AdRGD-CCL17, -CCL20, -CCL21, -CCL22, or control AdRGD-Null at an MOI of 10. These transduced tumor cells were intradermally inoculated into H-2 haplotype-matched mice, and tumor growth was compared with that of intact tumors. As shown in Fig. 3, the tumorigenicity of B16BL6 and CT26 cells was hardly affected by transfection with the control vector, whereas OV-HM cells transfected with AdRGD-Null exhibited a slight delay of tumor growth as compared with intact OV-HM cells. Among 20 combinations of chemokine and tumor cells, an obvious tumor-suppressive effect was recognized in mice inoculated with CCL19/B16BL6, XCL1/B16BL6, or CCL22/OV-HM cells. In contrast, the in vivo growth of CCL27/B16BL6, CCL20/CT26, CCL22/CT26, XCL1/CT26, and CCL20/OV-HM cells was the same as that of the control vector-transfected cells, and only a slight delay of tumor growth was observed in five B16BL6 groups (CCL17, CCL20, CCL21, CCL22, and CX3CL1), five CT26 groups (CCL17, CCL19, CCL21, CCL27, and CX3CL1), and two OV-HM groups (CCL17 and CCL21). Importantly, CCL22/OV-HM cells not only demonstrated considerable retardation in tumor growth but were also completely rejected in 9 of 10 mice. In the rechallenge experiment, these cured mice were intradermally injected with 1×10^6 parental OV-HM cells or irrelevant B16BL6 cells at 3 months after the initial challenge. Five of six mice rechallenged with OV-HM cells remained tumor-free for more than 2 months, whereas rechallenging with B16BL6 cells perfectly developed palpable tumors in three additional mice within 2 weeks (data not shown). These results indicate the generation of long-term specific immunity against OV-HM tumor in mice that could once reject CCL22/OV-HM cells.

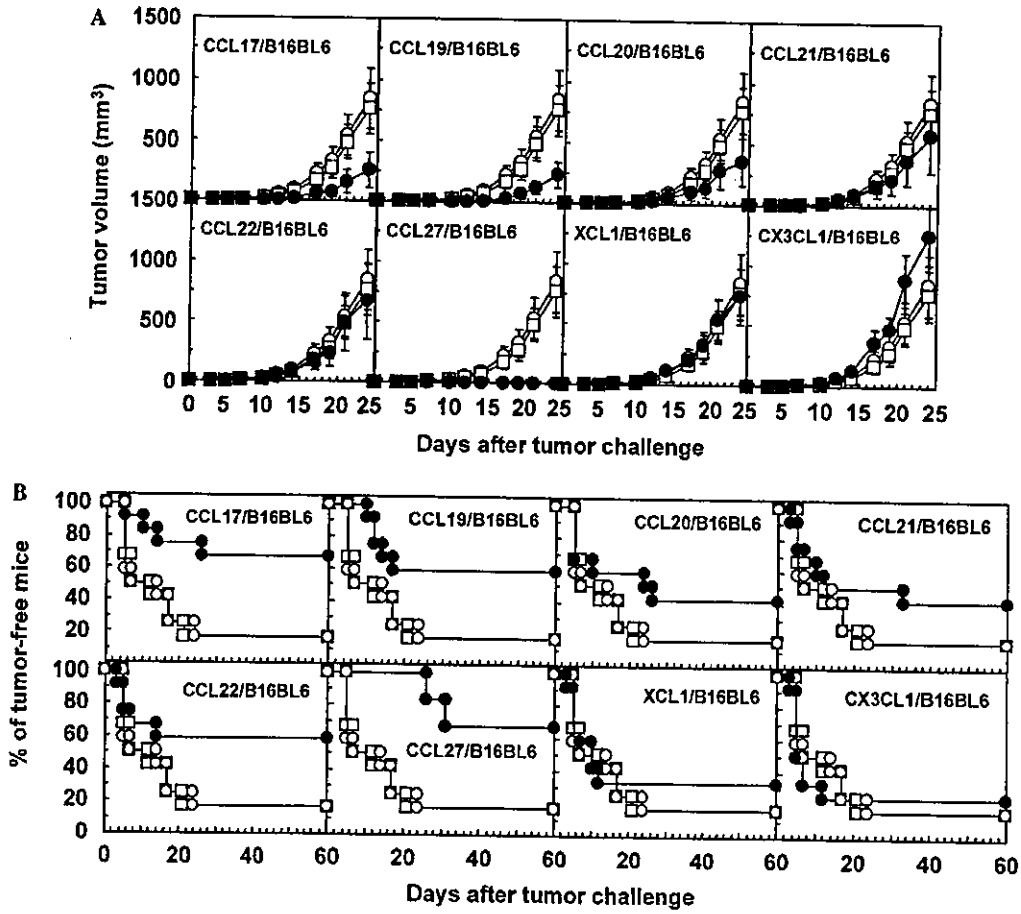

Fig. 4. Growth and rejection ratio of B16BL6 cells transduced with the chemokine gene in mice primed with melanoma-associated antigen. C57BL/6 mouse bone marrow-derived DCs were transfected with AdRGD-gp100 at an MOI of 50 for 2 h and they were intradermally injected into the right flank of syngeneic mice at 5×10^5 cells. At 1 week after the vaccination, these mice were intradermally inoculated in the left flank with 2×10^5 B16BL6 cells transfected with each chemokine-expressing AdRGD at an MOI of 400 for 24 h (●). Likewise, intact B16BL6 cells (○) or AdRGD-Luc-transfected B16BL6 cells (□) were inoculated in the gp100-primed mice, which were used as control groups. (A) The tumor volume was assessed three times per week. Each point represents the mean ± SE of results obtained from 12 mice. (B) Data are expressed in terms of the percentage of mice without visible tumor against the total mice tested in each group.

Growth and rejection ratio of chemokine gene-transfected B16BL6 cells in gp100-primed mice

For the purpose of examining the influence of chemokine against tumor growth in hosts specifically sensitized with tumor-associated antigen, B16BL6 cells transfected with chemokine-expressing AdRGD were inoculated into mice that were vaccinated with DCs presenting gp100, one of the identified melanoma-associated antigens. As shown in Fig. 4A, CCL17-, CCL19-, or CCL27-transfection was very effective for tumor growth suppression in gp100-primed mice, whereas AdRGD-Luc-transfected B16BL6 cells did not show any difference in tumor growth as compared with intact cells. A remarkable enhancement was observed in the complete rejection ratio at 2 months after tumor inoculation in the CCL22-transfected group as well as in the CCL17, CCL19, and CCL27 groups (Fig. 4B). Also, transfection with AdRGD-CCL20 or -CCL21 moderately improved the rejection ratio of B16BL6 cells in gp100-primed mice. XCL1 did not show a notable difference in both the growth and the rejection ratio of B16BL6 cells as compared with the control groups, and CX3CL1-transfected cells showed a tendency to promote tumor growth as compared with the intact B16BL6 cells.

Discussion

The application of chemokines to cancer immunotherapy has recently attracted great attention, because of their chemoattractant activity for a variety of immune cells as well as the angiostatic activity of some chemokines such as CXCL9 and CXCL10. In addition, it has been known that some tumor cells express a lower level of chemokines than normal cells [22]. Therefore, we may obtain novel cancer gene immunotherapy capable of demonstrating an excellent therapeutic effect, if a specific chemokine is adequately expressed at a local tumor site by gene transduction. The tumor-suppressive activity of several chemokines was observed in actuality in various experimental tumor models using the in vitro transfection method [8,23–27]. We also previously demonstrated that a CC family chemokine, CCL27, could suppress OV-HM tumor growth via transfection into the tumor cells due to the local recruitment of T cells and natural killer (NK) cell, whereas the transfection of CX3CL1 did not show a significant effect [19]. However, there are few reports comparing the antitumor activity of a specific chemokine between distinct tumor models.

Thus, we screened the potential anti-tumor activity of CCL17, CCL19, CCL20, CCL21, CCL22, CCL27, XCL1, and CX3CL1 in three murine tumor models by in vitro transfection. In order to efficiently transduce the chemokine gene into tumor cells, we constructed the AdRGDs carrying an expression cassette containing each murine chemokine cDNA by an improved in vitro ligation method. AdRGD can enhance gene transduction efficiency against a variety of tumor cells as compared with conventional Ad because of the expression of the RGD sequence, the αv-integrin-targeting peptide, at the HI-loop in their fiber knob [11–13]. Moreover, the improved in vitro ligation method enables speedy construction of a series of AdRGDs for screening by easy insertion of the expression cassette for the concerned gene into E1-deletion site [15,16]. With respect to the RT-PCR analysis and in vitro chemotaxis assay, transfection using our eight AdRGDs encoding the chemokine gene allowed tumor cells to express each corresponding chemokine mRNA and secrete a specific chemokine protein in a biologically active form (Figs. 1 and 2). Murine B16BL6 melanoma, murine CT26 colon carcinoma, and murine OV-HM ovarian carcinoma cells were transfected with chemokine-expressing AdRGDs at the MOI, which was suitable for adequately introducing a reporter gene into each tumor cell in preliminary examinations. To address the possibility of growth suppression depending on the cytotoxicity by AdRGD itself or secreted chemokine, we evaluated the viability of tumor cells transfected with each AdRGD at 48 h after transfection by MTT assay. The in vitro growth of the transfected cells was essentially identical to that of the intact cells with the exception of the OV-HM cells transduced with AdRGD-CCL19 or -XCL1 (data not shown). Therefore, CCL19 and XCL1 were excluded from the in vivo experiment using OV-HM cells.

Although a slight delay in tumor growth was observed in most of the combinations of tumor cells and chemokines, only CCL19/B16BL6, XCL1/B16BL6, and CCL22/OV-HM cells demonstrated a notable tumor-suppressive activity in immunocompetent mice as compared with the control vector-transfected cells (Fig. 3). In particular, CCL22-transfection was highly efficacious for the repression of OV-HM tumor growth, since complete rejection was observed in 9 of 10 mice. Furthermore, five of six cured mice could resist rechallenge with parental OV-HM cells, indicating the generation of a long-term tumor-specific immunity by rejection of CCL22/OV-HM cells. CCL22 exhibits a strong chemoattractant activity for a variety of immune cells including T cells, NK cells, and DCs. Guo et al. [28] also reported that the intratumoral injection of conventional Ad encoding human CCL22 resulted in a marked tumor regression in a murine 3LL lung carcinoma model with significant cytotoxic T lymphocyte (CTL) activity. However, CCL22-transfection did not show an antitumor effect in both B16BL6 and CT26 cells, and the chemokine that could demonstrate an obvious suppressive effect common to tumor cells of all three kinds was not found even if the results of CCL27/OV-HM and

CX3CL1/OV-HM cells, which were examined in our previous work [19], were included. In addition, some chemokines such as CCL17, CCL20, CCL21, and CX3CL1 failed to induce a notable suppressive effect against all three kinds of tumors although their chemoattractant activity for immune cells was reported. These complicated phenomena suggest that the anti-tumor effect via chemokine expression might be affected by several factors, for example, (1) the immunogenicity of the tumor cells, (2) the quantity and population ratio of the immune cells accumulated in tumor tissue, and (3) the activation state and deviation of the immune system in host.

