochemical sections were used to assess the numbers of CD3⁺ (a and b), CD4⁺ (c and d) and CD8⁺ (e and f) cells infiltrating into tumor parenchyma by counting six fields per specimen under $\times 400$ -magnification. The data represent the mean \pm s.d. of results from three tumors. Statistical analysis was carried out using Welch's *t*-test: * $P < 0.05$, ** $P < 0.01$.

esis were rarely observed in the livers from 4 of 6 mice (Figure 6b and g), and four of six specimens of spleen exhibited augmentation of extramedullary hematopoiesis (Figure 6c and g). On the other hand, in the group treated with the AdRGD-IL12 and AdRGD-CCL27 combination, the extramedullary hematopoiesis in the liver was not observed, whereas lymphocyte infiltration and lung hemorrhages were less evident compared with the group treated with AdRGD-IL12 alone (Figure 6d, e and g). In addition, the enhancement of extramedullary hematopoiesis was not observed in splenic examinations of mice treated with the AdRGD-IL12 and AdRGD-CCL27 combination (Figure 6f and g). These results indicate that IL-12 disseminated from local tumor sites into the systemic circulation might cause undesired histopathological changes in mice treated with AdRGD-

IL12 alone, and that the combination of AdRGD-CCL27 at low doses was a promising approach for suppressing the adverse effects in IL-12 gene therapy for cancer.

Discussion

The development of cancer immunotherapy aims for sufficient induction of the tumor-specific immune responses to a level capable of tumor rejection and regression, and involves three criteria for the immunological elimination of established tumor mass: (i) sufficient numbers of immune cells capable of recognizing tumor-associated antigens (TAAs) must be generated *in vivo*; (ii) these cells must distribute to local tumor tissue and infiltrate into the tumor parenchyma; and (iii)

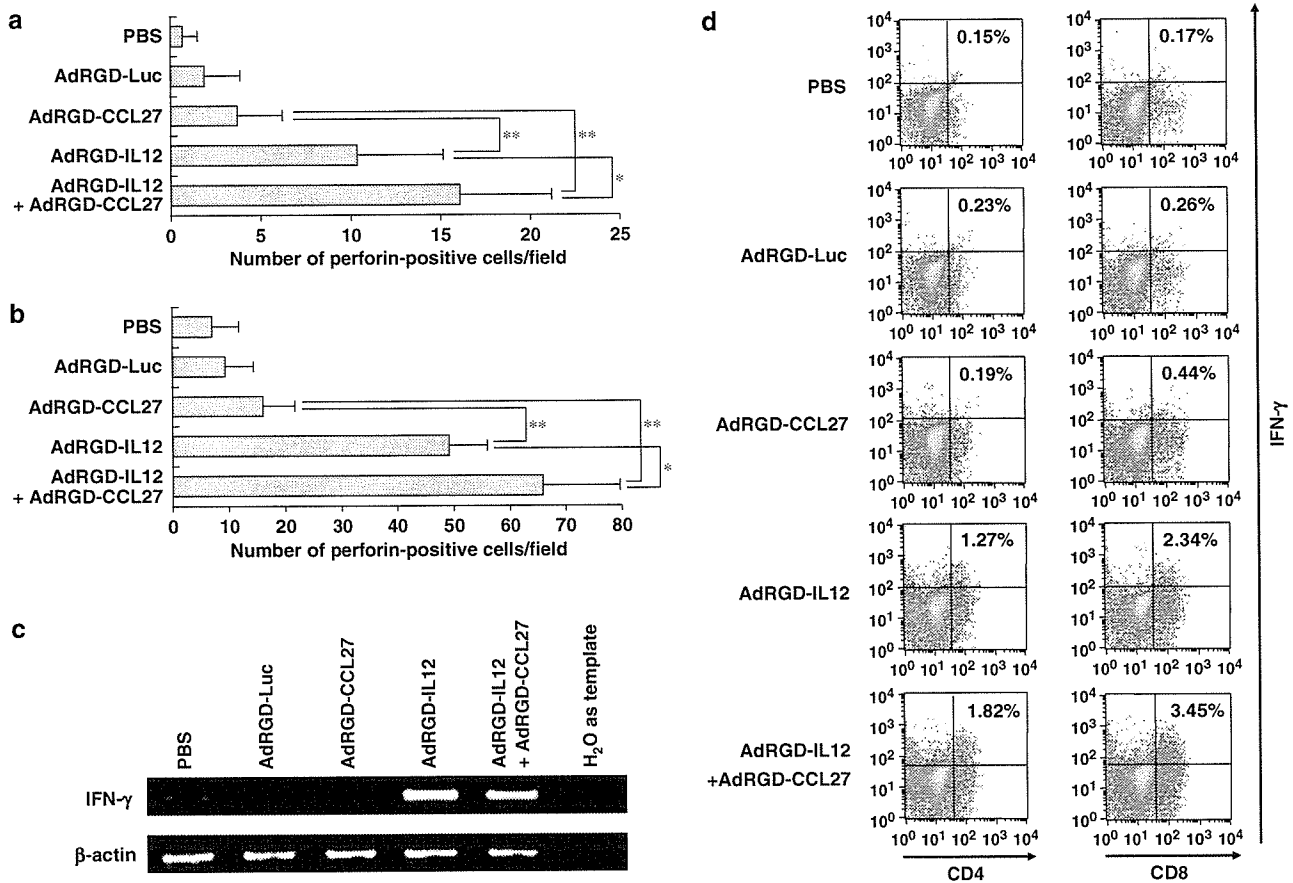


Figure 5 Increases in activated infiltrating lymphocytes in OV-HM and Meth-A tumors injected with the IL-12/CCL27 combination. (a and b) The number of perforin-positive cells infiltrating into tumor parenchyma was assessed by counting six fields per specimen of immunohistochemical sections against perforin under $\times 400$ magnification. The data represent the mean \pm s.d. of results from three tumors. Statistical analysis was carried out using Welch's *t*-test: * $P < 0.05$, ** $P < 0.01$. (c) On day 6 after the intratumoral injections, total RNA was isolated from the OV-HM tumors collected from treated mice, and then RT-PCR, specific for IFN- γ and β -actin transcripts, was performed as described in the Materials and methods section. The PCR products were electrophoresed through a 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. (d) On day 6 after the intratumoral injections, single-cell suspensions were prepared by enzymatic digestion of Meth-A tumors as described in the Materials and methods section. Isolated cells were stained with mAbs specific for CD4, CD8 or IFN- γ , and then analyzed by flow cytometer. The percentage value in each panel expresses the percentage of CD4/IFN- γ or CD8/IFN- γ double positive cells.

