RESEARCH ARTICLE

Dendritic cells transduced with gp100 gene by RGD fiber-mutant adenovirus vectors are highly efficacious in generating anti-B16BL6 melanoma immunity in mice

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Dendritic cells (DCs) are the most potent professional antigen-presenting cells for the initiation of antigen-specific immune responses, and antigen-loaded DCs have been regarded as promising vaccines in cancer immunotherapy. We previously demonstrated that RGD fiber-mutant adenovirus vector (AdRGD) could attain highly efficient gene transduction into human and murine DCs. The aim of the present study is to demonstrate the predominance of *ex vivo* genetic DC manipulation using AdRGD in improving the efficacy of DC-based immunotherapy targeting gp100, a melanoma-associated antigen (MAA). Vaccination with murine bone marrow-derived DCs transduced with AdRGD encoding gp100 (AdRGD-gp100/mBM-DCs) dramatically improved resistance to B16BL6 melanoma challenge and pulmonary metastasis as compared with immunization with

conventional Ad-gp100-transduced mBM-DCs. The improvement in antimelanoma effects upon immunization with AdRGD-gp100/mBM-DCs correlated with enhanced cytotoxic activities of natural killer (NK) cells and B16BL6-specific cytotoxic T lymphocytes (CTLs). Furthermore, *in vivo* depletion analysis demonstrated that CD8⁺ CTLs and NK cells were the predominant effector cells responsible for the anti-B16BL6 immunity induced by vaccination with AdRGD-gp100/mBM-DCs, and that helper function of CD4⁺ T cells was necessary for sufficiently eliciting effector activity. These findings clearly revealed that highly efficient MAA gene transduction to DCs by AdRGD could greatly improve the efficacy of DC-based immunotherapy against melanoma. Gene Therapy (2003) 10, 1891–1902. doi:10.1038/sj.gt.3302090

Keywords: dendritic cell; adenovirus vector; fiber-mutant; gp100; melanoma; immunotherapy

Introduction

The goal of cancer vaccines is to raise the cytotoxic immune response, in which antitumor cytotoxic T lymphocytes (CTLs) play a central role, to a level capable of tumor rejection and regression. It is well established that potent priming and subsequent activation of antitumor CTLs are required for the processing and presentation of tumor-associated antigens (TAAs) as peptide fragments in the context of appropriate major histocompatibility complex (MHC) class I molecules by antigen-presenting cells (APCs).¹ The APC system is represented by dendritic cells (DCs), which possess structural and functional capabilities that are specialized for processing and presenting antigens as peptide fragments to T cells.^{2–4} Owing to their unique priming

and stimulating ability to naive T cells, DCs offer new perspectives in immune intervention strategies against cancer. For this novel anticancer therapy, which is based on the activation of tumor-specific CTLs using DCs as APCs, optimal *ex vivo* TAA delivery into DCs is a key issue for attaining effective therapeutic efficacy. To date, multiple approaches have been designed for delivery of TAAs to DCs, including loading with peptides, full-length proteins, and tumor cell lysates, transfection with mRNA, fusion between DCs and tumor cells, and gene transfer via nonviral or recombinant viral vectors.^{5,6} While the use of peptides still ensures antigen specificity and safety, the peptide sequence and the MHC allele presenting that peptide must be known and expressed by the individual, thereby limiting the population of patients in whom T cells can be activated. On the other hand, genetic modification of DCs with specific sequences of TAA genes offers major advantages as opposed to other approaches. Following the transduction of TAA genes into DCs, full-length TAA proteins are expected to be physiologically processed and presented via MHC class I molecules by DCs. This, therefore, is a

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mean for activating specific antitumor CD8⁺ CTLs with the full antigenic determinants spectrum. Furthermore, TAA gene-modified DCs may also present MHC class II-restricted epitope(s) to CD4⁺ T cells, and can express TAA continuously and MHC-peptide complexes for long periods.⁷⁻⁹

Among various techniques for transferring the TAA gene to DCs, the *ex vivo* engineering of DCs using adenovirus vector (Ad) provides encouraging results.¹⁰⁻¹² However, the low or lack of coxsackie-adenovirus receptor (primary Ad-receptor) expression on the DC surface makes sufficient gene transduction to DCs by conventional Ad difficult.¹³⁻¹⁶ Likewise, the cytopathic effect of high-dose Ad on DCs remains an impediment to this strategy. We recently reported that RGD fiber-mutant Ad (AdRGD), which targets α_v -integrin during attachment to cells by insertion of the RGD peptide into the HI loop of the fiber knob,¹⁷ exhibited highly efficient foreign gene transduction in murine and human DCs compared to conventional Ad.^{15,16,18} Moreover, a murine DC line (DC2.4 cells) transduced with ovalbumin (OVA) gene by AdRGD could elicit a more effective OVA-specific immune response in vaccinated mice than a cell line transduced by conventional Ad.¹⁸ Although these results suggest that efficient gene transduction by AdRGD would improve the efficacy of DC-based gene immunotherapy, the use of primary DCs and the targeting of natural TAA rather than a continuously proliferating DC line and highly immunogenic model antigen is desirable in order to establish the predominance of AdRGD in DC-based immunotherapy.