We considered that not only the accumulation but also the activation of immune cells in tumor tissue is very important in cancer immunotherapy using chemokines, because several approaches that combined chemokines with cytokines or costimulatory molecules resulted in the synergic enhancement of anti-tumor activity as compared with the application of chemokine alone [29–32]. DCs, unique antigen-presenting cells capable of priming and stimulating naive T cells, not only play a critical role in establishing antigen-specific adaptive immune responses but also regulate the innate immune system [33–35]. Because of these properties, DCs loaded with tumor-associated antigen are ideal for generating a primary immune response against cancer as "nature's adjuvant" [33,36]. We previously reported that the vaccination of DCs transfected with gene coding gp100, one of the melanoma-associated antigens, by AdRGD could induce anti-B16BL6 tumor immunity based on increasing cytotoxic activities of NK cells and gp100-specific CTLs [21]. When chemokine-transfected B16BL6 cells were inoculated into mice vaccinated with gp100-expressing DCs, CCL17, CCL19, CCL22, and CCL27 could promote resistance to tumor formation (Fig. 4). Upon comparing the outcomes in Figs. 3A and 4, CCL19 demonstrated B16BL6 tumor-suppressive activity in both intact and gp100-primed mice, whereas the enhancement of the anti-tumor effect by CCL17, CCL22, or CCL27 was observed only in gp100-primed mice. Surprisingly, the anti-tumor activity of XCL1 detected in intact mice was lost in gp100-primed mice, and the CX3CL1/B16BL6 tumor grew more rapidly than the control tumor in gp100-primed mice. We speculated that the weak anti-B16BL6 tumor activity of XCL1 or CX3CL1 was masked by vaccine efficacy of gp100-expressing DCs, and that the angiogenic activity of CX3CL1 [37] might be emphasized in a tumor-specifically sensitized host.

Collectively, our data suggested that the tumor-suppressive activity of chemokine was greatly influenced by the kind of tumors and the activation state of the immune cells, and that a search for an effective chemokine for cancer immunotherapy should be performed in an experimental model that can reflect clinical status, including the immunogenicity of tumors, the state of the host's immune system, and the combination of other treatments, as much as possible.

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RNA interfering approach for clarifying the PPARγ pathway using lentiviral vector expressing short hairpin RNA

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Abstract Peroxisome proliferator-activated receptor γ (PPARγ) plays a central role in adipocyte differentiation and insulin sensitivity. Although PPARγ also appears to regulate diverse cellular processes in other cell types such as lymphocytes, the detailed mechanisms remain unclear. In this study, we established a lentivirus-mediated short hairpin RNA expression system and identified a potent short hairpin RNA which suppresses PPARγ expression, resulting in marked inhibition of preadipocyte-to-adipocyte differentiation in 3T3-L1 cells. Our PPARγ-knockdown method will serve to clarify the PPARγ pathway in various cell types in vivo and in vitro, and will facilitate the development of therapeutic applications for a variety of diseases.

Key words: Peroxisome proliferator-activated receptor γ; RNA interference; Short hairpin RNA; Lentiviral vector; Adipocyte

1. Introduction

The peroxisome proliferator-activated receptor (PPAR) family was discovered as an orphan nuclear receptor, and three different subtypes were subsequently identified, namely PPARα, PPARδ/β and PPARγ. PPARγ is abundantly expressed in adipose tissue and plays a key role in adipocyte differentiation and insulin sensitivity [1]. Recently, our group and other researchers reported that PPARγ is also an attractive therapeutic target as it can play an important role in immune responses, especially in transcriptional regulation of inflammatory responses [2–5].

The biological role of PPARγ had been widely investigated by using PPARγ-deficient mice generated by targeted disruption of the PPARγ gene. Since homozygous PPARγ-deficient mice ($PPAR\gamma^{-/-}$) are embryonic lethal due to placental dysfunction [1], heterozygous mice ($PPAR\gamma^{+/-}$) have been used to investigate the role of PPARγ in vivo experiments. However, $PPAR\gamma^{+/-}$ mice seem to be of limited use in some experiments, because PPARγ also appears to regulate diverse cellular processes in cells that show lower levels of PPARγ expression in comparison to adipose tissue [6,7].

RNA interference (RNAi) is a powerful technique for selectively silencing the expression of genes. Recent work has provided a system for the stable expression of short interfering RNA (siRNA) in mammalian cells, which is generally based on the expression of short hairpin RNA (shRNA) under the control of the RNA polymerase III promoter [8–11]. The technique has allowed for the development of a new approach for achieving targeted gene silencing of disease-associated genes in animal models as well as in cultured cells.

Lentiviral vectors (LVs) are a promising tool for exogenous gene transfer among gene transfer vehicles, because LVs have the advantages of infecting non-dividing cells and being stably integrated into the host genome resulting in long-term expression of transgene [12–16]. Furthermore, recent reports have demonstrated that virus-mediated RNAi could provide long-term silencing in mammalian cells [9,17,18]. In the present study, we attempted to develop a technique for suppressing the expression of PPARγ in vivo and in vitro. We established a lentivirus-mediated shRNA expression system and identified a potent shRNA target sequence in the coding region of PPARγ mRNA. This approach has enabled us to clarify a novel role of PPARγ.

2. Materials and methods

2.1. Vector construction

Vectors were constructed using standard cloning procedures. H1-RNA promoter was amplified from human genomic DNA (Clontech, Palo Alto, CA, USA) using the following primers: 5′-CCATGGAATTCGAACGCTGACGTC-3′ and 5′-GCAAGCTTAGATCTGTGGTCTCATACAGAACTTATAAGATTCCC-3′. The amplified polymerase chain reaction (PCR) product was inserted into the *Eco*RI-*Bgl*II site of pHM5 [19], generating pHM5-H1. pHM5-H1 was designed to express shRNA upon the insertion of an appropriate sequence into the *Bgl*II/*Xba*I site (Fig. 1A). Oligonucleotides encoding

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Abbreviations: LV, lentiviral vector; shRNA, short hairpin RNA; MOI, multiplicity of infection; PPAR, peroxisome proliferator-activated receptor; GPDH, glycerol-3-phosphate dehydrogenase; BRL, rosiglitazone (BRL-49653)

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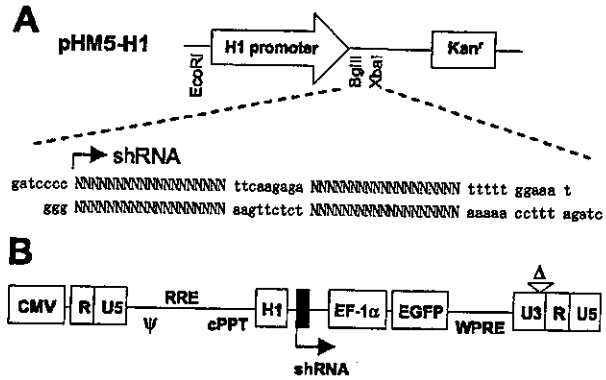

Fig. 1. Vector construction. A: pHM5-H1 was constructed as described in Section 2. Oligonucleotides encoding both strands of the targeting sequence, a spacer sequence which provided a loop structure and a transcriptional termination signal T5 were annealed and inserted into *BglII*/*XbaI* sites in pHM5-H1. B: Schematic representation of self-inactivating (SIN) LV plasmid (CS-H1-shRNA-EG). CMV: cytomegalovirus promoter, Ψ: packaging signal, RRE: rev responsive element, cPPT: central polypurine tract, H1: human H1 promoter, EF-1α: human elongation factor 1α subunit gene promoter, EGFP: enhanced green fluorescent protein, WPRE: woodchuck hepatitis virus posttranscriptional regulatory element. Δ: deleting 133 bp in the U3 region of the 3′ long terminal repeat.

both strands of the targeting sequence were annealed and inserted into *BglII*/*XbaI* sites of pHM5-H1 (Fig. 1A and Table 1). The sequence was verified on a DNA sequencer (ABI Prism 310, Applied Biosystems) and the cassette containing the H1 promoter plus the shRNA was transferred to a self-inactivating (SIN) LV construct, generating CS-H1-shRNA-EG (Fig. 1B).

2.2. Preparation of LV expressing shRNA (LV-shRNA)

LVs pseudotyped with vesicular stomatitis virus G glycoprotein (VSV-G) were prepared according to a previously described method [15,20,21]. Briefly, 293T cells were transfected with four plasmids: packaging construct (pMDLg/pRRE), VSV-G-expressing construct (pMD.G), Rev-expressing construct (pRSV-Rev), and SIN vector construct (CS-H1-shRNA-EG). Vector supernatant was concentrated by ultracentrifugation, and the pellet was resuspended in Hanks' balanced salt solution. Vector titers, which can be detected by enhanced green fluorescent protein (EGFP) expression under the control of a human elongation factor 1α subunit gene promoter, were determined by infection of HeLa P4 cells with serial dilutions of the vector stocks, followed by fluorescence-activated cell sorter (FACS) analysis for EGFP-positive cells.

2.3. Cell culture and infection of LV-shRNA

3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The 3T3-L1 cells were infected with viral stocks at a multiplicity of infection (MOI) of 50 or 200, followed by FACS analysis for EGFP expression. Transduction efficiencies were 66.57% ± 1.44 at 50 MOI and 91.64% ± 1.07 at 200 MOI, expressed as S.E.M. The transduced 3T3-L1 cells were grown and then used in subsequent experiments.

2.4. Differentiation protocol

Induction of adipocyte differentiation was performed essentially as described [22]. Two days after confluence (day 0), the medium was replaced with differentiation medium containing rosiglitazone (BRL, 1 μM), insulin (INS, 150 nM), dexamethasone (DEX, 1 μM) and 3-isobutyl-1-methylxanthine (IBMX, 100 μM), which was changed every 3 days thereafter until analysis.

2.5. Measurement of adipocyte differentiation

Differentiation of 3T3-L1 preadipocytes to adipocytes was monitored by measurement of intracellular lipid accumulation using Oil red O staining and glycerol-3-phosphate dehydrogenase (GPDH) activity on day 9. Cultured cells were fixed for 2 h with 10% formalin in isotonic phosphate buffer and then washed with distilled water. The cells were then stained by complete immersion in a working solution (0.3%) of Oil red O for 4 h. Excess dye was removed by exhaustive washing with water. The GPDH activity was measured using a GPDH assay kit (Hokudo, Hokkaido, Japan).

