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RESEARCH ARTICLE

Augmentation of the migratory ability of DC-based vaccine into regional lymph nodes by efficient CCR7 gene transduction

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Although dendritic cell (DC)-based immunotherapy is considered a promising approach for cancer treatment, a large quantity of DC vaccine is required for effective sensitization/activation of immune cells because of the poor migratory ability of administered DCs into regional lymphoid tissue. In this study, we created a DC vaccine sufficiently transduced with CC chemokine receptor-7 gene (CCR7/DCs) by applying RGD fiber-mutant adenovirus vector (AdRGD), and investigated its immunological characteristics and therapeutic efficacy. CCR7/DCs acquired strong chemotactic activity for CC chemokine ligand-21 (CCL21) and exhibited an immunophenotype similar to mature DCs but not immature DCs with regard to major histocompatibility complex/costimulatory molecule-expression levels and allogenic T cell proliferation-stimulating ability, while maintaining inherent endocytotic activity. Importantly, CCR7/DCs injected intradermally into mice could accumulate in draining lymph nodes about 5.5-fold more efficiently than control AdRGD-applied DCs. Reflecting these properties of CCR7/DCs, DC vaccine genetically engineered to simultaneously express endogenous antigen and CCR7 could elicit more effective antigen-specific immune response in vivo using a lower dosage than DC vaccine transduced with antigen alone. Therefore, the application of CCR7/DCs having positive migratory ability to lymphoid tissues may contribute to reduction of efforts and costs associated with DC vaccine preparation by considerably reducing the DC vaccine dosage needed to achieve effective treatment by DC-based immunotherapy.
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Introduction

Immunotherapy using dendritic cells (DCs), which play a critical role in control of both acquired and innate immune responses in the living body, is studied energetically in many research organizations aiming to immunologically eradicate cancer. In addition, several cancer immunotherapy protocols using DC vaccine introduced with tumor-associated antigen (TAA) advanced to the clinical study phase.[1–4] However, since currently available DC-based immunotherapy has not demonstrated exceptional therapeutic effects in these clinical studies, the development of a novel approach capable of improving the efficacy of this promising strategy for cancer treatment is eagerly awaited.

DCs are widely distributed over peripheral tissues, where they catch invading antigens by full endocytotic activity, characteristic of an immature state. The phenotype of DCs internalizing antigens changes to a mature state in response to these inflammatory stimuli. Subsequently, they process the antigens into the peptides presented on major histocompatibility complex (MHC) molecules, migrate into draining lymph nodes (LNs) via afferent lymphatic venules, and induce primary immune responses through antigen presentation to T cells.[5–7] On the basis of these serial immune mechanisms, the degree of administered DC vaccine accumulation in lymphoid tissues is a factor in enhancing or restricting therapeutic effects in DC-based immunotherapy.

In recent years, identification and functional analysis of chemokines/chemokine receptors, which regulate relevant leukocyte migration and invasion into tissues, have progressed remarkably, and the chemokine–chemokine receptor coupling in DC migration from peripheral tissue to lymphoid tissue has been elucidated. Gunn *et al*[8] found that DC migration to secondary lymphoid tissues was inhibited in CC chemokine ligand-21 (CCL21) expression-defective *plt/plt* mice. Likewise, Förster *et al*[9] reported that inhibition of DC migration to secondary lymphoid tissues occurred in CC chemokine receptor-7 (CCR7)-knockout mice. Based on these results, the association between CCL21, which is pro-

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duced and secreted constitutively in lymphoid tissues and lymphatic vessels, and CCR7, a seven-transmembrane domain G-protein-coupled receptor whose expression is enhanced on the surface of maturing DCs, has been shown to play a central role in control of DC migration from the peripheral tissue to lymphoid tissues. Therefore, DCs, which are not only introduced with antigens but also exhibit enhanced CCR7 expression, may positively migrate to lymphoid tissue and efficiently activate the host's immune system after administration to a living body. Efficient CCR7-gene transduction to DCs is proposed as a preparatory method for this novel 'lymphoid tissue-directivity DC' vaccine.

Efficient gene transduction to DCs is difficult in any conventional vector system including adenovirus vector (Ad), which could transfect in a wide variety of cells and tissues.[10] In this regard, we succeeded in establishing highly efficient gene transduction to DCs by applying RGD fiber-mutant Ad (AdRGD),[11–14] and clarified that vaccination with DCs transduced with TAA gene using AdRGD induced considerable antitumor effect based on activation of TAA-specific cytotoxic T lymphocytes (CTLs) in mice.[12,14] Our results not only revealed that AdRGD is very useful in antigen gene delivery to DCs, but also opened up new potentiality for genetically enhancing the immunological functions of DCs by making use of the predominance of AdRGD in gene transduction efficiency to DCs. Thus, in the present study, we first constructed an AdRGD-carrying CCR7 gene (AdRGD-CCR7), and investigated the immunological properties and vaccine efficacy of murine bone marrow-derived DCs modified with AdRGD-CCR7 in order to create a 'lymphoid tissue-directivity DC' vaccine.

Results

Gene transduction into DCs by AdRGD-CCR7

We examined the cytopathic effects of gene transduction using AdRGD-CCR7 to DCs by MTT assay. AdRGD-CCR7 did not injure DCs with a vector dose of 100 multiplicities of infection (MOI) or less, whereas viability of DCs was slightly reduced by using AdRGD-CCR7 at 200 MOI (data not shown). Thus, CCR7 gene expression was evaluated by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis in DCs transduced with AdRGD-CCR7 at 100 MOI or less. The CCR7 mRNA level of lipopolysaccharide-stimulated DCs (LPS/DCs) or Luc/DCs transduced with AdRGD-Luc (luciferase-expressing control vector) at 50 MOI did not show a remarkable change in comparison with that of mock (immature) DCs at 24 h post-treatment (Figure 1a).

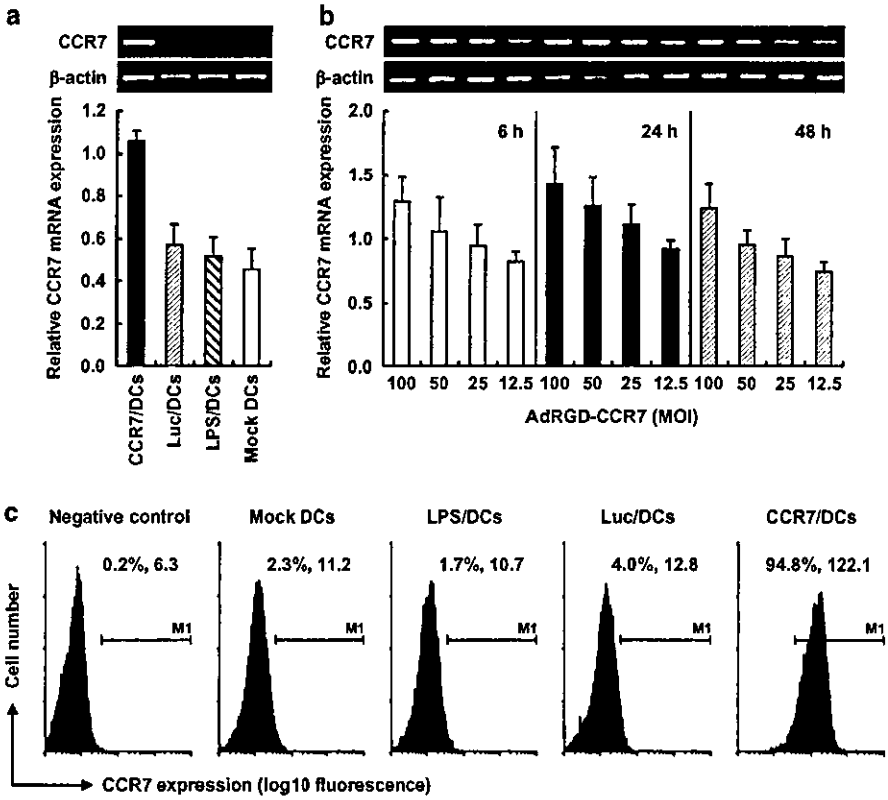

Figure 1 RT-PCR and flow cytometric analysis for mouse CCR7 expression levels. DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI (a and c) or the indicated MOI (b). These transduced cells, LPS-stimulated DCs, and mock DCs were cultured for 24 h (a and c) or the indicated period (b) in GM-CSF-free medium. (a and b) Total RNA was isolated from these DCs, and then mouse CCR7 mRNA expression was assessed by RT-PCR analysis. Relative CCR7 mRNA expression was calculated as ratio of the densitometric units of PCR products derived from CCR7 transcripts to the densitometric units of PCR products derived from β-actin transcripts. Data are presented as mean±s.d. of results from three independent experiments. (c) Flow cytometric analysis was performed by using anti-mouse CCR7 antibody. Negative control represents mock DCs stained by second antibody alone. The data are representative of two independent experiments, and the % value and the numerical value indicated in the upper part of each panel express % of M1-gated cells and mean fluorescence intensity (MFI), respectively.

On the other hand, CCR7/DCs prepared with AdRGD-CCR7 at 50 MOI were able to express CCR7 mRNA at a level more than double that of mock DCs. In addition, we analyzed the changes in CCR7 mRNA expression over time in CCR7/DCs transduced at various vector doses (Figure 1b). It was revealed that CCR7 mRNA expression in CCR7/DCs increased in a vector dose-dependent manner, and highest levels were observed at 24 h after gene transduction. Moreover, flow cytometric analysis using anti-mouse CCR7 polyclonal antibody showed that most of the CCR7/DCs, which were prepared with AdRGD-CCR7 at 50 MOI and then cultured for 24 h, expressed CCR7 protein on their surface, whereas few CCR7-positive cells were detected in mock DCs, LPS/DCs, and Luc/DCs (Figure 1c).

Next, we evaluated the chemotactic activity of CCR7/DCs for CCL21 by *in vitro* chemotaxis assay in order to confirm functional CCR7 expression on the cell surface. Under transductional conditions at an MOI of 50, the number of migrating CCR7/DCs cultured for 24 h markedly increased with increasing CCL21 concentration, whereas Luc/DCs, LPS/DCs, and mock DCs remained at low levels despite CCL21 stimulation (Figure 2a). Furthermore, as was observed for RT-PCR analysis in Figure 1b, the chemotactic activity of CCR7/DCs for CCL21 was enhanced with increasing AdRGD-CCR7 dose during transduction (Figure 2b). These results clearly demonstrated that functional CCR7, which could promote migration of DCs in response to a CCL21 concentration gradient, was expressed on the CCR7/DC surface, and that transduction of the chemokine receptor gene by AdRGD could modify chemokine responsiveness of DCs.

