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Efficient Antigen Gene Transduction Using Arg-Gly-Asp Fiber-Mutant Adenovirus Vectors Can Potentiate Antitumor Vaccine Efficacy and Maturation of Murine Dendritic Cells[1]

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ABSTRACT

Dendritic cells (DCs), the most effective antigen-presenting cells, are being studied as adjuvants or antigen delivery vehicles for eliciting T-cell-mediated antitumor immunity. Gene delivery to DCs provides an intracellular source of antigen for efficient and persistent loading to MHC class I molecules capable of activating $CD8^+$ CTLs, which play a central role in antitumor immunity. We previously reported that the fiber-mutant adenovirus vector (Ad) harboring the Arg-Gly-Asp (RGD) sequence in the HI loop of its fiber knob could more efficiently transduce the *LacZ* gene into both murine DC lines and normal human DCs than conventional Ad. In the present study, we compared immunological properties and vaccine efficacy of DC2.4 cells, an immature murine DC line, transduced with an ovalbumin (*OVA*) gene by fiber-mutant Ad (Ad-RGD-OVA) or conventional Ad (Ad-OVA). Ad-RGD-OVA-infected DC2.4 cells could more efficiently present OVA peptides via MHC class I molecules in a vector particle-dependent manner and induce OVA-specific CTL response by vaccination than Ad-OVA-infected DC2.4 cells. This result was correlated with the efficiency of gene transduction into DC2.4 cells by both types of Ad. Moreover, vaccination with Ad-RGD-OVA-infected DC2.4 cells could achieve an equal or greater antitumor effect against challenge with E.G7-OVA tumor cells with lower doses of Ad on infection or fewer cells for immunization than the vaccination procedure using Ad-OVA-infected DC2.4 cells. In addition, the maturation of DC2.4 cells was promoted by efficient expression of the antigen gene by the Arg-Gly-Asp fiber-mutant Ad. Flow cytometric analysis indicated enhanced expression of MHC class I and II molecules as well as CD80, CD86, CD40, and CD54, and reverse transcription-PCR analysis revealed increased levels of interleukin 12 p40 mRNA. However, infection by Ad-OVA or Ad that did not contain the cDNA of interest (Ad-Null and Ad-RGD-Null) contributed little to phenotypical changes in DC2.4 cells. On the basis of these results, we propose that DC manipulation using the Arg-Gly-Asp fiber-mutant Ad system could advance the development of more effective vaccines and allow for more convenient administration of DC-based gene immunotherapy.

INTRODUCTION

DCs[3] represent a family of so-called professional APCs that play a crucial role in the generation of T-cell-mediated immune response (1, 2). Because DCs can process intracellular and internalized antigens, present them to naive $CD4^+$ and $CD8^+$ T cells, and consequently generate a strong immune response against these antigens, DC-based immunotherapy has been studied widely as a potential approach for vaccinating against or treating cancers (3–5).

Over the past several years, a number of tumor-associated antigens have been structurally and genetically defined (6–8), and DCs have been genetically engineered to present antigenic peptides via MHC class I and possibly class II molecules (9–11). In fact, several reports on the murine system have described tumor regression mediated by the induction of antitumor CTLs after transfer of DCs transduced with an antigen gene (12, 13). Arthur *et al.* (14) reported that gene delivery to DCs by Ad was more efficient than that by other transfection techniques, including lipofection, electroporation, and calcium phosphate coprecipitation. However, extremely high doses of Ad, which may be cytopathic, are required for sufficient gene transduction into DCs because DCs express little or no CAR on their surface (15–17). The internalization of Ad into target cells is mediated by two steps: the fiber knob of Ad particles initially attaches to CAR on the cell surface (18), and then $\alpha_v\beta_3$- or $\alpha_v\beta_5$-integrins interact with the Arg-Gly-Asp motif in the Ad-penton base and facilitate internalization of the virion (19). Therefore, the manipulation of Ad, which alters Ad tropism and leads to the highly efficient delivery of foreign genes into DCs at noncytotoxic doses, can greatly advance DC-based gene immunotherapy.

We previously demonstrated that the fiber-mutant Ad harboring the Arg-Gly-Asp sequence in the HI loop of the fiber knob could more efficiently transduce the *LacZ* gene into both murine DC lines and normal human DCs than could conventional Ad and reasoned that this fiber-mutant Ad system might target α_v-integrins during the first attachment to DCs (15). In the present report, we compare the effects of DCs transduced with the *OVA* gene by fiber-mutant or conventional Ads on antigen presentation via MHC class I molecules, induction of OVA-specific CTL response, and antitumor effect to evaluate the usefulness of the Arg-Gly-Asp fiber-mutant Ad system for DC-based gene immunotherapy. Furthermore, we examine whether Ad infection influences DC maturation, which is accompanied by elevated expression of MHC molecules and adhesion and/or costimulatory molecules such as CD40, CD54, CD80, and CD86, and production of T-cell-stimulatory cytokines, including IL-12.

