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Tumor Necrosis Factor α-Gene Therapy for an Established Murine Melanoma Using RGD (Arg-Gly-Asp) Fiber-mutant Adenovirus Vectors

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Although adenovirus vectors (Ad) provide high-level transduction efficacy to many cell types, extremely high doses of Ad are required for sufficient gene transduction into several tumors, including melanoma. Here, we demonstrated that the expression of coxsackie-adenovirus receptor, a primitive Ad-receptor, was very low in murine and human melanoma cells. We also found that fiber-mutant Ad containing the Arg-Gly-Asp (RGD) sequence in the fiber knob remarkably augmented gene transduction efficacy in melanoma cells by targeting α_v-integrins. In addition, intratumoral injection of RGD fiber-mutant Ad containing the tumor necrosis factor α gene (Ad-RGD-TNFα) revealed dramatic anti-tumor efficacy through hemolytic necrosis in an established murine B16 BL6 melanoma model. Ad-RGD-TNFα required one-tenth the dosage of Ad-TNFα to induce an equal therapeutic effect. These results suggest that α_v-integrin-targeted Ad will be a very powerful tool for the advancement of melanoma gene therapy.

Key words: Adenovirus vector — Fiber-mutant — Melanoma — Tumor necrosis factor α — Gene therapy

The frequency of malignant melanoma, by far the most fatal skin cancer, has increased by a factor of approximately 15 in the past 60 years. The factors underlying this rapid increase are incompletely understood, although ultraviolet radiation is strongly implicated.[1)] The prognosis of patients with melanoma is generally poor even after surgery, chemotherapy, or radiotherapy, and the melanomas commonly metastasize.[2,3)] Various gene therapy approaches for melanoma, using suicide gene,[4–6)] cytokine gene,[6–10)] or anti-angiogenic gene,[11,12)] are being developed to inhibit tumor-growth and metastasis. To treat malignant tumors successfully through gene therapy, high-quality vector systems are required for efficient gene transduction.

Among the various methods of gene transduction, adenovirus vectors (Ad) provide high-level transduction efficacy to a broad range of cell types.[13,14)] The internalization of Ad into target cells is mediated by at least two distinct cell receptors: the fiber knob of Ad particles initially attaches to a coxsackie-adenovirus receptor (CAR) on the cell surface,[15)] then $\alpha_v\beta_3$- or $\alpha_v\beta_5$-integrins subsequently interact with the Arg-Gly-Asp (RGD) motif in the Ad-penton base and facilitate internalization of the virion.[16)] Although the Ad system should theoretically be applicable to melanoma gene therapy, extremely high doses of Ad are required for sufficient gene transduction into melanoma because of the low expression of CAR on melanoma cells.[17)] We previously demonstrated that compared with conventional Ad, the fiber-mutant Ad harboring the RGD sequence in the HI loop of the fiber knob could more efficiently transduce foreign genes into human glioma cells[18)] or dendritic cells[19,20)] that expressed little or no CAR on their surface. We reasoned that this fiber-mutant Ad system might target α_v-integrins during the first attachment to target cells. Therefore, this fiber-mutant system is an attractive strategy for altering Ad tropism for efficient gene transduction into melanoma.

In the present study, we firstly confirmed, using reverse transcription-polymerase chain reaction (RT-PCR), that the relative resistance of melanoma cells to Ad-mediated gene transfer is due to low CAR expression, and that melanoma cells expressed adequate levels of α_v-integrins. Next, we compared the gene transduction efficiency and gene expression efficacy of conventional Ad and α_v-integrin-targeted Ad on melanoma cells. Furthermore, the

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Abbreviations: Ad, adenovirus vector; CAR, coxsackie-adenovirus receptor; FBS, fetal bovine serum; GFP, green fluorescent protein; HE, hematoxylin and eosin; PBS, phosphate-buffered saline; RGD, Arg-Gly-Asp; RT-PCR, reverse transcription-polymerase chain reaction; TNFα, tumor necrosis factor α.

anti-tumor effects of intratumoral treatment using conventional or RGD fiber-mutant Ad carrying tumor necrosis factor α (TNFα) gene was investigated in the murine B16 BL6 tumor model.