Immunological properties of CCR7/DCs

We analyzed the immunological characteristics of CCR7/DCs prepared with AdRGD-CCR7 at 50 MOI. At first, the expression levels of MHC/costimulatory molecules in CCR7/DCs cultured for 24 h were analyzed by flow cytometry (Figure 3a). In comparison with mock DCs, CCR7/DCs and Luc/DCs exhibited upregulated expression of all tested surface marker molecules, which play critical roles in the sensitization/activation of T cells, as is seen in mature LPS/DCs. In particular, the expression levels of CD40 and CD86 were dramatically enhanced by gene transduction using AdRGD. This result agreed with our previous report demonstrating that transduction using AdRGD, irrespective of the type of inserted transgene, could enhance the expression of MHC/costimulatory molecules on DCs.[13] Moreover, CCR7/DCs were able to stimulate proliferation of allogenic naive T cells in mixed leukocyte reaction (MLR) more effectively than mock DCs, and the stimulatory ability of CCR7/DCs, Luc/DCs, and LPS/DCs was equal at a responder/stimulator ratio of 5 (Figure 3b).

The level of fluorescein isothiocyanate (FITC)-dextran uptake in CCR7/DCs was estimated as an index of their endocytotic activity (Figure 3c). Excellent endocytosis for FITC-dextran was observed in mock DCs incubated at 37°C, whereas fluorescence intensity derived from internalized FITC-dextran was drastically decreased by 4°C incubation or LPS-driving maturation. On the other hand, FITC-dextran-uptake levels in CCR7/DCs and Luc/DCs, which were cultured for 24 h after gene transduction, were comparable to those in mock DCs.

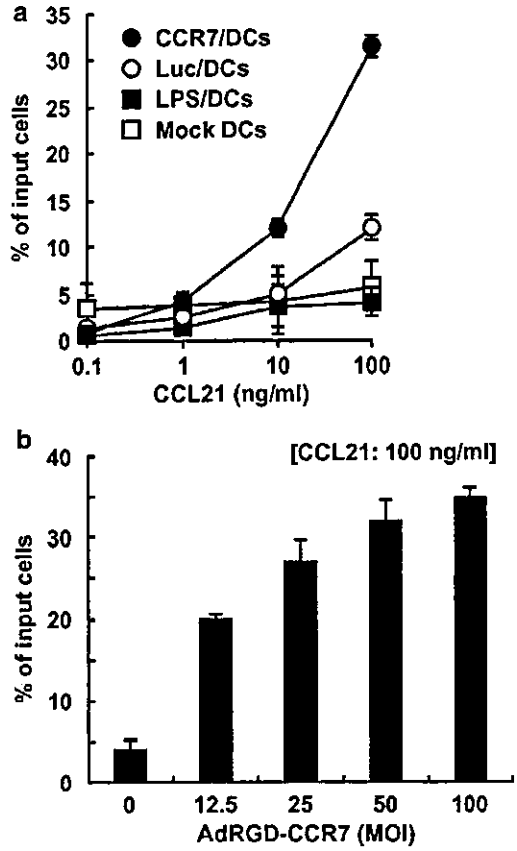

Figure 2 Chemotactic activity of CCR7/DCs in response to CCL21. DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI (a) or the indicated MOI (b). These transduced cells, LPS-stimulated DCs, and mock DCs were cultured for 24 h in GM-CSF-free medium. In vitro chemotaxis assay was performed by a Chemotaxicell-24 installed on a 24-well culture plate. CCL21 solution was added in the lower compartment at the indicated concentration, and DCs were placed in the upper chamber at 10^6 cells. After 4 h incubation, the number of cells that migrated to the lower compartment was counted on a NucleoCounter. Data are presented as mean ± s.d. of four independent cultures.

Taken together, these results demonstrate that gene transduction using AdRGD-CCR7 did not eliminate the antigen-presenting cell (APC) function of DCs; rather, CCR7/DCs acquired an immunophenotype similar to mature DCs while maintaining high endocytotic capacity. Additionally, these immunological characteristics of CCR7/DCs were similar at 48 h post-transduction (data not shown).

Accumulation of CCR7/DCs in regional LN

DCs derived from enhanced green fluorescent protein-transgenic (EGFP-Tg) mice were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI and then cultured for 24 h. These EGFP-positive CCR7/DCs, Luc/DCs, and mock DCs were intradermally injected into the flank of wild-type mice, and their accumulation in the draining inguinal LN was compared at 48 h post-administration by flow cytometric analysis (Figure 4). In all mice, the EGFP-positive DCs were not detected in the inguinal LNs contralateral to the DC-administration site (data not shown). More than double the number of EGFP-positive DCs was detected in regional LN cells prepared from mice injected with Luc/DCs, as compared

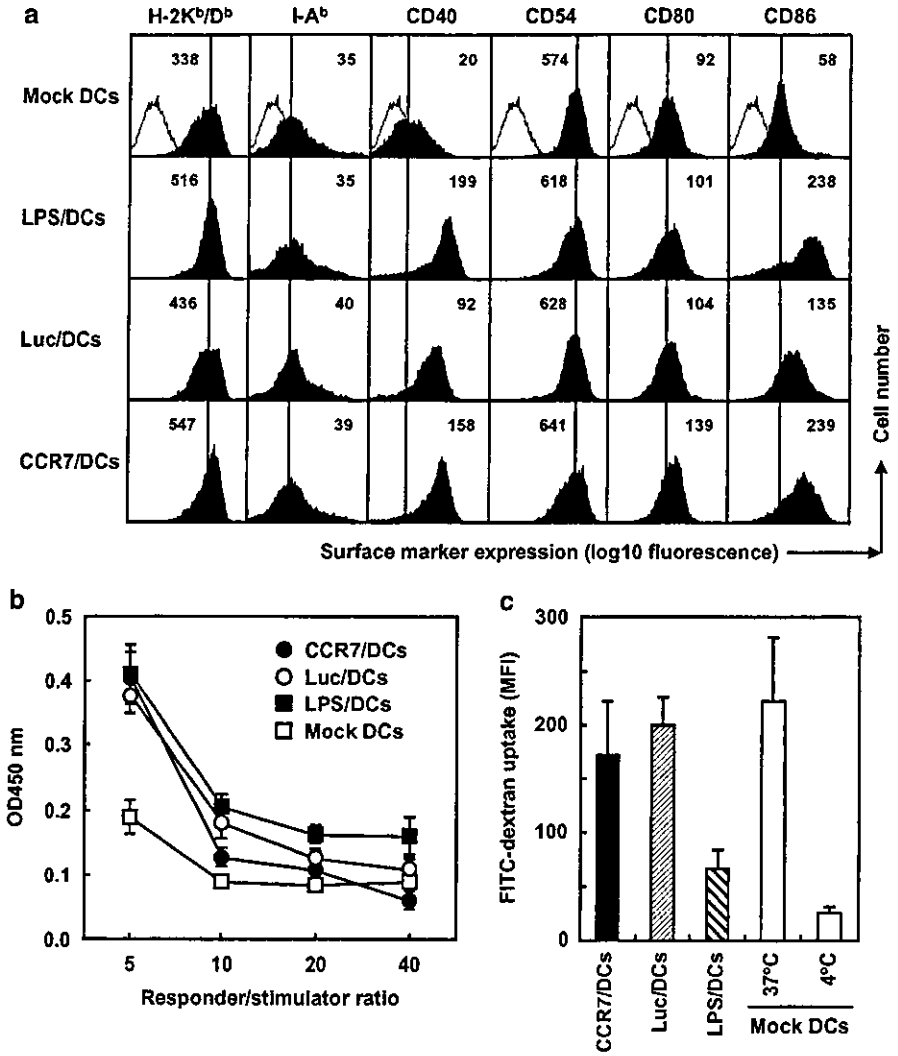

Figure 3 Immunological characteristics of CCR7/DCs. C57BL/6 DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI, and then were cultured for 24 h. (a) These transduced cells, LPS-stimulated DCs, and mock DCs were stained by indirect immunofluorescence using monoclonal antibodies of the indicated specificities (solid histogram). Dotted histograms represent cells stained by phycoerythrin-conjugated streptavidin alone. Values indicated in the upper part of each panel represent MFI of flow cytometric analysis. The data are representative of three independent experiments. (b) Naive BALB/c T lymphocytes were co-cultured with CCR7/DCs, Luc/DCs, LPS/DCs, or mock DCs at the indicated responder/stimulator ratio for 3 days. Cell cultures were pulsed with BrdU during the last 18 h, and then T-cell proliferation was assessed by BrdU-ELISA. Results are expressed as mean ± s.e. of three independent cultures using T cells prepared from three individual mice. (c) CCR7/DCs, Luc/DCs, LPS/DCs, and mock DCs were incubated with PBS containing 1 mg/ml FITC-dextran at 4 or 37°C. After 1 h, cells were washed five times with ice-cold PBS and uptake of FITC-dextran was assessed by flow cytometry. MFI of the flow cytometric analysis is presented in the bar chart. Data are presented as the mean ± s.d. of four independent cultures.

with the mock DC-treated group. This phenomenon probably reflected the maturation status of Luc/DCs as shown in Figure 3a and b. Importantly, CCR7/DCs could migrate approximately 5.5- and 15-fold more efficiently into the regional LN than Luc/DCs and mock DCs, respectively, clearly demonstrating that CCR7 gene transduction using AdRGD was useful technology to accelerate the accumulation of DC vaccine in regional LN.

Vaccine efficacy of DCs co-transduced with antigen and CCR7

In order to evaluate the potency of CCR7/DCs as vaccine carriers, we prepared the DC vaccine co-transduced with CCR7 gene and ovalbumin (OVA), model antigen, gene using AdRGD. As shown in Figure 5, DCs combined with AdRGD-OVA and AdRGD-CCR7 could present OVA peptides via MHC class I molecules at levels equal to DCs transduced with AdRGD-OVA alone. On the other hand, the OVA-presentation level in DCs co-transduced with AdRGD-OVA and AdRGD-Luc decreased by half. These data revealed that CCR7 gene transduction did not affect the MHC class I-presentation pathway for antigens endogenously and simultaneously expressed in DCs, and suggested that the proteins accumulating in the cytoplasm and the membrane-localized receptors, such as luciferase and CCR7, respectively, might induce different methods for processing co-expressed endogenous antigens in DCs.

We compared the antitumor effects of DCs co-transduced with gp100, a melanoma-associated antigen, and CCR7 (gp100+CCR7/DCs) and DCs transduced with AdRGD-gp100 alone (gp100/DCs) in the murine

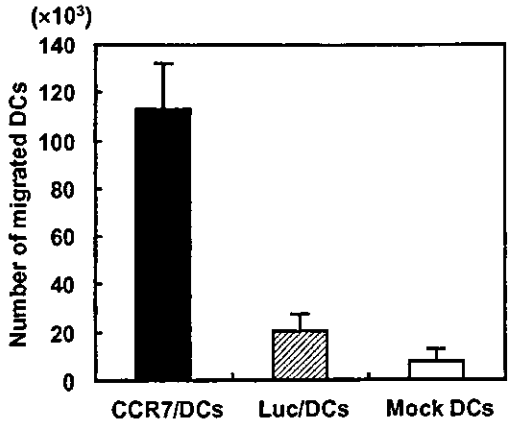

Figure 4 Migration of CCR7/DCs from administration site to draining LN. EGFP-Tg DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI, and then were cultured for 24 h. These transduced cells and mock DCs were intradermally injected into the left flank of C57BL/6 mice at 2×10^6 cells/50 μl. After 2 days, the draining inguinal LNs were collected from these mice, and a single-cell suspension was prepared and stained by indirect immunofluorescence using anti-CD11c monoclonal antibody. The abundance of EGFP⁺CD11c⁺ DCs was assessed by flow cytometric analysis acquiring 500 000 events. The number of DCs that had migrated into draining LNs was calculated by multiplying the EGFP⁺CD11c⁺ DC frequency by the total number of isolated LN cells. Data are presented as mean±s.e. of results from four mice.