the tumor-infiltrating immune cells must be activated to manifest appropriate effector mechanisms, such as direct cell lysis and cytokine secretion, capable of causing tumor destruction. It is widely believed that T cells represent the most potent antitumor effector cells and various vaccine strategies including administration of TAA-component vaccines,²⁶ TAA-coding DNA vaccines,²⁷ genetically modified tumor cell-based vaccines,^{28,29} or TAA-delivered DC-based vaccines,³⁰⁻³² can both induce and amplify TAA-restricted CTLs and Th cells in patients with cancer. However, excellent therapeutic efficacy, evidenced by marked tumor regression and complete response, has not been reported in a clinical setting to date. One potential cause of these disappointing results is insufficient investigation and understanding of methods that improve accumulation of immune effector cells in tumor tissue, because most conventional studies of cancer immunotherapy have focused on efficient induction and activation of immune effector cells. Even if effector cells exhibiting the ability to kill tumor cells were adequately induced in a patient, the

efficacy of cancer immunotherapy would be considerably limited if these effector cells were unable to infiltrate tumor tissue and thereby come in contact with tumor cells. Therefore, innovative approaches capable of better controlling the trafficking and biodistribution of immune effector cells are considered critical for overcoming the limitations of current therapies.

As chemokines regulate leukocytic migration and infiltration of local sites, they are now considered important molecules for augmenting tumor-infiltrating immune cells in cancer immunotherapy. In fact, recent studies in several murine tumor models have provided experimental evidence that introduction of chemokines into the tumor environment results in the recruitment of relevant leukocyte subsets and decreases tumorigenicity of malignant cells.¹⁶⁻²³ However, most of these studies used *ex vivo* gene transfection methods that are not suitable for use within clinical settings, especially for the treatment of patients with established malignancies, and few reports have showed that direct *in vivo* transduction with chemokine genes alone could induce complete

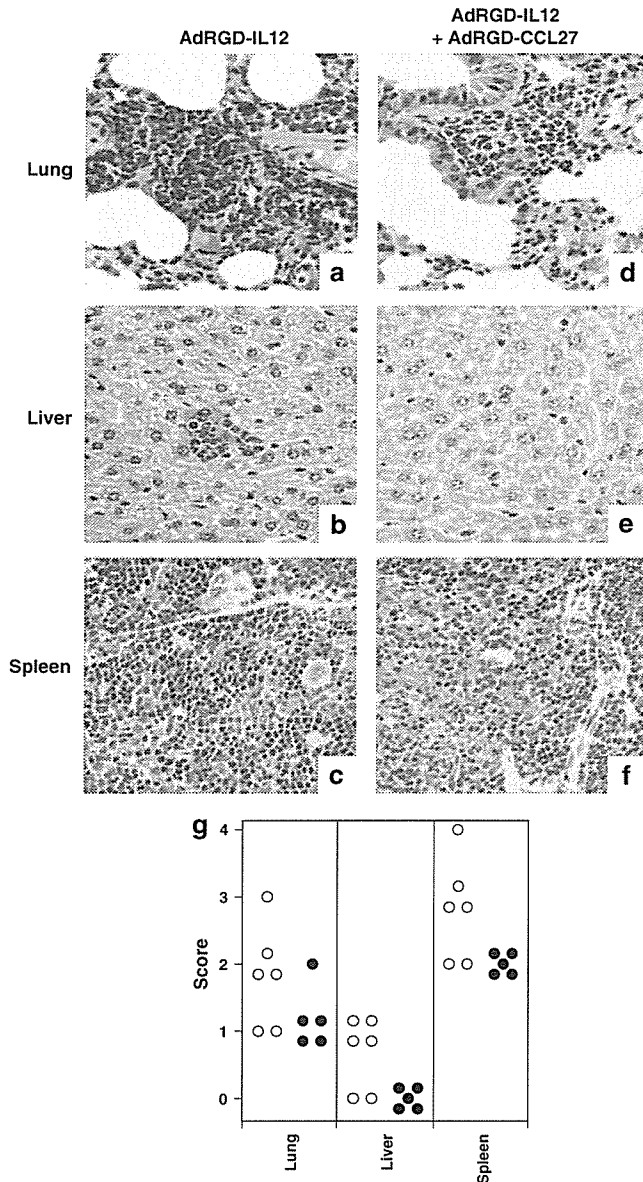


Figure 6 Histopathological examination of lung, liver and spleen in Meth-A tumor-bearing mice treated with the IL-12/CCL27 combination intratumoral injection. BALB/c mice were intradermally inoculated with 2×10^6 Meth-A cells into the flanks. The tumors (7–9 mm in diameter) were injected with AdRGD-IL12 alone or the AdRGD-IL12 and AdRGD-CCL27 combination in a ratio of 9:1 at the same dose totaling 2×10^7 PFU. Three months later, lung (a and d), liver (b and e) and spleen (c and f) were harvested from mice in which the primary tumors were judged to have completely regressed, and then HE staining was performed using paraffin-embedded tissue sections. Original magnifications are $\times 300$. (g): Histopathological changes (lymphocyte infiltration in lung and extramedullary hematopoiesis in liver and spleen) were scored as: 0, no; 1, rare; 2, mild; 3, moderate; 4, severe. (○) AdRGD-IL12 alone; (●) AdRGD-IL12 plus AdRGD-CCL27. For extramedullary hematopoiesis in liver and spleen, significant differences ($P < 0.05$) were observed between AdRGD-IL12 plus AdRGD-CCL27 group and AdRGD-IL12 alone group in Mann–Whitney *U*-test.