2.6. RNA isolation and reverse transcription (RT) PCR analysis

Total RNA was extracted from the 3T3-L1 cells infected with each kind of LV-shRNA using Tri-Reagent (Sigma). First-strand cDNA was generated from 1 μg of RNA by using oligo(dT_{12-18}) primer (Invitrogen) and SuperScript III RNase H Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. The reverse transcription reaction mix was amplified with the following pair of oligonucleotides specific for murine PPARα, PPARδ, PPARγ2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): PPARα, 5′-CGACAAGTGTGATCGGAGCTGCAAG-3′ and 5′-GTTGAAGTTCTTCAGGTAGGCTTC-3′; PPARδ, 5′-GGCCAACGGCAGTGGCTTCGTC-3′ and 5′-GGCTGCGGCCTTAGTACATGTCCT-3′; PPARγ2, 5′-GCTGTTATGGGTGAAACTCTG-3′ and 5′-ATAAGGTGGAGATGCAGGTTC-3′; GAPDH, 5′-GCTCACTGGCATGGCCTTC-3′ and 5′-ACCACCCTGTTGCTGTAGC-3′ [23]. The sample was amplified in the linear phase, optimized for each gene (PPARα: 38 cycles; PPARδ: 30 cycles; PPARγ2: 36 cycles; GAPDH: 23 cycles). All PCR products were electrophoresed on 2% agarose gel using 0.5×Tris-borate-EDTA buffer and visualized using ethidium bromide. The gel image was captured by a digital camera, and densitometric analysis was performed using NIH Image software.

2.7. Western blot analysis

Cultured cells were homogenized in Tris–HCl buffer containing a cocktail of protease inhibitors and insoluble materials were then removed by centrifugation at 4°C. The solubilized lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions at a concentration of 5 μg protein of sample per lane. Detection of PPARα, PPARδ, PPARγ and GAPDH was respectively performed with anti-PPARα polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PPARδ polyclonal antibody (Santa Cruz Biotechnology), anti-PPARγ monoclonal antibody (Santa Cruz Biotechnology) and anti-GAPDH polyclonal antibody (Trevigen, Gaithersburg, MD, USA) according to a previously described method [4]. The band intensity was quantified using NIH Image software.

2.8. Statistical analysis

All results are expressed as mean ± S.E.M. Statistical comparisons were made with Student's *t*-test or Scheffé's method after analysis of variances. The results were considered significantly different at $P < 0.05$.

Table 1
LV vectors used in this study

LV vector	shRNA target gene	shRNA target sequence
LV-shRNA-P11	PPARγ1 and 2	CAGCTCTACAACAGGCCTC
LV-shRNA-P12	PPARγ1 and 2	ATGGCCATTGAGTGCCGAG
LV-shRNA-P13	PPARγ1 and 2	TAAATGTCAGTACTGTCGG
LV-shRNA-P14	PPARγ1 and 2	TTGGCGGAGATCTCCAGTG
LV-shRNA-P15	PPARγ1 and 2	GTCTGCTGATCTGCGAGCC
LV-shRNA-P16	PPARγ1 and 2	TCACCATTTGTCATCTACG
LV-shRNA-P17	PPARγ1 and 2	GTTTGAGTTTGCTGTGAAG
LV-shRNA-P18	PPARγ1 and 2	ATGAGCCTTCACCCCCTGC
LV-shRNA-P19	PPARγ1 and 2	GATCTGCGAGCCCTGGCAA
LV-shRNA-P21	PPARγ2	ACTCTGGGAGATTCTCCTG
LV-shRNA-P22	PPARγ2	CCTTCGCTGATGCACTGCC
LV-shRNA-Lu	Luciferase	ACGCTGAGTACTTCGAAAT
LV-shRNA-Scramble	No target gene	GCGCGCTTTGTAGGATTCG
LV-EG	–	–

LV-EG has no shRNA-expressing cassette. All vectors carry an EGFP-expressing cassette as a marker gene so that the cells transduced with LV-shRNAs can be identified by green fluorescence.

3. Results and discussion

To develop an effective PPARγ-knockdown method, we constructed an LV-based siRNA system in which shRNA encoding both strands of the targeting sequence is expressed under the control of human H1 promoter [24]. A human H1 promoter was cloned to generate pHM5-H1, and oligonucleotide encoding shRNA against PPARγ mRNA was inserted (Fig. 1A). Subsequently, the cassette containing the H1 promoter plus the shRNA was transferred to the SIN LV construct (Fig. 1B). Using a shRNA target sequence against firefly luciferase, we previously demonstrated that our LV-based siRNA system effectively suppressed the target gene in mammalian cells (data not shown).

PPARγ exists as two isoforms, termed PPARγ1 and PPARγ2,

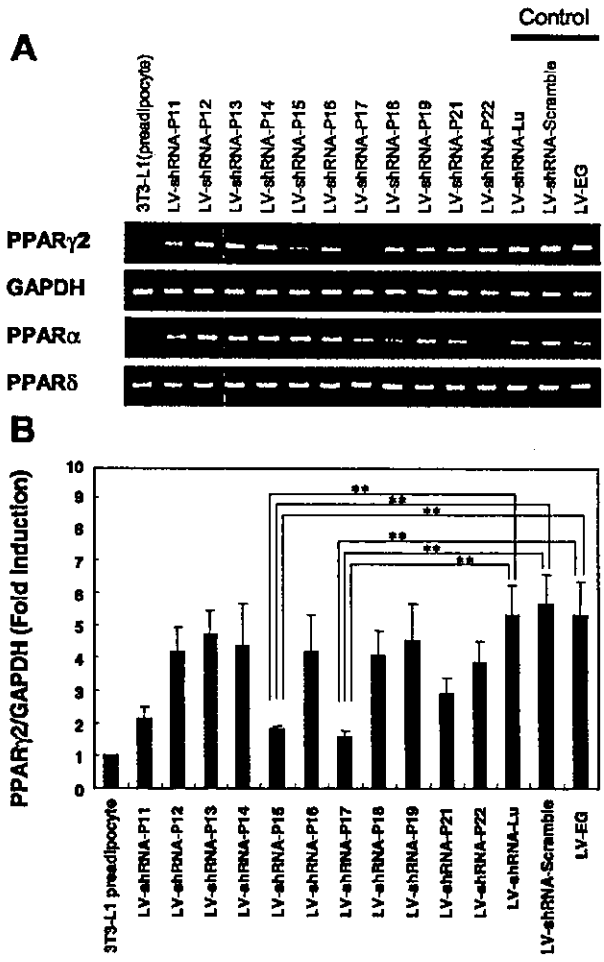

Fig. 2. Alteration of PPAR family mRNA levels in 3T3-L1 cells transduced with LV-shRNAs. A: 3T3-L1 preadipocytes were infected with each LV-shRNA (200 MOI) and then subjected to the differentiation protocol. Two days after the induction of adipocyte differentiation, mRNA levels of PPARγ2, PPARα, PPARδ, and GAPDH were determined by RT-PCR analysis. Results are representative gel images. B: Densitometric quantitation for PPARγ and GAPDH from three to four independent experiments. Each PPARγ value was normalized to the values for GAPDH and expressed as fold induction over the basal level detected in 3T3-L1 preadipocytes (bars, S.E.M.). $^{**}P < 0.01$ for LV-shRNA-P15 and -P17 compared with LV-shRNA-Lu, LV-shRNA-Scramble or LV-EG.

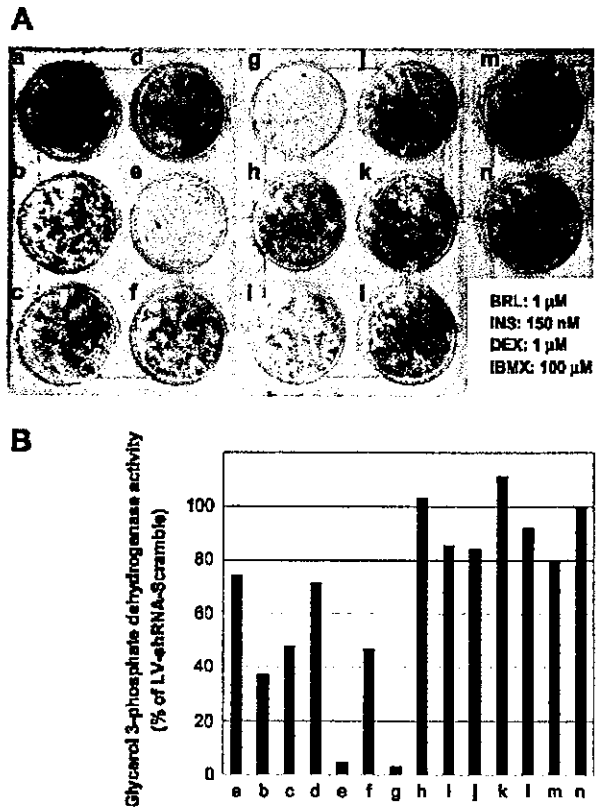

Fig. 3. Effect of LV-shRNAs on adipocyte differentiation. A: Differentiation of 3T3-L1 preadipocytes (infected with LV-shRNA; 200 MOI) to adipocytes was monitored by measurement of intracellular lipid accumulation using Oil red O staining on day 9. B: GPDH activity was measured on day 9. Data were expressed as percentage of the GPDH activity of 3T3-L1 cells which were infected with LV-shRNA-Scramble (200 MOI). a: LV-shRNA-P11; b: LV-shRNA-P12; c: LV-shRNA-P13; d: LV-shRNA-P14; e: LV-shRNA-P15; f: LV-shRNA-P16; g: LV-shRNA-P17; h: LV-shRNA-P18; i: LV-shRNA-P19; j: LV-shRNA-P21; k: LV-shRNA-P22; l: LV-EG; m: LV-shRNA-Lu; n: LV-shRNA-Scramble. Similar results were obtained in two independent experiments.

which are produced by a combination of different promoters and alternative splicing. PPARγ2 has an N-terminal extension of 30 amino acids and is very highly expressed in adipocytes [22,25]. We selected 11 target sequences in the coding region of PPARγ mRNA and constructed LV-shRNAs against PPARγ (Table 1). In the present study, LV-shRNA-Lu, LV-shRNA-Scramble and LV-EG were used as controls.

To find the most effective shRNA target sequence against PPARγ, we analyzed the silencing of PPARγ in 3T3-L1 cells during preadipocyte-to-adipocyte differentiation in which PPARγ is known to be a master regulator of adipogenesis [1,26,27]. The expression of PPARγ increases during the differentiation process and activation of PPARγ protein by its ligand leads to adipogenesis through the activation of the adipogenic gene cascade. The 3T3-L1 preadipocytes transduced with each of the LV-shRNAs, i.e. 3T3-L1 cells expressing shRNAs, as listed in Table 1, were exposed to differentiation medium (DM) 2 days after confluence (day 0). Initially, silencing of PPARγ expression was examined by RT-PCR after 2 days of culture in DM (Fig. 2). Although 3T3-L1 cells transduced with LV-shRNA-Lu, -Scramble and LV-EG showed

significant increases in the levels of PPARγ mRNA, 3T3-L1 cells transduced with LV-shRNA-P15 and -P17 retained low levels of PPARγ mRNA comparable to the level in preadipocytes maintained in normal culture medium. In contrast, the expression levels of GAPDH, PPARα and PPARδ were not altered by LV-shRNA-P15 or -P17. The other LV-shRNAs against PPARγ caused moderate decreases in the levels of PPARγ mRNA.

