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Efficient Gene Transduction by RGD-fiber Modified Recombinant Adenovirus into Dendritic Cells

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Dendritic cells (DC) are important antigen-presenting cells in the development of an anti-tumor T cell response. To extend the range of current immuno/gene therapies, we tested luciferase-expressing RGD-adenovirus (Ad) (Ad5lucRGD)-mediated transduction into DC. Phenotypically characterized DC were generated from peripheral blood $CD14^+$ cells by incubation with granulocyte-macrophage colony-stimulating factor, interleukin-4 and tumor necrosis factor α. On the 7th day of culture, the cells became mature DC with a $CD1a^+$, $CD11c^+$, $CD80^+$, $CD83^+$, $CD86^+$, human leukocyte antigen (HLA)-DR^+, $CD14^-$ phenotype. The expression of $\alpha_v\beta_3$ integrin was enhanced on day 3 and returned to the basal level on day 7. We then compared the transduction efficiency of an Ad5lucRGD system to that using conventional Ad, in cells harvested on days 1, 3 and 7 of culture. Luciferase activity was negligible in AdCMVLuc, but remarkable in cells processed with Ad5lucRGD. Activity was maximal in cells that had been cultured for 3 days. Recombinant Ad5 fiber knob protein blocked AdCMVLuc- and Ad5lucRGD-mediated gene transduction by 90% and 20%, respectively. Surface markers and cytokine production were not affected by Ad5lucRGD-mediated transduction.

Key words: Adenoviridae — Gene tranfer — Dendritic cell — Integrins — Coxsackie-adenovirus receptor

Dendritic cells (DC) are important in the induction of anti-tumor immunity.[1)] Clinical trials have therefore been initiated to generate activated DC by culturing precursor cells with cytokine cocktails such as that used for tumor antigen pulsing and gene transduction procedures.[2–5)] Adenovirus (Ad) vectors have generally been applied for gene transduction, partly because of their ability to efficiently deliver genes to a broad array of target cells.[6,7)] However, the utility of Ad vectors is limited when the expression level of Coxsackie virus or Ad receptors (CAR) on target cells is low, such as on most immunocompetent cells.[8–10)] On the other hand, recombinant Ad that contains fibers incorporating an RGD peptide (Ad5lucRGD) may augment gene delivery to target cells via a CAR-independent cell-entry mechanism,[11)] especially its attachment. If so, this system might be a useful tool with which to develop immuno/gene therapy. In this study we tested the transduction efficiency of Ad5lucRGD with DC generated from peripheral blood $CD14^+$ cells.

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MATERIALS AND METHODS

Cell lines 293 cells (human kidney epithelial cells transformed with Ad5 DNA) and A549 cells (human alveolar carcinoma cells) were obtained from American Type Culture Collection (Rockville, MD). 293 cells were maintained with Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 2 m*M* L-glutamine, 1% pyruvate, 2% bicarbonate, 100 U/ml streptomycin (GIBCO BRL, Gaithersburg, MD) and 10% fetal calf serum (FCS) (Dainippon Pharmaceutical Co., Ltd., Osaka) and A549 were maintained in DMEM supplemented with 10% FCS (Dainippon Pharmaceutical Co., Ltd.). These cells were incubated in a water-vapor-saturated atmosphere containing 5% CO_2 at 37°C.

Preparation of $CD14^+$ cells from peripheral blood and cell culture Buffy coat, derived from 400 ml of whole blood drawn from a healthy volunteer, was supplied by the Japanese Red Cross Blood Center (Tokyo). We obtained mononuclear cells (MNC) using Lympho-sepal density-gradient medium (Immuno-Biological Laboratories, Gunma). $CD14^+$ cells were then isolated from MNC by using the magnetic cell sorting (MACS) system. Over

3×10^7 $CD14^+$ cells of >90% purity were generally isolated from 5×10^8 MNC. We derived DC from $CD14^+$ monocytes as reported by Pickl *et al.*[12] Briefly, isolated $CD14^+$ cells were cultured with RPMI-1640 supplemented with 2 m*M* L-glutamine, 1% pyruvate, 2% bicarbonate, 100 U/ml penicillin, 100 U/ml streptomycin (GIBCO BRL) and 10% FCS in the presence of 50 ng/ml rhGM-CSF, 1000 U/ml rhIL-4 and 50 U/ml rhTNF-α (all from Sigma, St. Louis, MO). Cells were incubated in a water-vapor-saturated atmosphere containing 5% CO_2 at 37°C.