regression of pre-existing tumor mass. Our previous work also demonstrated that whereas intratumoral injections of AdRGD carrying one chemokine, CCL19, could attract massive numbers of T lymphocytes in

established murine B16BL6 melanomas, the antitumor efficacy of this treatment was very modest, resulting in only a slight delay of tumor growth.²³ These results strongly suggested that the augmentation of tumor-infiltrating immune cells due to chemokine gene transduction in itself is insufficient, and that subsequent activation of the recruited immune cells by another treatment is required for the induction of excellent antitumor response. On the other hand, in a number of animal models, multifunctional cytokines, such as IL-2 and IL-12, demonstrate strong antitumor activity by promoting the proliferation and activation of T and NK cells, and by overcoming some immunosuppressive factors in tumor tissues. Although the utility of recombinant protein therapy or gene therapy using these immunomodulators has been widely inspected in clinical trials, a major drawback involves serious adverse effects caused by high systemic peak concentrations associated with large or frequent doses.^{11,12} Therefore, establishing a combinational approach, which can maximize the benefits of both chemokines and immunostimulating cytokines, is necessary when designing optimal immunotherapy for malignancies. Dilloo *et al.*⁵³ reported that *in vitro* cotransfection with the chemokine XCL1 and the cytokine IL-2 into A20 tumor cells using retrovirus vectors greatly expanded tumor-infiltrating T cells attracted by XCL1 and protected against the growth of established tumors in a CD4⁺ and CD8⁺ T-cell-dependent manner. Also, Emtage *et al.*⁵⁴ demonstrated that intratumoral injection with double-recombinant adenoviral vectors expressing XCL1 along with either IL-2 or IL-12 could elicit potent and long-lasting antitumor responses. These approaches, termed ‘attraction-expansion’, are based on the assumption that if more immune cells could be recruited to the tumor site by chemokines and subsequently activated by cytokines, the antitumoral immune response could then be significantly enhanced.

In the present study, we selected as a co-stimulator, a chemokine CCL27 – which in our *ex vivo* transfection model²¹ could attenuate tumorigenicity of murine OV-HM ovarian carcinoma cells by augmenting tumor-infiltrating T and NK cells – for promoting the antitumor efficacy and improving the safety of IL-12 gene therapy for established tumors. OV-HM tumors are relatively responsive to IL-12-based therapy; in contrast, systemic administration of IL-12 fails to inhibit Meth-A tumor growth because of a lack of immune cell migration and infiltration into tumor stroma.^{14,15} In the present therapeutic experiments using AdRGDs capable of efficient gene transduction in various tumors, the IL-12 and CCL27 combinational treatment demonstrated excellent antitumor effects against both established OV-HM and Meth-A tumors compared with treatment with either vector alone, and, more importantly, induced complete tumor regression in all tested OV-HM tumor-bearing mice and in 12 of 13 Meth-A tumor-bearing mice. Moreover, the antitumor responses elicited by the combinational therapy with AdRGD-IL12 and AdRGD-CCL27 were tumor-specific and long lasting as determined by rechallenge experiments and hence demonstrated a protective immunity. These data indicate that synergic effects arising from the IL-12/CCL27 combination may promote more efficacious and safer IL-12 gene therapy for cancer at doses lower than the maximum tolerance dosage.

Therefore, we also investigated the antitumoral mechanisms associated with the synergy of IL-12 and CCL27 by *in vivo* depletion assays and immunohistochemical analysis. The immune system is roughly classified into cellular immunity and humoral immunity, and an optimal immune response is achieved by the balanced activation of both systems. Cellular immune responses including the activation of NK cells and CTLs play a more important role in elimination of tumor cells by tumor immunity than humoral immune responses accompanied by antibody production from B cells. The antitumor effects in the IL-12/CCL27 combinational therapy were T-cell-dependent, with the effector activity of CD8⁺ CTLs, rather than with the NK activity, mainly contributing to the regression of the pre-existing tumors. This finding reasonably accounts for the generation of systemic long-term specific immunity in mice receiving the IL-12/CCL27 combinational therapy because T-cell-dependent immunity possesses both effector and memory phases. In addition, tumors injected with the IL-12/CCL27 combination showed more enhanced accumulation of CD3⁺ T cells than those injected with AdRGD-IL12 alone, whereas treatment with AdRGD-CCL27 alone was the most effective. This result demonstrates that CCL27 is a strong recruiter for T cells into tumor tissue, and is consistent with our previous data in an *ex vivo* transfection model.²¹ In T-cell subset analysis, the IL-12/CCL27-treated group induced the infiltration of CD8⁺ CTLs – the major effector cells in the *in vivo* depletion assays – into tumor parenchyma more efficiently than the group treated with AdRGD-CCL27 alone even though the total vector dosage of AdRGD-CCL27 in the former was only one-tenth of that in the latter. Moreover, the infiltrating immune cells in tumors treated with AdRGD-IL12 alone or IL-12/CCL27 together were highly activated, as determined by the number of perforin-positive cells and IFN- γ -producing T cells in tumor tissue. On the other hand, most of the immune cells recruited by injection with AdRGD-CCL27 alone were in an inactivated or naive state, explaining why treatment with AdRGD-CCL27 alone did not show significant antitumor effects in spite of its ability to augment T-cell-infiltration. IL-12 can activate signal transducer and activator of transcription (STAT)4 via binding to its receptor, and STAT4 plays an important role within the microenvironment for Th1-biased immune responses, by expressing a set of genes such as IFN- γ and IL-18 receptor α subunit.³⁵ In addition, the cellular immune response including effector activity of CTLs and NK cells is dominantly activated by the helper function of Th1 cells.³⁶ On the basis of our current results, we determined that the synergic antitumor responses in the IL-12/CCL27 combinational therapy reflected both the CCL27-induced augmentation of tumor-infiltrating immune cells and the subsequent IL-12-stimulated enhancement of effector activity.