MATERIALS AND METHODS

Cells and Animals. DC2.4 cells, which were previously characterized as an immature murine DC line ($H\text{-}2^b$; Ref. 20), were generously provided by Dr. K. L. Rock (Department of Pathology, University of Massachusetts Medical School, Worcester, MA) and were grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 μM nonessential amino acid, 50 μM 2-ME, and antibiotics. CD8-OVA 1.3 cells, a T-T hybridoma against OVA^+ $H\text{-}2K^b$ (21), were maintained in DMEM supplemented with 10% FBS, 50 μM 2-ME, and antibiotics. EL4 murine thymoma cells ($H\text{-}2^b$) were cultured in RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, and antibiotic. E.G7-OVA cells

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[3] The abbreviations used are: DC, dendritic cell; APC, antigen-presenting cell; Ad, adenovirus vector; CAR, Coxsackie-adenovirus receptor; OVA, ovalbumin; IL, interleukin; FBS, fetal bovine serum; 2-ME, 2-mercaptoethanol; RGD, Arg-Gly-Asp; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; LPS, lipopolysaccharide; PE, phycoerythrin; RT-PCR, reverse transcription-PCR; E/T, effector:target.

(OVA cDNA transfectant of EL4 cells) were maintained in RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, and 400 μg/ml G418. The packaging cell line, 293 cells, was grown in DMEM supplemented with 10% FBS and antibiotics.

Female C57BL/6 mice, 7–8 weeks of age, were purchased from SLC Inc. (Hamamatsu, Japan) and held under specified pathogen-free conditions. All of the experimental procedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

Preparation of Recombinant Ads. The replication-deficient Ad used in the present study was based on adenovirus serotype 5 and contained a deletion in early regions 1 and 3. The Arg-Gly-Asp sequence was introduced into the HI loop of the fiber knob using the two-step method developed by Mizuguchi *et al.* (22). Both conventional and fiber-mutant Ads, which carried the desired coding cDNA under the control of the cytomegalovirus promoter, were constructed by an improved *in vitro* ligation method as described (23, 24). Both types of Ad were propagated in 293 cells, purified by two rounds of CsCl density centrifugation, dialyzed, and stored at −80°C. Vector particle titer was spectrophotometrically determined by the method of Maizel *et al.* (25).

Evaluation of Gene Transduction Efficiency of Recombinant Ad against DC2.4 Cells. DC2.4 cells cultured on 24-well plates were infected with conventional or Arg-Gly-Asp fiber-mutant Ads (Ad-GFP or Ad-RGD-GFP) carrying GFP cDNA for 1.5 h at various numbers of vector particles/cell in 400 μl of FBS-free medium. A 1-ml aliquot of culture medium was then added to each well. Two days later, gene transduction efficiency was assessed by flow cytometry on a FACScalibur flow cytometer using CellQuest software (Becton Dickinson, Tokyo, Japan), acquiring 10,000 events by forward and side scatter gating to exclude cell debris.

Antigen Presentation Assay. DC2.4 cells were seeded on a 96-well flat-bottomed culture plate at a density of 1×10^4 cells/well and cultured overnight. Cells were infected with conventional or fiber-mutant Ads (Ad-OVA or Ad-RGD-OVA), which carried OVA cDNA, for 1.5 h at various numbers of vector particles/cell in 50 μl of FBS-free medium; 150 μl of culture medium were then added to each well. Two days later or, in some cases, 6 and 24 h later, each well was washed twice with PBS, and then cells were cocultured with specific T-T hybridoma against OVA^+ $H\text{-}2K^b$ (Ref. 21; CD8-OVA 1.3 cells; 1×10^5 cells/well) at 37°C for 20 h. We assessed the response of stimulated CD8-OVA 1.3 cells 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).

Viral Transduction into DC2.4 Cells for *in Vivo* Immunization. DC2.4 cells were infected with Ad-OVA, Ad-RGD-OVA, or Ad-RGD-LacZ for 1.5 h at 1000 or 4000 vector particles/cell. After 2 days of cultivation, the cells were treated with mitomycin C (50 μg/ml) at 37°C for 30 min to inhibit proliferation. Ad-infected DC2.4 cells were then washed twice and resuspended in PBS prior to immunization.

CTL Assay. Ad-infected (4000 vector particles/cell) or uninfected DC2.4 cells were injected once intradermally into C57BL/6 mice at 1×10^6 cells/100 μl. At 1 week after immunization, the spleens were excised, and single cell suspensions were prepared. The splenocytes were restimulated *in vitro* using E.G7-OVA cells, which were treated with 50 μg/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 μM 2-ME, and antibiotics. Five days later, target cells (E.G7-OVA cells and EL4 cells) were Eu-labeled and a Eu-release assay was performed as described previously (26). Specific cytotoxic activity was determined using the following formula: specific lysis (%) = [(experimental Eu release − spontaneous Eu release)/(maximum Eu release − spontaneous Eu release)] × 100. Spontaneous Eu release from the target cells was <10% of maximum Eu release by detergent in all assays. The SE was <5% in triplicate cultures.