The differentiation of 3T3-L1 preadipocytes to adipocytes can be monitored by measurement of intracellular lipid accumulation and GPDH (an important enzyme in triglyceride synthesis) activity [28–30]. Intracellular lipid accumulation was dramatically reduced in the LV-shRNA-P15- and -P17-infected 3T3-L1 cells as shown by Oil red O staining (Fig. 3A, e: LV-shRNA-P15; g: LV-shRNA-P17). GPDH activity also demonstrated that LV-shRNA-P15 and LV-shRNA-P17 express a potent shRNA which suppresses PPARγ mRNA expression, resulting in marked inhibition of preadipocyte-to-adipocyte differentiation (Fig. 3B). We also confirmed that the expression of PPARγ-inducible genes, such as uncoupling protein-1 and adipocyte fatty acid binding protein, were inhibited in 3T3-L1 cells transduced with LV-shRNA-P15 and LV-shRNA-P17 in the presence of the PPARγ-specific ligand, BRL (unpublished data).

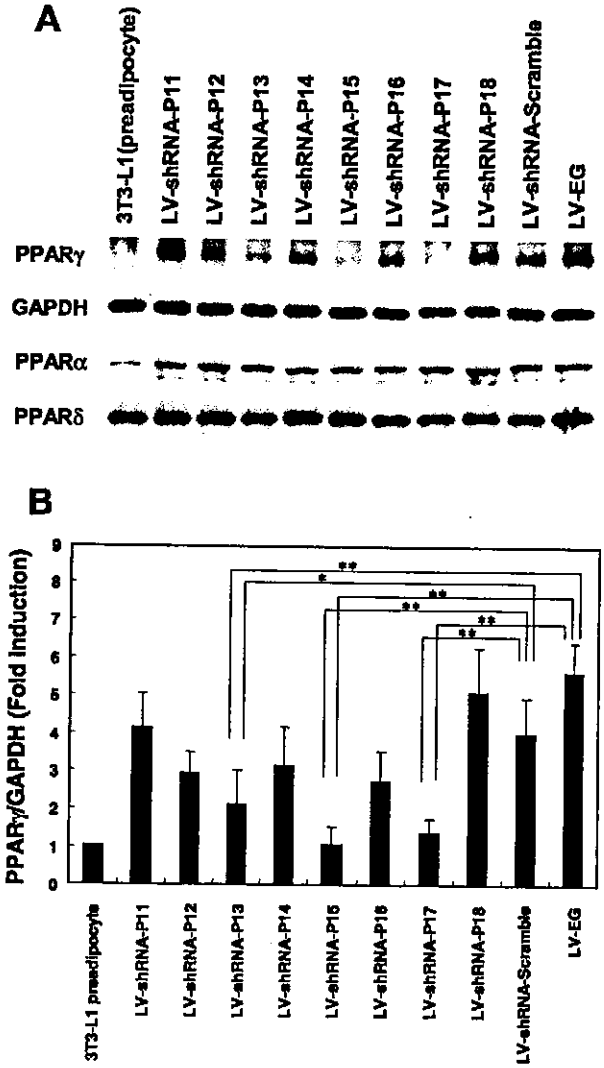

Fig. 4. Alteration of PPAR family protein levels in 3T3-L1 cells transduced with LV-shRNAs (200 MOI). A: Four days after the induction of adipocyte differentiation, the whole cell extract was analyzed by Western blotting with antibodies against PPARγ, PPARα, PPARδ and GAPDH. Results are representative of three individual experiments. B: Densitometric quantitation for PPARγ and GAPDH from three individual experiments. Each PPARγ value was normalized to the values for GAPDH and expressed as fold induction over the basal level detected in 3T3-L1 preadipocytes (bars, S.E.M.). $^{**}P < 0.01$ for LV-shRNA-P13, -P15 and -P17 compared with LV-shRNA-Scramble or LV-EG. $^{*}P < 0.05$ for LV-shRNA-P13 compared with LV-EG.

A recent study demonstrated that if the degree of complementarity to its target is reduced, siRNA can function as microRNAs which affect translational suppression without cleavage [31]. An important objective of this study was to determine whether the silencing effect of PPARγ caused by these LV-shRNAs was specific for PPARγ. In fact, several shRNA target sequences used in this study partially correspond to PPARα or PPARδ. Western blotting analysis demonstrated that PPARγ protein levels were significantly decreased in the LV-shRNA-P15- and LV-shRNA-P17-infected 3T3-L1 cells, while LV-shRNAs did not alter the amount of PPARα, PPARδ or GAPDH protein (Fig. 4). These results were consistent with the result from RT-PCR analysis (Fig. 2).

Furthermore, we examined 3T3-L1 cells exposed to either LV-shRNA-Scramble, -P15 or -P17 by fluorescent microscopy for EGFP expression to identify cells not infected with those vectors, i.e. the 3T3-L1 cells not expressing the shRNA encoded by LV-shRNA-P15 or -P17 (Fig. 5). In the case of LV-shRNA-Scramble, which expresses control shRNA, the differentiation of preadipocytes to adipocytes was not affected by infection with LV. In contrast, all of the cells infected with LV-shRNA-P15 or -P17 retained their fibroblast-like morphology. Taken together, these results indicate that our LV-shRNA-based PPARγ-knockdown method resulted in decreased PPARγ expression and specific inhibition of the PPARγ pathway, even in the case of adipocyte differentiation in which PPARγ expression is strongly induced by DM and PPARγ protein is effectively activated by the PPARγ-specific ligand used in this study, BRL.

Accessibility of the siRNA might depend on the secondary structure of the target mRNA. However, a clear correlation between either secondary structure or GC content and effectiveness of target sites has not yet been recognized. Although we designed 11 different shRNAs against PPARγ, we have not found any correlation between several factors that have been implicated in the accessibility of transcriptional/translational regulatory elements and effectiveness of target sites of shRNA until now.

In the present study, we developed a promising tool for suppressing the expression of PPARγ. Our PPARγ-knockdown method will serve to clarify the role of the PPARγ pathway in various cell types in vivo and in vitro, and will facilitate the development of therapeutic applications for a variety of diseases.

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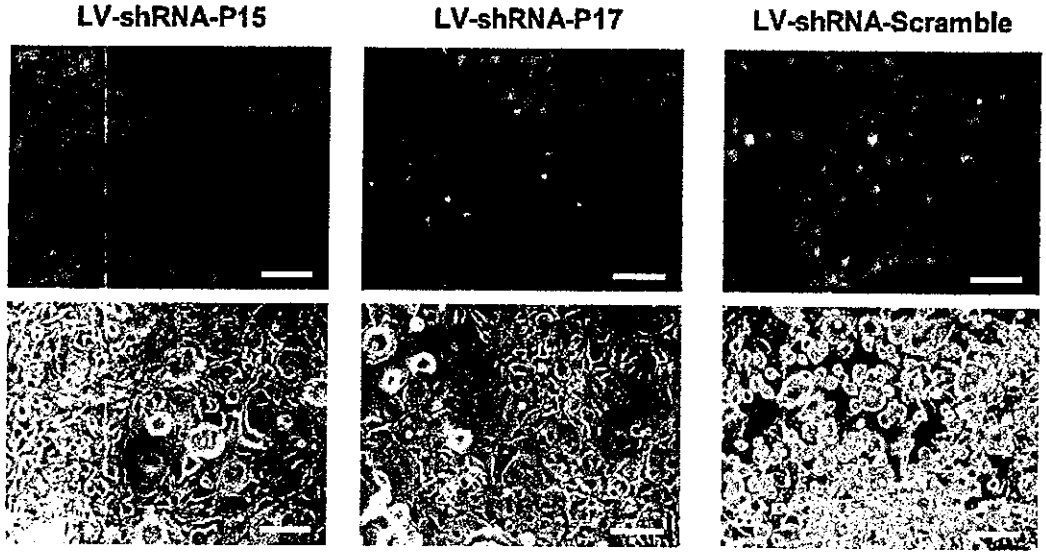

Fig. 5. Identification of the 3T3-L1 cells transduced with LV-shRNA. Brightfield and fluorescent microscopy images collected from the same field. The LV-shRNA-infected cells, which expressed EGFP, were detected as green fluorescence (upper panels) and morphologically identified mature adipocyte with a voluminous spherical shape and a large accumulation of intracytoplasmic lipid vesicles (lower panels). Bars represent 100 μm.

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Design and synthesis of a peptide-PEG transporter tool for carrying adenovirus vector into cells☆

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Abstract—The adenovirus vector is a promising carrier for the efficient transfer of genes into cells via the coxackie-adenovirus receptor (CAR) and integrins (αvβ3 and αvβ5). The clinical use of the adenovirus vector remains problematic however. Successful administration of this vector is associated with side effects because antibodies to this vector are commonly found throughout the human body. To make the adenovirus vector practicable for clinical use, it is necessary to design an auxiliary transporter. The present study describes the use of Arg-Gly-Asp(RGD)-related peptide, a peptide that binds to integrins, as an auxiliary transporter to aid efficient transport of adenovirus vector. Furthermore, poly(ethylene glycol) (PEG) was also used as a tool to modify the adenovirus such that the risk of side effects incurred during clinical application was reduced. The present study describes the design, preparation and use of (acetyl-Tyr-Gly-Gly-Arg-Gly-Asp-Thr-Pro-$_\beta$Ala)$_2$Lys-PEG-$_\beta$Ala-Cys-NH_2[(Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC] as an efficient peptide-PEG transporter tool for carrying adenovirus vector into cells. (Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC was coupled with 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester and the resulting 6-[(Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC-succinimido]hexanoic acid *N*-hydroxysuccinimide ester reacted with adenovirus. The modified adenovirus with the peptide-PEG hybrid exhibited high gene expression even in a CAR-negative cell line, DC2.4.