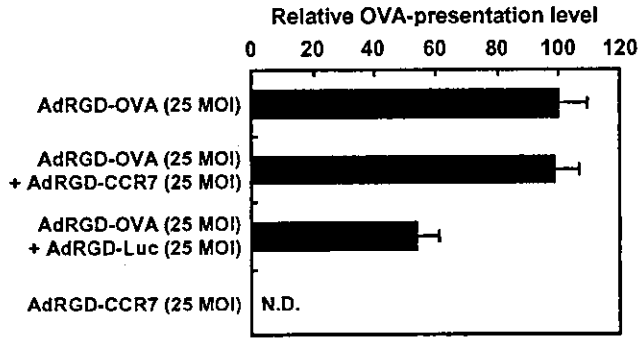

Figure 5 Antigen presentation on MHC class I molecules by CCR7/DCs. DCs were transduced with the indicated combination of AdRGD-OVA, AdRGD-CCR7, and AdRGD-Luc. These cells were co-cultured with CD8-OVA 1.3 cells for 20 h. IL-2 levels released from stimulated CD8-OVA 1.3 cells into culture supernatants were determined by ELISA, and relative OVA-presentation level via MHC class I molecules in each transduced DC was expressed as a percentage of the group using DCs transduced with AdRGD-OVA alone. Data represent the mean±s.d. of three independent cultures. ND: IL-2 secreted from CD8-OVA 1.3 cells was not detectable.

B16BL6 melanoma model. Obvious growth suppression of B16BL6 tumor challenge was achieved in mice vaccinated with gp100/DCs, as shown in our previous report,[14] whereas immunization with mock DCs or CCR7/DCs was not effective (Figure 6). This vaccine efficacy of gp100/DCs depended on the administered DC dosage. Notably, equal antitumor effects were observed in groups vaccinated with 2×10^5 gp100+CCR7/DCs and with 5×10^5 gp100/DCs, and vaccination with 5×10^5 gp100+CCR7/DCs caused extensive inhibition of B16BL6 tumor growth. Thus, we assessed B16BL6-specific CTL activity in mice vaccinated with gp100+CCR7/DCs or gp100/DCs by europium

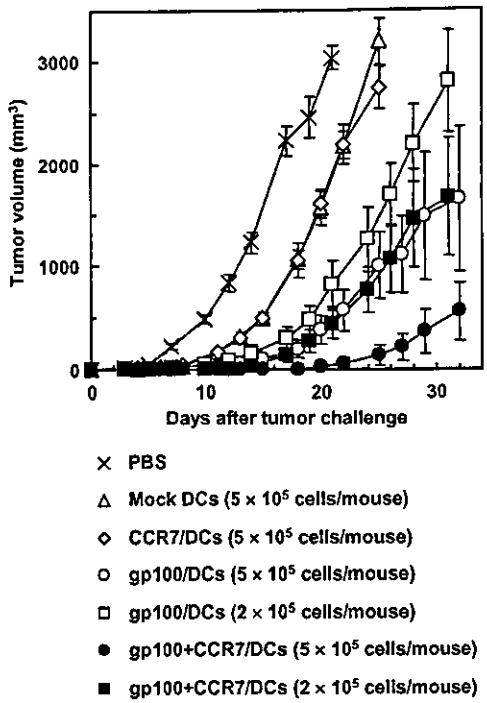

Figure 6 Vaccine efficacy of DCs co-transduced with CCR7 and gp100 gene against B16BL6 melanoma challenge. CCR7/DCs, gp100/DCs, and gp100+CCR7/DCs were prepared using corresponding vectors at 25 MOI, and then cultured for 24 h. C57BL/6 mice were immunized by intradermal injection of transduced DCs into the left flank at the indicated cell dosage, and then 4×10^5 B16BL6 melanoma cells were inoculated into the right flank of the mice at 1 week post-vaccination. The tumor sizes were assessed using microcalipers three times per week. Each point represents the mean±s.e. of 5–10 mice.

(Eu)-release assay (Figure 7a). The effector cells prepared from mice vaccinated with gp100/DCs could strongly injure B16BL6 cells, as we reported previously.[14] In addition, the effector cells in all groups did not induce lysis of YAC-1 cells, which are highly susceptible to NK activities, and H-2 haplotype-matched irrelevant EL4 thymoma cells (data not shown). B16BL6-specific cytotoxic activity increased in splenocytes from mice vaccinated with gp100+CCR7/DCs, depending on administered DC-dosage, and cytotoxicity exceeded that observed in the group treated with gp100/DCs at same dosage (5×10^5 cells/mouse). Furthermore, re-stimulated splenocytes from mice vaccinated with gp100+CCR7/DCs at 2 or 5×10^5 cells/mouse exhibited higher frequency of interferon-γ (IFN-γ)-producing cells in ELISPOT assay in comparison with those from 5×10^5 gp100/DCs-immunized mice (Figure 7b). Taken together, these data indicated that the induction of a TAA-specific immune response can be potentiated by the improved migration of TAA-loaded DC vaccine from the administration site to lymphoid tissue.

Discussion

The development of a vaccine for cancer treatment aims for sufficient induction of the tumor-specific immune response, in which antitumor CTLs play a central role, to a level capable of tumor rejection and regression. The

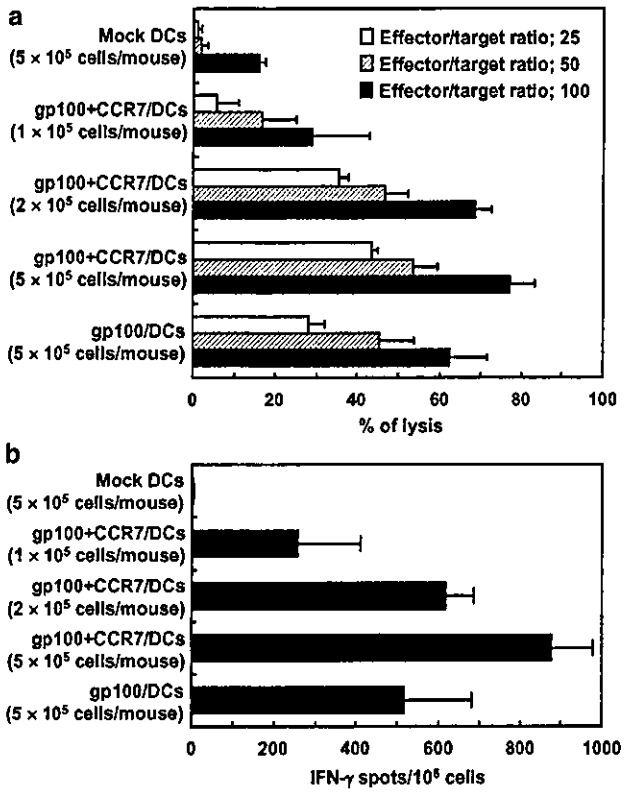

Figure 7 CTL activity and the frequency of IFN-γ-producing cells in splenocytes from mice immunized with DCs cotransduced with CCR7 and gp100 gene by AdRGD. gp100/DCs and gp100+CCR7/DCs were prepared using corresponding vectors at 25 MOI, and then cultured for 24 h. These transduced cells and mock DCs were vaccinated once intradermally into C57BL/6 mice at the indicated cell dosage. At 1 week after immunization, nonadherent splenocytes were prepared from these mice, and then were re-stimulated in vitro for 5 days with IFN-γ-stimulated and MMC-inactivated B16BL6 cells. (a) A cytolytic assay using the re-stimulated splenocytes (effector cells) was performed against IFN-γ-stimulated B16BL6 cells (target cells). The data represent the mean±s.e. of four independent cultures from four individual mice. (b) IFN-γ-producing cells in re-stimulated splenocytes were evaluated by mouse IFN-γ ELISPOT assay. The data represent the mean±s.d. of results from four mice.

efficient priming and subsequent activation of antitumor CTLs requires the processing and presentation of TAAs as peptide fragments in the context of appropriate MHC class I molecules by APCs.[15] DCs are the most potent APCs and are uniquely capable of presenting novel antigens to naive T cells to initiate and modulate immune responses.[5–7] Owing to these properties, TAA-loaded DCs are considered promising vaccine carriers in immune intervention strategies against cancer. However, very few DCs in currently available DC-based immunotherapies are capable of migrating from an administration site to regional lymphoid tissue,[16–18] where they present MHC class I- and II-restricted peptides to naive T cells, because optimal DC conditioning for enhancing migratory ability is not yet established. Therefore, increasing the migratory ability of a DC vaccine toward lymphoid tissues would remarkably improve the efficacy of DC-based immunotherapy because priming/activation of immune effector cells would be significantly promoted. In the present study, we focused on the chemokine receptor, CCR7, which manages facilitated DC migration to lymphoid tissues, and attempted to create a DC vaccine that sufficiently expressed CCR7 on the surface by gene transduction.

A vector system that can effectively deliver a foreign gene to DCs is essential for preparing a genetically modified-DC vaccine. We have demonstrated that AdRGD can enhance gene transduction efficiency against murine and human DCs as compared with conventional Ad because of the expression of the RGD sequence, the αv-integrin-targeting peptide, at the HI loop in the fiber knob.[11–14] Therefore, we constructed AdRGD-CCR7, which carried the expression cassette containing mouse CCR7 cDNA under the control of the cytomegalovirus promoter, and confirmed the vector's performance. RT-PCR analysis indicated that transfection using AdRGD-CCR7 allowed murine bone marrow-derived DCs to express abundant CCR7 mRNA, and CCR7 mRNA expression in CCR7/DCs was the highest at 24 h after gene transduction. Furthermore, CCR7/DCs cultured for 24 h exhibited sufficient CCR7 protein expression on the cell surface in flow cytometric analysis and demonstrated strong migratory ability toward CCL21, a CCR7 ligand secreted constitutively from lymphoid tissues, depending on both CCL21 concentration in a 4 h chemotaxis assay and AdRGD-CCR7 dose in transfection. That is, efficient CCR7-gene transduction into DCs by AdRGD reinforced surface expression of CCR7, which sensed a CCL21 concentration gradient and transmitted intracellular signals associated with migration in their original biologically active form. Although DCs generally enhance the expression of CCR7 and acquire migratory ability to lymphoid tissues by various maturation stimuli, an increase in CCR7 mRNA levels and enhancement of migratory activity toward CCL21 were not observed in LPS/DCs, contrary to our expectation. Granucci *et al*[19] reported that DCs stimulated with LPS significantly decreased their intrinsic migratory ability and increased the antigen uptake function at 1–2 h post-stimulation. This phenomenon probably represents the *in vivo* stage during which DCs remain at an inflammation site caused by the components of invading bacteria, such as LPS, and capture antigens by full endocytosis. In addition, Granucci *et al*[19] demonstrated that, at around 4 h after LPS activation, DCs recovered their migratory ability and started to progressively lose antigen uptake function until they reached the mature stage in which LPS/DCs showed poor antigen uptake and migratory activity. Therefore, because in the present study we tested the responses 24 h after LPS stimulation, low CCR7 mRNA levels and poor migratory response to CCL21 by LPS/DCs may have been observed because tests were run after the optimal time point, suggesting that the control of DC-migratory activity by LPS stimulation is difficult.