Tumor Rejection Assay. C57BL/6 mice were immunized by intradermal injection of Ad-infected (1000 or 4000 vector particles/cell) or uninfected DC2.4 cells into the right flank at 1×10^5 or 1×10^6 cells/100 μl. At 1 week after vaccination, 1×10^6 E.G7-OVA tumor cells were inoculated into the left flanks of the mice. Tumor size was assessed using microcalipers and was expressed as tumor volume calculated by the following formula (27): tumor volume (mm^3) = (major axis) × (minor axis)^2 × 0.5236. Mice containing tumors >10 mm or tumor volume >400 mm^3 were euthanized. On day 21 after tumor challenge, all survivors were euthanized.

FACS Analysis of Ad-infected DC Phenotype. DC2.4 cells were infected with Ad-OVA, Ad-RGD-OVA, or Ad not containing the cDNA of interest (Ad-Null and Ad-RGD-Null) for 1.5 h at 4000 vector particles/cell. DC2.4 cells treated with 10 μg/ml LPS (Nacalai Tesque, Inc., Kyoto, Japan) and 100 units/ml recombinant mouse IFN-γ (Pepro Tech EC LTD., London, England) for 24 h were used as positive controls for phenotypical DC maturation. At 24 h after infection, cell phenotype was confirmed by FACS analysis. Briefly, 100,000 cells in 100 μl of staining buffer (PBS containing 0.1% BSA and 0.01% NaN_3) were incubated for 30 min on ice with the anti-FcγRII/III monoclonal antibody (2.4G2) to block nonspecific binding of the subsequently used antibody reagents. The cells were resuspended in 100 μl of staining buffer and incubated for 30 min on ice, using the manufacturer's recommended amounts of biotinylated antibodies: 28-8-6 ($H\text{-}2K^b/D^b$), AF6-120.1 ($I\text{-}A^b$), 16-10A1 (CD80), GL1 (CD86), 3/23 (CD40), and 3E2 (CD54). The cells were then resuspended in 100 μl of staining buffer containing PE-conjugated streptavidin at a 1:200 dilution, and nonspecific binding was measured using PE-conjugated streptavidin alone. After incubation for 30 min on ice, 10,000 events of the stained cells were analyzed for surface phenotype, using FACScalibur and CellQuest software. Between all incubation steps, cells were washed three times with staining buffer. The immunoreagents used in the present study were all purchased from PharMingen (San Diego, CA).

RT-PCR Analysis for Mouse IL-12 p40. DC2.4 cells were infected with Ad-Null, Ad-RGD-Null, Ad-OVA, or Ad-RGD-OVA for 1.5 h at 4000 vector particles/cell. DC2.4 cells treated with 10 μg/ml LPS for 24 h were used as positive controls for DC maturation. At 24 h after infection, total RNA was isolated from the cells by TRIZOL reagent (Life Technologies) according to the manufacturer's instructions. Reverse transcription proceeded for 60 min at 42°C in 100 μl of a reaction mixture containing 10 μg of total RNA, 10 μl of 10× PCR buffer, 20 μl of 25 mM $MgCl_2$, 40 μl of 2.5 mM deoxynucleotide triphosphate mixture, 1 μM random hexamer, 1 μM oligo(dT), and 200 units of ReverTra Ace (TOYOBO Co., LTD., Osaka, Japan). PCR amplification of the mouse IL-12 p40 transcripts was performed in 50 μl of a reaction mixture containing 5 μl of reverse-transcribed material, 1.25 units of *Taq* DNA polymerase (TOYOBO Co., LTD.), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate mixture, and 0.5 μM primers. The sequences of the specific primers were as follows (28): forward, 5′-CTCACCTGTGACACGCCTGA-3′; reverse, 5′-CAGGACACTGAATACTTCTC-3′. After denaturation for 5 min at 95°C, 40 cycles of denaturation for 45 s at 95°C, annealing for 60 s at 48°C, and extension for 120 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 UV radiation. EZ Load (Bio-Rad, Tokyo, Japan) was used as a 100-bp molecular ladder. The expected PCR product size was 431 bp. To ensure the quality of the procedure, RT-PCR was performed on the same samples using specific primers for β-actin.

RESULTS

Gene Transduction Efficiency of Ad-GFP and Ad-RGD-GFP in DC2.4 Cells. The efficiency of foreign gene transduction and expression in DC2.4 cells was evaluated by flow cytometry using GFP-expressing Ads (Ad-GFP and Ad-RGD-GFP). As shown in Fig. 1, GFP expression in DC2.4 cells infected with Ad-GFP or Ad-RGD-GFP increased in a vector particle-dependent manner and resulted in 10- and 7-fold differences in mean fluorescence intensity at 4000 and 8000 vector particles/cell, respectively. Furthermore, Ad-RGD-GFP could deliver GFP gene into >90% of DC2.4 cells at 8000 vector particles/cell, whereas only half of the cells infected with Ad-GFP were positive for GFP expression under the same infection conditions. Cell viability was not affected by infection with either Ad type under these experimental conditions (data not shown).