Flow cytometric characterization of cultured cells Cultured cells were characterized by flow cytometry using a FACScan cytometer (Becton Dickinson, Sunnyvale, CA). All monoclonal antibodies (MoAbs) were commercially obtained and used with appropriate isotype controls. The reagents for $\alpha_v\beta_3$ (clone LM609) and $\alpha_v\beta_5$ (clone P1F6) were obtained from Chemicon International, Inc., Temecula, CA; human leukocyte antigen (HLA)-D and CD11c were from Becton Dickinson (San Jose, CA); CD14, CD80, CD86 were from Pharmingen (San Diego, CA) and CD83 and CD1a were from Immunotech (Marseilles, Cedex, France). On days 1, 3 and 7, cultured cells were harvested and resuspended in 10% FCS-RPMI1640 medium at a density of 1×10^5/tube. After centrifugation for 5 min at 2000 rpm, the cells were resuspended in 50 μl of phosphate-buffered salts (PBS) containing 1 μl of antibodies and left for 30 min at 4°C. The cells were washed twice with PBS, then resuspended in 10 μl of PBS containing the second-stage antibodies, $F(ab')_2$ of fluorescence isothiocyanate (FITC)- or phosphatidylethanolamine (PE)-conjugated goat anti-mouse IgG, at a dilution of 1:100 at 4°C for 30 min. The cells were washed twice, resuspended in PBS and stored at 4°C until analysis. Data are shown as forward scatter (FSC), side scatter (SSC), FL-1 indicating $\alpha_v\beta_3$ or $\alpha_v\beta_5$, and FL-2 indicating CD14, CD1a or CD83.

Virus preparation The conventional Ad vector AdCMV-Luc contains a firefly luciferase-expressing cassette, whereas Ad5lucRGD contains both recombinant fiber-RGD protein and a firefly luciferase-expressing cassette.[13] These vectors were propagated on 293 cells and purified by centrifugation in CsCl gradients by a standard protocol. The titer of infectious virus particles on 293 cells was determined by the method of Saito *et al.*[14]

Virus infection and quantitation of expressed luciferase Cultured $CD14^+$ cells were harvested on days 1, 3 and 7 and resuspended in 10% FCS-RPMI medium at a density of 1.5×10^5/tube. After centrifugation, cells were resuspended in 500 μl of infection medium including recombinant Ad at a multiplicity of infection (MOI) of 1 and 3 and incubated for 2 h at 37°C. The infected cells were washed twice with 10% FCS-RPMI medium, re-suspended in 1 ml of 10% FCS-RPMI, seeded in 24-well culture plates and incubated at 37°C. On the following day, the cells were collected and luciferase activity was assayed using a Promega luciferase assay System (Promega, Madison, WI) according to the manufacturer's protocol. Luciferase expression was assessed using a luminometer (Berthold, Badwildbad, Germany).

Effect of Ad5lucRGD on the maturation of cultured $CD14^+$ cells On day 3, cultured cells were incubated with Ad5lucRGD at an MOI of 1, then culture was continued for the next 4 days, and the expression of DC markers was tested by FACS. To evaluate the influence of Ad5lucRGD virus infection on the production of cytokines, the culture supernatants were harvested on day 7 and the concentrations of interferon-γ (IFNγ) and interleukin-12 (IL-12) were measured using an ELISA system.

CAR expression on cultured cells We evaluated the time-dependent expression of CAR on cultured $CD14^+$ cells. Recombinant soluble fiber knob protein (100 μl) combined with a sequence encoding amino-terminal 6-His was added to a pellet of cultured $CD14^+$ cells. After washing twice with PBS, 50 μl of anti-6-His mouse monoclonal antibody, tetra-sigma His antibody (10× dilution, Qiagen, Valencia, CA), was added as a second antibody. After washing twice, the cells were stained with FITC-conjugated goat anti-mouse IgG. The positive controls were HeLa cells.

Recombinant fiber proteins and blocking studies Recombinant Ad5 fiber knob protein was expressed in *Escherichia coli* and purified by immobilized metal ion affinity chromatography (IMAC) on Ni-nitrilotriacetic acid (NTA)-Sepharose (Qiagen) as described.[11] Cultured $CD14^+$ cells (day 3) were incubated in either 10% fetal bovine serum (FBS)-RPMI1640 or 10% FBS-RPMI1640 containing recombinant Ad5 fiber knob[11, 13] at 100 μg/ml for 10 min at room temperature, then AdCMVLuc or Ad5lucRGD was added at a final concentration of 1 or 3 MOI for 2 h at 37°C. The unbound virus was aspirated and fresh 10% FBS-RPMI1640 was added. After incubation at 37°C for 24 h, the cells were lysed and luciferase activity was determined.