In our experimental models, mice achieving complete regression of the primary tumors because of AdRGD-IL12 treatment alone showed some histopathological changes within lung, liver and spleen, for example lymphocyte infiltration and extramedullary hematopoiesis. Although the mechanisms associated with these peripheral adverse effects remain unclear, we speculate that IL-12 spreading into the systemic blood circulation from tumor tissues potentiated peripheral hematopoiesis because the administration of recombinant IL-12 results

in significant splenic hyperplasia with increased progenitor cells, increased circulating progenitor cells and bone marrow hypoplasia with decreased progenitor cells in a time- and dose-dependent manner.^{37,38} On the other hand, the IL-12/CCL27 combinational treatment could reduce the side effects observed after complete tumor regression, suggesting that most of the IL-12 produced in tumor tissues might have been utilized for local activation of tumor-infiltrating immune cells; this improved therapeutic safety was probably related to the consumption of intratumoral IL-12 by recruited immune cells.

In conclusion, our results revealed that cotransduction with IL-12 and CCL27 genes into established tumors by using AdRGD could promote antitumor responses through both the recruitment and activation of tumor-infiltrating immune cells, and would greatly contribute to the development of an efficacious and safe cancer immunogene therapy. At present, in order to expand our combinational cancer immunogene therapy to metastatic tumors, in which intratumoral injection of vectors is physically difficult, we are pushing forward with the development of vectors, which can be transductionally and transcriptionally targeted to tumors and also with methods to specifically deliver therapeutic genes into tumor cells by systemic administration.^{39–41}

Materials and methods

Cell lines and mice

Murine ovarian carcinoma OV-HM cells (H-2^{b/k}) and murine fibrosarcoma Meth-A cells (H-2^d) were kindly provided by Dr Hiromi Fujiwara. Murine colon adenocarcinoma CT26 cells (H-2^d) were kindly provided by Professor Nicholas P Restifo (National Cancer Institute, Bethesda, MD, USA). OV-HM and CT26 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Meth-A cells were maintained by intraperitoneal passage in syngeneic BALB/c mice. HEK293 cells, the helper cell line for AdRGD-propagation, were purchased from ATCC (Manassas, VA, USA). B16BL6 cells (H-2^b), a melanoma cell line originating from C57BL/6 mice, were distributed from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). HEK293 cells and B16BL6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Female (C57BL/6 \times C3H/He) F1 (B6C3F1) mice, BALB/c mice and BALB/c nude mice were purchased from Japan SLC Inc. (Hamamatsu, Japan), and were used at 6–8 weeks of age. Animal experimental procedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

Vectors

Because most malignant tumors express little coxsackie-adenovirus receptor, which is the primary receptor for conventional adenoviral vectors, we utilized AdRGDs, which are infectivity-enhanced adenoviral vectors with an RGD-4C motif in the adenoviral fiber-knob region. Replication-deficient AdRGD was based on the adenovirus serotype 5 backbone with deletions of E1 and E3 regions. The RGD-4C sequence for α v-integrin-targeting was inserted into the HI loop of the fiber knob using a two-step method, as previously described.⁴² AdRGD-

IL12,¹³ AdRGD-CCL27,²¹ and AdRGD-Luc⁴² under the control of the cytomegalovirus promoter were previously constructed by an improved *in vitro* ligation method.^{42–44} All recombinant AdRGDs were propagated in HEK293 cells, purified by two rounds of cesium chloride gradient ultracentrifugation, dialyzed, and stored at -80°C . Titers of infective AdRGD particles (PFU) were evaluated by the end-point dilution method using HEK293 cells.

Tumor inoculation and intratumoral administration of vectors in animal experiments

B6C3F1 mice, BALB/c mice or BALB/c nude mice were intradermally inoculated with 10^6 OV-HM cells or 2×10^6 Meth-A cells into the flank. After established tumor diameters reached 7–9 mm, AdRGD-IL12 alone, AdRGD-CCL27 alone or AdRGD-IL12 plus AdRGD-CCL27 in a ratio of 1:9, 1:1 or 9:1 were intratumorally injected at a total of 2×10^7 PFU in 50 μl PBS. AdRGD-Luc was used as a control vector. Tumor growth was monitored twice or three times a week by measurement of the major axis of the tumors using microcalipers. The data were expressed as a relative tumor size to the initial tumor size. The mice were euthanized when the tumor measurements were greater than 20 mm. On day 90 after treatment, mice without palpable tumor masses were judged to be individuals that could potentially achieve complete regression.

Tumor rechallenge experiments in mice achieving complete regression of primary tumors

Three or six months after treatment with the combination of AdRGD-IL12 and AdRGD-CCL27 in a 9:1 ratio totaling 2×10^7 PFU, mice that achieved complete regression of primary tumors were rechallenged with 10^6 OV-HM cells, 3×10^5 B16BL6 cells, 10^6 Meth-A cells or 3×10^5 CT26 cells by intradermal injection into the opposite flank of the first tumor inoculation. On day 60 after tumor rechallenge, the tumor-free mice were judged to be individuals that had achieved complete tumor rejection.

In vivo depletion analysis

Ascites from BALB/c nude mice intraperitoneally injected with GK1.5 hybridoma (anti-CD4)⁴⁵ or 53–6.72 hybridoma (anti-CD8)⁴⁶ were kindly provided by Professor Hiroshi Yamamoto (Department of Immunology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan). Mice bearing OV-HM or Meth-A tumor were intratumorally injected with the combination of AdRGD-IL12 and AdRGD-CCL27 in a ratio of 9:1 totaling 2×10^7 PFU on day 0, and intraperitoneally injected seven times on days -3 , -2 , -1 , 0 , 5 , 10 and 15 with 100- μl GK1.5 or 53–6.72 ascites, or 40- μl rabbit anti-asialoGM1 antiserum (Wako Pure Chemical Industries, Ltd, Osaka, Japan). The depletion of T-cell subsets and NK cells was confirmed by flow cytometric analysis of peripheral blood and splenic cells. Tumor growth was monitored as described above. The mice were euthanized when the tumor measurements were greater than 20 mm.