MHC Class I-restricted Antigen Presentation of DC2.4 Cells Infected with Ad-OVA or Ad-RGD-OVA. To compare MHC class I-restricted antigen presentation between DC2.4 cells infected with Ad-OVA and Ad-RGD-OVA, we performed an antigen presentation assay on day 2 after infection by measuring the production of IL-2 by CD8-OVA 1.3 cells specific for OVA peptides bound to MHC class

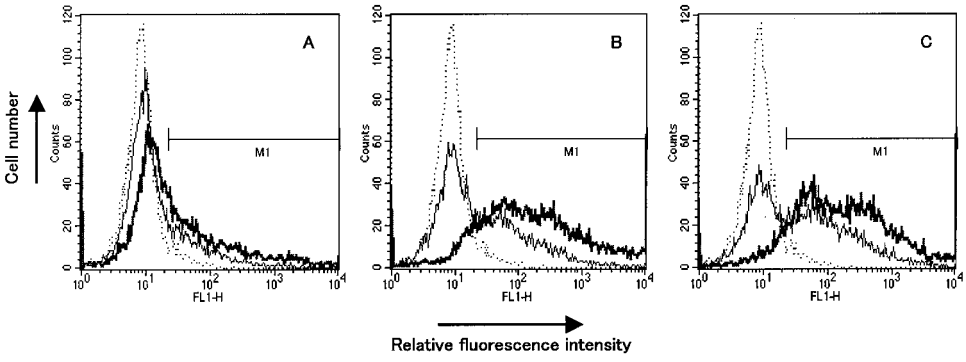

Fig. 1. GFP expression in DC2.4 cells transduced with GFP cDNA by Arg-Gly-Asp fiber-mutant or conventional Ad. DC2.4 cells were infected with Ad-GFP (*thin lines*) or Ad-RGD-GFP (*thick lines*) at 1000 (*A*), 4000 (*B*), or 8000 (*C*) vector particles/cell for 1.5 h. Two days later, GFP expression in cells was evaluated by flow cytometric analysis. *Dotted lines* represent untransfected DC2.4 cells.

Ad (vector particles/cell)		Mean of fluorescence intensity	% M1-gated
–		10.12	4.27
Ad-GFP	1000	28.47	19.66
	4000	77.33	40.14
	8000	123.44	52.93
Ad-RGD-GFP	1000	158.89	40.00
	4000	768.26	85.97
	8000	888.17	90.60

I molecules (Fig. 2*A*). DC2.4 cells infected with Ad-RGD-OVA efficiently presented OVA peptides on MHC class I molecules in a vector particle-dependent manner. In addition, the MHC class I-restricted antigen presentation of Ad-RGD-OVA-infected DC2.4 cells was >10-fold higher at 4000 vector particles/cell than that of Ad-OVA-infected cells. The difference in antigen presentation between DC2.4 cells infected with conventional and Arg-Gly-Asp fiber-mutant Ads correlated with the gene transduction efficiency of both Ad types, as shown in Fig. 1.

MHC class I-restricted antigen presentation of Ad-RGD-OVA-infected DC2.4 cells was affected by cultivation time after infection (Fig. 2*B*). DC2.4 cells cultured for 48 h postinfection at 4000 vector particles/cell more effectively stimulated IL-2 release from CD8-OVA 1.3 cells than those cultured for 6 or 24 h postinfection. We determined that after a 48 h of postinfection culture, DC2.4 cells would sufficiently express OVA and thereby most efficiently present OVA peptides via MHC class I molecules because β-galactosidase activity in DC2.4 cells infected with Ad-RGD-LacZ at 4000 vector particles/cell peaked on day 2 postinfection (data not shown). On the basis of this result, we used DC2.4 cells cultured for 2 days post-Ad infection for vaccination in subsequent *in vivo* experiments.

CTL Response in Mice Immunized with DC2.4 Cells Infected with Ad-OVA or Ad-RGD-OVA. We investigated OVA-specific CTL responses in mice vaccinated with 1×10^6 DC2.4 cells infected with 4000 Ad vector particles/cell (Fig. 3). At an E/T ratio of 100, splenocytes prepared from mice immunized with Ad-RGD-OVA- or Ad-OVA-infected DC2.4 cells injured E.G7-OVA cells that presented OVA peptide on MHC class I molecules. In addition, immunization with Ad-RGD-OVA-infected DC2.4 cells could more efficiently induce a cytotoxic response against E.G7-OVA cells than could immunization with Ad-OVA-infected DC2.4 cells. These cytotoxic effects were ascribable to OVA-specific CTLs because both the cytotoxicity against E.G7-OVA cells after immunization with Ad-RGD-LacZ-infected or uninfected DC2.4 cells and the cytotoxicity against EL4 cells after all immunizations were negligible. Therefore, it was demonstrated that the highly efficient transduction of antigen gene into DCs by Arg-Gly-Asp fiber-mutant Ad could lead not only to superior MHC class I-restricted antigen presentation on DCs, but also to more effective induction of antigen-specific CTL response on *in vivo* DC immunization.