Gene therapy is a new field of clinical treatment for intractable diseases. A key aspect of gene therapy, and a major determinant of its success, lies in the vector used for transgenesis. Adenovirus vectors (Ad) are widely used as vectors for gene therapy experiments[2] since they exhibit highly efficient transduction and gene expression. Ad infection is performed in two steps; firstly Ad binds to its receptor, coxackie-adenovirus receptor (CAR),[3] followed by receptor-mediated endocytosis via αvβ3 and αvβ5 integrins.[4] Both of these integrins are known as a receptor of peptides containing the Arg-Gly-Asp (RGD) sequence. Ad is able to transfer genes efficiently into both dividing and nondividing cells, but some problems remain in terms of its clinical application. Side effects are common because antibodies to Ad are commonly found within the human body (Fig. 1).[5]

Poly(ethylene glycol) (PEG) is a low toxicity polymer and its hybrid formation (conjugation) with a protein is a method known to improve certain characteristics of the chosen protein (such as response to an antibody, response to enzymatic degradation, solubility to aqueous and organic solvents and prolongation of biological activities). As a result of this new methodology, the formation of a protein-PEG hybrid has become known as 'pegylation', a term that is now commonly used. Several studies have investigated the pegylation of Ad;[6] results demonstrated that pegylated Ad exhibited enhanced circulation and half-life in blood depending on the rate of pegylation. Transduction by the pegylated Ad was not disturbed in the presence of its antibody.[7] However the ability of the pegylated Ad to penetrate into cells

Keywords: Adenovirus vector; RGD; Poly(ethylene glycol); Transduction; Peptide synthesis.
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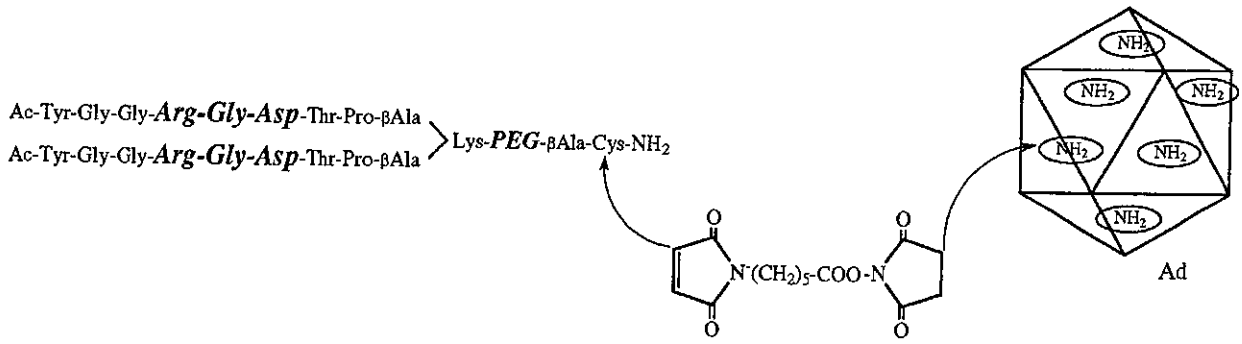

Figure 1. Design of an efficient peptide-PEG transporter tool for carrying adenovirus vector into cells via integrins.

through the coxackie-adenovirus receptor (CAR) was reduced by the steric hindrance of the associated PEG chains. To overcome this problem, an Arg-Gly-Asp(RGD)-related peptide, Tyr-Gly-Gly-Arg-Gly-Asp-Thr-Pro (YGGRGDTP),[8] was considered as a tool to allow Ad to penetrate into cells via its receptors (αvβ3 and αvβ5 integrins). The peptide was reported to show good endocytotic ability and RGD sequence in the peptide was reported to be necessary to exhibit this activity. Since the final synthetic product will be reacted with Ad by the active ester method, the RGD-related peptide should not have side chains, which will be acylated by the active ester method. YGGRGDTP has no such side chain (Fig. 2).

In an attempt to ensure efficient affinity between the peptide and integrins, a bivalent peptide derivative through Lys (K) was designed (Fig. 1). In order to prepare a hybrid of the peptide and PEG, an amino acid type PEG (aaPEG) was utilized. To introduce the peptide-PEG hybrid to Ad, a heterofunctional cross-linking reagent with amine and sulfhydryl reactivity, 6-maleim-

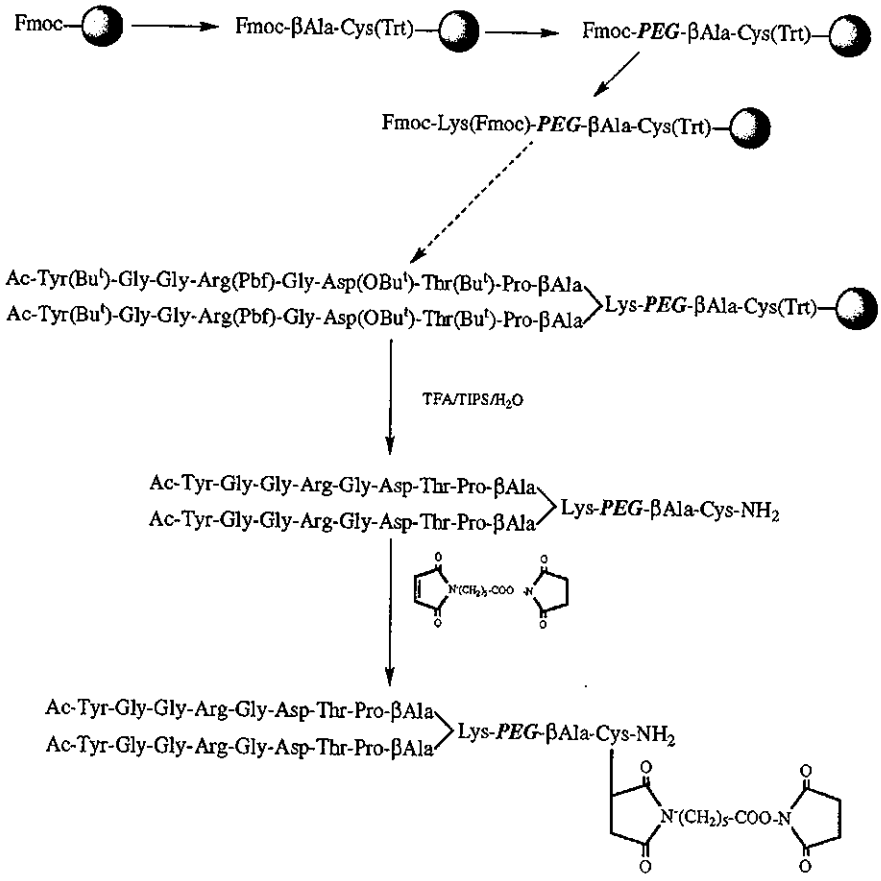

Figure 2. Synthetic scheme for the preparation of the PEG-(RGD-peptide) hybrid used to carry Ad into cells.

Table 1. Synthetic protocol for the solid-phase synthesis of Ac-YGGRGDTP)$_2$K-PEG-$_\beta$AC

Step	Reagent	Volume	Period	Time
1	20% Piperidine/DMF	5 mL	5 min	1
		5 mL	0.5–1 h	1
2	DMF (wash)	5 mL	3 min	5
3	Fmoc-amino acid derivative	0.75 mmol		
	0.5 M HBTU/HOBt/DMF	1.5 mL	2–4 h	
	2 M DIEA/NMP	0.75 mL		
4	DMF (wash)	5 mL	3 min	5

idohexanoic acid *N*-hydroxysuccinimide ester[9] (MHS), was utilized. As a result, Cys was also incorporated in the hybrid. βAla ($_\beta$A) was also incorporated into the hybrid as a spacer. The final hybrid, (acetyl-Tyr-Gly-Gly-Arg-Gly-Asp-Thr-Pro-$_\beta$Ala)$_2$Lys-PEG-$_\beta$Ala-Cys-NH_2, (Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC amide, was thus designed (Fig. 2). The hybrid was synthesized by manual solid-phase methodology using fluorenylmethoxycarbonylamino acids (Fmoc-amino acids) on Rink amide resin (0.67 mequiv L/g, PE BioSystems. 370 mg, 0.25 mmol)[10] according to the protocol shown in Table 1. The following amino acids were purchased from Watanabe Chemical Industry Ltd (Japan) and Peptide Institute Inc. (Japan); Fmoc-Arg(Pbf)-OH (Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl), Fmoc-Cys(Trt)-OH (Trt: trityl), Fmoc-Tyr(Bu^t)-OH (Bu^t: *tert*-butyl), Fmoc-Thr(Bu^t)-OH, Fmoc-Asp(OBu^t)-OH, N^α,N^ε-diFmoc-Lys-OH, Fmoc-Gly-OH, Fmoc-$_\beta$Ala-OH and Fmoc-Pro-OH. Fmoc-aaPEG-OSu (–OSu: *N*-hydroxysuccinimide ester) (MW 3400) was purchased from Shearwater polymers Inc. and MHS was purchased from Dojindo Laboratories, Japan). All of the above materials were used as supplied without any further purification. As described in the protocol (Table 1), Fmoc groups were removed by 20% piperidine/dimethylformamide (DMF) treatment and coupling reactions were performed with 0.5 M 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/DMF in the presence of 1-hydroxybenzotriazole (HOBt) in *N*-methylpyrrolidinone (NMP). Since Fmoc-aaPEG-OSu did not react with $_\beta$Ala-Cys(Trt)-Rink amide resin, HBTU, HOBt and diisopropylethylamine (DIEA) were added to the reaction mixture. The reaction did not proceed without the addition of HBTU, HOBt and DIEA. Since the deprotection reaction of the Fmoc group decreased in rate after the introduction of Fmoc-aaPEG, the following deprotection procedure was performed for 1 h. After the introduction of Lys, the amount of each added reagent was doubled, and each step involving the introduction of Fmoc-amino acid was performed using a double coupling reaction. The amino group of the *N*-terminal Tyr was acetylated with acetic anhydride. The synthetic (Ac-Tyr(Bu^t)-Gly-Gly-Arg(Pbf)-Gly-Asp-(OBu^t)-Thr(Bu^t)-Pro-$_\beta$Ala)$_2$Lys-aaPEG-$_\beta$Ala-Cys(Trt)-Rink amide resin was then treated with a mixture of trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIPS) (95:2.5:2.5), and the resulting crude peptide-PEG hybrid, (Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC, 462 mg), was purified by HPLC (Fig. 3). The purified hybrid (55 mg, 10 mmol)[11] dissolved in PBS (pH 7.4, 1 mL), and the

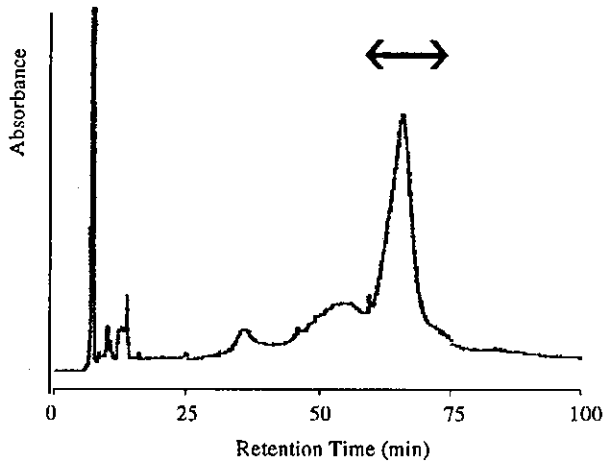

Figure 3. HPLC profile of synthetic crude (Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC-SHS. Column: DAISOPAK SP-120-5-ODS-B (20 × 250 mm). Flow rate: 10 mL/min. Eluent: CH_3CN/H_2O containing 0.05% CF_3COOH. Gradient: 10:90 → 70:30 (60 min). OD at 220 nm.