Melanoma is one of the few cancers for which multiple TAAs capable of eliciting a specific cellular immune response have been identified. These include gp100, MART-1/Melan-A, tyrosinase, and tyrosinase-related protein.¹⁹⁻²³ Among these defined melanoma-associated antigens (MAAs), which are nonmutated self-differentiation antigens associated with melanin synthesis in normal melanocytes, gp100 appears to be a promising target antigen. Several human MHC class I-restricted peptides from gp100 have been identified, and vaccination strategies using synthetic gp100-derived peptides in conjunction with chemical adjuvant or autologous DCs have been evaluated in early phase I/II clinical trials.^{24,25} The murine B16 melanoma model expresses most mouse homologs of these human MAAs, so it can be used to evaluate vaccine strategies in a preclinical setting. However, mice vaccinated with viral vectors encoding murine gp100 do not develop measurable murine gp100-specific immunity, and are apparently tolerant to murine gp100.^{26,27} In contrast, immunization with human gp100-expressing viral vectors can overcome self-tolerance to gp100 in C57BL/6 mice because this approach induces human gp100-specific and also murine B16 melanoma-crossreactive T-cell responses.^{26,27} Furthermore, immunization with DCs transduced *ex vivo* with human gp100 gene using conventional Ad generates more effective anti-B16 melanoma immunity than *in vivo* direct injection of human gp100-expressing Ad.¹⁰

In the current research, we attempted to demonstrate the predominance of AdRGD in DC-based immunotherapy using murine bone marrow-derived DCs (mBM-DCs) and targeting gp100. We found that mBM-DCs were highly susceptible to human gp100 gene transduction by AdRGD, and that *ex vivo* DC engineering

with gp100 gene using AdRGD, as compared with conventional Ad, could generate more potent anti-B16BL6 melanoma immunity, as determined by protection against tumor challenge, reduction of lung metastasis, and analysis of effector activity. In addition, the efficacy of AdRGD-applied DC-based immunotherapy resulted from eliciting natural killer (NK) cells and a specific CD8⁺ CTLs response via a CD4⁺ T cell-dependent mechanism.

Results

Cytopathic effect of AdRGD-gp100- or Ad-gp100 transduction against mBM-DCs

Immature mBM-DCs from C57BL/6 mice were expanded *in vitro* for 8–14 days in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF). Flow cytometric analysis indicated that the resulting cell population was negative for B220 and CD14, and that more than 85% of these cells displayed characteristic DC surface markers, including I-A^b, CD80, CD86, and CD11c (data not shown). In order to investigate cytopathic effects of AdRGD-gp100- or Ad-gp100 transduction on mBM-DCs, we performed an MTT assay at 48 h post-transduction (Figure 1). Although cytotoxicity after transduction with each vector was not observed at a multiplicity of infection (MOI) of less than 50, the viability of AdRGD-gp100- or Ad-gp100-transduced mBM-DCs (AdRGD-gp100/mBM-DCs or Ad-gp100/mBM-DCs, respectively) tended to decrease at a MOI of 100 or more. In addition, cell damage caused by AdRGD-gp100 transduction was slightly more serious than that by Ad-gp100 transduction at a high MOI, and the viability of AdRGD-gp100/mBM-DCs fell to 80% at an MOI of 200. Flow cytometric analysis using an annexin-V and propidium iodide staining kit demonstrated that the manner of cell death under high-dose transductional conditions was partially apoptosis (data not shown). On the basis of these results, we set the MOI

Figure 1 Cytopathic effect of AdRGD-gp100 and Ad-gp100 on gene transduction into mBM-DC. mBM-DCs were infected with AdRGD-gp100 or Ad-gp100 at the indicated MOI, and then were cultured on 96-well plates at 1×10^5 cells/100 μ l in GM-CSF-free culture medium. After 2 days, cell viability was assessed by MTT assay. Each point represents the mean $\pm$ s.d. of triplicate cultures.

for each vector used for DC modification in the following experiments as 50 or less, which did not affect the cell viability.

Human gp100 expression in AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs

Expression levels of human gp100 mRNA in AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) at 48 h after transduction (Figure 2a). Human gp100-specific PCR products increased in an MOI-dependent manner in both AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs, whereas control vector-transduced mBM-DCs (AdRGD-LacZ/mBM-DCs) did not express any detectable gp100 mRNA. In addition, AdRGD-gp100/mBM-DCs showed much higher levels of gp100 mRNA expression than Ad-gp100/mBM-DCs. In order to confirm cytoplasmic expression of human

gp100 protein, we performed flow cytometric analysis by the intracellular staining method using HMB50 antibody (Figure 2b). Under transductional conditions at an MOI of 50, more than 95% of AdRGD-gp100/mBM-DCs could express gp100 protein in their cytoplasm, whereas only 8.3% of Ad-gp100/mBM-DCs were gp100-positive. These data clearly demonstrate that AdRGD is a very potent vector system for *ex vivo* genetic modification of DCs, as we showed in our previous studies using reporter genes.^{15,16,18}

Allogenic and syngeneic T-cell proliferation-stimulating ability of AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs

Next, we performed allogenic and syngeneic mixed leukocyte reaction (MLR) to compare T-cell proliferation-stimulating ability of AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs. Ad-gp100/mBM-DCs could stimulate allogenic naive T cells more effectively than intact (immature) mBM-DCs, and the proliferation level of responder cells stimulated by Ad-gp100/mBM-DCs was comparable with that by lipopolysaccharide (LPS)-driving mature mBM-DCs (Figure 3a). Moreover, AdRGD-gp100/mBM-DCs could more strongly induce allogenic T-cell proliferation than Ad-gp100/mBM-DCs. On the other hand, both AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs, as well as intact mBM-DCs, were not able to stimulate syngeneic naive T-cell proliferation (Figure 3b). However, only AdRGD-gp100/mBM-DCs could induce the significant proliferation of syngeneic gp100-primed T cells, which were purified from C57BL/6 mouse vaccinated beforehand with 1×10^6 AdRGD-gp100/mBM-DCs, at responder/stimulator ratio of 5 (Figure 3c). These results suggested that AdRGD-gp100/mBM-DCs possessed the more potent allostimulating ability than Ad-gp100/mBM-DCs, and that they could expand syngeneic T cells in the gp100-specific manner. We determined that the T-cell proliferation-stimulating ability of AdRGD-gp100/mBM-DCs is most likely related to not only to the sufficient presentation of processed gp100 peptides via MHC molecules but also the enhancement of both costimulatory molecules expression and interleukin (IL)-12 secretion on AdRGD-gp100/mBM-DCs, which we observed previously.¹⁶