Protection against Challenge with E.G7-OVA Tumor Cells by Immunization of DC2.4 Cells Infected with Ad-OVA or Ad-RGD-OVA. E.G7-OVA tumor cells were intradermally inoculated into C57BL/6 mice at a lethal dose (1×10^6 cells/mouse) on day 7 postvaccination with Ad-infected or uninfected DC2.4 cells. Tumor volumes and ratios of tumor-free mice on days 9 and 21 after challenge, respectively, are summarized in Table 1. Although a potent anti-E.G7-OVA tumor effect was observed in the group immunized with 1×10^6 DC2.4 cells infected with 4000 Ad-OVA vector particles/cell, this vaccine efficacy was attenuated by the decrease of either Ad dose on infection of DC2.4 cells or cell number on immunization. On the other hand, tumor formation was completely blocked in mice immunized with 1×10^5 or 1×10^6 DC2.4 cells infected with 4000 Ad-RGD-OVA vector particles/cell. In addition, mice immunized with 1×10^6 DC2.4 cells infected with 1000 Ad-RGD-OVA vector particles/cell exhibited marked vaccine efficacy that was comparable to the outcome seen after immunization with 1×10^6 DC2.4 cells infected with 4000 Ad-OVA vector particles/cell. These results dem-

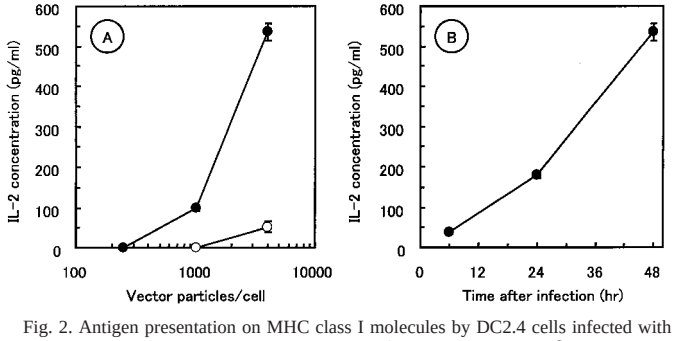

Fig. 2. Antigen presentation on MHC class I molecules by DC2.4 cells infected with Ad-OVA or Ad-RGD-OVA. *A*, DC2.4 cells were infected with Ad-OVA (○) or Ad-RGD-OVA (●) at 250, 1000, or 4000 vector particles/cell for 1.5 h. Two days later, antigen presentation was determined using CD8-OVA 1.3 cells as described in "Materials and Methods." *B*, DC2.4 cells were infected with Ad-RGD-OVA at 4000 vector particles/cell for 1.5 h. After cultivation for the indicated time, an antigen presentation assay was performed. Each point represents the mean ± SE (*bars*) of three independent cultures.

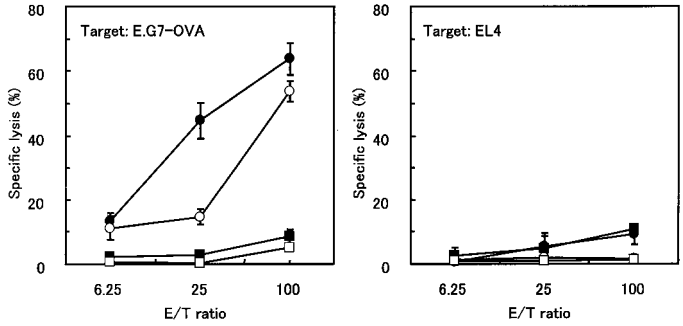

Fig. 3. OVA-specific CTL responses in mice immunized with Ad-OVA- or Ad-RGD-OVA-infected DC2.4 cells. C57BL/6 mice were immunized by intradermal injection of 1×10^6 DC2.4 cells transduced by Ad-OVA (○), Ad-RGD-OVA (●), or Ad-RGD-LacZ (■). Similarly, intact DC2.4 cells (□) were intradermally injected into mice. One week later, mice were sacrificed, their spleens were removed, and splenocytes were prepared. After *in vitro* restimulation with E.G7-OVA cells for 5 days, cytotoxic effects of splenocytes against E.G7-OVA cells or EL4 cells were evaluated by Eu-release assay. Each point represents the mean ± SE (*bars*) of 3–5 mice.

onstrate that highly efficient transduction using the Arg-Gly-Asp fiber-mutant Ad system can reduce the Ad dose for antigen gene delivery to DCs and the amount of DCs used for immunization. Thus, this system yields greater vaccine efficacy than the use of DCs transduced by conventional Ads.

Alteration in Surface Marker Expression and IL-12 p40 mRNA Expression in Ad-infected DC2.4 Cells. Phenotypical changes in Ad-infected DC2.4 cells were examined by FACS analysis using specific antibodies against MHC molecules and adhesion and/or co-stimulatory molecules (Fig. 4). Ad-RGD-OVA-infected DC2.4 cells markedly enhanced the expression of the MHC class I and class II molecules as well as CD80, CD86, CD40, and CD54, a response comparable with phenotypical changes in DC2.4 cells matured after treatment with a combination of LPS and IFN-γ. In contrast, only a slight increase was observed in MHC class I molecules and CD40 expression in DC2.4 cells infected with Ad-RGD-Null, Ad-Null, or Ad-OVA. These data suggested that Ad infection had little direct effect on phenotypical changes in DC2.4 cells, but that DC maturation was promoted by efficient transduction and expression of antigen gene by Arg-Gly-Asp fiber-mutant Ad.