MATERIALS AND METHODS

Cell lines and mice Murine melanoma B16 BL6 cells and HeLa cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Human melanoma A2058 cells, HEK293 cells, A549 cells, and Caco-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics. EL4 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol, and antibiotics. Colon 26 cells were grown in RPMI 1640 medium supplemented with 10% FBS and antibiotics.

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

Vectors The replication-deficient Ad used in the present study was based on adenovirus serotype 5 and contained a deletion in early regions 1 and 3. The RGD sequence was introduced into the HI loop of the fiber knob using the two-step method developed by Mizuguchi *et al*.[18] Both conventional and RGD fiber-mutant Ad, which carried the desired coding cDNA under the control of the cytomegalovirus promoter, were constructed by an improved *in vitro* ligation method as described.[21, 22] Human TNFα gene derived from pCATNF2,[23] *Escherichia coli* β-galactosidase (LacZ) gene derived from pCMVβ (Clontech, Palo Alto, CA), and green fluorescent protein (GFP) gene derived from pQBI 63 (Takara, Tokyo) were used as cDNA sources. Both types of Ad were propagated in HEK293 cells, purified by two rounds of CsCl density centrifugation, dialyzed, and stored at −80°C. Vector particle titer was spectrophotometrically determined by the method of Maizel *et al*.[24]

RT-PCR analysis RT-PCR of the CAR and integrin transcripts was performed as described previously.[19] In brief, RT proceeded for 60 min at 42°C in a 100 μl reaction mixture containing 10 μg of total RNA isolated using TRIZOL reagent (Life Technologies, Tokyo), 10 μl of 10× PCR buffer, 20 μl of 25 mM $MgCl_2$, 40 μl of 2.5 mM dNTP mix, 1 μM random hexamer, 1 μM oligo(dT), and 200 U of ReverTra Ace (Toyobo Co., Ltd., Osaka). PCR amplification proceeded in 50 μl of a reaction mixture containing 5 μl of RT-material, 1.25 U of *Taq* DNA polymerase (Toyobo Co., Ltd.), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.5 μM primers. The sequences of the specific primers, PCR conditions, and the expected PCR product sizes are summarized in Table I. "EZ Load" (BIO-RAD, Tokyo)

Table I. Primer Sequences Used for PCR Amplification

Species	Gene		Sequence (5′ to 3′)	Denaturation	Annealing	Extension	Cycle No.	Product size
Mouse	CAR	Forward	TGATCATTTTGTATTCTGGA	for 45 s at 94°C	for 60 s at 50°C	for 90 s at 72°C	25	211 bp
		Reverse	TTAACAAGAACGGTCAGCAG					
	α_v-integrin	Forward	CCAGCCTGGGATTGTAGAAG	for 45 s at 94°C	for 60 s at 53°C	for 90 s at 72°C	40	105 bp
		Reverse	ACTCCAGTGGGTCATCTTTG					
	β_3-integrin	Forward	TCTGGCTGTGAGTCCTGTGT	for 45 s at 94°C	for 60 s at 55°C	for 90 s at 72°C	40	115 bp
		Reverse	GCCTCACTGACTGGGAACTC					
	β_5-integrin	Forward	TCGTGTGAAGAATGCCTGTT	for 45 s at 94°C	for 60 s at 53°C	for 90 s at 72°C	40	126 bp
		Reverse	GCTGGACTCTCAATCTCACC					
	β-actin	Forward	TGTGATGGTGGGAATGGGTCAG	for 45 s at 94°C	for 45 s at 60°C	for 120 s at 72°C	20	514 bp
		Reverse	TTTGATGTCACGCACGATTTCC					
Human	CAR	Forward	AGCCTTCAGGTGCGAGATGTTACG	for 30 s at 94°C	for 60 s at 52°C	for 120 s at 72°C	20	366 bp
		Reverse	TACGACAGCAAAAGATGATAAGAC					
	α_v-integrin	Forward	GAGCAGCAAGGACTTTGGG	for 60 s at 94°C	for 60 s at 60°C	for 60 s at 72°C	30	619 bp
		Reverse	GGGTACACTTCAAGACCAGC					
	β_3-integrin	Forward	GAGGATGACTGTGTCGTCAG	for 30 s at 94°C	for 60 s at 58°C	for 120 s at 72°C	35	232 bp
		Reverse	CTGGCGCGTTCTTCCTCAAA					
	β_5-integrin	Forward	CAGGATGGGGAGAACCAGAGC	for 60 s at 94°C	for 90 s at 55°C	for 90 s at 72°C	30	510 bp
		Reverse	CTGGTCATCTTTCACGATGGT					
	β-actin	Forward	CCTTCCTGGGCATGGAGTCCTG	for 60 s at 94°C	for 90 s at 55°C	for 90 s at 72°C	20	210 bp
		Reverse	GGAGCAATGATCTTGATCTTC					