Vaccine efficacy of AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs against B16BL6 tumor challenge

C57BL/6 mice received a single intradermal injection of 1×10^6 mBM-DCs transduced with AdRGD-gp100, Ad-gp100, or AdRGD-Null at an MOI of 50, and then these mice were inoculated with 2×10^5 B16BL6 melanoma cells at 1 week postimmunization. The mice immunized with AdRGD-Null/mBM-DCs showed no significant protection against the inoculated B16BL6 cells. The results in Figure 4 demonstrated that immunization with Ad-gp100/mBM-DCs markedly inhibited B16BL6 melanoma growth when compared to the AdRGD-Null/mBM-DCs control group. In addition, a more significant inhibitory effect on tumor growth could be observed in mice after vaccination with AdRGD-gp100/mBM-DCs than in mice vaccinated with Ad-gp100/mBM-DCs. Four of six mice immunized with AdRGD-gp100/mBM-DCs

Figure 2 Human gp100 expression in AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs. mBM-DCs were infected with AdRGD-gp100, Ad-gp100, or AdRGD-LacZ at the indicated MOI. After 48 h cultivation, human gp100 gene expression was assessed by RT-PCR analysis (a) and flow cytometric analysis with an intracellular staining method using HMB50 antibody (b). (a) PCR for human gp100 and mouse β -actin transcripts was performed on the same RT samples using each specific primer set to ensure the quality of the procedure. (b) the % value and the numerical value in the parenthesis express % of M1-gated cells and mean fluorescence intensity, respectively.

Figure 3 Allogenic and syngeneic T-cell proliferation-stimulating ability of AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs. C57BL/6 mBM-DCs were infected with AdRGD-gp100 or Ad-gp100 at an MOI of 50, and cultured for 2 days. Naive BALB/c T lymphocytes (a), naive C57BL/6 T lymphocytes (b), or gp100-primed C57BL/6 T lymphocytes (c) were cocultured with AdRGD-gp100/mBM-DCs, Ad-gp100/mBM-DCs, LPS/mBM-DCs, or intact mBM-DCs at different responder/stimulator ratios 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 $\pm$ s.e. of three independent cultures using T cells prepared from three individual mice.

remained tumor free for 60 days after B16BL6 cells challenge, whereas only one of six mice immunized with Ad-gp100/mBM-DCs were tumor free. Furthermore, immunization with AdRGD-gp100/mBM-DCs was specifically effective only against B16BL6 melanoma because mice vaccinated by AdRGD-gp100/mBM-DCs, as well as mice immunized with phosphate-buffered saline (PBS), did not exhibit any protective effect against syngeneic irrelevant EL4 lymphoma challenge (data not shown). These results reveal that vaccination with Ad-gp100/mBM-DCs could provide protection against B16BL6 tumor challenge, but less effectively than vaccination with AdRGD-gp100/mBM-DCs. Therefore, our data suggest that highly efficient MAA

Figure 4 Vaccine efficacy of AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs against B16BL6 melanoma challenge. AdRGD-gp100/mBM-DCs, Ad-gp100/mBM-DCs, and AdRGD-Null/mBM-DCs were prepared using corresponding vectors at an MOI of 50. C57BL/6 mice were immunized by intradermal injection of transduced mBM-DCs into the right flank at 1×10^6 cells, and then 2×10^5 B16BL6 melanoma cells were inoculated into the mouse left flank after 1 week postvaccination. The size of tumors was assessed using microcalipers three times per week. Each point represents the mean $\pm$ s.e. from six mice.

expression by application of AdRGD to *ex vivo* DC engineering could significantly enhance protective and specific antimelanoma immunity in DC-based immunotherapy.

Inhibitory effect of AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs on B16BL6 pulmonary metastasis

DC-based immunotherapy is expected to satisfactorily demonstrate its efficacy in prevention of metastasis and recurrence after surgical removal of the primary cancer rather than in regression of advanced primary cancer mass. Thus, we compared the anti-B16BL6 pulmonary metastasis effects of AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs using both the vaccine and therapeutic protocols (Figure 5). In response to the vaccine protocol, B16BL6 metastatic nodules in lung were reduced by a single immunization with AdRGD-gp100/mBM-DCs or Ad-gp100/mBM-DCs in an MOI-dependant manner, and more potent inhibitory effects were observed in the AdRGD-gp100/mBM-DCs group. The antimetastasis effect of AdRGD-gp100/mBM-DCs prepared at an MOI of 1 was comparable with that of Ad-gp100/mBM-DCs prepared at an MOI of 25, and more than 99% inhibition was attained in mice immunized with AdRGD-gp100/mBM-DCs prepared at an MOI of 25. Likewise, three immunizations with AdRGD-gp100/mBM-DCs could more effectively inhibit established pulmonary metastasis in the therapeutic protocol after intravenous injection of B16BL6 cells than Ad-gp100/mBM-DCs. These data indicate that the immunization with MAA genemodified DCs using AdRGD can greatly improve the efficacy against melanoma metastasis not only when the animals were vaccinated in advance but also when micrometastasis already existed in peripheral tissue distant from the immunization site.

Figure 5 Inhibition of B16BL6 pulmonary metastasis by immunization with AdRGD-gp100/mBM-DCs or Ad-gp100/mBM-DCs. AdRGD-gp100/mBM-DCs, Ad-gp100/mBM-DCs, and AdRGD-Null/mBM-DCs were prepared using corresponding vectors at the indicated MOI. Experimental procedure of both vaccine and therapeutic protocols is described in the 'Materials and methods' section. Results are expressed as mean $\pm$ s.e. from six mice.