DCs are strong initiators of defense mechanisms that combat infectious diseases and cancer, but, in their role as APCs, DCs are also involved in immune suppression and immune tolerance.[20–23] In their induction of a positive immune response, immature DCs capture invading antigens at peripheral locations and are then activated by inflammatory stimuli, such as interleukin (IL)-1β, tumor necrosis factor-α, and bacterial components, to migrate to T-cell-rich areas in regional LN. In these processes, DCs enhance the expression of MHC/costimulatory molecules, which is essential for T-cell surface sensitization for effective induction of T-cell-

dependent primary immune responses. On the other hand, in negative immune regulation, immature DCs take up resident biological materials or apoptotic bodies and present processing peptides without acquiring activated phenotypes during migration to regional LN.[24,25] Therefore, a full analysis and understanding of immunological characteristics of DC vaccine is imperative for the development of DC-based immunotherapy because the polarity of the immune response is greatly influenced by the activated state of DCs during T-cell sensitization. CCR7/DCs created in the present study maintained inherent endocytotic activity, suggesting that CCR7/DCs administered into tumor tissue can capture necrotic or apoptotic tumor cell fragments and may induce TAA-specific immune responses. Yanagawa *et al*[26] reported that internalization of FITC-dextran in murine mature DCs increased in the presence of CCR7 ligand, CCL19 or CCL21. These results support the notion that CCL21, secreted from lymphatic vessels located near a tumor, may encourage uptake of tumor cell fragments by CCR7/DCs injected into the tumor tissue. Although high endocytotic activity is a characteristic feature of immature DCs, flow cytometric analysis indicated that CCR7/DCs enhanced expression of MHC/costimulatory molecules and allo-MLR suggested the reinforcement of T-cell proliferation-stimulating ability. These results agreed with our previous report demonstrating that transduction using AdRGD could enhance the maturation status of DCs,[13] strongly suggesting that CCR7 gene transduction using AdRGD did not inhibit the original APC characteristics of DCs and that CCR7/DCs can function as a useful vaccine possessing sufficient T-cell activation ability *in vivo*.

Although a technique to label DCs with radioisotopes or fluorescent substances is frequently used for evaluating *in vivo* kinetics of administered DC vaccines,[27–32] release via exosomes or leakage of the labeling material from DCs complicates the analysis. Eggert *et al*[33] reported that application of EGFP-Tg mouse-derived DCs avoided this problem and enabled simple evaluation and analysis of the kinetics of administered DCs without the need for labeling. Thus, we investigated the migratory ability of CCR7/DCs by flow cytometric analysis of regional LN cells prepared from mice intradermally injected with transduced EGFP-Tg mouse-derived DCs. At 48 h post-injection, regional LNs that served as administration sites for CCR7/DCs were obviously larger than contralateral LNs under macroscopic observation, and the frequency of $EGFP^+CD11c^+$ DCs in regional LN cells was 1.93 ± 0.54, 0.57 ± 0.19, and $0.12 \pm 0.04\%$ (mean $\pm$ s.e.) for CCR7/DCs-, Luc/DCs-, and mock DCs-injected mice, respectively. In addition, we could prepare more cells from regional LN in CCR7/DCs-injected mice than from the Luc/DC or mock DC groups, suggesting that not only administered DCs but also other immune cells, such as T cells, might accumulate in regional LN in mice injected with CCR7/DCs.

Previously, we demonstrated that immunization with DCs efficiently transduced with gp100 gene by AdRGD could significantly inhibit the growth and lung metastasis of murine B16BL6 melanoma *in vivo*.[14] In order to actually evaluate the predominancy of CCR7-transduced DCs as a vaccine carrier *in vivo*, we investigated anti-B16BL6 melanoma effects in mice vaccinated with DCs genetically engineered to simultaneously express gp100 and CCR7. Although antigen presentation via MHC class I molecules in the experiment using AdRGD-OVA was the same for DCs transduced with OVA gene alone and co-transduced with OVA and CCR7 gene, immunization with gp100+CCR7/DCs could more effectively suppress growth of B16BL6 tumors than vaccination with gp100/DCs. Furthermore, DC vaccine co-transduced with the TAA gene and the CCR7 gene effectively induced TAA-specific and IFN-γ-producing CTLs using a lower dosage than conventional DC vaccine transduced with TAA gene alone. These data suggest that augmentation of lymphoid tissue directivity of DCs by efficient CCR7-gene transduction can reduce the DC vaccine dosage that is needed to elicit efficacious therapeutic effects in DC-based immunotherapy. In other words, this method may considerably reduce the cost, time, and effort for DC vaccine preparation and relieve the patient from the burden of frequent blood drawing for DC isolation.

In conclusion, our data strongly suggested that *in vivo* kinetic control of DC vaccine, namely the establishment of an active DC delivery system to lymphoid tissues, was very useful for improving the efficacy of DC-based immunotherapy. We expect that superior lymphoid tissue accumulation of DCs transduced with the CCR7 gene is advantageous for a vaccine carrier because of efficient activation of immune effector cells in regional LNs and a rapid supply of activated effector cells to the whole body. Recently, optimized DC conditioning, which includes stimulation of antigen-presenting DCs with various cytokines or ligand molecules, has been investigated for the development of more effective DC-based immunotherapy. Several reports demonstrated that DC stimulation by prostaglandin E_2[34–36] or the pre-induction of inflammatory response at the DC-administration site[37] was effective in promoting migration of administered DCs to lymphoid tissues. Further research and development of 'lymphoid tissue-directivity DC' vaccine based on these data and our results may greatly improve efficacy and lead to clinical application of DC-based immunotherapy.

Materials and methods

Cell lines and mice

A murine B16BL6 melanoma cell line ($H\text{-}2^b$) was cultured in minimum essential medium supplemented with 7.5% fetal bovine serum (FBS) and antibiotics. The helper cell line, 293 cells, was grown in Dulbecco's modified Eagle medium supplemented with 10% FBS and antibiotics. CD8-OVA 1.3 cells,[38] specific T–T hybridoma against OVA^+ $H\text{-}2K^b$ (kindly provided by Dr CV Harding; Department of Pathology, Case Western Reserve University, Cleveland, OH, USA), were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol, and antibiotics. Female C57BL/6 mice ($H\text{-}2^b$) and female BALB/*c* mice ($H\text{-}2^d$), aged 7–8 weeks, were purchased from SLC Inc. (Hamamatsu, Japan). EGFP-Tg mice, C57BL/6 TgN(act-EGFP)OsbC14-Y01-FM131,[39] were kindly provided by Dr M Okabe (Genome Information Research Center, Osaka University, Suita, Japan). All mice were held under specific pathogen-free conditions and the 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 and E3 regions. The RGD sequence for αv-integrin targeting was inserted into the HI loop of the fiber knob using a two-step method as described previously.[40] The expression cassette containing mouse CCR7 cDNA derived from pBluescript SK(+)/mCCR7 under the control of the cytomegalovirus promoter was inserted into the E1-deletion site to construct AdRGD-CCR7, using an improved *in vitro* ligation method as described previously.[40–42] Luciferase-expressing AdRGD (AdRGD-Luc), OVA-expressing AdRGD (AdRGD-OVA), and gp100-expressing AdRGD (AdRGD-gp100) were constructed previously.[12–14] All recombinant AdRGDs were propagated in 293 cells, purified by two rounds of cesium chloride gradient ultracentrifugation, dialyzed, and stored at −80°C. Titers of infective AdRGD particles were evaluated by the end-point dilution method using 293 cells.

Mouse bone marrow-derived DCs

DCs were prepared according to the method of Lutz *et al*,[43] with slight modification. Briefly, bone marrow cells flushed from the femurs and tibias of C57BL/6 or EGFP-Tg mice were seeded at 5×10^6 cells per sterile 100-mm bacterial grade culture dish in 10 ml of RPMI 1640 containing 10% FBS, 40 ng/ml recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF; kindly provided by KIRIN Brewery Co., Ltd, Tokyo, Japan), 50 μM 2-mercaptoethanol, and antibiotics. On day 3, another 10 ml of culture medium was added to the dish for medium replenishment. On day 6, 10 ml of the culture supernatant was collected and centrifuged at 1500 rpm for 5 min at room temperature, and the pellet was resuspended in 10 ml of fresh culture medium, and then returned to the original dish to conserve unattached cells. Eight-day-old DCs (nonadherent cells) were harvested and used as intact immature DCs in subsequent experiments. DCs cultured for another 24 h with media containing 1 μg/ml LPS (Nacalai Tesque, Inc., Kyoto, Japan) were used as phenotypically mature DCs.

Viral transduction into DCs

DCs were suspended at a concentration of 5×10^6 cells/ml in FBS-free RPMI 1640 and placed in a 15-ml conical tube. Each AdRGD was added at various MOI, the suspension was mixed well, and the tube was incubated at 37°C for 2 h with occasional gentle agitation. The cells were washed three times with phosphate-buffered saline (PBS) and resuspended in a suitable solution.

Semiquantitative RT-PCR analysis

CCR7/DCs, Luc/DCs, and mock DCs were cultured on 100-mm bacterial grade culture dishes in GM-CSF-free culture medium. Mouse CCR7 gene expression was assessed by semiquantitative RT-PCR analysis as follows. Total RNA was isolated from these cells and LPS/DCs using Sepasol-RNA I Super (Nacalai Tesque, Inc.) according to the manufacturer's instructions, and then 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 5 × RT buffer, 5 mM $MgCl_2$, 1 mM dNTP mix, 1 μM random primer (9-mer), 1 μM oligo$(dT)_{20}$, and 100 U ReverTra Ace (TOYOBO Co., Ltd, Osaka, Japan). PCR amplification of the CCR7 and β-actin transcripts was performed in 50 μl of a reaction mixture containing 1 μl of RT material, 5 μl 10 × PCR buffer, 1.25 U *Taq* DNA polymerase (TOYOBO Co., Ltd), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.4 μM primers. The sequences of the specific primers were as follows; mouse CCR7: forward, 5′-aca gcg gcc tcc aga aga aca gcg g-3′; reverse, 5′-tga cgt cat agg caa tgt tga gct g-3′; mouse 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 2 min at 95°C, 20 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 60°C, 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 through a 3% agarose gel, stained with ethidium bromide, and visualized under UV radiation. The expected PCR product sizes were 345 bp (CCR7) and 514 bp (β-actin). Quantification of PCR products was performed by densitometry analysis, and the relative CCR7 mRNA expression level was calculated as the ratio of the densitometric units of CCR7 PCR-products to the densitometric units of β-actin PCR products.