was used as a 100 bp molecular ruler. To ensure the validity of the procedure, RT-PCR was also performed on the samples using specific primers for β-actin.

Ad-binding assay Ad-LacZ and Ad-RGD-LacZ were labeled with Cy3 (Amersham Pharmacia Biotech, Tokyo) as described.[25] The Cy3-labeling efficiency, the ratio of Cy3-fluorescence intensity to number of Ad particles, was equal for both Ad types (data not shown). Ad-binding of B16 BL6 cells was confirmed by flow cytometry. Briefly, 10^6 cells in 50 μl of staining buffer (phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and 0.05% NaN_3) were incubated for 30 min on ice with 25 μg of anti-mouse α_v-integrin monoclonal antibody (H9.2B8; Phamingen, San Diego, CA) or hamster IgG isotype control (A19-4; Phamingen), and then 200 μl of staining buffer containing 1×10^5 vector particles/cell of Cy3-labeled Ad was added. After incubation for 30 min on ice, cells were washed three times with staining buffer, and 30 000 events of the stained cells were analyzed using a FACScalibur flow cytometer and CellQuest software (Becton Dickinson, Tokyo) at an FL2 voltage of 550. The relative value of Ad-binding of B16 BL6 cells was determined using the following formula: fluorescence intensity/cell = (mean fluorescence intensity of tested sample) − (mean fluorescence intensity of B16 BL6 cells alone). The mean fluorescence intensity of B16 BL6 cells alone (background) was 28.86±1.24.

Analysis of gene transfer B16 BL6 cells and A2058 cells cultured on 24-well plates were infected with conventional or RGD fiber-mutant Ad (Ad-GFP or Ad-RGD-GFP), which carried GFP cDNA, for 1.5 h at various numbers of vector particles/cell in 400 μl of FBS-free medium. After washing twice with PBS, a 1-ml aliquot of culture medium was added to each well. Two days later, gene transduction efficiency and gene expression efficacy were assessed by flow cytometry on a FACScalibur using CellQuest software, acquiring 20 000 events by forward and side scatter gating to exclude cell debris.

***In vivo* tumor therapy model** B16 BL6 cells were intradermally inoculated into C57BL/6 mice at 2×10^5 cells/mouse. Six days later, the mice were divided into eight groups (n=5 or 6/group) and injected with Ad-RGD-TNFα (10^{10}, 10^9, or 10^8 vector particles), Ad-TNFα (10^{10}, 10^9, or 10^8 vector particles), Ad-RGD-LacZ (10^{10} vector particles), or PBS in a 50-μl volume into tumors of 5–7 mm in diameter. Tumor diameter was measured using microcalipers, and tumor volume was calculated by applying the following formula: tumor volume (mm^3)=(major axis)×(minor axis)^2×0.5236.[26] Mice containing tumors > 20 mm were euthanized. On day 90 after tumor challenge, all survivors were euthanized.

Histology B16 BL6 tumors were removed on days 2 and 3 after Ad-treatment, placed in neutral 10% formalin/PBS, and embedded in paraffin. Sections (5 μm) were prepared for hematoxylin and eosin (HE) staining and histopathological examination.