Cytolytic activity of NK cells and CTLs from mice immunized with AdRGD-gp100/mBM-DCs or Ad-gp100/mBM-DCs

In order to attain sufficient therapeutic efficacy in cancer immunotherapy, it is important to efficiently prime and activate effector cells belonging to the cellular immune system, which can directly recognize and eliminate tumor cells, rather than activate the humoral immune system. Thus, we examined the cytolytic activities of NK cells and CTLs in mice intradermally immunized with 1×10^6 AdRGD-gp100/mBM-DCs or Ad-gp100/mBM-DCs by Eu-release assay. At 1 week after immunization of C57BL/6 mice with various vaccines, the splenocytes were used in a cytolytic assay against YAC-1 cells, and were restimulated *in vitro* with inactivated B16BL6 cells, which were treated with interferon (IFN)- γ to promote the expression of their MHC class I molecules, for CTL expansion.

As shown in Figure 6, the splenic NK activity increased after immunization with AdRGD-gp100/mBM-DCs, while effector cells from mice immunized with Ad-gp100/mBM-DCs exhibited equivalent NK activity to those from mice immunized with intact mBM-DCs or PBS. These data suggested that the enhancement of nonspecific immunity might be involved

Figure 6 NK activity of splenocytes from mice immunized with AdRGD-gp100/mBM-DCs or Ad-gp100/mBM-DCs. AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs were prepared using corresponding vectors at an MOI of 50. Transduced or intact mBM-DCs were vaccinated once intradermally into C57BL/6 mice at 1×10^6 cells. At 1 week after immunization, nonadherent splenocytes were prepared from these mice, and directly used in cytolytic assays against YAC-1 cells. Each point represents the mean $\pm$ s.e. from three independent cultures using splenocytes prepared from three individual mice.

Figure 7 B16BL6-specific CTL response in mice immunized with AdRGD-gp100/mBM-DCs or Ad-gp100/mBM-DCs. AdRGD-gp100/mBM-DCs, Ad-gp100/mBM-DCs, and AdRGD-LacZ/mBM-DCs were prepared using corresponding vectors at an MOI of 50. Transduced or intact mBM-DCs were vaccinated once intradermally into C57BL/6 mice at 1×10^6 cells. At 1 week after immunization, nonadherent splenocytes were prepared from these mice, and then were restimulated *in vitro* for 5 days with IFN- γ -stimulated and mitomycin C-inactivated B16BL6 cells. A cytolytic assay was performed using B16BL6 cells stimulated with or without IFN- γ , EL4 cells, and YAC-1 cells. Each point represents the mean $\pm$ s.e. of three independent cultures using restimulated splenocytes prepared from three individual mice.

in the improvement of anti-B16BL6 melanoma response by AdRGD-gp100/mBM-DCs immunization. On the other hand, the cytolytic effects on B16BL6 cells by

in vitro restimulated effector cells were promoted in mice immunized with Ad-gp100/mBM-DCs as compared with mice immunized with intact mBM-DCs. However, the AdRGD-gp100/mBM-DCs group exhibited the most effective cytolytic activity (Figure 7). When B16BL6 cells stimulated with IFN- γ were used as target cells, the cytolytic activities in the groups immunized with AdRGD-gp100/mBM-DCs or Ad-gp100/mBM-DCs were still more obvious than those in the other groups. This cytolytic activity was caused by B16BL6-specific CTLs because the effector cells prepared from mice immunized with AdRGD-LacZ/mBM-DCs did not injure the B16BL6 cells and no cytolytic effects against syngeneic EL4 cells and NK-sensitive YAC-1 cells were detected in any group. Therefore, consistent with the protective and therapeutic effect against B16BL6 tumor growth and metastasis, the mice immunized with AdRGD-gp100/mBM-DCs exhibited a B16BL6 melanoma-specific CTL response, which was more potent than that induced by immunization with Ad-gp100/mBM-DCs.

Taken together, these results suggest that immunization with MAA gene-modified DCs using AdRGD, as compared with those using conventional Ad, could more efficiently prime and activate effector cells, nonspecific NK cells, and MAA-specific CTLs, which is subsequently related to rejection and regression of melanoma.

Figure 8 Determination of immune subsets responsible for the protective immunity induced by vaccination with AdRGD-gp100/mBM-DCs. C57BL/6 mice were immunized once with 1×10^6 mBM-DCs transduced with AdRGD-gp100 at an MOI of 50, and inoculated with 1×10^5 B16BL6 cells at 1 week after immunization. Antibodies, GK1.5 (anti-CD4), 53-6.72 (anti-CD8), or anti-asialoGM1, were intraperitoneally injected according to the indicated schedule. Depletion of T-cell subsets and NK cells was monitored by flow cytometry, which showed >90% specific depletion in splenocytes at the time of immunization or tumor challenge in each administration schedule. Each point represents the mean $\pm$ s.e. of six mice.

Effect of T-cell subsets- or NK cell-depletion on vaccine efficacy of AdRGD-gp100/mBM-DCs

In order to investigate what subset of immunocompetent cells was involved in the induction and effector phases and improve vaccine efficacy by immunization with AdRGD-gp100/mBM-DCs, we performed *in vivo* depletion analysis using specific antibodies against CD4, CD8, and asialoGM1. The antibody administration schedule for this experiment is shown in the lower part of Figure 8. Upon depletion during immunization (induction phase), intraperitoneal administration of each antibody was started 3 days before AdRGD-gp100/mBM-DCs immunization. These mice then received two administrations every other day. Flow cytometry showed >90% specific depletion in splenocytes from these mice at immunization, and the number of specific cells recovered was at least 70% at the time of B16BL6 tumor challenge (data not shown). On the other hand, to deplete specific cell types during tumor challenge (effector phase), antibody administration was started 3 days before B16BL6 tumor challenge. This was followed by four additional administrations performed every other day. These mice showed >90% specific depletion in splenocytes at tumor challenge, and 50–70% recovery of specific cells was observed on day 12 post-tumor challenge (data not shown).