heterofunctional cross-linkage reagent (MHS 3.1 mg, 10 mmol) dissolved in dimethylsulfoxide (DMSO, 0.1 mL), were combined and the mixture stirred for 0.5 h. Since the product of this reaction, [(Ac-YGGR-GDTP$_\beta$A)$_2$K-PEG-$_\beta$AC-SHS)] (SHS: 6-succimidohexanoic acid *N*-hydroxysuccinimide ester) was easily hydrolyzed in water, the reaction mixture was frozen immediately and kept in a freezer to await the next reaction step. Purification of the reaction product by HPLC was attempted, but was not successful since the *N*-hydroxysuccinimide ester portion of the product hydrolysed easily in the presence of water. We observed that 50% of MHS was hydrolyzed at pH 7.4 after 1.5 h at room temperature (Fig. 4). Ad, which has luciferase expression ability, was modified with (Ac-YGGR-GDTP$_\beta$A)$_2$K-PEG-$_\beta$AC-SHS solution at 37 °C for 45 min with gentle stirring and the transduction efficiency of the resulting modified Ad (RGDpep-PEG-Ad) via receptor-mediated endocytosis was examined with A549 (CAR + and integrins-positive) and DC2.4 (CAR – and integrins-positive) cell lines using a Luciferase Assay System Kit (Promege, USA) and a Microlumat Plus LB 96 instrument (Perkin–Elmer, USA), after

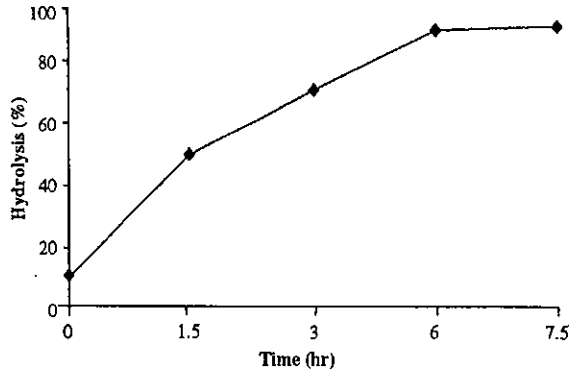

Figure 4. Hydrolysis of 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (MHS) in water at pH 7.4.

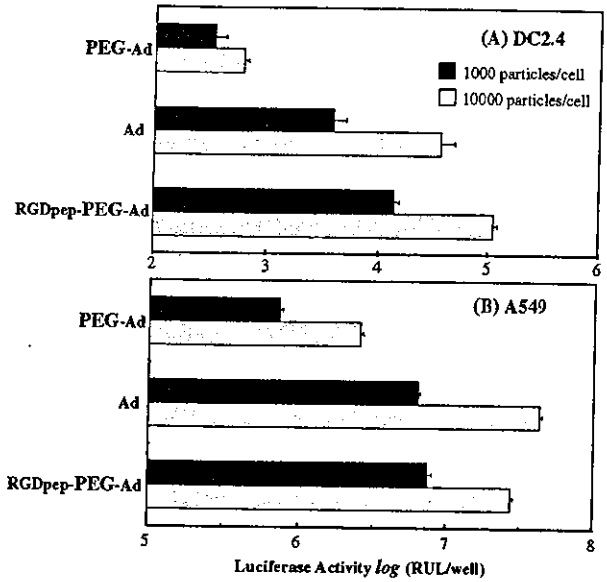

Figure 5. Transduction efficiency of RGDpep-PEG-Ad into DC2.4 (CAR −) cells and A549 (CAR +) cells. (A) DC2.4 cells (2×10^{10} cells) and (B) A549 cells (2×10^{10} cells) were transduced with 1000 and 10,000 particles/cells of Ad, PEG-Ad and RGDpep-PEG-Ad, respectively. Luciferase expression was measured after 24 h. Each point represents mean ± SD ($n = 3$).

cells were first lysed with Luciferase Cell Culture Lysis Reagent (Promega, USA). Luciferase activity was described as relative light unit per well (RLU/well). We thus measured the luciferase activity of cells transducted with Ad, PEG-Ad[7] RGDpep-PEG-Ad, as shown in Figure 5. A549 cells that had been infected with Ad or RGDpep-PEG-Ad exhibited luciferase activity in similar proportions. On the other hand, the luciferase activity of DC2.4 cells that had been infected with Ad was much lower than that of DC2.4 cells that had been infected with RGDpep-PEG-Ad. Furthermore, infection of cells with RGDpep-PEG-Ad was not blocked in the presence of an antibody to Ad (data is not shown). These results indicate that RGDpep-PEG-Ad could be transducted into cells via the integrins and was protected from the antibody by its pegylated structure.

In summary, we designed and prepared (Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC as an efficient auxiliary transporter tool for carrying Ad into cells. Although (Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC-SHS could not be purified by HPLC owing to its instability in water, quality of this tool when constructed in situ was still sufficient to modify Ad. Various active esters of 6-maleimidohexanoic acid are presently being examined in order to obtain a stable 6-[(Ac-YGGRGDTP$_\beta$A)$_2$K-PEG-$_\beta$AC-succinimido]hexanoic acid active ester in water.

Recently, Ogawara et al.[12] reported a procedure that modified Ad in two steps, using PEG and cyclic RGD peptide. These authors prepared PEG-Ad and then combined cyclic RGD peptide (Ansynth, Netherlands) with the pegylated Ad. Our own future studies will investigate peptide-PEG transporters, which can modify Ad in just one step.

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11. Yield 78 mg (6%, calculated from NH_2 content of the used resin). Amino acid ratios in an acid hydrolysate: Asp, 0.80; Gly, 3.21; Arg, 0.87; Thr, 1.06; Pro, 1.02; Tyr, 0.91; Lys, 0.50. Peptide content calculated from the amino acid analysis, 0.15 mmol/g.
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quate levels of αv integrins, which was confirmed by RT-PCR (data not shown). As shown in Fig. 1, A2058 cells and B16BL6 cells infected using Ad-RGD carrying the luciferase gene induced significantly enhanced gene expression compared to that induced by the Ad-Luc.

Subsequently, the gene expression of Ad-RGD was evaluated *in vivo*. Ad-RGD or the conventional Ad encoding luciferase gene was injected intravenously in tumor-bearing mice and the luciferase activity in each organ was measured. After insertion of the RGD peptide into the HI loop of the fiber, the Ad-RGD showed a significantly increased luciferase activity compared to that induced by a conventional Ad vector in liver, lung, brain, and B16BL6 tumor, while it showed almost similar gene expression in spleen and heart (Fig. 2). Hence, the enhanced gene transfer in tissues, especially in brain and tumor makes this vector a useful and powerful carrier for efficient gene transduction and gene therapy.

Experimental

1. Cell lines and animals

B16BL6 mouse melanoma cells were maintained in Minimal Essential Medium (MEM) supplemented with 7.5% heat-inactivated Fetal Bovine Serum (FBS). The human embryonic kidney (HEK) 293 cells and A2058 human melanoma cells were cultured in DMEM supplemented with 10% FBS. All the cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2. The C57BL/6 female mice (4 weeks old) were purchased from SLC Inc. (Shizuoka, Japan). All the experimental procedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

2. Procedures

2.1. Construction of adenovirus vectors encoding RGD peptide in the fiber

The replication-deficient adenovirus vectors used in this study were constructed from the adenovirus serotype 5 backbone with deletions of E1 and E3 and the expression cassette in the E1 region (Mizuguchi et al. 2001a). The integrin-targeting RGD sequence was inserted into the HI loop of the fiber knob using the two-step method. The fiber-mutant adenovirus vector, Ad-RGD-Luc carrying the luciferase gene under the control of the cytomegalovirus (CMV) promoter, was constructed by an improved *in vitro* ligation method as described previously (Mizuguchi and Kay 1998). A conventional vector encoding luciferase gene (Ad-Luc) was also developed. The Ad vectors were propagated in HEK 293 cells and purified by cesium chloride gradient ultracentrifugation, and their titer was determined by plaque-forming assay.

2.2. Gene expression with Ad-RGD-Luc or conventional Ad-Luc in vitro

The A2058 human melanoma cells and B16BL6 mouse melanoma cells were infected with Ad-Luc or Ad-RGD-Luc at 1250, 2500, 5000, and 10000 viral particles/cell for 48 h. Subsequently, the cells were washed, collected, and their luciferase activity was measured using the Luciferase Assay System (Promega, USA) and Microlumat Plus LB96 (Perkin Elmer, USA) after the cells were lysed with the Luciferase Cell Culture Lysis (Promega, USA) according to the manufacturer's instruction.

2.3. Gene expression with Ad-RGD-Luc or conventional Ad-Luc in vivo

The C57BL/6 mice were intradermally inoculated with 2×10^5 B16BL6 melanoma cells. After 6 days, 2×10^9 viral particles/mouse of Ad-Luc or Ad-RGD-Luc were injected into the tail vein and the organs were harvested after 2 days. Subsequently, the luciferase activity in organ homogenates was measured using the method described in section 2.2.

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High gene expression of the mutant adenovirus vector, both *in vitro* and *in vivo*, with the insertion of integrin-targeting peptide into the fiber

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In the present study, a first-generation adenovirus (Ad) vector was modified with the RGD peptide inserted into the fiber. The insertion of an integrin-targeting sequence into the Ad vector notably enhanced the luciferase expression in the Coxsackie virus and Adenovirus Receptor-deficient A2058 and B16BL6 melanoma cells. The results of an *in vivo* study with tumor-bearing mice also showed that Ad-RGD-Luc had enhanced gene expression in many organs and in the B16BL6 tumor compared to that induced by a conventional Ad vector after intravenous injection.

Adenovirus (Ad) vectors are widely used as carriers for gene therapy, both *in vitro* and *in vivo* (Asaoka et al. 2000; Gao et al. 2003; Gao et al. 2004). Recombinant Ad vectors can produce large amounts of gene products in a variety of dividing and nondividing cells. It has been reported that the initial process of Ad infection involves at least two sequential steps.