Immunohistochemical examination of tumor sections

Tumor-bearing mice were killed 6 days after the intratumoral injection of AdRGD. For immunohistochemical analysis, the fresh tumor nodules were

embedded in OCT compound (Sakura Finetechnical Co., Ltd, Tokyo, Japan), and frozen in liquid nitrogen. Frozen sections (6 μm in thickness) were fixed in 4% paraformaldehyde, washed with PBS and then stored at -80°C until needed for subsequent procedures. The immunostaining procedures, which included blocking for endogenous peroxidase activity with peroxidase blocking solution (Dako Japan Co., Ltd, Kyoto, Japan), blocking for nonspecific binding of the subsequently used immunoreagents with 5% bovine serum albumin, incubation with optimal dilution of antibody, incubation of reagent(s) for detection, and development with 3,3'-diaminobenzidine, were carried out on an automated immunostaining system (Autostainer Plus, Dako Japan). Between all incubation steps, the tumor sections were washed with Tris-buffered saline containing Tween-20. The following were used as primary antibodies: rabbit anti-human CD3 polyclonal antibody (Dako Japan), rat anti-mouse CD4 monoclonal antibody (mAb) (RM4-5; BD Biosciences, San Jose, CA, USA), rat anti-mouse CD8 mAb (KT15; Serotec Co., Ltd, Sapporo, Japan) and rat anti-mouse perforin mAb (CB5.4; Abcam, Paris, France). Rabbit anti-human CD3 polyclonal antibody was detected with ENVISION+ Rabbit/HRP (Dako Japan). Other primary antibodies were detected with HRP-conjugated anti-rat Ig (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and CSA II System (Dako Japan). The sections were finally counterstained with hematoxylin. The numbers of immunostained cells in six fields per specimen were counted under a light microscope using $\times 400$ -magnification.

RT-PCR analysis for IFN- γ expression levels in tumor tissues

OV-HM tumors were collected 6 days after intratumoral injection of AdRGD, and total RNA was isolated using TRI Reagent (SIGMA-ALDRICH Japan, Tokyo, Japan) according to the manufacturer's instructions. RT proceeded for 60 min at 50°C in a 20 μl reaction mixture containing 2 μg total RNA treated with DNase I, 4 μl 5 \times RT buffer, 0.1 M dithiothreitol, 0.1 mM dNTP mix, 0.5 μg oligo (dT)₂₀, 1 μl RNase inhibitor and 1 μl SuperScript III (Invitrogen, Carlsbad, CA, USA). PCR amplification of IFN- γ and β -actin transcripts was performed in a 50 μl reaction mixture containing 2 μl of RT-material, 5 μl 10 \times PCR buffer, 0.25 μl Ampli Taq DNA polymerase (TOYOBO Co., Ltd, Osaka Japan), 1 μl dimethylsulfoxide, 0.2 mM dNTP and 0.4 μM primers. The sequences of the specific primers were as follows; murine IFN- γ : forward, 5'-gct ttg cag ctc ttc ctc at-3'; reverse, 5'-tga gct cat tga atg ctt gg-3'; murine β -actin: forward, 5'-tgt gat ggt ggg aat ggg tca g-3'; reverse, 5'-ttt gat gtc acg cac gat ttc c-3'. After denaturation for 4 min at 96°C , 3 sequential steps, denaturation for 30 s at 96°C , annealing for 30 s at 60°C and extension for 30 s at 72°C , were repeated for 30 cycles, ending with a final extension step for 4 min at 72°C . The PCR products was electrophoresed through a 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet radiation. The expected PCR product sizes were 379 bp (IFN- γ) and 514 bp (β -actin).

Flow cytometric analysis for tumor-infiltrating T cells

Meth-A tumors were dissected 6 days after intratumoral injection of AdRGD, and then chopped into small pieces

using a razor blade before incubation with a mixture of collagenase (1 mg/ml, Wako Pure Chemical Industries, Ltd) dissolved in Hanks' balanced salt solution for 60 min at 37°C. The cells were passed through a 70- μ m nylon strainer to remove any debris, recovered by centrifugation and resuspended in complete medium. Then, the cell suspension was overlaid on 40 and 80% Percoll gradient and centrifuged at 2000 r.p.m. for 20 min at room temperature. After centrifugation, cells deposited at the interface were harvested and washed two times with complete medium. Phenotype of T cells isolated from tumors treated with various AdRGD was confirmed by flow cytometric analysis. Briefly, 10⁶ cells were incubated on ice for 20 min with 100 μ l of staining buffer (PBS containing 2% FBS and 0.09% NaN₃) containing anti-Fc γ RII/III mAb (93, rat IgG_{2a, κ} ; eBioscience, San Diego, CA, USA) to block nonspecific binding of the subsequently used mAbs, and then the cells were stained with FITC-conjugated anti-mouse CD4 mAb (GK1.5, rat IgG_{2b, κ} ; eBioscience) or FITC-conjugated anti-mouse CD8 mAb (53-6.7, rat IgG_{2a, κ} ; eBioscience). After incubation for 20 min, cells were washed with staining buffer, fixed with Cytofix/Cytoperm Buffer (BD Biosciences) for 15 min and permeabilized with Perm/Wash Buffer (BD Biosciences). Intracellular IFN- γ was stained with PE-conjugated anti-mouse IFN- γ mAb (XMG1.2, rat IgG_{1, κ} ; BD Biosciences). After incubation for 20 min, the stained cells were analyzed for CD4/CD8 and IFN- γ expression by a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

Histopathological examination of tissue sections

Tumor-regressed mice were killed 3 months after the intratumoral injection with AdRGD-IL12 alone or the combination of AdRGD-IL12 and AdRGD-CCL27 in a ratio of 9:1 totaling 2 \times 10⁷ PFU. The lungs, liver and spleen were harvested from each of these mice, placed in neutral 10% formalin/PBS and embedded in paraffin. Sections (5 μ m) were prepared for hematoxylin and eosin (HE) staining, and then histopathological examination was performed at Applied Medical Research Laboratory (Osaka, Japan).