As shown in Figure 8, the depletion of CD8⁺ T cells or NK cells during the effector phase abrogated the protective immunity induced by immunization with AdRGD-gp100/mBM-DCs, whereas mice whose CD4⁺ T cells were depleted during the effector phase could resist B16BL6 tumor challenge as well as nondepletion group. In contrast, mice depleted of CD4⁺ T cells during the induction phase failed to reject B16BL6 tumor challenge. However, the depletion of CD8⁺ T cells or NK cells during the induction phase did not influence vaccine efficacy of AdRGD-gp100/mBM-DCs. These results confirmed that CD8⁺ CTLs and NK cells were predominant effector cells in anti-B16BL6 melanoma immunity induced by immunization with AdRGD-gp100/mBM-DCs, and that the activation of effector cells was greatly dependent on the helper function of CD4⁺ T cells activated during the induction phase.

Discussion

In general, DCs capture antigens in the periphery and then migrate to T-cell areas in lymphoid organs to prime the antigen-specific immune responses.^{4,28} Owing to these features and establishment of methods for expansion of human and rodent DCs on a large scale from hematopoietic precursors in the presence of appropriate cytokine cocktails,^{29–31} DCs have attracted great attention as vehicles for the delivery of cancer vaccines. The efficacy of different viral vectors, including retroviral vector,^{8,32} Ad,^{8–12} and lentiviral vector,^{33,34} has been evaluated for genetic modification of DCs. Among them, Ad was confirmed as a good candidate because of its high efficiency and minimum risk associated with insertional mutagenesis.³⁵ Owing to the life span and migration period of DCs administered to a living body,^{36,37} the indication that only transient gene expression can be attained by Ad-mediated gene transfer will not impact DC-based immunotherapy. Genetic DC

modification using Ad to express TAA has been actually documented to be efficacious to induce antitumor immunity in animal models.^{10–12} Nevertheless, the antitumor immunity induced by TAA gene-modified DC vaccine is still limited in clinical trials,^{38,39} suggesting that the breakthrough capable of improving the efficacy of DC-based immunotherapy will need to be explored for wide application of this novel cancer treatment. The antitumor efficacy of immunization with DCs genetically modified with TAA gene is still far from satisfactory in translational research and early phase clinical trials for a variety of reasons and is partly due to the relative resistance of DCs against gene transduction. From the perspective of the exploitation of DC-based immunotherapy, we previously reported a method using AdRGD that enhances the *ex vivo* gene transduction efficiency of murine and human DCs.^{15,16,18}

In recent years, the cloning of various MAAs interacting with the melanocyte differentiation and melanogenesis pathways has opened new possibilities for the development of active immunotherapy designed to cause the rejection of malignant melanoma.^{19–23} gp100, an MAA, is consistently expressed in both murine and human melanomas,^{27,40} and has been shown to be highly immunogenic and an important target for active-specific immunotherapy in humans.⁴¹ However, mice injected with Ad²⁶ or recombinant vaccinia virus²⁷ encoding murine gp100, which was cloned from a B16 cDNA library, failed to produce any detectable T-cell responses or protective immunity against murine B16 melanoma. In contrast, immunization of mice with human gp100 gene, which shares 75.5% nucleotide and 77% amino-acid identity to murine gp100, using Ad elicited T cells specific for human gp100. However, these T cells also crossreacted with B16 tumor *in vitro* and could induce some level of protective tumor immunity in C57BL/6 mice against B16 challenge.^{26,27} Hence, the primary aim of the present study was to demonstrate that the highly efficient transduction of mBM-DCs with a natural MAA, human gp100 gene, by AdRGD can improve the therapeutic efficacy of DC-based vaccine in the mouse B16BL6 melanoma model.

When the cytopathic effect of AdRGD-gp100- or Ad-gp100 transduction on mBM-DCs was evaluated at first, neither vector type affected cell viability at the lower MOI of 50. Although AdRGD-gp100 damaged mBM-DCs more than Ad-gp100 at an MOI of 100 or more, this phenomenon resulted from the presence of more AdRGD-gp100 particles capable of infecting and invading mBM-DCs than Ad-gp100 particles. In fact, expression of both gp100 mRNA and gp100 protein was notably higher under noncytotoxic transductional conditions in AdRGD-gp100/mBM-DCs than in Ad-gp100/mBM-DCs. Surprisingly, nearly all AdRGD-gp100/mBM-DCs could express gp100 protein in their cytoplasm under transductional conditions at an MOI of 50, and the percentage of gp100-positive cells between AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs resulted in a 10-fold difference. Although data were not shown, we confirmed that AdRGD was able to transduce the reporter gene into mBM-DCs more efficiently under the same transductional conditions, based on MOI or on the number of vector particles, than type 35 fiber-modified Ad whose high gene transduction efficiency in human DCs was reported by Rea *et al.*⁴² Therefore, we

are fully confident that AdRGD has the highest capability to genetically modify DCs *ex vivo* among the various vector systems established to date. Furthermore, AdRGD-gp100/mBM-DCs could stimulate allogenic naive T cells more effectively than Ad-gp100/mBM-DCs and also mature LPS/mBM-DCs, and the proliferation of syngeneic gp100-primed T cells was observed only in cocultivation with AdRGD-gp100/mBM-DCs. We previously demonstrated that antigen presentation level via MHC molecules in mBM-DCs transduced with the OVA gene by AdRGD was much greater than that in mBM-DCs transduced with conventional Ad.¹⁶ Likewise, we found that AdRGD transduction could enhance the expression of MHC and costimulatory molecules and the secretion of IL-12 in mBM-DCs as compared with conventional Ad transduction.¹⁶ Thus, we expected that AdRGD-gp100/mBM-DCs could stimulate more repertoires of T-cell receptors via gp100 peptides–MHC complexes than Ad-gp100/mBM-DCs and LPS/mBM-DCs because they may sufficiently present peptides produced by processing of gp100 proteins that are expressed in large quantities within the cytoplasm. Also, they could provide secondary signals for activation and proliferation of naive T cells via costimulatory molecules whose expression levels were promoted by AdRGD transduction. In addition, IL-12 secreted from mature AdRGD-gp100/mBM-DCs might participate in potent T-cell stimulation because IL-12 is a major Th1-driving cytokine.