Flow cytometric analysis for mouse CCR7

CCR7/DCs and Luc/DCs were prepared by using corresponding vectors at 50 MOI. These transduced DCs, LPS/DCs, and mock DCs were cultured on 100-mm bacterial grade culture dishes in GM-CSF-free culture medium for 24 h. The cells (1×10^6) were fixed by incubation for 10 min in 2% paraformaldehyde, and then incubated with 100 μl staining buffer (PBS containing 0.1% bovine serum albumin and 0.01% NaN_3) containing the anti-FcγRII/III monoclonal antibody (2.4G2; rat $IgG_{2b,\kappa}$; BD Biosciences, San Jose, CA, USA) to block nonspecific binding of the subsequently used antibody reagents. After 1 h, the cells were incubated overnight with anti-mouse CCR7 polyclonal antibody (goat Ig; ImmunoDetect Inc., Fayetteville, NY, USA) at a 1:10 dilution. Successively, cells were incubated for 2 h with FITC-conjugated rabbit anti-goat Ig (DakoCytomation, Kyoto, Japan) at a 1:100 dilution. Finally, 30 000 events of the stained cells were analyzed for mouse CCR7 protein expression by a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, Tokyo, Japan). Between all incubation steps, cells were washed three times with staining buffer.

In vitro chemotaxis assay

CCR7/DCs, Luc/DCs, and mock DCs were cultured for 24 or 48 h in GM-CSF-free medium. Chemotactic activity of these DCs and LPS/DCs for CCL21 was measured by an *in vitro* chemotaxis assay across a polycarbonate membrane with 5-μm pores (Chemotaxicell-24; Kurabo Industries Ltd, Osaka, Japan). Recombinant murine CCL21 (PeproTech EC Ltd, London, England) dissolved in an assay medium (RPMI 1640 containing 0.5% bovine serum albumin and 20 mM HEPES) was added to a 24-well culture plate. DCs were suspended with the assay medium and were placed in a Chemotaxicell-24 installed on each well at 10^6 cells. Cell migration was allowed for 4 h at 37°C in a 5% CO_2 atmosphere. Cells that migrated into the lower compartment were counted using a NucleoCounter™ (ChemoMetec, Allerød, Denmark), and the chemotactic activity was expressed in terms 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; 10^6 cells) × 100.

Analysis of surface marker expression

All immunoreagents used in this experiment were purchased from BD Biosciences. CCR7/DCs and Luc/DCs were prepared by using corresponding vectors at 50 MOI. At 24 h after transduction, immunophenotype of transduced DCs, LPS/DCs, and mock DCs was confirmed by flow cytometric analysis. Briefly, 10^6 cells in 100 µl staining buffer were incubated for 30 min on ice with the 2.4G2 monoclonal antibody to block nonspecific binding of the subsequently used immunoreagents. The cells were resuspended in 100 µl staining buffer and incubated for 30 min on ice using the manufacturer's recommended amounts of biotinylated monoclonal antibodies: 28-8-6 (anti-H-2K^b/D^b), AF6-120.1 (anti-I-A^b), 3/23 (anti-CD40), 3E2 (anti-CD54), 16-10A1 (anti-CD80), and GL1 (anti-CD86). The cells were then resuspended in 100 µl staining buffer containing phycoerythrin-conjugated streptavidin at a 1:200 dilution, and nonspecific binding was measured using phycoerythrin-conjugated streptavidin alone. After incubation for 30 min on ice, 30 000 events of the stained cells were analyzed for surface phenotype by flow cytometry. Between all incubation steps, cells were washed three times with staining buffer.

Allogenic MLR

C57BL/6 DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI, and then cultured for 24 h. Allogenic naive T cells were purified from BALB/*c* splenocytes as nylon wool nonadherent cells, and were used as responder cells at 1×10^5 cells/well in 96-well plates. CCR7/DCs, Luc/DCs, LPS/DCs, or mock DCs (stimulator cells) were inactivated by 50 µg/ml mitomycin C (MMC) for 30 min and added to responder cells in varying cell numbers. Cells were co-cultured in 100 µl RPMI 1640 supplemented with 10% FBS, 50 µM 2-mercaptoethanol, and antibiotics at 37°C and 5% CO_2 for 3 days. Control wells contained either stimulator cells alone or responder cells alone. Cell cultures were pulsed with 5-bromo-2'-deoxyuridine (BrdU) during the last 18 h, and then proliferation of responder cells was evaluated by Cell Proliferation ELISA, BrdU (Roche Diagnostics Co., Indianapolis, IN, USA).

Antigen uptake assay

CCR7/DCs and Luc/DCs were prepared by using corresponding vectors at 50 MOI. These cells, LPS/DCs, and mock DCs were cultured for 24 h, and then resuspended at 5×10^6 cells/ml in ice-cold PBS containing 1 mg/ml FITC-dextran (MW: 77 000; Sigma Chemical Co., St Louis, MO, USA). The cells were incubated at 37 or 4°C to determine background uptake. After 1 h, the cells were washed extensively with ice-cold PBS and analyzed by flow cytometry.

In vivo migration assay

EGFP-Tg mouse-derived DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI, and then cultured for 24 h. These transduced cells and mock DCs were intradermally injected into the left flank of wild-type C57BL/6 mice at 2×10^6 cells/50 µl. After 2 days, the draining inguinal LNs were collected from these mice, and then a single-cell suspension was prepared. The isolated LN cells were blocked by 2.4G2 monoclonal antibody and stained by indirect immunofluorescence with biotinylated anti-CD11c monoclonal antibody (HL3; BD Biosciences) and PerCP-conjugated streptavidin. The stained cells were analyzed by flow cytometry acquiring 500 000 events, and the number of migrated DCs into draining LNs was calculated by multiplying the EGFP$^+$CD11c$^+$ cell frequency by the total number of isolated LN cells.

Antigen-presentation assay

C57BL/6 DCs were transduced with various combinations of AdRGD-OVA, AdRGD-CCR7, and AdRGD-Luc each at 25 MOI, and then seeded on a 96-well flat-bottom culture plate at a density of 1×10^5 cells/well. These cells were co-cultured with 1×10^5 cells/well CD8-OVA 1.3 cells at 37°C for 20 h. The response of stimulated CD8-OVA 1.3 cells was assessed by determining the amount of IL-2 released into an aliquot of culture medium (100 µl) using a murine IL-2 ELISA KIT (Biosource International, Camarillo, CA, USA). Relative OVA-presentation level via MHC class I molecules in each transduced DC was calculated by the following formula: (relative OVA-presentation level) = (IL-2 level in tested group)/(IL-2 level in group using DCs transduced with AdRGD-OVA alone) × 100.

Tumor protection assay

DCs were transduced with AdRGD-CCR7 alone, AdRGD-gp100 alone, or a combination of AdRGD-CCR7 and AdRGD-gp100, each at 25 MOI, and then cultured for 24 h. These transduced DCs and mock DCs were intradermally injected into the left flank of C57BL/6 mice at 2 or 5×10^5 cells/50 µl. At 1 week after the vaccination, 4×10^5 B16BL6 melanoma cells were intradermally inoculated into the right flank of the mice. 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) × (minor axis; mm)^2 × 0.5236. The mice were euthanized when one of the two measurements was greater than 20 mm.

Eu-release assay and ELISPOT assay for B16BL6-specific CTLs

DCs were transduced with AdRGD-gp100 alone or a combination of AdRGD-CCR7 and AdRGD-gp100 at 25 MOI, and then cultured for 24 h. These transduced DCs and mock DCs were administered once intradermally into the left flank of C57BL/6 mice at 1, 2, or 5×10^5 cells/50 µl. At 1 week after immunization, nonadherent splenocytes were prepared from these mice and re-stimulated *in vitro* using B16BL6 cells, which were cultured in media containing 100 U/ml recombinant murine IFN-γ (PeproTech EC Ltd) for 24 h and inactivated with 50 µg/ml MMC at 37°C for 30 min, at an effector/stimulator ratio of 10 in RPMI 1640 supplemented with 10% FBS, 50 µM 2-mercaptoethanol, and antibiotics. After 5 days, the splenocytes were collected and used as CTL effector cells. Target cells, IFN-γ-stimulated B16BL6 cells, were Eu-labeled and a Eu-release assay

was performed as described previously.[44] Cytolytic activity was determined using the following formula: (% of lysis) = ((experimental Eu release−spontaneous Eu release)/(maximum Eu release−spontaneous Eu release)) × 100. Spontaneous Eu release of the target cells was <10% of maximum Eu release by the detergent. In addition, IFN-γ-producing cells in the re-stimulated splenocytes were detected by using Mouse IFN-γ ELISPOT Kit (BD Biosciences) according to the manufacturer's instruction. Splenocytes from unimmunized mice had a frequency of three IFN-γ spots/10^6 cells with or without re-stimulation.

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Brief Report

Adenovirus Vector-Mediated Doxycycline-Inducible RNA Interference

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ABSTRACT

RNA interference (RNAi) is a powerful tool for the knockdown of gene expression. Here, we report on the development of an adenovirus (Ad) vector-mediated doxycycline (Dox)-inducible small interfering RNA (siRNA) expression system. We used this siRNA system to control the expression of p53 and c-Myc in human cancer cells. Coinfection of Ad vectors containing the siRNA expression system under the control of the Dox-inducible H1 promoter and Ad vectors expressing a tetracycline repressor inhibited the expression levels of p53 and c-Myc in a dose-dependent manner with both Dox and viral dose. Regulated silencing of p53 and c-Myc expression was obtained. Because an Ad vector-mediated inducible RNAi system can efficiently transduce a variety of cell types *in vitro* and *in vivo*, and the degree of loss of gene expression can be modulated according to the dose of Dox, this expression system should be a useful tool for both basic research on the analysis of gene function and therapeutic applications of RNAi.

INTRODUCTION

RNA INTERFERENCE (RNAi) mediates the sequence-specific suppression of gene expression in a wide variety of eukaryotes by double-stranded RNA homologies to the target gene (McManus and Sharp, 2002). In mammalian cells, small interfering RNA (siRNA; 19- to 29-nucleotide RNA) leads to the inhibition of target gene expression in a sequence-specific manner (Elbashir *et al.*, 2001). Vector-based siRNA systems have also been developed with RNA polymerase III (Pol III) promoters, such as the small nuclear RNA U6 promoter or the human RNase P RNA H1 promoter, to express siRNA (Brummelkamp *et al.*, 2002; Lee *et al.*, 2002; Miyagishi and Taira, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002). Because Pol III promoters, however, are constitutive and ubiquitous, knockdown of the target gene in an inducible or cell-specific manner is more difficult than with the RNA polymerase II (Pol II) promoters. An inducible RNAi system becomes a more powerful tool for the analysis of gene function, because the loss-of-function or phenotype change can be analyzed according to the degree of loss of gene expression.