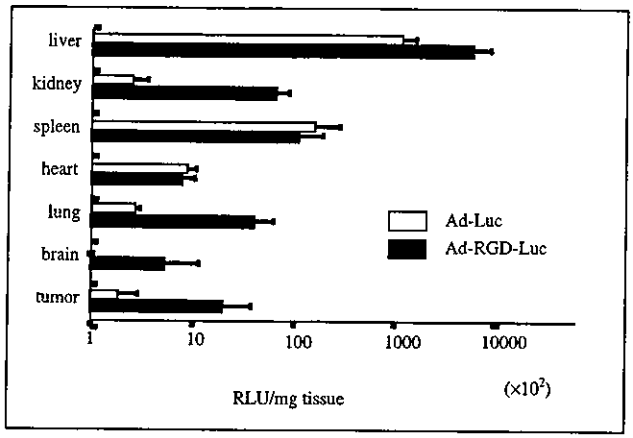

Fig. 2: Luciferase activity in organs after i.v. injection of Ad-Luc and Ad-RGD-Luc. The C57BL/6 mice were intradermally inoculated with 2×10^5 B16BL6 melanoma cells. After six days, 2×10^9 viral particles/mouse of Ad-Luc or Ad-RGD-Luc were injected into tail vein, respectively and the organs were harvested after 48 h. Then luciferase activity in organ homogenates was measured. Data are presented as the mean ± SD of relative light units (RLU)/mg tissue determined from four mice

The first step is the attachment of the virus to the cell surface, which occurs by binding of the fiber knob to the Coxsackie virus and Adenovirus Receptor (CAR) (Bergelson et al. 1997; Tomko et al. 1997). Following this, in the second step, the interaction between the RGD motif of the penton base with αv integrins, the secondary host-cell receptors, facilitates internalization by receptor-mediated endocytosis (Wickham et al. 1993; 1994). In other words, if the surface of host cells lack CAR, it is difficult to obtain an efficient gene transfer into those cells using a conventional Ad vector. For overcoming the low gene expression in CAR negative cells through Ad vectors, we developed a fiber-mutant Ad vector with an integrin-targeting RGD peptide by a simple *in vitro* method (Mizuguchi et al. 2001a). We anticipated that the fiber-mutant Ad system might target αv integrins during the first attachment to host cells. Therefore, this fiber-mutant system is an intriguing strategy for altering Ad tropism to enable efficient gene transduction into cells expressing little or no CAR. In the present study, we evaluated gene expression in A2058 human melanoma cells and B16BL6 mouse melanoma cells that are deficient in CAR and express ade-

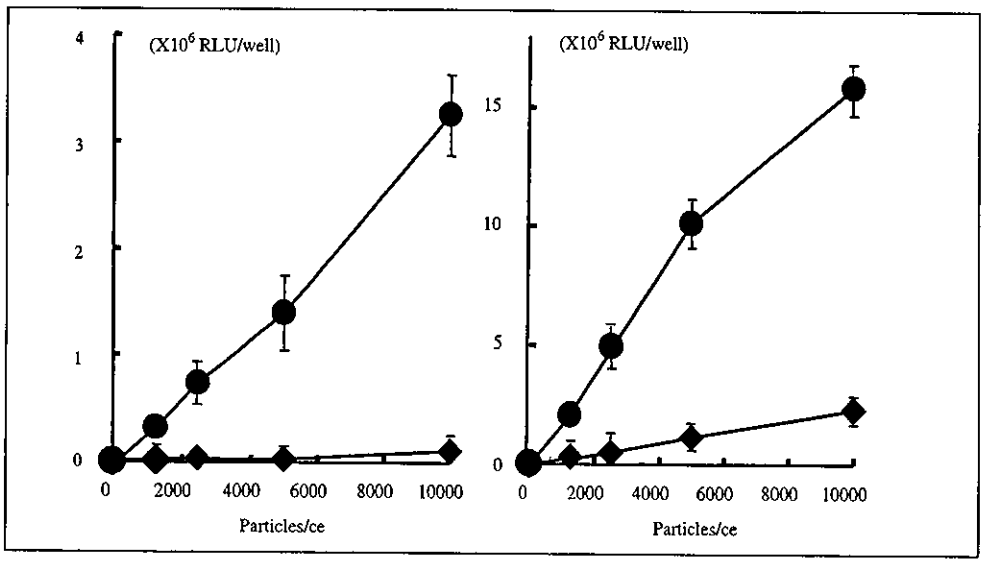

Fig. 1:
Luciferase expression of Ad-Luc or Ad-RGD-Luc infected A2058 and B16BL6 melanoma cells. A2058 cells (right) and B16BL6 cells (left) were transduced with Ad-Luc (◆) or Ad-RGD-Luc (●) respectively at the indicated viral particles/cell for 48 h. Subsequently, the cells were washed, collected, and their luciferase activity was measured. Data are presented as the mean ± SD of relative light units (RLU)/well determined from the three experiments

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Tumor-suppressive activities by chemokines introduced into OV-HM cells using fiber-mutant adenovirus vectors

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In this study, fiber-mutant adenovirus vectors encoding chemokines, Ad-RGD-mCCL17, Ad-RGD-mCCL21 and Ad-RGD-mCCL22 were constructed. The insertion of integrin-targeting RGD sequence into fiber knob of adenovirus vectors notably enhanced the infection efficiency into tumor cells. Among three chemokine-encoding vectors evaluated, Ad-RGD-mCCL22 showed significant tumor-suppressive activity via transduction into OV-HM cells.

Cytokine or chemokine encoded by a viral vector is currently regarded as a promising way of cancer gene immunotherapy. Chemokines consist of a superfamily of small secreted proteins that attract their target cells by interacting with G protein-coupled receptors expressed on these cells. Researchers have paid attention to chemotactic activity of chemokines for immune cells, and have expected that they may be able to play a pivotal role in cancer treatment, because the basis and premise of immunotherapy is the accumulation of immune cells in tumor tissues. More than 40 chemokines have been identified so far (Yoshie et al. 2001), but only a few have been demonstrated as candidates for cancer therapy by using as sole agents or with adjuvant (Gao et al. 2003; Maric and Liu 1999).

In the present report, three CC family chemokines, thymus and activation-regulated chemokine/CCL17, secondary lymphoid-tissue chemokine/CCL21 and macrophage-derived chemokine/CCL22 have been studied. CCL17 and CCL22 are chemotactic for memory CD4 + T cells via CCR4 while CCL21 induces migration of T cells, B cells, dendritic cells and NK cells via CCR7 (Campbell et al. 1999, Nagira et al. 1997). CCL22 was also shown to have a chemoattractant activity for dendritic cells, NK cells and T cells (Godiska et al. 1997). We hypothesized that if tumor cells could be genetically modified *in vitro* to produce chemokines *in vivo*, the chemokines would accumulate immune cells in the tumor. The facilitated interaction of immune cells with the tumor cells *in vivo* might induce anti-tumor activity. To test this hypothesis, we developed a recombinant adenovirus vector with a fiber mutation containing the integrin-targeting Arg-Gly-Asp (RGD) sequence in the fiber knob (Mizuguchi et al. 2001b). As shown in the Table, This vector has been demonstrated to possess higher transduction efficacy to OV-HM cells, a mouse ovary carcinoma line (Hashimoto et al. 1989), compared to that of conventional adenovirus vector.

Table: EGFP expression in OV-HM cells infected with Ad-EGFP and Ad-RGD-EGFP

	250 PT % gated	500 PT % gated
Non-infection	0.94%	0.94%
Ad-EGFP	47.1%	76.3%
Ad-RGD-EGFP	98.7%	99.6%

OV-HM cells were infected with 250 or 500 particles/cell of Ad-EGFP or Ad-RGD-EGFP for 48 h and EGFP expression was measured by flow cytometric analysis

In this study, we infected OV-HM with fiber-mutant adenovirus vectors encoding mCCL17, mCCL21 or mCCL22 and examined their expression by RT-PCR. The migration assay of chemokine-encoding vectors was also conducted *in vitro*. The results demonstrated that the efficient production of biologically active mCCL17, mCCL21 and mCCL22 could be detected in the culture supernatants of cells infected with these vectors, and the vectors could efficiently migrate the specific receptor-expressing cells (data not shown). Then OV-HM cells infected with Ad-RGD-mCCL17, Ad-RGD-mCCL21, Ad-RGD-mCCL22 or Ad-RGD-NULL (the control vector only) were intradermally inoculated into B6C3F1 mice to evaluate their effects on tumor growth *in vivo*. As shown in the Fig., OV-HM infected with Ad-RGD-mCCL22 showed significant suppression in tumor growth. On the other hand, OV-HM infected with either Ad-RGD-mCCL17 or Ad-RGD-mCCL21 did not show any difference in tumor growth from that infected with Ad-RGD-NULL. In rechallenge experiment, mice that had complete regression were intradermally injected with OV-HM or B16/BL6 cells 90 days after the initial challenge. Results demonstrated that 100% of mice rechallenged with OV-HM remained tumor-free. In contrast, all of the mice rechallenged with B16/BL6 developed palpable tumors within 2 weeks (data not shown). These results indicated the generation of specific immunity against OV-HM in mice that rejected OV-HM expressing mCCL22. To exclude the possibility that the growth suppression of the tumor cells by Ad-RGD-mCCL22 was due to the cytotoxicity of adenovirus or

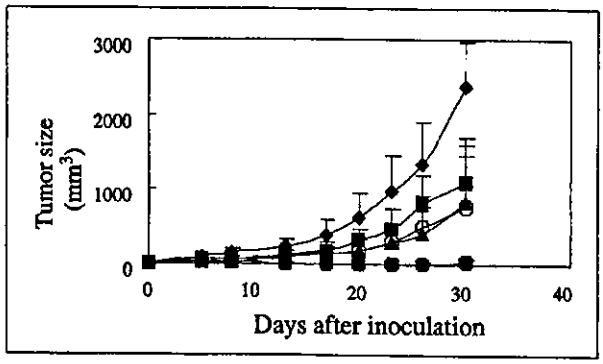

Fig.: Tumor-suppressive activity of Ad-RGD-mCCL22
—◆— No-treat, —■—Ad-RGD-NULL, —▲— Ad-RGD-mCCL17, —●— Ad-RGD-mCCL22, —○— Ad-RGD-mCCL21

chemokine, OV-HM cells transfected with Ad-RGD-mCCL17, Ad-RGD-mCCL21, Ad-RGD-mCCL22 or Ad-RGD-NULL were cultured for 48 h, and the cell viability was measured by the MTT assay. The *in vitro* growth of the cells infected with these vectors was essentially identical to that of control cells (data not shown).

In summary, our study suggests that CCL22, a CC family chemokine, may be a good candidate for cancer gene immunotherapy.

Experimental

1. Cell lines and animals

OV-HM ovarian carcinoma cells were kindly provided by Dr. Hiromi Fujiwara (School of Medicine, Osaka University, Japan) and were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS. Human embryonic kidney (HEK) 293 cells were cultured in DMEM supplemented with 10% FBS. All the cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Female B6C3F1 mice (6–8 week of age) were purchased from SLC Inc. (Hamamatsu, Japan). All of the experimental procedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

2. Procedures

2.1. Infection of chemokines into OV-HM cells using fiber-mutant adenovirus vectors

Replication-deficient adenovirus vectors used in this study were based on the adenovirus serotype 5 backbone with deletions of E1 and E3 and the expression cassette in the E1 region (Mizuguchi et al. 2001a). The integrin-targeting RGD sequence was inserted into the HI loop of the fiber knob using a two-step method (Mizuguchi et al. 2001b). Fiber-mutant adenovirus vectors, Ad-RGD-mCCL17, Ad-RGD-mCCL21 and Ad-RGD-mCCL22 carrying the murine chemokine cDNA under the control of the cytomegalovirus promoter, were constructed by an improved *in vitro* ligation method as described (Mizuguchi and Kay 1998). The Ad-RGD-NULL vector, serving as a negative control, is identical to the Ad-RGD-chemokine vectors without the chemokine gene in the expression cassette. The adenovirus vectors were propagated in HEK 293 cells and purified by cesium chloride gradient ultracentrifugation, and their titer was determined by plaque-forming assay.

2.2. Tumor rejection in mice and subsequent rechallenge by tumor reinoculation

1×10^6 OV-HM cells that had been infected with Ad-RGD-mCCL17, Ad-RGD-mCCL21 or Ad-RGD-mCCL22 at a MOI (Multiplicity of Infection) of 10 for 24 h were inoculated intradermally into the flank of mice. The length and width of the tumor were measured twice a week. Animals were euthanized when one of the two measurements were greater than 15 mm. Three months after complete regression of primary tumors, mice were rechallenged with freshly isolated OV-HM tumor cells or B16/BL6 melanoma cells by intradermal injection of 1×10^6 cells into the flank.