We next examined the effect of Ad infection on expression levels of mouse IL-12 p40 mRNA in DC2.4 cells by RT-PCR analysis (Fig. 5). PCR products derived from IL-12 p40 mRNA were not detected in intact, Ad-Null-infected, or Ad-RGD-Null-infected DC2.4 cells. In contrast, transcription of IL-12 p40 in DC2.4 cells was up-regulated by Ad-OVA or Ad-RGD-OVA infection as well as LPS treatment. Our results suggested that the difference in IL-12 p40 mRNA levels between DC2.4 cells infected with OVA expression cassette-containing Ads and nonexpression Ads may arise as a result of expression of antigenic proteins in DCs on Ad-mediated gene transfer, which may be a factor in DC maturation. However, further analysis is required for confirmation of this theory.

DISCUSSION

Effective induction of antigen-specific CTL response is more important for antitumor immunity than the enhancement of the humoral immune response. Because priming of CTL responses requires MHC class I-restricted presentation of the relevant antigen by professional APCs capable of providing costimulation, vaccines and immunotherapies, which can preferentially deliver antigens to the MHC class I pathway in APCs, are critical for establishing effective host tumor immunity. MHC class I-presented peptides are derived in most situations exclusively from endogenous antigens synthesized by cells, including DCs. Gene delivery to DCs provides an intracellular source of antigen for efficient and persistent loading to MHC class I molecules. However, DCs are relatively resistant to gene transduction even when Ads, which can provide high-level transduction efficacy to a broad range of cells, are applied. Previous reports have indicated that the relative resistance of DCs to Ad-mediated gene transfer is attributable to a lack or low levels of CAR expression, which is an essential first step for Ad entry into target cells (15–17). Additionally, we designed a fiber-mutant Ad containing the Arg-Gly-Asp sequence in the HI loop of the fiber knob based on the Ad entry pathway and successfully used this fiber-mutant Ad system for efficient LacZ gene expression in DCs (15).

In the present study, we initially compared the efficiency of gene transduction into DC2.4 cells, an immature murine DC line (20), between a Arg-Gly-Asp fiber-mutant Ad (Ad-RGD-GFP) and a conventional Ad (Ad-GFP; Fig. 1). Although Ad-GFP delivered GFP gene into only ~50% of DC2.4 cells at 8000 vector particles/cell, >80% of Ad-RGD-GFP-infected DC2.4 cells were positive at an excess of 4000 vector particles/cell. In addition, the application of Ad-RGD-GFP increased the efficacy of GFP-expression in DC2.4 cells by 5–10-fold over Ad-GFP application. We therefore established that Arg-Gly-Asp fiber-mutant Ad could promote not only the level of gene expression, as indicated by the total activity of Ad-treated DCs, but also the ratio of cells that were subjected to foreign gene transfer. We believe that this advantage of the Arg-Gly-Asp fiber-mutant Ad system will allow better use of limited DCs collected from patients on antigen delivery for DC-based gene immunotherapy. Moreover, DC2.4 cells infected with Ad-RGD-OVA could effectively present OVA peptides via MHC class I molecules in a vector particle- and a time-after-infection-dependent manner (Fig. 2). This finding demonstrated that the levels of MHC class I-restricted presentation by DCs transduced by Arg-Gly-Asp fiber-mutant Ad directly reflected the levels of antigen expression in cells. In a previous report, we indicated that DCs loaded with OVA proteins complexed with Lipofectin, a cationic liposome, could present OVA peptides via MHC class I molecules. We determined that this phenomenon was caused by the enhancement of exogenous OVA delivery into the classical MHC class I pathway in DCs as a consequence of efficient internalization and accumulation of OVA by endocytosis, and perhaps phagocytosis, and the leakage of OVA from endosomes to cytosol (29). When MHC class I-restricted OVA presentation was compared under optimal conditions for antigen delivery, Ad-RGD-OVA-infected DC2.4 cells were significantly more effective than DC2.4 cells loaded with Lipofectin-OVA complexes (data not shown). Therefore, the data strongly suggested that the levels of antigen expression in DCs on transduction using Arg-Gly-Asp fiber-mutant Ad were substantially higher than the levels achieved by accumulation of exogenous antigenic proteins by phagocytosis.

We also evaluated vaccine efficacy using Ad-RGD-OVA-infected

Table 1 *Summary of anti-E.G7-OVA tumor effect by immunization with DC2.4 cells infected with Ad-RGD-OVA or Ad-OVA*

Ad treatment for DC2.4 cells (vector particles/cell)	Number of administered DC2.4 cells	Tumor volume[a] (mm^3)	Tumor-free mice[b]
Ad-RGD-OVA (4000)	1×10^6	0.0 ± 0.0	6/6
Ad-RGD-OVA (4000)	1×10^5	0.0 ± 0.0	6/6
Ad-RGD-OVA (1000)	1×10^6	17.0 ± 11.1	4/6
Ad-OVA (4000)	1×10^6	20.4 ± 16.2	4/6
Ad-OVA (4000)	1×10^5	72.6 ± 30.3	0/6
Ad-OVA (1000)	1×10^6	48.4 ± 19.2	1/6
Controls	1×10^6	144.8 ± 48.2	0/6

[a] Day 9 after tumor challenge; tumor volume (mm^3) = (major axis) × (minor axis)^2 × 0.5236. Data are means ± SE of 6 mice.
[b] Day 21 after tumor challenge; (tumor-free mice)/(tumor-challenged mice).