RESULTS AND DISCUSSION

Ad-mediated gene transduction is a powerful technology for human gene therapy. However, the efficiency of gene transfer using the presently available Ad, which is derived from human adenovirus serotype 5, varies widely depending on the tissue of origin of the target cells. Several tumor lines, including melanoma,[17] glioma,[18, 27, 28] and gastrointestinal carcinoma,[29, 30] which are prime targets for gene therapy, require high Ad doses for sufficient gene expression. Because Ad over-dosage might induce adverse effects in clinical gene therapy, establishment of a strategy to enhance the efficacy of Ad-mediated gene transfer against these tumors is urgently needed.

In the present study, we investigated mRNA levels of CAR and integrins, which act as Ad-receptors, in murine B16 BL6 melanoma cells and human A2058 melanoma cells by RT-PCR (Fig. 1). CAR PCR products were barely detectable in either melanoma cell line, whereas murine EL4 thymoma and human 4 cell lines (HEK293, HeLa, A549 and Caco-2) adequately expressed CAR mRNA. In contrast, α_v-, β_3-, and β_5-integrin PCR products were found in both melanoma cell lines. These findings support the notion that low level CAR expression is a major cause of the relative resistance of melanoma to Ad-mediated gene transfer, and that the modification of the Ad-fiber knob to target α_v-integrins during cell attachment is an attractive strategy for altering Ad tropism for efficient

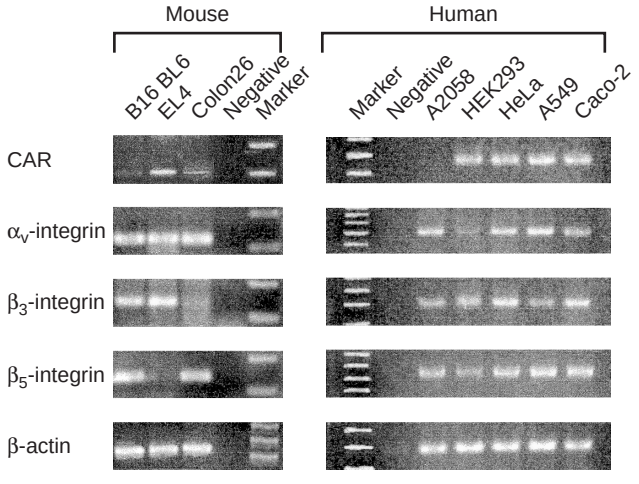

Fig. 1. RT-PCR analysis of CAR, α_v-integrin, β_3-integrin, and β_5-integrin in murine and human cell lines. Total RNA was prepared from three murine cell lines and five human cell lines, and then RT-PCR was performed as described in "Materials and Methods" and Table I.

gene transduction into melanoma. An Ad-binding assay against B16 BL6 cells, which used Cy3-labeled Ad and anti-mouse α_v-integrin monoclonal antibodies, confirmed that the RGD fiber mutant Ad could infect cells by recognizing α_v-integrins (Fig. 2). In the presence of IgG isotype control, Ad-RGD-LacZ, which contained an oligonucleotide corresponding to the RGD peptide in the fiber coding region, bound five-fold more B16 BL6 cells than conventional Ad-LacZ. However, the increased binding by RGD fiber-mutant Ad was counteracted in the presence of anti-mouse α_v-integrin monoclonal antibodies, whereas Ad-LacZ-binding was not affected.