In the present study, we developed a doxycycline (Dox)-inducible siRNA expression system utilizing the H1 promoter containing a tetracycline operator (*tetO*) sequence. This vector system was constructed by modifying the Pol II promoter-based gene regulation system, using the tetracycline repressor (TetR), which was developed by Yao *et al.* (1998). In the absence of Dox, TetR binds the *tetO* sequence in the modified H1 promoter, thus preventing transcription. In contrast, TetR does not bind the *tetO* sequence in the presence of Dox, thus allowing transcription. Therefore, target gene expression is turned off in the absence of Dox, but is turned on in its presence.

As a delivery system for the inducible-siRNA expression cassette, the adenovirus (Ad) vector was employed because of its

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numerous attractive characteristics. Recombinant Ad vector has been extensively used to deliver foreign genes to a variety of cell types and tissues both *in vitro* and *in vivo*. Ad vector can be easily grown to high titer, and can efficiently transfer genes into both dividing and nondividing cells. Furthermore, several types of improved Ad vector systems, such as tropism-modified vectors, have been developed (Curiel, 1999; Wickham, 2000; Koizumi *et al.*, 2003a,b). An Ad vector-mediated inducible siRNA expression system would be an effective strategy to use for the basic analysis of gene function and have potential for therapeutic use. In the present study, we demonstrate the efficiency of Ad vector-mediated inducible RNAi against two endogenous genes: *p53* and c-*myc*.

MATERIALS AND METHODS

Cells

293 cells were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). A549 cells were cultured with F12-K nutrient mixture (Kaighn's modification) medium supplemented with 10% FCS. HepG2 cells were cultured with minimum essential medium supplemented with 10% FCS.

Plasmid and virus

H1 promoter was amplified from human genomic DNA (BD Biosciences Clontech, Palo Alto, CA), using the following primers: 5′-ccatggaattcgaacgctgacgtc-3′ and 5′-gcaagcttagatctgtgtctcatacagaacttataaattccc-3′. The amplified polymerase chain reaction (PCR) product was inserted into the *Eco*RI–*Bgl*II site of pHM5 (Mizuguchi and Kay, 1999), generating pHM5-H1. H1 promoter containing the *tetO* sequence was amplified from pHM5-H1, using the following primers: 5′-tttgccagaattcgaacgctgacgtcatcaacccg-3′ and 5′-ttggaagatctctatcactgataggga-cttataagattcccaaatccaaagacatttcacgtttatg-3′ (*tetO* sequence is underlined). The amplified PCR product was inserted into the *Eco*RI–*Bgl*II site of pHM5-H1, generating pHM5-H1tetO. pHM5-H1 and pHM5-H1tetO are designed to express short hairpin RNA (shRNA) on the insertion of an appropriate sequence into the *Bgl*II–*Xba*I site. To insert the target sequence that encodes *p53* and c-*myc* shRNA, oligonucleotides for *p53* (5′-gatccccgactccagtggtaatctacttcaagagagtagattaccactg-gagtcttttggaaat-3′ and 5′-ctagatttccaaaaagactccagtggtaatctactc-tcttgaagtagattaccactggagtcggg-3′) (Brummelkamp *et al.*, 2002) and c-*myc* (5′-gatccccgatgaggaagaaatcgatgttcaagagacatcgatt-tcttcctcatcttttggaaat-3′ and 5′-ctagatttccaaaaagatgaggaag-aaatcgatgtctcttgaacatcgatttcttcctcatcggg-3′) (loop sequences are underlined) (van de Wetering *et al.*, 2003) were synthesized, annealed, and cloned into the *Bgl*II and *Xba*I sites of pHM5-H1 and pHM5-H1tetO, generating pHM5-H1-p53, pHM5-H1-Myc, pHM5-H1tetO-p53, and pHM5-H1tetO-Myc, respectively. The sequence was verified with a DNA sequencer (ABI PRISM 310; Applied Biosystems, Foster City, CA).

Ad vectors were constructed by an improved *in vitro* ligation method (Mizuguchi and Kay, 1998, 1999). Briefly, pHM5-H1-p53, pHM5-H1-Myc, pHM5-H1tetO-p53, and pHM5-H1tetO-Myc were digested with I-*Ceu*I and PI-*Sce*I, and then ligated with I-*Ceu*I- and PI-*Sce*I-digested pAdHM15-RGD (Mizuguchi *et al.*, 2001). The resulting plasmids were digested with *Pac*I and transfected into 293 cells plated in a 60-mm dish with SuperFect (Qiagen, Valencia, CA), according to the manufacturer's instructions. Viruses (Ad-H1-p53, Ad-H1-Myc, Ad-H1tetO-p53, and Ad-H1tetO-Myc) were prepared as described previously (Mizuguchi and Kay, 1998). Ad vectors containing only the H1 promoter sequence (without a target sequence) (Ad-H1 and Ad-H1tetO) were similarly prepared. Ad-TR, the Ad vector expressing TetR, had been previously prepared (Xu *et al.*, 2003a). Ad-null contains no transgene in the E1 deletion region. Virus was purified by $CsCl_2$ gradient centrifugation, dialyzed with a solution containing 10 m*M* Tris (pH 7.5), 1 m*M* $MgCl_2$, and 10% glycerol, and stored in aliquots at −70°C. Determination of virus particle titer and infectious titer (plaque-forming units; PFU) was accomplished spectrophotometrically by the method of Maizel *et al.* (1968) and by the method of Kanegae *et al.* (1994), respectively. The PFU-to-particle ratio was 1:56 for Ad-H1-p53, 1:58 for Ad-H1-Myc, 1:56 for Ad-H1tetO-p53, 1:65 for Ad-H1tetO-Myc, 1:36 for Ad-H1, 1:50 for Ad-H1tetO, 1:24 for Ad-TR, and 1:57 for Ad-null.

Adenovirus-mediated gene transduction

A549 and HepG2 cells (2×10^5 cells) were seeded into a 12-well dish. The next day, they were transduced with the Ad vectors for 1.5 hr. The cells were cultured with medium containing various concentrations of Dox (BD Biosciences Clontech), a derivative of tetracycline. Tet system-approved FCS (BD Biosciences Clontech), a tetracycline-free serum that has been determined to be optimal for the tetracycline-controllable expression system, was used as the FCS.

Western blotting for p53 and c-Myc proteins

Cell extracts were prepared in lysis buffer (25 m*M* Tris [pH 7.5], 1% Triton X-100, 0.5% sodium deoxycholate, 5 m*M* EDTA, 150 m*M* NaCl) containing a cocktail of protease inhibitors (Sigma, St. Louis, MO). The protein content was measured with an assay kit from Bio-Rad (Hercules, CA), using bovine serum albumin as the standard. Protein samples (10 μg) were electrophoresed on 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gels under reducing conditions, followed by electrotransfer to Immobilon-P membranes (Millipore, Bedford, MA). After blocking in Block Ace (Dainippon Pharmaceuticals, Osaka, Japan), the filters were incubated with antibodies against p53 (Santa Cruz Biotechnology, Santa Cruz, CA), c-Myc (Santa Cruz Biotechnology), and actin (Oncogene Research Products, San Diego, CA), followed by incubation in the presence of peroxidase-labeled goat anti-mouse IgG antibody (American Qualex Antibodies, San Clemente, CA) or peroxidase-labeled goat anti-mouse IgM antibody (Oncogene Research Products). The filters were developed using chemiluminescence (ECL Western blotting detection system; Amersham Biosciences, Piscataway, NJ). Signals were read with an LAS-3000 (Fujifilm, Tokyo, Japan), and quantified by Image Gauge software (Fujifilm).

Northern blot for p53 *and* c-myc *siRNAs*

Total RNA was isolated with ISOGEN reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruc-

tions. To determine the levels of *p53* and c-*myc* siRNAs, 20 μg of total RNA, which was denatured with formamide, was separated on 15% polyacrylamide gels containing 7 *M* urea and electrotransferred to Hybond-N+ membrane (Amersham Biosciences). Loading was checked by ethidium bromide staining. Hybridization was performed with Rapid-Hyb buffer (Amersham Biosciences). Probes which were antisense oligonucleotide (19 bp) of target sequence, were labeled with a MEGALABEL DNA 5′-end labeling kit (TaKaRa Bio, Shiga, Japan). Signals were read with a BAS-2500 (Fujifilm).

RESULTS AND DISCUSSION

Using a combination of Ad vectors and an siRNA expression system is clearly an advantage in gene transfer experiments and therapeutic applications. An inducible siRNA expression system is more desirable, because the degree of gene silencing can be controlled by adjusting the dose or concentration of the inducer. In this study, we developed Ad vectors containing a Dox-inducible siRNA expression system. For inducible siRNA expression, the *tetO* sequence was placed between the TATA box and transcription start site of the H1 promoter (the sequence is described in Materials and Methods). Various Ad vectors, in which target sequences were inserted to express shRNA under the control of the H1 promoter and a mutant H1 promoter containing the *tetO* sequence, were constructed and are shown in Fig. 1. For proof of concept, the expression of endogenous genes *p53* and c-*myc* was silenced.

First, to examine the feasibility of the Ad vector-mediated siRNA expression system, p53 and c-Myc expression was constitutively knocked down by infection with Ad vectors containing the normal H1 promoter or a mutant H1 promoter containing the *tetO* sequence. A549 and HepG2 cells were infected with various concentrations of Ad vector (Ad-H1-p53, Ad-H1-Myc, Ad-H1tetO-p53, Ad-H1tetO-Myc, Ad-H1, Ad-H1tetO, or Ad-null), and cultured without Dox for 3 days. Levels of p53 and c-Myc protein expression were examined by Western blotting (Fig. 2). Expression of actin was also measured as an internal control. Expression of p53 and c-Myc in