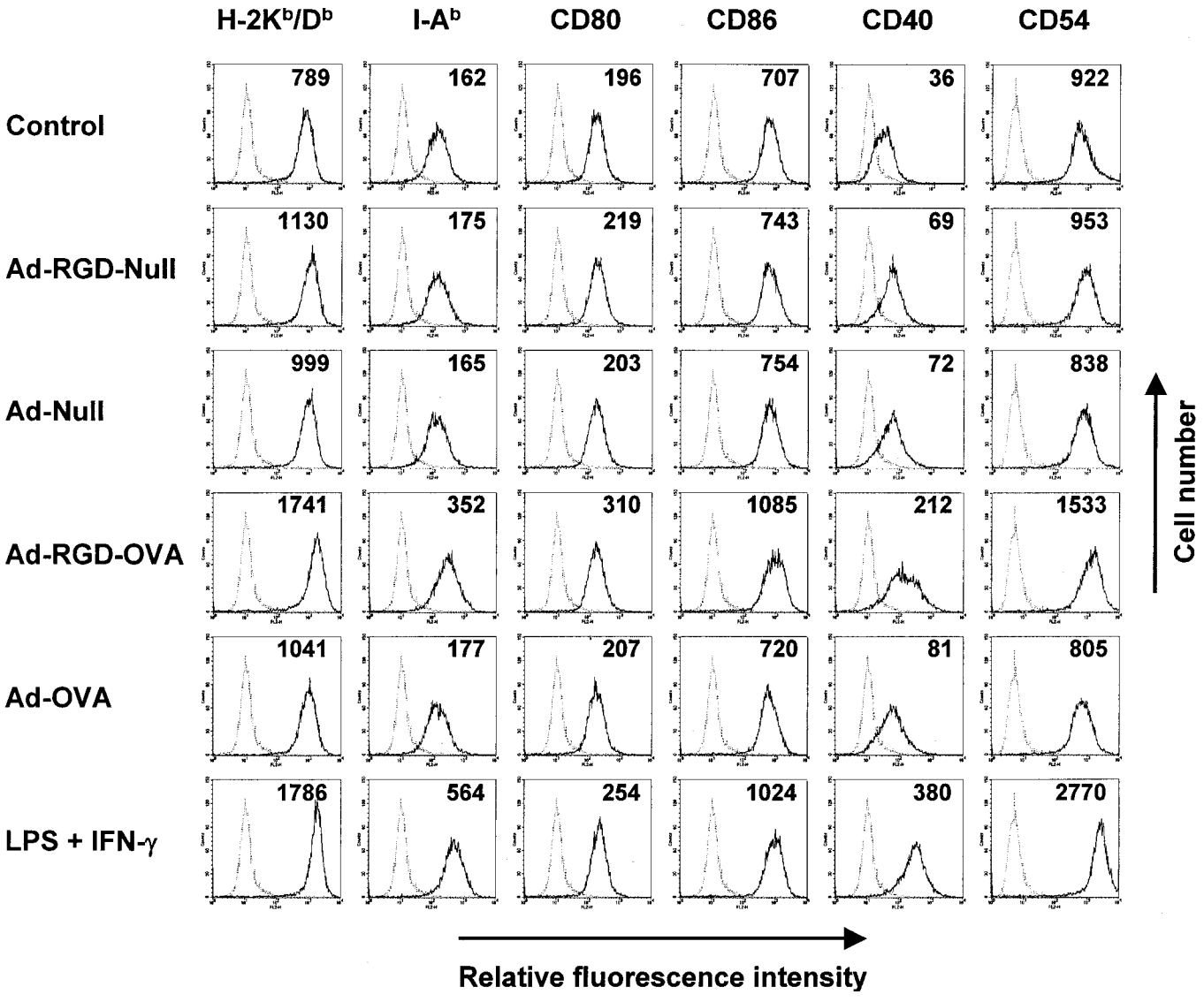

Fig. 4. Immunofluorescence analysis of Ad-infected DC2.4 cells. DC2.4 cells were infected with Ad-RGD-Null, Ad-Null, Ad-OVA, or Ad-RGD-OVA at 4000 vector particles/cell for 1.5 h. DC2.4 cells treated with 10 μg/ml LPS plus 100 units/ml IFN-γ for 24 h were used as positive controls for phenotypical DC maturation. At 24 h after infection, cells were stained by indirect immunofluorescence using biotinylated monoclonal antibodies of the indicated specificities followed by PE-conjugated streptavidin as described in "Materials and Methods." *Dotted lines* represent cells stained by PE-conjugated streptavidin only. Value in the *upper right-hand corner* of each *panel* represents the mean fluorescence intensity in flow cytometry analysis in the presence of specific antibodies.