To compare the transfection efficiency of Ad-RGD-GFP and Ad-GFP in melanoma cell lines, we performed flow cytometric analysis (Fig. 3 and Table II). Table II shows that both the transduction efficiency (% M1-gated) and the expression efficacy (mean fluorescence intensity) of the GFP reporter gene in B16 BL6 cells and A2058 cells infected with Ad-RGD-GFP or Ad-GFP increased in an Ad-dose dependent manner. Of the B16 BL6 cells infected with Ad-RGD-GFP at 10 000 vector particles/cell, 65% were GFP-positive−10-fold higher than in Ad-GFP-infected B16 BL6 cells under the same conditions. Ad-RGD-GFP at 10 000 vector particles/cell successfully transferred the GFP gene into more than 98% of A2058 cells, whereas A2058 cells infected with Ad-GFP at 10 000 vector particles/cell showed only 73% GFP-gene transduction efficiency. At 10 000 vector particles/cell, the mean fluorescence intensity in Ad-RGD-GFP-infected B16 BL6 cells and A2058 cells was 3-fold and 14-fold higher, respectively, than that of Ad-GFP-infected cells. In addition, 1000 Ad-RGD-GFP vector particles/cell exhibited equal or greater transduction efficiency and expression efficacy as that obtained by infection with 10 000 Ad-GFP vector particles/cell in both melanoma cell lines. Taken together, these results demonstrated that the RGD fiber-mutant Ad can target α_v-integrins during cell attachment, and thus increase the transduction efficiency and

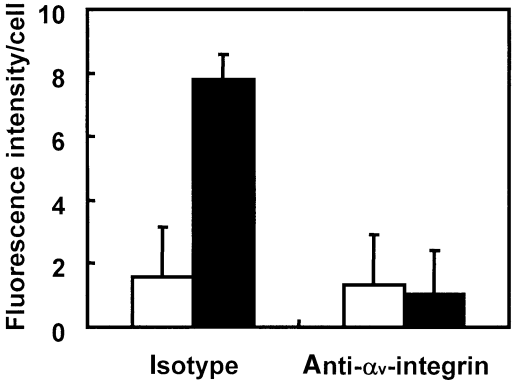

Fig. 2. Relative Ad-binding to B16 BL6 cells. B16 BL6 cells were incubated with anti-mouse α_v-integrin monoclonal antibody or hamster IgG isotype control for 30 min on ice. Then, the cells were resuspended in staining buffer containing Cy3-labeled Ad-LacZ (open column) or Ad-RGD-LacZ (closed column). After incubation for 30 min on ice, cells were washed and analyzed using a flow cytometer. The relative value of Ad-binding against B16 BL6 cells was determined using the following formula: fluorescence intensity/cell=(mean fluorescence intensity of tested sample)−(mean fluorescence intensity of B16 BL6 cells' self). Data are presented as means±SD of four experiments.

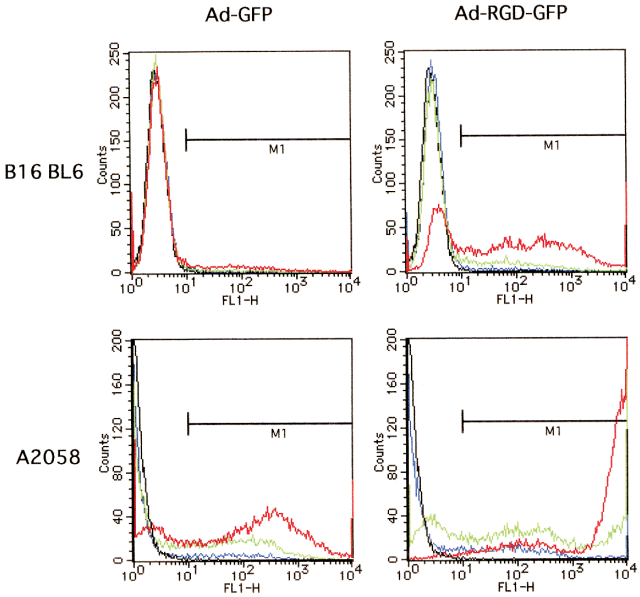

Fig. 3. The transduction efficiency and the expression efficacy of GFP genes on melanoma cells by RGD fiber-mutant or conventional Ad. B16 BL6 cells or A2058 cells were infected with Ad-GFP or Ad-RGD-GFP at 100 (blue lines), 1000 (green lines), or 10 000 (red lines) vector particles/cell for 1.5 h. Two days later, GFP expression in cells was evaluated by flow cytometry. Black lines represent untransfected cells.