As expected, a single vaccination with AdRGD-gp100/mBM-DCs could provide C57BL/6 mice with much higher resistance against tumor growth and lung metastasis from subsequent B16BL6 challenge than Ad-gp100/mBM-DCs. Moreover, in the therapeutic protocol that called for three DC immunizations after intravenous injection of B16BL6 cells, mice immunized with AdRGD-gp100/mBM-DCs could more largely reduce the number of metastatic nodules than those in the Ad-gp100/mBM-DCs group. The difference between AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs in vaccine and therapeutic efficacy against B16BL6 melanoma reflected the gene transduction efficiency and the maturation status to mBM-DCs by AdRGD-gp100 and Ad-gp100. As the prognosis of patients with melanoma is generally poor even after surgery, chemotherapy, or radiotherapy, and the melanomas commonly metastasize,^{43,44} the fact that immunization with AdRGD-gp100/mBM-DCs could demonstrate more potent therapeutic efficacy against pre-existing B16BL6 metastasis is significant. Taken together, our results indicate that highly efficient MAA gene transduction to DCs using AdRGD leads directly to the generation of very effective APCs and an improvement in the efficacy of DC-based immunotherapy against melanoma.

As DCs are uniquely capable of presenting novel antigens to naive T cells, previous studies of immune responses after immunization using antigen-loaded DCs have focused on generating antigen-specific CTLs. However, several recent reports confirm that DCs can activate NK cells and play an important role in crosstalk between innate and acquired immune responses.^{45,46} We, therefore, attempted to analyze the mechanism behind the anti-B16BL6 tumor effects of immunization with AdRGD-gp100/mBM-DCs. Splenocytes from mice immunized with AdRGD-gp100/mBM-DCs could injure

YAC-1 cells more effectively than those from intact mice, whereas NK activity did not change upon immunization with Ad-gp100/mBM-DCs or intact mBM-DCs. Although data were not shown, we confirmed that the increase in NK activity in mice immunized with AdRGD-gp100/mBM-DCs disappeared 2 weeks postimmunization. We speculated that the increase in NK activity during the early stage of immunization with AdRGD-gp100/mBM-DCs was caused by upregulation of IL-12 secretion from mBM-DCs transduced with AdRGD as observed in our previous work.¹⁶ Recently, Miller *et al*⁴⁷ also suggested that the enhancement in NK activity in mice immunized with Ad-transduced DCs depended partially on IL-12 levels secreted from immunized DCs. On the other hand, the increase in B16BL6-specific CTL activity was detected in mice immunized with Ad-gp100/mBM-DCs, and more effective cytolytic activity against B16BL6 cells was observed in effector cells prepared from mice immunized with AdRGD-gp100/mBM-DCs. These data revealed that highly efficient transduction of MAA genes to DCs using AdRGD could elicit both NK activity and melanoma-specific CTL activity more effectively than the use of conventional Ad in DC-based immunotherapy. Furthermore, the depletion of CD4⁺ T cells during the induction phase greatly reduced the inhibition of B16BL6 tumor growth, whereas vaccine efficacy of AdRGD-gp100/mBM-DCs was not affected by CD8⁺ T cell- or NK cell depletion during the induction phase. These data provided three important observations related to anti-B16BL6 tumor immunity generated by the immunization with AdRGD-gp100/mBM-DCs: (1) sufficient activation of the effector cells capable of demonstrating excellent anti-B16BL6 tumor effects was greatly dependent on CD4⁺ T cells activated beforehand by administered AdRGD-gp100/mBM-DCs; (2) despite endogenous antigen presentation by gene transfer, AdRGD-gp100/mBM-DCs might present not only MHC class I-restricted epitope(s) but unidentified class II-restricted epitope(s) that are recognized by CD4⁺ T cells; (3) the period when AdRGD-gp100/mBM-DCs could prime and activate CD4⁺ T cells after immunization was relatively short because we confirmed that the number of CD4⁺ T cells recovered to more than 70% on day 7 postimmunization in CD4⁺ T-cell-depleted mice during the induction phase. Wan *et al*⁴⁸ argued that in addition to acting as helpers during the induction phase, CD4⁺ T cells are major effector cells in anti-B16 tumor immunity induced by immunization with DCs transduced with human gp100 using conventional Ad. However, CD4⁺ T-cell depletion during the effector phase did not impact the protective effect against B16BL6 tumor growth in mice vaccinated with AdRGD-gp100/mBM-DCs, whereas vaccine efficacy of AdRGD-gp100/mBM-DCs was clearly weakened by CD8⁺ T-cell or NK cell depletion during the effector phase. Therefore, in our model experiment, it was confirmed that the effector cells responsible for anti-B16BL6 tumor immunity were activated nonspecific NK cells and gp100-specific CD8⁺ CTLs.