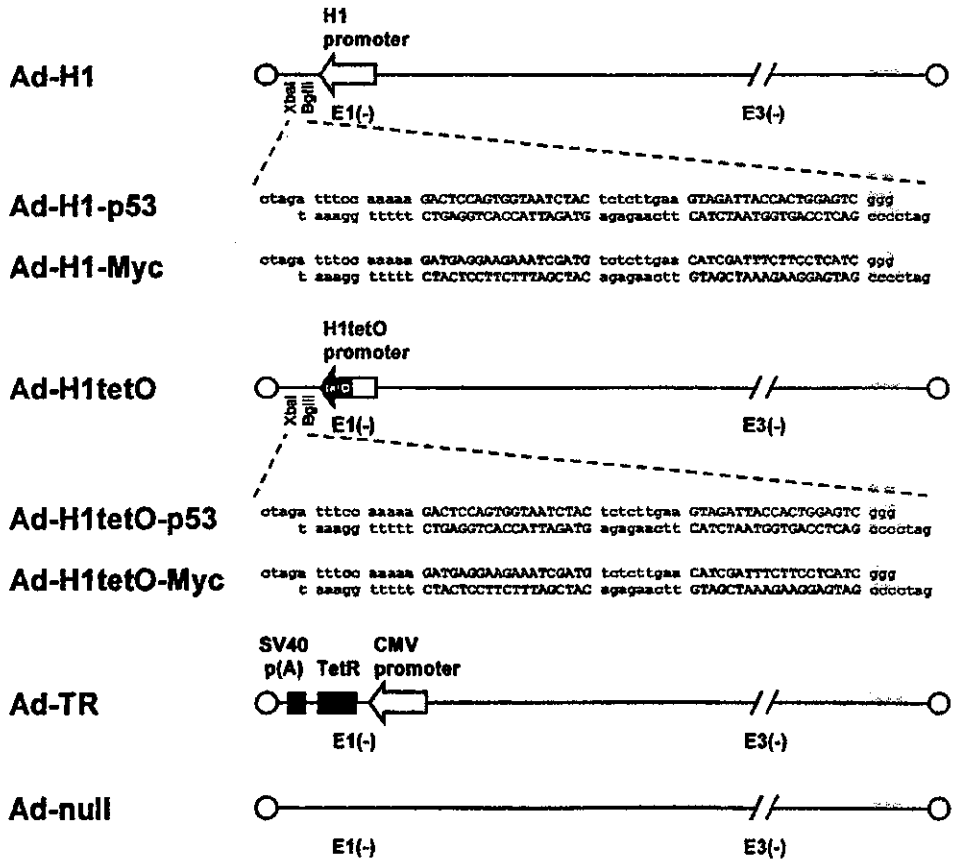

FIG. 1. Structure of Ad vectors used in the present study. The H1 promoter-based siRNA expression cassette was inserted into the E1 deletion region of the Ad genome. For inducible siRNA expression, a tetracycline operator (*tetO*) sequence was introduced downstream of the TATA box in the H1 promoter, as described in Materials and Methods. Target sequences against *p53* and c-*myc* genes are shown in upper case letters. Ad-TR is Ad vector containing a tetracycline repressor sequence under the control of the CMV promoter-enhancer. Ad-null is Ad vector without foreign genes in the E1 deletion region.

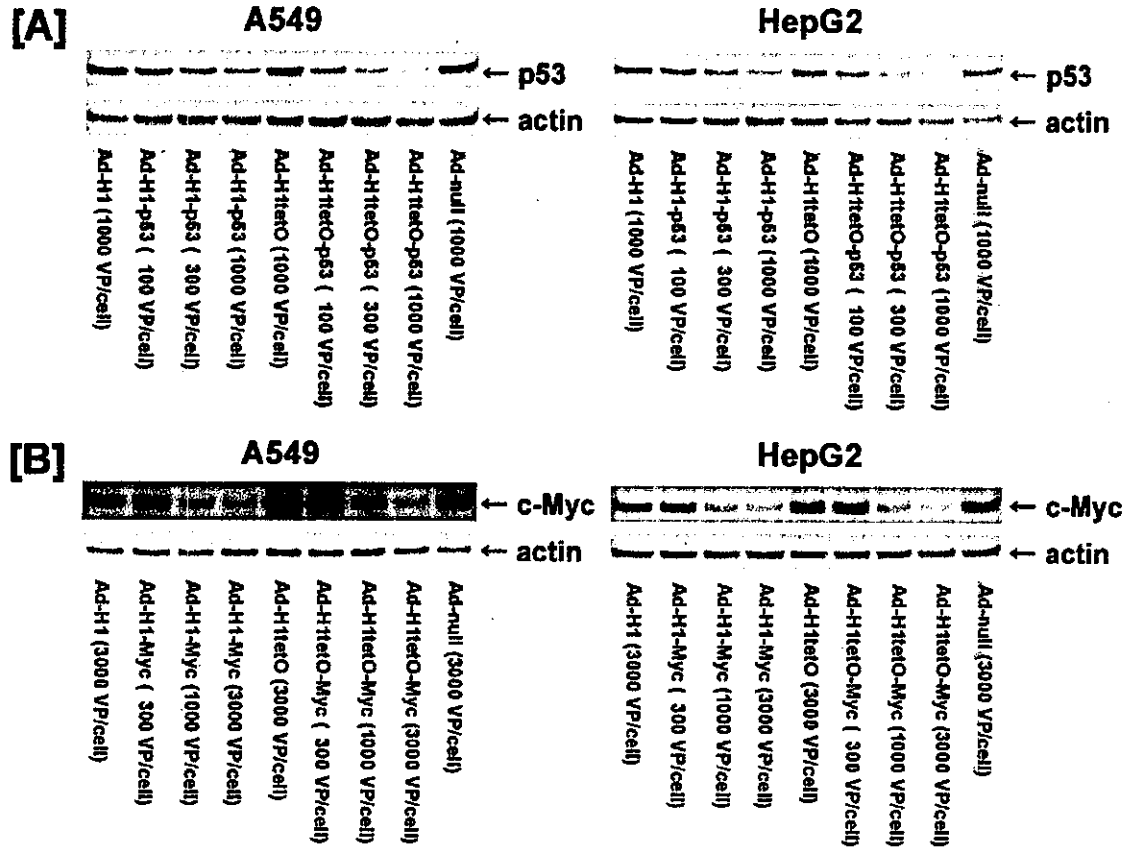

FIG. 2. Dose-dependent suppression of p53 and c-Myc protein expression by Ad vector-delivered siRNA. A549 and HepG2 cells were infected with each Ad vector for 1.5 hr, and then cultured for 3 days. Proteins were then extracted from the cells, and levels of p53 (**A**) and c-Myc (**B**) expression were examined by Western blotting. Actin bands served as an internal control for equal total protein loading.

A549 and HepG2 cells decreased in a dose-dependent manner with Ad vector carrying the siRNA expression cassette for p53 or c-Myc (Ad-H1-p53, Ad-H1-Myc, Ad-H1tetO-p53, or Ad-H1tetO-Myc). In the case of p53, a viral concentration of 1000 virus particles (VP)/cell seemed to be enough to knock down expression. Levels of p53 expression in cells treated with Ad-H1-p53 (1000 VP/cell) or Ad-H1tetO-p53 (1000 VP/cell) were decreased to 35–37 or 14–23%, respectively (Fig. 2A), relative to cells treated with Ad-null (1000 VP/cell), according to Image Gauge software (Fujifilm). In the case of c-Myc, a viral concentration of 3000 VP/cell was required to completely knock down expression, although a viral concentration of 1000 VP/cell enabled a moderate knockdown of expression. c-Myc protein expression in cells treated with Ad-H1-Myc (3000 VP/cell) or Ad-H1tetO-Myc (3000 VP/cell) was decreased to 14–44 or 16–35%, respectively (Fig. 2B), relative to cells treated with Ad-null and Ad-H1 (3000 VP/cell). The difference in degree of gene silencing may reflect the effectiveness of the siRNA sequence against each target gene. Compared with Ad-null, Ad-H1 and Ad-H1tetO did not show any effect on gene expression. These results indicate that the *tetO* sequence, placed between the TATA box and the transcription start site of the H1 promoter, does not interfere with promoter activity, and that Ad vectors containing the mutant H1 promoter, as well as the normal H1 promoter-mediated siRNA expression cassette, efficiently silence target gene expression.

Next, we examined whether regulated gene silencing is obtained by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR, the Ad vector expressing TetR, into A549 cells cultured with or without Dox (10 μg/ml). As shown in Fig. 3, in the presence of Dox the silencing effect on p53 expression decreased in proportion to the dose of Ad-TR. Efficient release of gene silencing was obtained with a 1:6 molar ratio of Ad-H1tetO-p53 to Ad-TR, although more Ad-TR might be required to completely release gene silencing. These results suggest that increased amounts of TetR are required to block transcription from the mutant H1 promoter, which contains the *tetO* sequence, in the presence of Dox (Fig. 3A). In the absence of Dox, p53 expression in cells was silenced by coinfection with Ad-H1tetO-p53 and Ad-TR. Therefore, for the regulated silencing of p53 expression, increased amounts of Ad-TR, compared with Ad-H1tetO-p53, were required. A similar result was observed in experiments on c-Myc expression (Fig. 3B), and also in HepG2 cells (both *p53* and *c-myc* genes; data not shown).

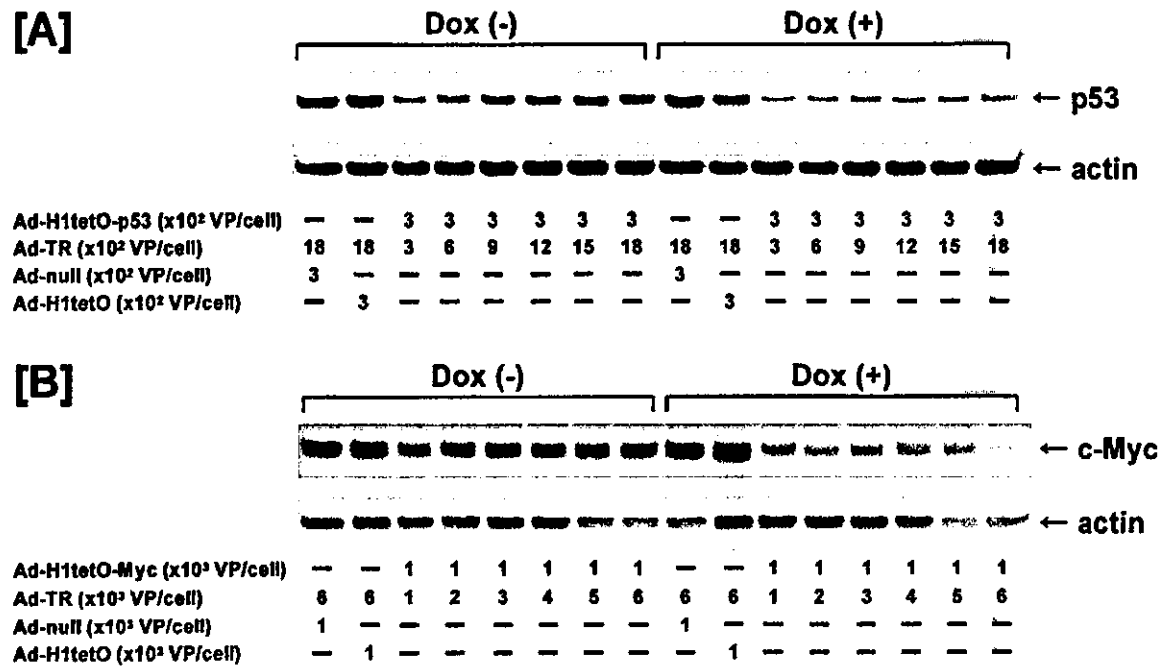

FIG. 3. Regulated suppression of p53 and c-Myc expression by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR. A549 cells were infected with the indicated amounts of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR for 1.5 hr, and then cultured with or without Dox (10 μg/ml) for 3 days. The cells were also infected with Ad-null or Ad-H1tetO plus Ad-TR. Proteins were then extracted from the cells, and levels of p53 (A) and c-Myc (B) expression were examined by Western blotting. Actin bands served as an internal control for equal total protein loading.