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Neutralizing Antibody Evasion Ability of Adenovirus Vector Induced by the Bioconjugation of Methoxypolyethylene Glycol Succinimidyl Propionate (MPEG-SPA)

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Although adenovirus vectors (Ad) which possesses high transduction efficiency are widely used for gene therapy in animal models, clinical use is very limited. One of the main reason is that nearly 80% of human beings possess anti-Ad antibodies. In this study, we tried to modify Ad with methoxypolyethylene glycol (MPEG) activated by succinimidyl propionate, and, the neutralizing antibody evasion ability of PEGylated Ad was evaluated. The results demonstrated that PEG-Ad showed stronger protection ability against anti-Ad neutralizing antibody compared to that with unmodified-Ad. Considering there are many people carrying neutralizing antibody against Ad and readministration of Ad was necessary for treating chronic diseases, this strategy, which was also applicable to other vectors, can be used for developing improved vectors.

Key words adenovirus vector; polyethylene glycol; antibody; gene therapy

Although, the gene therapy for cancer or an incurable disease has attracted considerable attention, clinical use is very limited by the problem of vectors for transgenics. Vectors based on human adenoviruses have been developed as efficient vehicles for therapeutic transgenes in a wide variety of animal models.[1—3] Gene manipulated adenovirus vectors (Ad) have commanded considerable attention as gene delivery carriers since high-titer replication-deficient recombinant viral preparations can be easily generated, the viruses can be engineered to accommodate large DNA inserts, and they can show high gene expression in a wide variety of dividing and nondividing cells.[4,5] However, first-generation adenoviruses possess a fundamental problem that both cellular and humoral immune responses prohibit gene transfer on readministration of Ad due to neutralization of viral particles by antibodies produced against capsid proteins.[6,7] Furthermore, many people carry immunity to Ad and a large amount of Ad administration causes side effects. Therefore, clinical application of Ad was very limited.[8—10] PEGylation, the covalent attachment of activated polyethylene glycol (PEG) to free lysine groups on Ad surface, is a promising strategy for overcoming these limitations. PEG-modification is a well-established technique for the modification of therapeutic peptides and proteins, and the validity of PEGylation has been reported by us and some other groups.[11,12] This method enabled transgenics in the presence of neutralizing antibodies of Ad without the necessity of recombining a gene in a vector like a gutless vector.[13,14] In this study, we used monomethoxypolyethylene glycol (MPEG) activated by succinimidyl propionate, which reacts preferentially with the ε-amino terminal of lysine residues, to covalently attached to the surface of Ad. And, we assessed the effects of biochemical modification of viral capsids with functionalized PEG on the neutralizing antibody against first-generation Ad.

MATERIALS AND METHODS

Cells and Animals HEK 293 cells, A549 human lung carcinoma cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Female ICR mice were purchased from Nippon SLC (Kyoto, Japan) and used at 6 weeks-old stage.

Adenovirus ad expressing firefly Luciferase under the control of cytomegalovirus (CMV) promoters were amplified in 293 cells, using a modification of established methods and purified from cell lysates by banding twice on CsCl gradients, dialyzed and stored at −80 °C. The Ad used in this study were constructed by an improved *in vitro* ligation method as described previously.[15] Viral particle titer was spectrophotometrically determined by the established method.[16]

PEGylation of Ad Activated methoxypolyethylene glycol succinimidyl propionate (MPEG-SPA, MW 5000, Shearwater Corporation) was used in this study (Fig. 1). Ad was reacted with 100 molar excess of mPEG-SPA for viral lysine residue at 37 °C for 45 min with gentle stirring (300 rpm). The particle size of PEG-Ad was measured by ZETASIZER 3000HS (Malvern, U.K.).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis SDS-PAGE analysis was conducted for detecting the PEGylated viral proteins. In short, the unmodified-Ad or PEG-Ad suspensions were mixed with the same volume of 2XSDS protein gel loading solution (Quality Biological, Inc. U.S.A.) and 5% (v/v) 2-

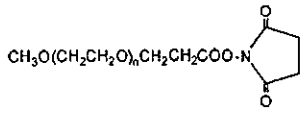

Fig. 1. mPEG-SPA Methoxypolyethylene Glycol Succinimidyl Propionate MW 5000.

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mercaptothanol was added to the final solution. After heating at 95 °C for 5 min, the mixture was added to 4—20% of polyacrylamide gel (PAG Mini 4/20) and electrophoresis was carried out using SDS-PAGE buffer. Full range Rainbow™ molecular weight marker (Amersham Life Science, U.S.A.) was used as the marker in this study. The gel was stained for viral hexon using Coomassie blue. The bioconjugation ratio of PEGylated Ad was calculated by the colority of the hexon band and the PEGylated hexon using NIH Image software.

Preparation of Ad Antiserum Ad antiserum was obtained from ICR mouse according to the approach described previously.[17,18] In brief, female ICR mouse (6 weeks old) was administered hypodermically with a dose of 10^{10} viral particles of conventional Ad with Freund's complete adjuvant in 100 μl of PBS. Another 10^{10} viral particles of conventional Ad were hypodermically administered with Freund's incomplete adjuvant after 2 and 4 weeks. Then serum of the mouse was collected after 1 week and filtered, and stored at −20 °C.

Transduction Efficiency of PEG-Ad and Unmodified-Ad into A549 Cells in the Presence or Absence of Ad Antiserum A549 cells (1×10^4 cells) were seeded into a 48-well plate with 500 μl medium. On the following day, the cells were transduced with 1000 particles/cell of unmodified-Ad or PEG-Ad respectively in a final volume of 500 μl in the presence of 0, 42, 125 ng protein/well of Ad antiserum. After 24 h cultivation, luciferase activity was measured using Luciferase Assay System (Promega, U.S.A.) and Microlumat Plus LB 96 (Perkin Elmer, U.S.A.) after cells were lysed with Luciferase Cell Culture Lysis Reagent (Promega, U.S.A.) according to the manufacturer's instruction.

RESULTS AND DISCUSSION

In this study, we tried to modify Ad with MPEG activated by succinimidyl propionate which was safety and widely used.[19] A simple and practicable method for constructing of PEG-Ad was employed. After reaction, the sizes of PEG-Ad and unmodified-Ad were measured. The results showed that the particle size of Ad was increased with PEGylation. The average viral particle size of PEG-Ad was about 10 nm bigger than that observed in the unmodified-Ad (Table 1). And SDS-PAGE analysis showed the presence of a new band of PEGylated viral capsid protein, hexon (Fig. 2). We also demonstrated that all Ad were conjugated by activated PEG, and there was no unmodified Ad mixed with the PEGylated ones (data not shown).

For evaluating the protection ability of PEG-Ad against the neutralizing antibodies, PEG-Ad and unmodified-Ad were added to A549 cells in the presence or absence of neutralizing antibodies against adenovirus capsid proteins, and the transduction levels were compared. Transduction efficiency of the unmodified-Ad was significantly reduced by the neutralizing antibodies (Fig. 3). In the presence of neutralizing antiserum of 42 ng, the luciferase gene expression of unmodified-Ad was less than half of that without antiserum, whereas PEG-Ad remained about 80% of its gene expression. In the presence of antibodies of 125 ng, the gene expression of unmodified-Ad was only 2.5% compared to that in the absence of antibodies, however, PEG-Ad showed more than 10-fold of antibody evasion ability compared to unmodified-Ad. As anticipated, the absolute level of gene expression of PEG-Ad was decreased to about 1/300 compared to that of unmodified-Ad in the absence of antibodies due to the effect that PEG chains prevented the interaction between Ad and coxsackie-adenovirus receptor (CAR). For developing novel Ad which possess a targeting ability, it is indispensable to block the route of Ad infection through CAR because of its broad expression in many tissues. So as the next step, we are trying to construct Ad which was conjugated by PEG with targeting molecule on the tip of the PEG. And we expect that this novel Ad will enhance the transduction efficiency of PEG-Ad at the same time keep the evading ability from the neutralizing antibodies.

One of the goals of Ad PEGylation is to provide a means to overcome the major limitation of Ad gene therapy, namely, antibody neutralization. The results presented here demonstrate that PEGylation can notably improve the ability of Ad to escape antibody neutralization *in vitro* by the steric hindrance of PEG chains. This antibody evasion ability is essential for clinical applications, because nearly 80% of human beings possess anti-Ad antibodies and readministration is indispensable in some cases like treating chronic diseases, such as cystic fibrosis and hemophilia. Furthermore, antibody evasion ability enabled decrease of the amount of medical re-

Table 1. Viral Particle Sizes of PEGylated Ad and Unmodified-Ad

Ratio (Ad:PEG)[a]	Vector size (nm)
1:0 (unmodified)	113.3±0.76
1:100 (PEG-Ad)	123.8±0.98

a) Amount of PEG to lysine residue of adenovirus vector capsid protein (mol:mol).

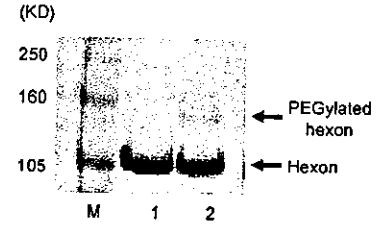

Fig. 2. SDS-PAGE Analysis of PEGylated Hexon

Lane M, protein marker; lane 1, unmodified-Ad; lane 2, PEG-Ad. Further details are described in Materials and Methods.

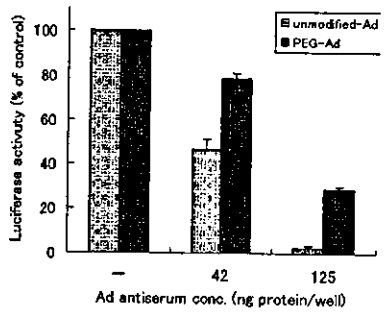

Fig. 3. Transduction Efficiency of Unmodified-Ad and PEG-Ad in the Presence or Absence of Ad Antiserum

A549 cells (1×10^4 cells) were transduced with 1000 particles/cell of unmodified-Ad and PEG-Ad in the presence or absence of Ad antiserum respectively. Luciferase expression was measured after 24 h. Each point was represented as mean ± S.D. ($n=3$).

quired to antibody carriers, and result in a reduction of side effects.

As next step, we are trying to evaluate its antibody evasion ability *in vivo* aiming for clinical use. Especially considering that the PEG-Ad used in this study was with low modification rate (34%, confirmed by SDS-PAGE, Fig. 2), we anticipated that highly modified PEG-Ad would protect it from neutralizing antibody more efficiently *via* stronger steric hindrance of PEG chains. Including of the other merits possessed by PEG-Ad, such as the extension of blood retention half-time and reduction of antigenicity. These approaches which are applicable to other vectors and other high compounds will promote development of the novel intelligent virus vectors.

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