In conclusion, our findings demonstrate that AdRGD is a very powerful vector system that improves the efficacy of DC-based immunotherapy using primary DCs and targeting natural TAA. Genetic manipulation of DCs using AdRGD not only potentiated the efficiency of TAA gene expression, but also could increase T-cell stimula-

tion by DCs to a greater extent than conventional Ad. As a result, a more powerful anti-B16BL6 tumor effect based on efficient activation of NK cells and CD8⁺ CTLs, which depends on the helper function of CD4⁺ T cells, was demonstrated in mice immunized with AdRGD-gp100/mBM-DCs. Several recent reports indicated that genetically modified DCs that express cytokine or CD40 ligand could induce effective antitumor immunity in mouse model.⁴⁹⁻⁵¹ If the AdRGD system is applied in these strategies, not only more potent efficacy can be attained due to highly efficient gene transduction into DCs, but the immunologic properties of the modified DCs can be analyzed in detail without sorting and collection of transduced live cells. Thus, AdRGD contributes to a significant advance in the exploitation of *ex vivo* DC manipulation for experimentally and clinically relevant vaccines.

Materials and methods

Cell lines and mice

Poorly immunogenic and highly metastatic B16BL6 melanoma cell line derived from C57BL/6 mice (H-2^b) was cultured in minimum essential medium supplemented with 7.5% fetal bovine serum (FBS) and antibiotics. EL4 cells, a T-lymphoma cell line of C57BL/6 origin, and YAC-1 cells, a lymphoma cell line highly sensitive to NK cells, were maintained in RPMI 1640 medium supplemented with 10% FBS, 50 μ M 2-mercaptoethanol, and antibiotics. The helper cell line, 293 cells, was grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics. Female C57BL/6 mice and female BALB/c mice (H-2^d), aged 7–8 weeks, were purchased from SLC Inc. (Hamamatsu, Japan). Female ICR-nu/nu mice, aged 7 weeks, were purchased from Charles River Japan, Inc. (Yokohama, 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.

Generation of mBM-DCs

Isolation and cultivation of mBM-DCs were performed according to the method of Lutz *et al*²⁹ and slightly modified. Briefly, bone marrow cells flushed from the femurs and tibias of C57BL/6 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 GM-CSF (PeproTech EC LTD., London, England), 50 μ M 2-mercaptoethanol, and antibiotics. On day 3, another 10 ml of the culture medium was added to the dish for medium replenishment. Then, every 3 days, 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. mBM-DCs (nonadherent cells), 8–14 days old, were harvested and used as intact immature mBM-DCs in subsequent experiments. mBM-DCs cultured for more than 24 h in media containing 1 μ g/ml LPS (Nacalai Tesque, Inc., Kyoto, Japan) were used as positive controls for phenotypical DC maturation.

Preparation of recombinant Ad

The replication-deficient AdRGD-gp100 and conventional Ad-gp100, which carried human gp100 cDNA derived from pAx1-CA h-gp100 (kindly provided by Dr Hirofumi Hamada, Sapporo Medical University, Sapporo, Japan) under the control of chicken β -actin promoter, were based on early regions 1- and 3-deleted adenovirus serotype 5, and were constructed by an improved *in vitro* ligation method as described.⁵²⁻⁵⁴ AdRGD-LacZ and AdRGD-Null, which were constructed previously,^{15,16,18} were used as control vectors in the present study. All recombinant viruses were propagated in 293 cells, purified by two rounds of CsCl density centrifugation, dialyzed, and stored at -80°C . Titers (tissue culture infectious dose₅₀; TCID₅₀) of infective Ad particles were evaluated by end-point dilution method using 293 cells.

Viral transduction into mBM-DCs

mBM-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 or Ad was added at various MOI (TCID₅₀/cell), 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 PBS and resuspended in a suitable solution.

Evaluation of viability of transduced mBM-DCs

AdRGD-gp100/mBM-DCs, Ad-gp100/mBM-DCs, and intact mBM-DCs were cultured on 96-well plates at 1×10^5 cells/100 μl in GM-CSF-free culture medium. After 48 h cultivation, cell viability was assessed by MTT assay and calculated by the following formula: (% of mBM-DC viability)=(MTT activity of transduced cells)/(MTT activity of intact cells) $\times 100$.

Analysis of gene expression levels of human gp100

AdRGD-gp100/mBM-DCs, Ad-gp100/mBM-DCs, and AdRGD-LacZ/mBM-DCs were cultured on 100-mm bacterial grade culture dishes in GM-CSF-free culture medium. After 2 days, human gp100 gene expression was assessed by RT-PCR analysis and flow cytometric analysis. RT-PCR analysis for human gp100 was performed as follows. Total RNA was isolated from transduced mBM-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 $\times$ RT buffer, 5 mM MgCl₂, 1 mM dNTP mix, 1 μM random hexamers, 1 μM oligo(dT), and 100 U ReverTra Ace (TOYOBO Co., LTD., Osaka, Japan). PCR amplification of the human gp100 and mouse β -actin transcripts was performed in 50 μl of a reaction mixture containing 1 μl of RT-material, 5 μl 10 $\times$ PCR buffer, 1.25 U Taq DNA polymerase (TOYOBO Co., LTD.), 1.5 mM MgCl₂, 0.2 mM dNTP, and 0.4 μM primers. The sequences of the specific primers were as follows; human gp100: forward, 5'-tgg aac agg cag ctg tat cc-3'; reverse, 5'-cct aga act tgc cag tat tgg c-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 94°C , 20 cycles of denaturation for 60 s at 94°C , annealing for 60 s at 58°C (human gp100) or 60°C (mouse β -actin),