Abbreviations

AdRGD, RGD fiber-mutant adenoviral vector; CCL, CC-chemokine ligand; CTL, cytotoxic T lymphocyte; DC, dendritic cell; FBS, fetal bovine serum; HE, hematoxylin and eosin; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; NK, natural killer; PBS, phosphate-buffered saline; PFU, plaque-forming unit; RT-PCR, reverse transcription-polymerase chain reaction; STAT, signal transducer and activator of transcription; TAA, tumor-associated antigen; Th, helper T

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University, Osaka, Japan) for providing murine IL-12 cDNA-containing plasmid, GK1.5 ascites and 53-6.72 ascites, to Dr Hiromi Fujiwara for providing OV-HM and Meth-A cells, to Professor Nicholas P Restifo (National Cancer Institute, Bethesda, MD, USA) for providing CT26 cells and to Mr Alexandre Learth Soares and Mr Feng Qiu (Department of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan) for their technical assistance. The present study was supported in part by grants from the Ministry of Health, Labour and Welfare of Japan, by a Grant-in-Aid for Scientific Research on Priority Areas (17016043) and Young Scientists (A) (18689007) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by grant from the Takeda Science Foundation.

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SHORT COMMUNICATION

Characterization of capsid-modified adenovirus vectors containing heterologous peptides in the fiber knob, protein IX, or hexon

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Adenovirus (Ad) vectors are widely used in gene therapy and in vitro/in vivo gene transfer because of their high transduction efficiency. However, Ad vector application in the gene therapy field is limited by poor transduction into cells not expressing the primary receptor, coxsackievirus and adenovirus receptor. To overcome this problem, several types of capsid-modified Ad vectors have been developed. The HI loop or C-terminus of the fiber knob, the C-terminus of the protein IX (pIX) and the hypervariable region 5 of the hexon are promising candidate locations for displaying foreign peptide sequences. In the present study, we constructed Ad vectors in which each of the above region was modified by a simple in vitro ligation-based method, and examined the characterization of each Ad vector containing the FLAG tag (DYKDDDDK) or RGD (CDCRGDCFC) peptide. Enzyme-

linked immunosorbent assay examining the surface expression of foreign peptides on the virus suggested that foreign peptides are exposed on virion surfaces in all types vectors and that the hexon was the most efficiently reacted, reflecting the copy number of the modification. However, in the case of the transduction efficiency of Ad vectors containing the RGD peptides, the modification of pIX and the hexon showed no effect. The modification of the HI loop of the fiber knob was the most efficient, followed by the modification of the C-terminus region of the fiber knob. These comparative analyses, together with a simple construction method for each modified Ad vector, could provide basic information for the generation of capsid-modified Ad vectors. Gene Therapy (2007) 14, 266–274. doi:10.1038/sj.gt.3302859; published online 28 September 2006

Keywords: adenovirus; capsid; fiber; protein IX; hexon

Introduction

Adenovirus (Ad) vectors based on Ad type 5 are widely used for gene transfer studies and clinical gene therapy trials, since they can achieve high transduction efficiency and transduce into both dividing and non-dividing cells.^{1,2} However, one of the hurdles confronting Ad-mediated gene transfer is that Ad infection is dependent on the expression levels of the coxsackievirus and adenovirus receptor (CAR) in the target cells. Ad vectors cannot transfer genes of interest into cells lacking CAR expression (i.e. many advanced tumor cells, peripheral blood cells, hematopoietic stem cells, dendritic cells, etc).^{3,4}

Genetic modification of the Ad capsid, such as its fiber, protein IX (pIX), or hexon, is an attractive strategy for altering the Ad tropism. Among these options, modification of the fiber proteins has been the most widely studied. Fiber proteins consist of three distinct domains: the tail, shaft and knob. Each domain has distinct

functions in host cell infection. The trimeric subunits of the C-terminal knob domain are responsible for binding to the host's primary cellular receptor, CAR.^{5,6} Incorporation of the RGD (Arg-Gly-Asp) peptide or a stretch of lysine residues (K7 [KKKKKKK] peptide), which target αv integrins or heparan sulfate proteoglycans on the cellular surface, respectively, into the HI loop or the C-terminal region of the fiber knob allows Ad tropism to be expanded (or changed) by binding the modified fiber protein with the cellular receptor.^{7–10}

The C-terminal region of pIX is another candidate location for capsid modification.¹¹ pIX is a minor structural protein contained in the Ad virion which enhances the structural integrity of the particles by stabilizing hexon-hexon interaction.^{12,13} It also plays roles in transcriptional activity and nuclear reorganization.¹⁴ The attractive characteristics of ligand insertion into the pIX region is that the C-terminus of pIX tolerates the insertion of large ligands.^{15–17} As Ad pIX resides at a deep and hidden position below the tops of the hexon capsomer, Ad vectors containing the ligand-pIX fusion protein, which incorporates an α -helical spacer to sufficiently lift the ligands and expose them at the surface of the capsid, were developed.¹⁸ Enhanced transduction was reported by Ad vectors containing the RGD peptide in the C-terminus of pIX with an α -helical spacer.¹⁸

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Hexons are the most abundant capsid proteins and comprise each geometrical face of the capsid. As hexons are mostly targeted by neutralizing antibodies,¹⁹ hexon modification has been reported to escape from neutralizing antibodies as well as to modify the tropism.²⁰ The hypervariable region (HVR) 5 of hexon loop L1 is a candidate location for incorporating foreign peptides without affecting the normal function of Ad type 5 as a gene transfer vector (i.e. viral growth, virus formation, virion stability, CAR-mediated infectivity).^{21,22} Vigne *et al.*²¹ have reported that Ad vectors containing the RGD peptides (DCRGDCF) at HVR5 of the hexon can infect cells via cellular α v integrin, independently of the CAR. However, Wu *et al.*²² have reported that the His tag sequence at HVR5 had no effect on the transduction efficiency of Ad vectors when the vectors were applied to cells expressing anti-His tag single-chain antibody (scFv).