Table II. Summary of Flow Cytometric Analysis of Melanoma Cells Infected with Ad-GFP or Ad-RGD-GFP

Ad (vector particles/cell)		% M1-gated		Mean fluorescence intensity	
		B16 BL6	A2058	B16 BL6	A2058
—		0.04	0.04	25.55	19.52
Ad-GFP	100	0.32	3.87	16.73	67.20
	1000	0.85	28.19	49.68	86.44
	10000	6.51	72.95	60.90	234.96
Ad-RGD-GFP	100	1.28	12.54	45.85	81.97
	1000	10.97	70.49	62.77	499.57
	10000	65.00	98.78	189.64	3401.91

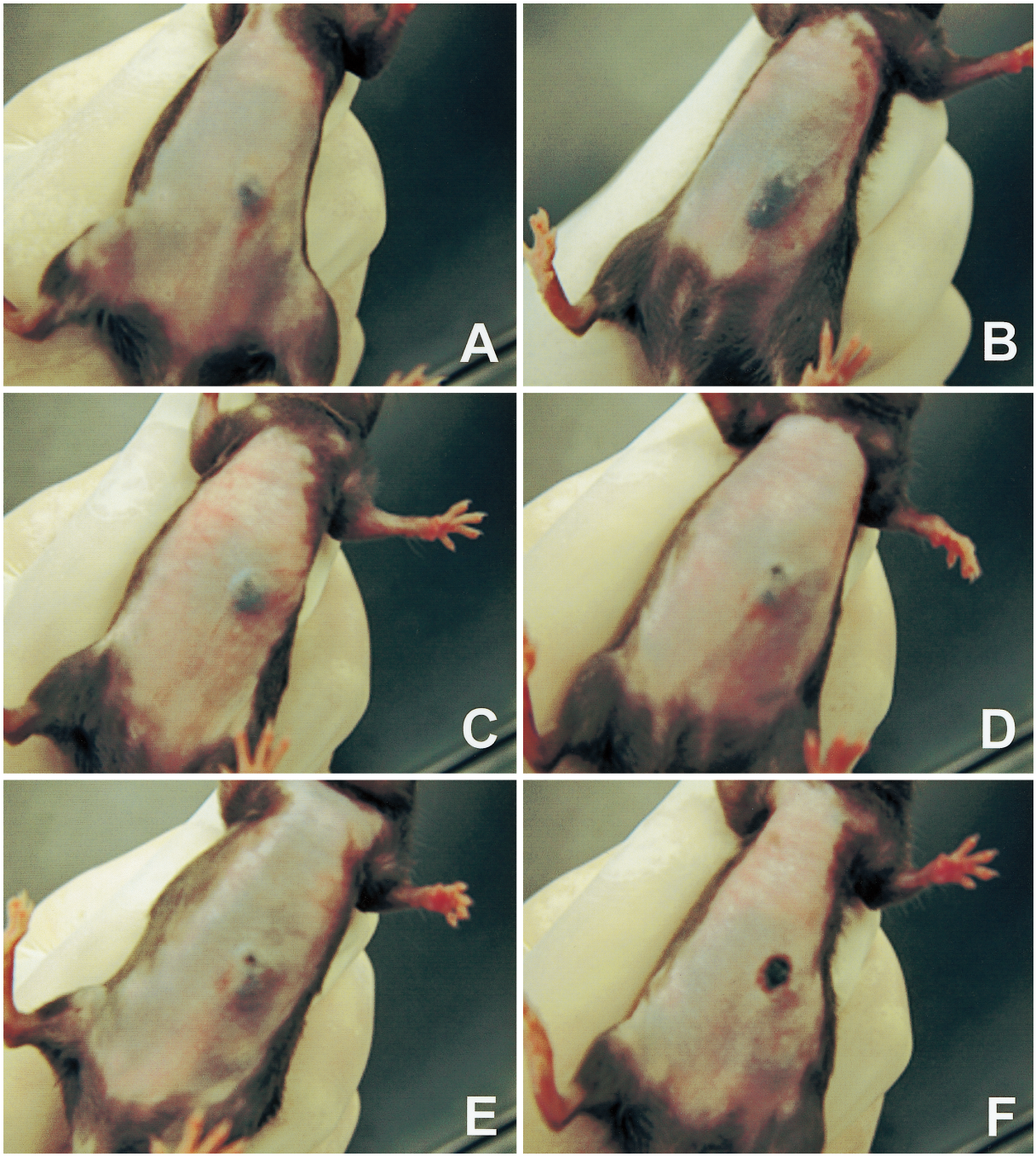

Fig. 4. Photographs of B16 BL6 tumors on day 2 after Ad-treatment. (A) PBS treatment; (B) 10^{10} Ad-RGD-LacZ treatment; (C) 10^{8} Ad-TNFα treatment; (D) 10^{9} Ad-TNFα treatment; (E) 10^{8} Ad-RGD-TNFα treatment; (F) 10^{9} Ad-RGD-TNFα treatment.

expression efficacy of foreign genes in target cells which expressed no or little CAR on their surface.

We next determined the anti-tumor effect of Ad-RGD-TNFα or Ad-TNFα treatment in the murine B16 BL6 tumor model. TNFα has been intensively studied because of the specific toxicity of this cytokine toward cells that undergo malignant transformation[31, 32] and toward new tumor blood vessels.[33, 34] However, its proinflammatory and immunoregulatory properties always presented a drawback to administration at high dosages in cancer ther-