RESULTS

Maturation of $CD14^+$ cells The results of a flow cytometric analysis of cells after incubation with granulocyte-macrophage coloney-stimulating factor (GM-CSF), IL-4 and tumor necrosis factor-α (TNF-α) are shown in Fig. 1. Levels of the DC markers CD1a and CD83 were enhanced on day 7, whereas that of the monocyte marker CD14 was decreased on day 3 and disappeared by day 7. Additionally, 7-day-cultured DCs also express the markers CD11c, CD80, CD86 and HLA-DR (data not shown); the morphology is shown in Fig. 2. These results suggested that the cultivation of $CD14^+$ cells in the presence of TNF-α, GM-CSF and IL-4 induced activated mature DC.[15]

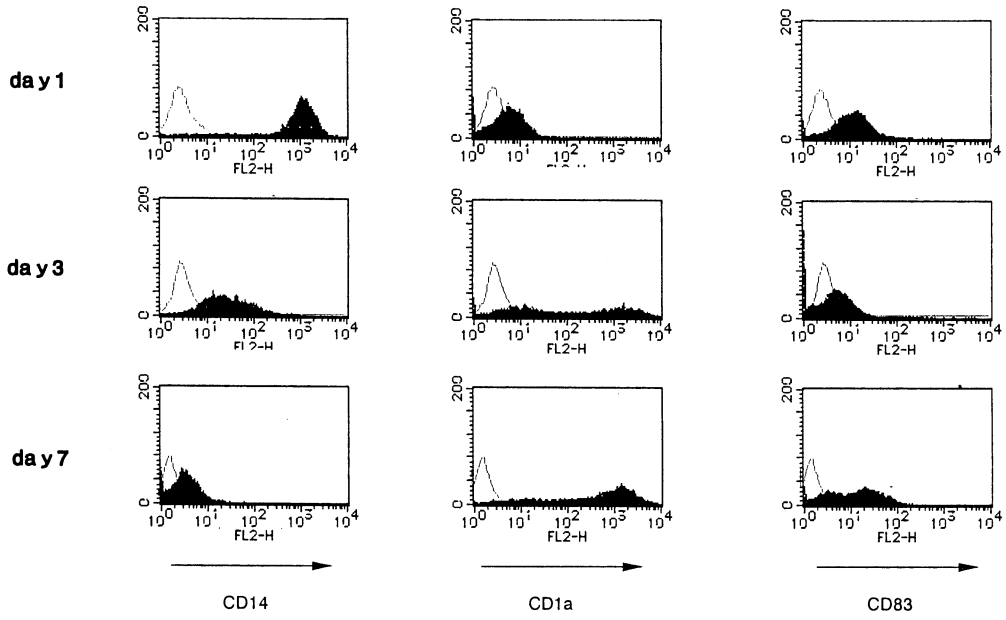

Fig. 1. Flow cytometric analysis of human dendritic cells cultured from $CD14^+$ leukocytes. Expression of CD14, CD1a and CD83 on cells cultured with GM-CSF, IL-4 and TNF-α on days 1, 3 and 7.

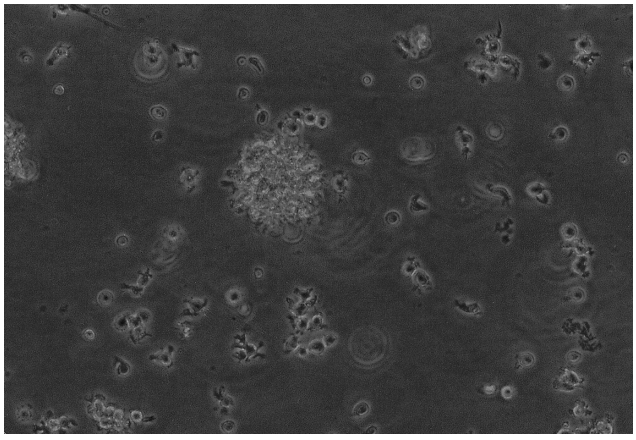

Fig. 2. Morphological appearance of 7-day-cultured $CD14^+$ cells.