One attractive point of pIX or hexon modification is that 240 or 720 molecules of foreign peptides per virion are displayed at pIX or the hexon, respectively, while only 36 molecules are displayed at the fiber (note that the fiber and hexon are composed of trimeric subunits). Therefore, pIX- or hexon-modified Ad vectors containing heterologous peptides might be more effective than fiber-modified Ad vectors.

In the present study, we first developed a simple method for generating pIX- or hexon-modified Ad vectors by using *in vitro* ligation-based plasmid construction. The functionalities of Ad vectors containing the FLAG tag or RGD peptide in the HI loop or C-terminus of the fiber knob, C-terminus of pIX, or the HVR 5 region of the hexon were systemically compared. These comparative analyses could provide basic information for generation of capsid-modified Ad vectors.

First, we constructed newer vector plasmids pAdHM56 and -62, which we used for the generation of pIX- or hexon-modified Ad vectors, respectively. These contain a unique *Xba*I site in the coding regions of the C-terminal of pIX or HVR5 of the hexon, respectively (Figure 1), where only a minimal *Xba*I recognition sequence (TCTAGA) was introduced. pAdHM56 and -62 also contain unique *I-Ceu*I/*Sva*I/*PI-Sce*I sites in the E1 deletion region; hence, a transgene expression cassette can be cloned into the E1 deletion region by simple *in vitro* ligation, as previously described.^{23,24} The shuttle plasmid pHM15-75A was constructed by introducing oligonucleotide 1/2, -3/4, -5/6, -7/8 (Table 1) into a derivative plasmid of pHM15.²⁵ pHM15-75A contains *Xba*I, *Avr*II, *Nhe*I and *Spe*I sites at both ends of a multicloning site and the coding sequence of a 75 Å α -helical spacer, as described by Vellinga *et al.*,¹⁸ in the same multicloning site (Figure 1a). Detailed information about the constructions of the vector and shuttle plasmids is available from the authors upon request.

We previously developed a simple method for generating fiber-modified Ad vectors.^{9,10} In that method, unique restriction enzyme sites (*Csp*45I and/or *Cla*I site) were introduced into the HI loop or C-terminal coding region of the fiber knob of the Ad vector plasmid, and the foreign DNA coding peptide of interest could easily be cloned into their regions by simple *in vitro* ligation.

In the present study, we expanded upon this system, so that heterologous peptide sequences could be inserted at the C-terminus region of pIX and the HVR5 region of

the hexon. Figure 1 shows a representative example for generating Ad-RGD(pIX/75)-L2 and Ad-RGD(hexon)-L2, luciferase-expressing Ad vectors containing the RGD peptides in the C-terminus of the pIX via a 75 Å α -helical spacer and in the HVR5 region of the hexon, respectively. By using pHM15-75A, the peptide of interest coding sequence together with the 75 Å α -helical spacer coding sequence could easily be introduced into the C-terminal coding region of pIX of pAdHM56. As *Xba*I, *Avr*II, *Nhe*I and *Spe*I produce compatible cohesive ends, *Avr*II, *Nhe*I and *Spe*I sites as well as the *Xba*I site can be used for cloning into the *Xba*I site. Oligonucleotides corresponding to the peptide of interest can also be directly introduced into the *Xba*I site of pAdHM56. For the generation of pAdHM56-RGD75-L2, *Xba*I-digested pAdHM56-L2, in which a luciferase expression cassette was introduced into the E1 deletion region of pAdHM56 using *I-Ceu*I and *PI-Sce*I sites,^{23,24} was ligated with *Avr*II-digested pHM15-75A-RGD, which was constructed by the introduction of oligonucleotides 9/10 corresponding to a GS linker plus the RGD-4C (CDCRGDCF) peptide into the *Csp*45I/*Asc*I sites of pHM15-75A (Figure 1a). pAdHM62-RGD-L2, a vector plasmid for the hexon-modified Ad vector containing the RGD peptide, was constructed by the ligation of *Xba*I-digested pAdHM62-L2 with oligonucleotides 11/12, which contain a binding site with an *Xba*I-digested fragment and correspond to the RGD-4C peptide (Figure 1b). Oligonucleotides were designed so that the positive recombinant plasmid lacked an *Xba*I site for convenience of selection. Sequencing of all inserted oligonucleotides in each plasmid verified that the clones contained the appropriate sequences.

pAdHM56-FLAG-L2, pAdHM56-FLAG75-L2, pAdHM56-His-L2, pAdHM56-His75-L2, pAdHM56-RGD-L2 and pAdHM62-FLAG-L2 were similarly constructed as shown in Figure 1 by using *Xba*I-digested pAdHM56-L2 or pAdHM62-L2 with oligonucleotides 11–20. Ad vectors were generated by the transfection of *Pac*I-digested linearized vector plasmids described above into 293 cells and were prepared as described previously.^{23,24} The conventional Ad vector (Ad-L2) and fiber-modified Ad vectors (Ad-RGD(HI)-L2 and Ad-RGD(C)-L2), had been previously constructed.^{9,10} Determination of virus particle titers (VP) and infectious titer was accomplished spectrophotometrically by the method of Maizel *et al.*²⁶ and by using an Adeno-X Rapid Titer Kit (Clontech, Palo Alto, CA, USA), respectively. The infectious titer-to-particle ratio was 1:10 for Ad-L2, 1:17 for Ad-RGD(HI)-L2, 1:28 for Ad-RGD(C)-L2, 1:9 for Ad-RGD(pIX)-L2, 1:23 for Ad-RGD(pIX/75)-L2, 1:44 for Ad-RGD(hexon)-L2. All capsid-modified Ad vectors except Ad-RGD(hexon)-L2 were readily propagated, with similar particle titers to those of the conventional Ad vector, Ad-L2. The growth rate of Ad-RGD(hexon)-L2 was similar to that of Ad-L2, but the yield was approximately 10 times lower. Ad vectors and vector plasmids used in the present study are summarized in Table 2. All capsid-modified Ad vectors contain CAR and integrin binding motifs in the fiber and the penton base.