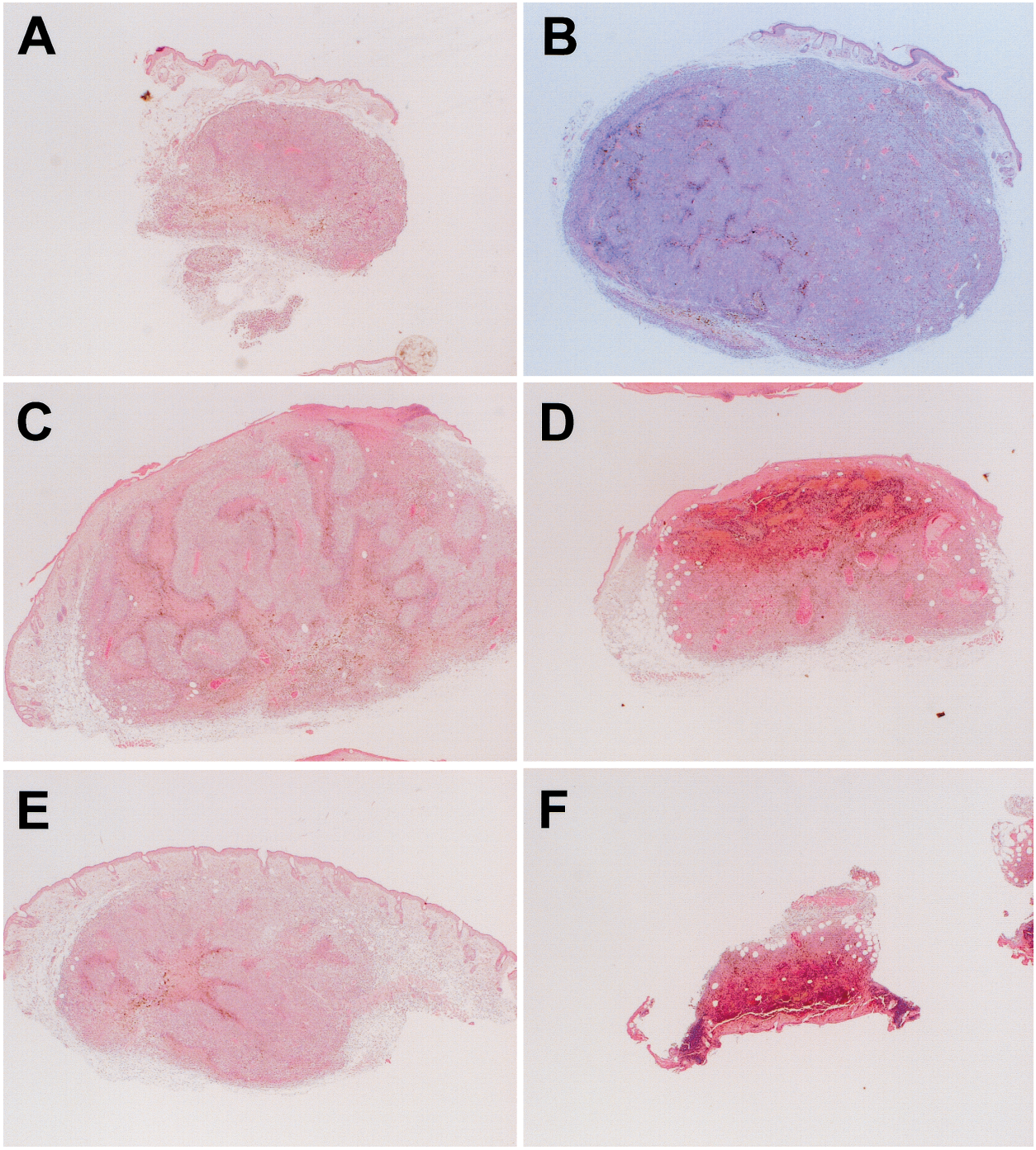

Fig. 5. HE staining of paraffin-embedded B16 BL6 tumor sections (original magnification, ×3.85). (A) On day 2 after PBS administration; (B) on day 2 after 10^{10} Ad-RGD-LacZ administration; (C) on day 2 after 10^{9} Ad-TNFα administration; (D) on day 2 after 10^{9} Ad-RGD-TNFα administration; (E) on day 3 after Ad-TNFα administration; (F) on day 3 after Ad-RGD-TNFα administration.

apy.[35] In order to attain locally elevated concentrations of TNFα while limiting the systemic use of high doses of TNFα, tumor cells must be genetically modified by insertion of the TNFα gene to secrete this cytokine. After intradermal inoculation of B16 BL6 cells into C57BL/6 mice, 10^{10}, 10^{9}, or 10^{8} vector particles of both Ad types were

Table III. Summary of Anti-B16 BL6 Tumor Effects after Ad-treatment

Treatment	VP[a)]	Tumor volume (mm^3)[b)]						Complete regression[c)]
PBS	—	1185	2447	2460	3060	3734	3825	0/6
Ad-RGD-LacZ	10^{10}	2173	2228	2497	2530	2771		0/5
Ad-RGD-TNFα	10^8	492	799	898	1043	1264	1417	0/6