Ad-TR expresses TetR from the conventional cytomegalovirus (CMV) promoter-enhancer (Xu *et al.*, 2003a). Addition of the intron A sequence to the CMV promoter-enhancer, or the use of a stronger promoter such as the hybrid promoter of the β-actin promoter and CMV enhancer (Niwa *et al.*, 1991; Xu *et al.*, 2001, 2003b), would result in higher expression of TetR, thus decreasing the concentration of Ad vector expressing TetR needed to obtain inducible gene silencing efficiently. These modifications for TetR expression would make the system more effective, and would therefore enable more widespread use of this system.

We then examined the Dox concentration responsiveness of Ad vector-mediated RNAi (Fig. 4). A549 cells were coinfected with Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR at a molar ratio of 1:6, and were cultured with medium containing various concentrations of Dox. A Dox concentration of 10^{-1} μg/ml was enough to completely suppress the expression of p53 and c-Myc. At a Dox concentration of 10^{-2} μg/ml, intermediate levels of knockdown of p53 and c-Myc expression were obtained. Ad-H1tetO-p53 plus Ad-TR and Ad-H1tetO-Myc plus Ad-TR in the presence of Dox did not interfere with c-Myc and p53 expression, respectively, suggesting that the suppressive effect was target gene specific. These results suggest that the degree of knockdown of target gene expression can be modulated by Dox concentration.

We next performed a Northern blot analysis of shRNA (siRNA) expression in the presence and absence of Dox (Fig. 5). Levels of shRNA and siRNA expression for *p53* in both A549 and HepG2 cells transduced with Ad-H1tetO-p53 plus Ad-TR in the absence of Dox were significantly reduced compared with those in transduced cells in the presence of Dox (Fig. 5A). The signal of shRNA and siRNA in the absence of Dox was faint. These observations were marked in the case of *c-myc* (Fig. 5B). These results suggested that shRNA expression was tightly regulated in the Ad vector-mediated Dox-inducible RNAi system.

While this work was in progress, a plasmid vector-mediated inducible siRNA expression system using TetR was reported by van de Wetering *et al.* (2003). The mutant H1 promoter in their system contains the *tetO* sequence at a different position (by 1 bp) compared with the position of the *tetO* sequence in the present study. Both positions for insertion of the *tetO* sequence in the H1 promoter seem to be functional for regulated transcription. A similar system using the mutant U6 promoter containing the *tetO* sequence and TetR for inducible RNAi has also been reported (Matsukura *et al.*, 2003). Furthermore, a tetracycline repressor-based system was reported by two groups. In the study by Wiznerowicz and Trono, the *tetO* sequence was placed upstream of the U6 promoter (Chen *et al.*, 2003; Wiznerowicz and Trono, 2003), whereas in the study by Chen *et al.*, the *tetO* sequence was placed in these three regions: (1) upstream of the U6 promoter, (2) between the distal promoter element and the core promoter (PSE) of the U6 promoter, and (3) between the PSE and TATA box of the U6 promoter (Chen *et al.*, 2003; Wiznerowicz and Trono, 2003).

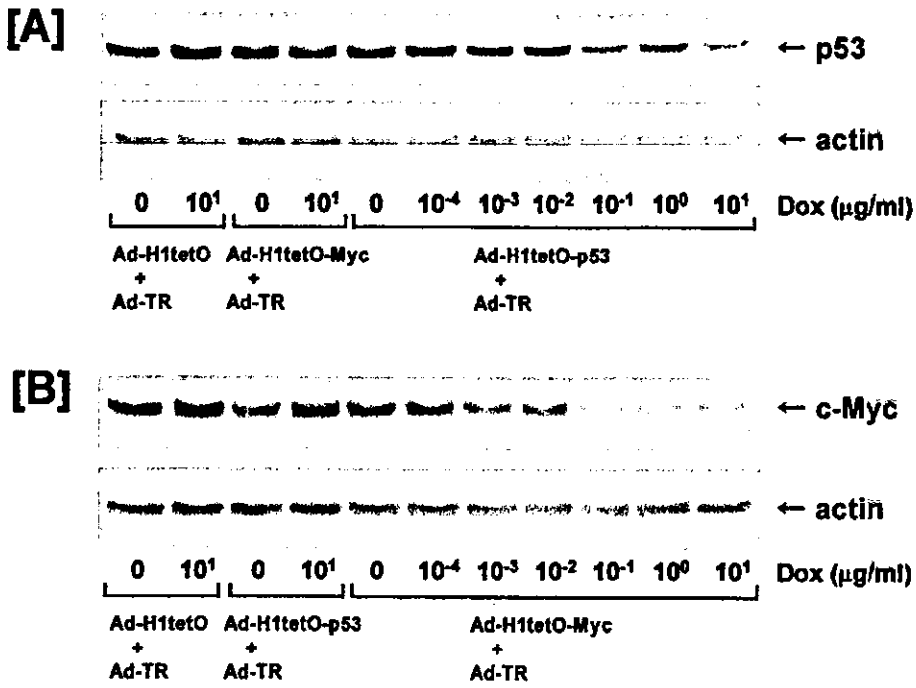

FIG. 4. Dox dose-dependent suppression of p53 and c-Myc expression by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR. A549 cells were infected with Ad-H1tetO-p53 (300 VP/cell) plus Ad-TR (1800 VP/cell) or with Ad-H1tetO-Myc (1000 VP/cell) plus Ad-TR (6000 VP/cell) for 1.5 hr, and then cultured with various concentrations of Dox for 3 days. The cells were also infected with Ad-H1tetO plus Ad-TR. Proteins were then extracted from the cells, and levels of p53 (A) and c-Myc (B) expression were examined by Western blotting. Actin bands served as an internal control for equal total protein loading.

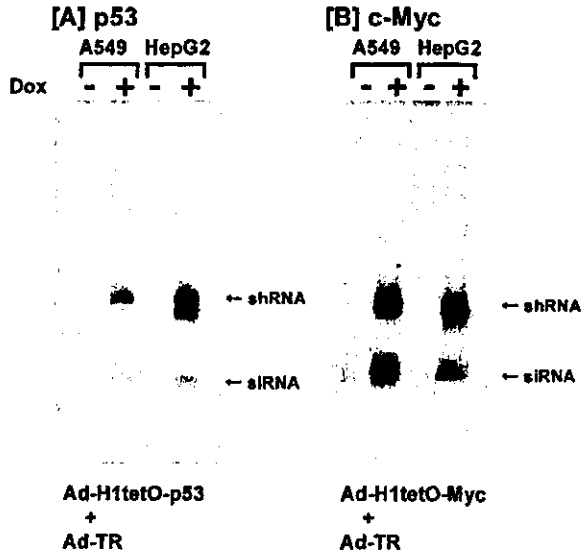

FIG. 5. Dox-inducible *p53* or c-*myc* siRNA expression by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR. A549 and HepG2 cells were infected with Ad-H1tetO-p53 (300 VP/cell) plus Ad-TR (1800 VP/cell) or with Ad-H1tetO-Myc (1000 VP/cell) plus Ad-TR (6000 VP/cell) for 1.5 hr, and then cultured with or without Dox (1 μg/ml) for 3 days. Total RNAs were then extracted from the cells, and levels of *p53* and c-*myc* siRNA expression were examined by Northern blotting.

Ad vector-mediated RNAi represents a new strategy for the study of gene function and therapeutic applications. Ad vector-mediated delivery of siRNA allows efficient transduction into a variety of cell types *in vitro* and *in vivo*. Several studies have reported Ad vector-mediated gene silencing using both the Pol II promoter, in which a mutant CMV promoter was used, and the Pol III (H1) promoter (Xia *et al.*, 2002; Zhao *et al.*, 2003). The combination of Ad vectors and an inducible siRNA expression system offers a superior strategy to researchers. To our knowledge, this study is the first to report on the development of Ad vector-mediated inducible RNAi. Various inducible siRNA expression systems, including a tetracycline repressor-based system, and a capsid-modified Ad vector to change viral tropism, can be easily combined. The system described here has great potential for therapeutic use as well as for a variety of applications, including the study of gene function.

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Review

Targeted Adenovirus Vectors

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ABSTRACT

Recombinant adenovirus (Ad) vectors continue to be the preferred vectors for gene therapy and the study of gene function because they are relatively easy to construct, can be produced at high titer, and have high transduction efficiency. However, in some applications gene transfer with Ad vectors is less efficient because the target cells lack expression of the primary receptor, coxsackievirus and adenovirus receptor (CAR). Another problem is the wide biodistribution of vector in tissue following *in vivo* gene transfer because of the relatively broad tissue expression of CAR. To overcome these limitations, various approaches have been developed to modify Ad tropism. In one approach, the capsid proteins of Ad are modified, such as with the addition of foreign ligands or the substitution of the fiber with other types of Ad fiber, in combination with the ablation of native tropism. In other approaches, Ad vectors are conjugated with adaptor molecules, such as antibody and fusion protein containing an anti-Ad single-chain antibody (scFv) or the extracellular domain of CAR with the targeting ligands, or chemically modified with polymers containing the targeting ligands. In this paper, we review advances in the development of targeted Ad vectors.

INTRODUCTION

ADENOVIRUS VECTORS have been expected to play a prominent role in gene therapy because of their extremely high transduction efficiency. However, one of the hurdles confronting gene transfer by adenovirus (Ad) vectors is their inefficient transduction to target cells lacking sufficient expression of the coxsackievirus and adenovirus receptor (CAR), the primary receptor; such cells include many advanced tumor cells, skeletal muscle cells, smooth muscle cells, peripheral blood cells, hematopoietic stem cells, dendritic cells, and so on. A high dose of vector is required to achieve efficient gene transfer to these cell types. This in turn increases unwanted side effects, such as vector-associated immunogenic toxicities.

Another hurdle confronting Ad vector-mediated gene transfer is their nonspecific distribution in tissue after *in vivo* gene transfer because of the relatively broad expression of CAR, α_v integrin (the secondary receptor), and heparan sulfate (the third receptor). This property imposes an increased risk of toxicity due to vector dissemination to nontargeted cells, such as antigen-presenting cells (e.g., macrophages and dendritic cells). This occurs even when Ad vectors are locally administered to the tissue of interest. Vector targeting to a specific tissue or cell type would enhance gene therapy efficacy and permit the delivery of lower doses, which should result in reduced toxicity.

Several approaches have been developed to overcome these hurdles, including genetic modification of Ad capsid proteins, such as fiber, penton base, hexon, and protein IX (pIX), and conjugation-based modification of virus such as antibody or bispecific fusion protein, and chemical modification by polymers containing the targeting ligands (Fig. 1). To improve gene transfer efficiency, modification of tropism is required. To target gene transfer, both the ablation of natural tropism and introduction of cell-specific tropism are required. In this paper we review approaches to developing targeted Ad vectors.

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