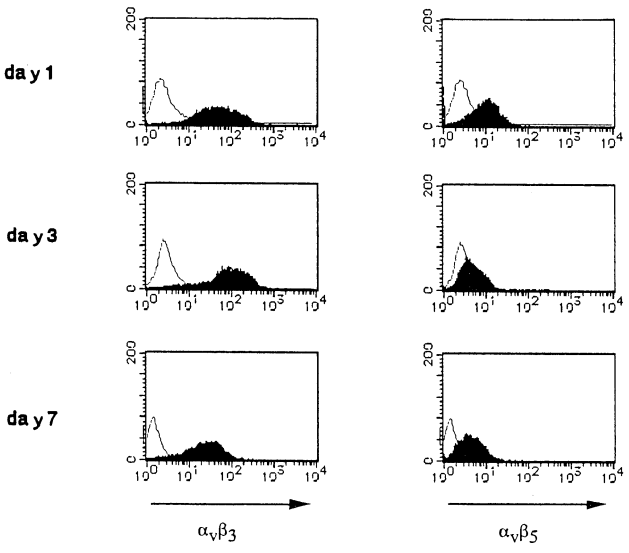

Fig. 3. Expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on cells incubated with GM-CSF, IL-4 and TNF-α on days 1, 3 and 7.

Expression of receptors and luciferase activity in cells

Under our culture conditions, $\alpha_v\beta_3$ integrin expression was enhanced on day 3 and returned to the basal level on day 7, whereas the expression of $\alpha_v\beta_5$ did not change during the same culture period (Fig. 3). The high level of CAR expression in cultured cells on day 1 gradually decreased and CAR disappeared by day 7 (Fig. 4).

The gene transduction by conventional Ad vector AdCMVLuc scarcely induced luciferase activity, but significant activity appeared in cells incubated with Ad5lucRGD. The expression levels of luciferase activity were 500, 6900 and 1900 relative luciferase unit (RLU) at an MOI of 1, and 450, 7200, and 2100 RLU at an MOI of 3 on days 1, 3 and 7, respectively (Fig. 5a). Fig. 5b shows the luciferase

activity of the virus-infected A549 cells. We also checked the transduction efficiency on K562, freshly isolated bone marrow cells and HUVEC. Ad5lucRGD showed at least 10−100 fold higher efficiency than AdCMVLuc. We confirmed AdCMVLuc can infect A549 in the same way as Ad5lucRGD, as reported previously.[16] Furthermore, we sorted CD1a$^+$ cells from 7-day-cultured CD14$^+$ cells using a magnetic cell sorting system (MACS system),[17] and compared the luciferase activity induced by AdCMVLuc with that induced by Ad5lucRGD. The level of luciferase activity of the cells infected by Ad5lucRGD was 1668 RLU, while that in the case of AdCMVLuc was 258 RLU. We also tested the infectious efficiency of the RGD-modified recombinant Ad (Ad5GFPRGD) using green fluorescent protein (GFP) expression assay on day 3 after gene transduction. While the efficiency of Ad5GFPRGD was higher than that of AdCMVGFP, the difference was not statistically significant.

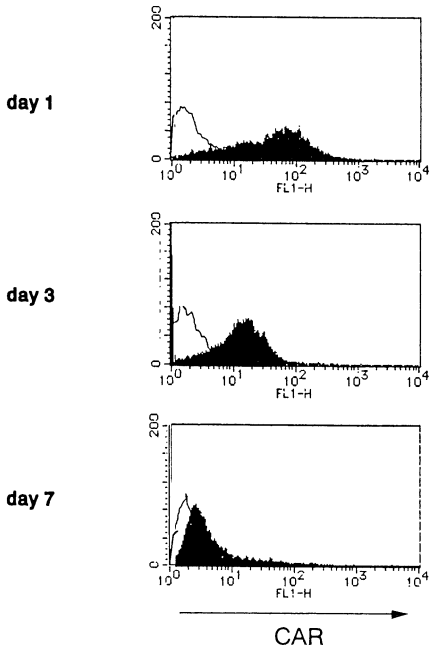

Fig. 4. Expression of CAR on cells incubated with GM-CSF, IL-4 and TNF-α on days 1, 3 and 7.