DC2.4 cells to show the usefulness of the Arg-Gly-Asp fiber-mutant Ad system in DC-based gene immunotherapy. Several reports have described that vaccination with DCs transduced with antigen genes (12, 13) or cytokine genes (30–32), using conventional Ad at relatively high doses, could induce potent tumor immunity in murine tumor models. However, because Ads are cytopathic at high doses, less Ad is preferred in DC-based gene immunotherapy to prevent damage to DCs from adenoviral toxicity. We observed that mice immunized with Ad-RGD-OVA-infected DC2.4 cells showed more effective OVA-specific CTL responses on day 7 postimmunization than mice immunized with Ad-OVA-infected DC2.4 cells (Fig. 3). In addition, vaccination with Ad-RGD-OVA-infected DC2.4 cells could achieve equal or greater growth inhibition of E.G7-OVA tumor at lower doses of Ad on infection or by use of fewer cells on immunization than in the vaccination procedure using Ad-OVA (Table 1). Therefore, the Arg-Gly-Asp fiber-mutant Ad system may contribute to the development of effective, safe, and clinically simple DC-based gene immunotherapies by reducing the Ad dose needed for gene delivery to DCs and by reducing the amount of DCs collected and prepared from patients for administration.

A number of reports have demonstrated that the ability of DCs to act as potent APCs for the induction of T cell responses is promoted

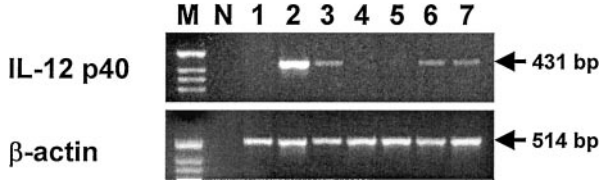

Fig. 5. RT-PCR analysis of murine IL-12 p40 in Ad-infected DC2.4 cells. DC2.4 cells were infected with four types of Ad at 4000 vector particles/cell and then cultured for 24 h. *Lane M*, 100-bp molecular ladder; *Lane N*, PCR using water as template; *Lane 1*, RT-PCR products from intact DC2.4 cells; *Lane 2*, RT-PCR products from B16/EMC cells (murine IL-12 transfectant); *Lane 3*, RT-PCR products from DC2.4 cells after treatment with LPS for 24 h; *Lane 4*, RT-PCR products from Ad-Null-infected DC2.4 cells; *Lane 5*, RT-PCR products from Ad-RGD-Null-infected DC2.4 cells; *Lane 6*, RT-PCR products from Ad-OVA-infected DC2.4 cells, *Lane 7*, RT-PCR products from Ad-RGD-OVA-infected DC2.4 cells.

by maturation. This is accompanied by elevated expression of MHC molecules and adhesion and/or costimulatory molecules such as CD40, CD54, CD80, and CD86, and production of T-cell-stimulatory cytokines, including IL-12 (33–35). DCs mature in response to stimulation by virus- and bacterium-derived materials, such as LPS (36, 37), CpG motif (38, 39), and double-strand RNA (40). We, therefore, investigated whether Ad infection influenced DC maturation. Ad-RGD-OVA infection enhanced the expression of MHC class I and II molecules as well as CD80, CD86, CD40, and CD54 on DC2.4 cells, although few phenotypical changes were observed in response to Ad-RGD-Null, Ad-Null, or Ad-OVA infection (Fig. 4). Furthermore, an increase in IL-12 p40 mRNA levels was observed in DC2.4 cells infected with Ad-RGD-OVA or Ad-OVA, but not in Ad-RGD-Null- or Ad-Null-infected DC2.4 cells (Fig. 5). Our data suggest that the sufficient expression of antigenic proteins in DCs by Arg-Gly-Asp fiber-mutant Ad infection might be more closely related to DC maturation than to the increase in Ad particles capable of invading DCs. Several researchers have reported that Ad infection could promote DC maturation (41–43), and Morelli *et al.* (44) argued that Ad-infected DCs matured via an nuclear factor-κB-dependent pathway, regardless of transgenic sequences and Ad genome transcription. On the other hand, Dietz *et al.* (45) demonstrated that the expression of CD83 and IL-12 p40 was unaffected by Ad infection and transferred gene expression. Further studies of the relationship between Ad infection and DC maturation are required for preparation of DCs that exhibit more effective immunological properties in DC-based gene immunotherapy.

In conclusion, DCs efficiently transduced with antigen gene using Arg-Gly-Asp fiber-mutant Ad could more effectively present antigenic peptides via MHC class I molecules and induce antigen-specific immune responses by vaccination than DCs transduced using conventional Ad. Furthermore, high expression of antigenic proteins on transduction using Arg-Gly-Asp fiber-mutant Ad could induce maturation of DCs, thereby priming and stimulating antigen-specific T cells more effectively. We are confident that DCs manipulated by the Arg-Gly-Asp fiber-mutant Ad system will prove to be a very useful and powerful tool for future clinical DC-based vaccination strategies.

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Efficient Antigen Gene Transduction Using Arg-Gly-Asp Fiber-Mutant Adenovirus Vectors Can Potentiate Antitumor Vaccine Efficacy and Maturation of Murine Dendritic Cells

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