and extension for 60 s at 72°C were repeated and followed by completion for 4 min at 72°C . The PCR product was electrophoresed on a 3% agarose gel, stained with ethidium bromide, and visualized under ultraviolet radiation. EZ Load (BIO-RAD, Tokyo, Japan) was used as a 100 bp molecular ruler. The expected PCR product sizes were 362 bp (human gp100) and 514 bp (mouse β -actin). An intracellular staining method was used for the detection of human gp100 protein in transduced mBM-DCs. Cells (1×10^6) were fixed by incubation for 10 min in 2% paraformaldehyde, and then cell membranes were permeabilized by incubation for 5 min in 1% saponin (Sigma Chemical Co., St Louis, MO, USA). The cells were incubated with 100 μl staining buffer (PBS containing 0.1% bovine serum albumin and 0.01% NaN₃) containing the anti-Fc γ RII/III monoclonal antibody (2.4G2; rat IgG_{2b,k}; Pharmingen, San Diego, CA, USA) to block nonspecific binding of the subsequently used antibody reagents. After 30 min, the cells were incubated for 60 min with 100 μl staining buffer containing the HMB50 antibody against human gp100 (mouse IgG; Neomarkers, Fremont, CA, USA), and then resuspended in 100 μl staining buffer containing fluorescein isothiocyanate-conjugated anti-mouse Ig κ (187.1; Pharmingen). After incubation for 30 min, 10 000 events of the stained cells were analyzed for human gp100 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.

MLR

AdRGD-gp100/mBM-DCs and Ad-gp100/mBM-DCs were prepared using corresponding vectors at an MOI of 50, and then cultured for 2 days. Three kinds of T cell, including allogenic naive T cells from intact BALB/c mouse, syngeneic naive T cells from intact C57BL/6 mouse, and syngeneic gp100-primed T cells from C57BL/6 mouse immunized intradermally with 1×10^6 AdRGD-gp100/mBM-DCs 1 week before, were purified from each mouse splenocytes as nylon wool nonadherent cells, and were used as responder cells at 1×10^5 cells/well in 96-well plates. Ad-transduced mBM-DCs, LPS/mBM-DCs, or intact mBM-DCs (stimulator cells) were inactivated by 50 $\mu\text{g}/\text{ml}$ mitomycin C for 30 min and added to responder cells in varying cell numbers. Cells were cocultured in 100 μl RPMI 1640 supplemented with 10% FBS, 50 μM 2-mercaptoethanol, and antibiotics at 37°C and 5% CO₂ 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).

Tumor protection assay

AdRGD-gp100/mBM-DCs, Ad-gp100/mBM-DCs, and AdRGD-Null/mBM-DCs were prepared using corresponding vectors at an MOI of 50. C57BL/6 mice were immunized by intradermal injection of transduced mBM-DCs into the right flank at 1×10^6 cells/50 μl . At 1 week after the vaccination, 2×10^5 B16BL6 melanoma

cells were inoculated into the mouse left flank. The size of tumors was assessed using microcalipers and was expressed as tumor volume calculated by the following formula: (tumor volume; mm³)=(major axis; mm) × (minor axis; mm)² × 0.5236. Mice containing tumors >20 mm were euthanized.

Lung metastasis assay

AdRGD-gp100/mBM-DCs, Ad-gp100/mBM-DCs, and AdRGD-Null/mBM-DCs were prepared using corresponding vectors at an MOI of 1, 5, 25, or 50. For the vaccine protocol, transduced or intact mBM-DCs were intradermally injected into the flank of C57BL/6 mice at 1×10^6 cells/50 μ l. At 1 week after the vaccination, 5×10^5 B16BL6 melanoma cells were injected into the tail vein. After 2 weeks lungs were excised from these mice, and then the number of metastatic nodules was counted. For the therapeutic protocol, C57BL/6 mice were intravenously injected with 5×10^5 B16BL6 cells, and immunized by intradermal injection of transduced or intact mBM-DCs into the flank at 1×10^6 cells/50 μ l on days 0, 3, and 7 post-tumor injection. At 1 week after the final immunization, lungs were removed from these mice, and the number of metastatic nodules was counted.

Eu-release assay for cytolytic activity of NK cells and CTLs

AdRGD-gp100/mBM-DCs, Ad-gp100/mBM-DCs, and AdRGD-LacZ/mBM-DCs were prepared using corresponding vectors at an MOI of 50. Transduced or intact mBM-DCs were administered once intradermally into C57BL/6 mice at 1×10^6 cells/50 μ l. At 1 week after immunization, nonadherent splenocytes were prepared from these mice and directly used as NK effector cells. The splenocytes were restimulated *in vitro* using B16BL6 cells, which were cultured in media containing 100 U/ml mouse IFN- γ (PeproTech EC LTD) for 24 h and inactivated with 50 μ g/ml mitomycin C at 37°C for 30 min, at an effector:stimulator ratio of 10:1 in RPMI 1640 supplemented with 10% FBS, 50 μ M 2-mercaptoethanol, and antibiotics. After five days, the splenocytes were collected and used as CTL effector cells. Target cells (IFN- γ -stimulated or untreated B16BL6, EL4 cells, and YAC-1 cells) were Eu-labeled and an Eu-release assay was performed as previously described.⁵⁵ 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 detergent in all assays.

In vivo depletion analysis

C57BL/6 mice were immunized once with 1×10^6 mBM-DCs transduced with AdRGD-gp100 at an MOI of 50, and inoculated with 1×10^5 B16BL6 cells at 1 week after immunization. At 3 days before vaccination or tumor challenge, the mice received a total of three (for vaccination) or five (for tumor challenge) intraperitoneal injections of 100 μ l ascites from ICR-nu/nu mice intraperitoneally injected with GK1.5 (anti-CD4) or 53-6.72 (anti-CD8) hybridoma (kindly provided by Dr Hiroshi Yamamoto, Osaka University, Suita, Japan) or 40 μ l rabbit anti-asialoGM1 antiserum (Wako Chemical Co., Osaka,

Japan) at a 1 day interval. Depletion of T-cell subsets and NK cells was monitored by flow cytometry, which showed >90% specific depletion in splenocytes at the time of immunization or tumor challenge in each administration schedule.

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