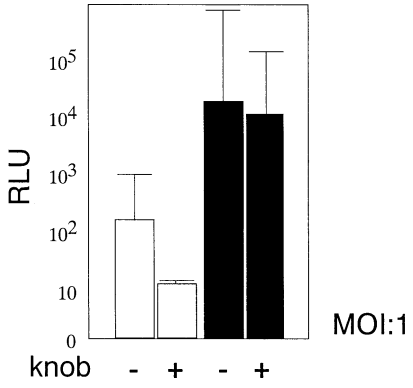

Fig. 6. Recombinant Ad5 fiber protein was added to cells prior to infection with the virus. Each point is the average of three independent measurements obtained in one experiment. □, MOI=1, AdCMVLuc; ■, MOI=1, Ad5lucRGD.

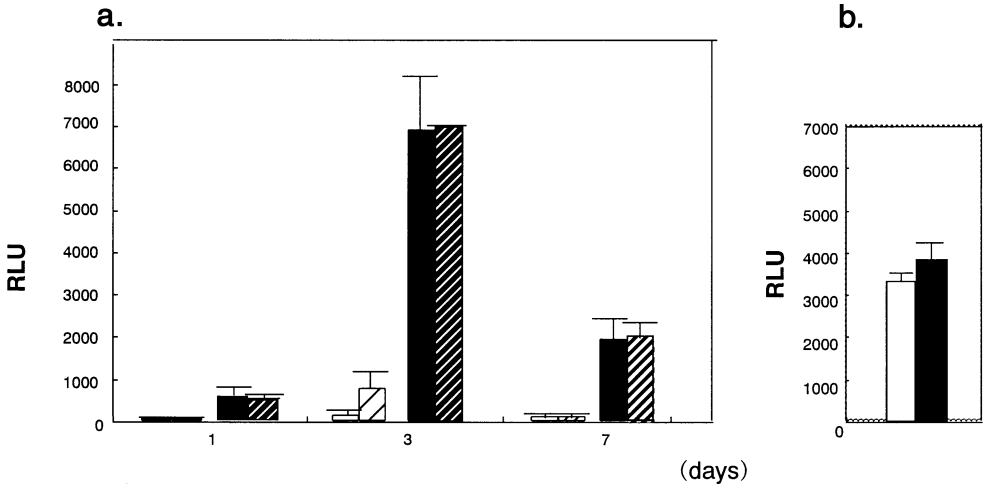

Fig. 5. a: Comparison of gene transfer to cultured DC mediated by AdCMVLuc and Ad5lucRGD. Dendritic cells were transduced with AdCMVLuc or Ad5lucRGD at an MOI of 1 and 3 on days 1, 3 and 7. b: Comparison of gene transfer to A549 mediated by AdCMVLuc and Ad5lucRGD. □, MOI=1, AdCMVLuc; ■, MOI=1, Ad5lucRGD; ▨, MOI=3, AdCMVLuc; ▨, MOI=3, Ad5lucRGD.

Blocking studies When Ad5lucRGD-mediated gene expression in cultured cells was blocked by recombinant Ad5 fiber knob protein,[18] which efficiently blocks virus binding to CAR, expression was reduced by only 20%. On the other hand, expression was reduced by 90% in conventional AdCMVLuc-mediated gene transfection (Fig. 6).

Effect of Ad5lucRGD on DC The expression of CD83, CD86 or HLA-DR did not change on day 7 of culture in either Ad5lucRGD- or control vector-infected cells, which were initially transduced on day 3, and the concentrations of IFNγ or IL-12 did not differ in DC infected with AdCMVLuc and with Ad5lucRGD (data not shown).

DISCUSSION

DC are professional antigen-presenting cells that develop in peripheral organs, where they are exposed to and capture antigens in the context of major histocompatibility complex (MHC) class I and II molecules to elicit an antigen-specific T cell response.[19–21] Other cell types, such as B-cells and macrophages, are also competent at capturing and presenting antigens. However, DCs are much more effective and are unique in their ability to prime naive T cells. Accordingly, DC express high levels of MHC class I and II, along with co-stimulatory molecules including B7.1 and B7.2 (CD80 and CD86, respectively) that are required for antigen presentation. Given their unique antigen-presenting properties, DC represents a particularly attractive cell component for the immunotherapy of diseases such as cancer.[19–24] Peptide/protein-pulsed DCs have been used in phase I/II clinical trials, with encouraging preliminary results.[25] The direct modification of antigen gene expression in DC could provide advantages over HLA-restricted peptide pulsing by extending the duration of antigen presentation on both class I and class II molecules. Additionally, the expression of transgenes for cytokines or chemokines could induce more potent immune responses than peptide alone. Successful T cell stimulation by human DC containing retrovirally transduced genes has been reported.[26–28] One limitation of gene transfer with retroviral vectors is that target cells need to be induced into the proliferating stage of the cell cycle. However, monocyte-derived DC shows poor proliferation during *in vitro* culture. Alternatively, the use of adenoviral vectors is likely to be advantageous for human gene therapy because of efficient transgene expression in non-dividing primary cell types.[29] The present study aimed to establish a technique that would enable highly efficient gene transfer into human peripheral blood DC that could be practically applied as a strategy of immuno/gene therapy.