We then examined whether foreign peptides are displayed in the HI loop of the fiber knob, the C-terminus of the fiber knob, the C-terminus of pIX or the HVR5 region of the hexon (Figure 2). To do this, we generated capsid-modified Ad vectors containing the

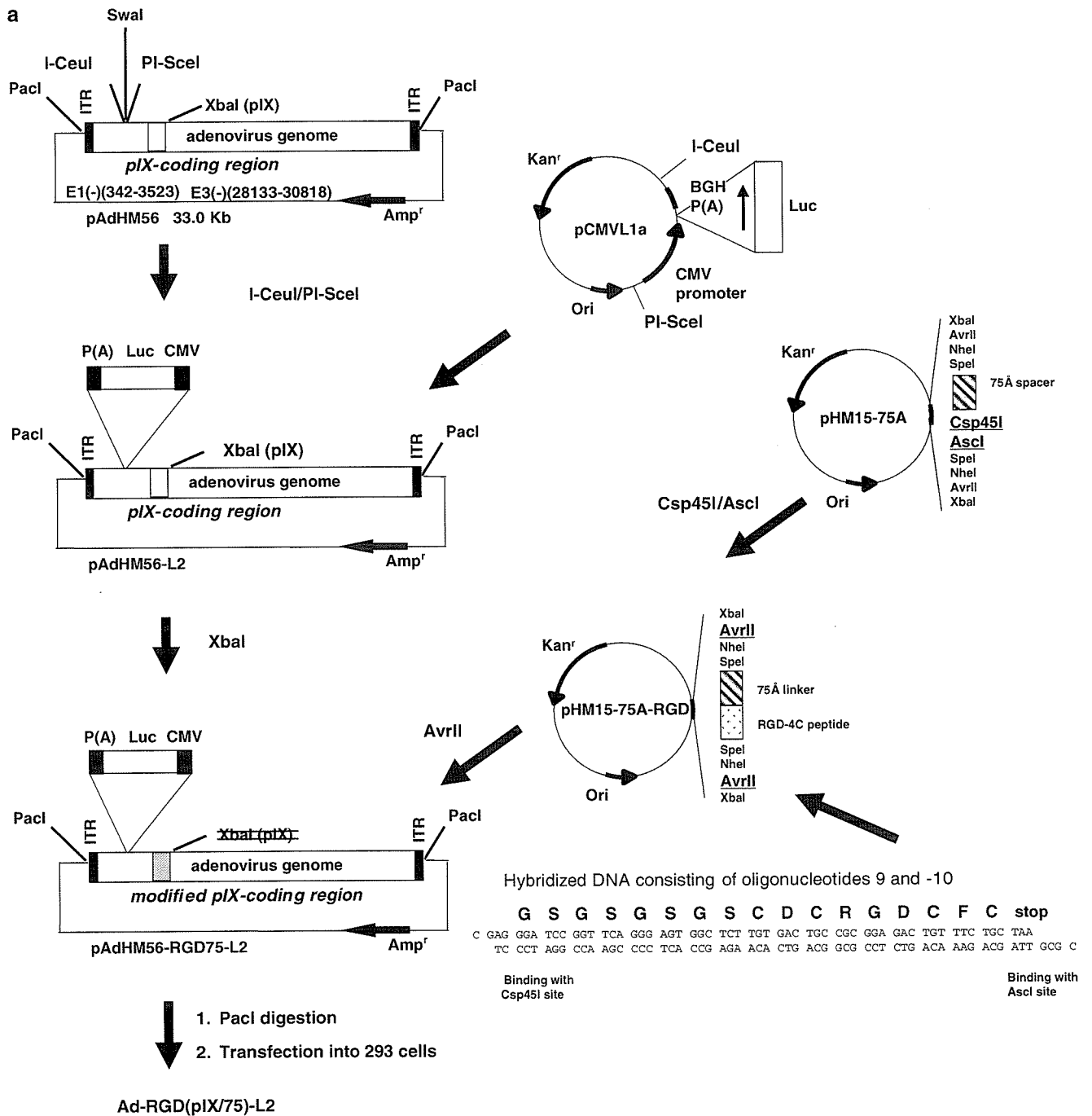


Figure 1 The construction strategy for pIX- or hexon-modified Ad vectors containing foreign peptides. (a) Construction of pIX-modified Ad vector. pAdHM56 was digested by I-CeuI/PI-SceI and ligated with I-CeuI/PI-SceI-digested pCMVL1a, which contains a CMV promoter-driven luciferase expression cassette, resulting in pAdHM56-L2. The shuttle plasmid pHM15-75A-RGD, which cloned oligonucleotides corresponding to the GS linker plus the RGD-4C peptide into pHM15-75A, was digested with AvrII and ligated with XbaI-digested pAdHM56-L2, resulting in pAdHM56-RGD75-L2. When AvrII, NheI or SpeI sites of the shuttle plasmid are used for cloning into the XbaI site of the vector plasmid, the positive recombinant plasmid lacks an XbaI site. Therefore, generation of the self-ligated plasmid is reduced by the digestion of the ligation sample with XbaI. A luciferase-expressing Ad vector containing the RGD peptide in the C-terminal of pIX with a 75 Å α -helical spacer, Ad-RGD(pIX/75)-L2, was produced by transfection of the PaclI-digested pAdHM56-RGD75-L2 into 293 cells. (b) Construction of hexon-modified Ad vector. pAdHM62-L2 was constructed by the ligation of I-CeuI/PI-SceI-digested pAdHM62 and I-CeuI/PI-SceI-digested pCMVL1a. Then, pAdHM62-L2 was digested with XbaI and ligated with an oligonucleotide corresponding to the linker (GS) and the RGD-4C peptide that contains a binding site with an XbaI-digested fragment, resulting in pAdHM62-RGD-L2. The oligonucleotide was designed so that the positive recombinant plasmid lacks an XbaI site. Generation of the self-ligated plasmid was reduced by the digestion of the ligation sample by XbaI. A luciferase-expressing Ad vector containing the RGD peptide in the HVR5 region of the hexon, Ad-RGD(hexon)-L2, was produced by transfection of the PaclI-digested pAdHM62-RGD-L2 into 293 cells.