We used the expression of luciferase as proof of infection and of effective expression of the transgene. As shown in Fig. 5, the enhanced luciferase activity was detected in the cultured $CD14^+$ cells treated with Ad5lucRGD. Infection by conventional Ad requires the adequate expression of CAR on the cell surface for attachment, and then interaction with integrin for internalization. Our results in Fig. 4 showed that CAR expression decreased through *in vitro* culture, reaching the negative control level on day 7. That might be the reason why conventional AdCMVLuc showed low transduction efficiency in our experiment. Interestingly, luciferase activity of $CD14^+$ cells cultured for 1 day with AdCMVLuc was extremely low even though these cells express CAR receptor. Previous reports suggested that integrins play a key role in internalization of Ad and their expression level directly reflected the extent of Ad-mediated transduction.[30] We guess that the basal levels of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ expression afforded low transduction efficiency. Other α_v integrins and other RGD-binding proteins, such as fibronectin, vitronectin, thrombospondin, and von Willebrand factor, which we did not examine, might also be important. Furthermore, although we checked the transduction efficiency at MOI=3 and more, the luciferase activity of Ad5lucRGD at higher MOI was not dramatically increased. One reason for this is that Ad5lucRGD-treated cells underwent apoptosis within 3 days after gene transduction at high MOI. For example, at MOI=10 more than 65% of Ad5lucRGD-treated cells showed apoptosis and the luciferase activity was almost the same as that at MOI=3. In preliminary experiments, we also treated cultured CD14-positive cells with AdRGDGFP and AdGFP at MOI=3 and 10 on day 3 and checked the GFP expression by FACS analysis on day 3 after gene transduction. In this analysis, we set the cut-off value so that 95% of control cells were GFP-negative. At this setting, less than 1% of AdGFP-treated cells were GFP-positive and 3–5% of AdRGDGFP-treated cells were positive. These differences were not statistically significant. At MOI=10, we detected about 16% positive cells in AdRGDGFP-treated cells and 11% positive cells in Ad-GFP-treated cells (no significant difference). One reason why we could not detect a significant difference between AdRGDGFP-treated cells and Ad-GFP-treated cells might be that many AdRGDGFP-treated cells died on day 3 although almost all the Ad-GFP-treated cells were alive. We think that RGD-fiber-modified Ad provide very high transduction efficiency and might be toxic to cultured dendritic cells.

In view of recent reports suggesting "cross-talk by apoptotic bodies," in which intact DC[31–33] treated with apoptotic cells expressing tumor antigen induced strong anti-tumor immunity, the apoptotic cells produced by Ad treatment might be a strong inducer of anti-tumor immunity.

We need further studies to elucidate the molecular mechanisms of Ad transduction. Our results in Fig. 6 confirmed that an alternative, CAR-independent cell entry pathway is involved in transduction by Ad5lucRGD. This

may explain the significantly improved infection efficiency using Ad5lucRGD. The enhanced expression of $\alpha_v\beta_3$ probably contributes further to the high rate of infection with Ad5lucRGD on day 3, since interaction between cellular integrins and various proteins containing an RGD tripeptide has been well established. This interaction plays an important role in a variety of fundamental biological processes, including cell adhesion and viral infection. Another advantage of Ad in *ex vivo* manipulation of cells for human use is their inability to integrate into the genome and change the oncogenic potential of manipulated cells. We demonstrated that infected DCs are equivalent to mature blood-derived DC with regard to phenotype, morphology, and functions. Further, the cell processing steps, including gene transduction, in our strategy are easier than those reported previously by others. In summary, Ad5lucRGD was extremely effective for gene transfer and expression in human DC. Further analysis of the cell entry mechanism of Ad5-RGD and evaluation of cytotoxic T lymphocyte (CTL) induction/enhancement using a tumor antigen expression vector Ad5-RGD are in progress.

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