	10^9	46	141	271	472	573	613	0/6
	10^{10}	0	0	0	0	4	20	2/6
Ad-TNFα	10^8	706	1546	1696	1780	2387	2599	0/6
	10^9	462	598	938	1107	1984		0/5
	10^{10}	0	0	0	907	†	†	1/6

a) Number of vector particles administered.
b) Day 21 after tumor challenge, tumor volume (mm^3)=(major axis)×(minor axis)^2×0.5236.
c) Day 90 after tumor challenge, tumor-free mice/all tested mice.
†: Sudden death on day 9 after Ad-treatment.

injected intratumorally. As shown in Fig. 4E, administration of 10^9 Ad-TNFα induced partial necrosis in the tumors on day 2 post-treatment, whereas in the groups injected with 10^8 Ad-TNFα (Fig. 4C) or 10^{10} Ad-LacZ (Fig. 4B), the tumor surface did not differ from that in the PBS-injected group (Fig. 4A). On the other hand, tumors injected with 10^9 Ad-RGD-TNFα showed distinguished necrosis on the surface (Fig. 4F), and partial necrosis was observed in the 10^8 Ad-RGD-TNFα-treated group (Fig. 4D).

Histopathological examination of B16 BL6 tumor sections stained with HE revealed clearly superior effects of Ad-RGD-TNFα to those of Ad-TNFα. In PBS-injected (Fig. 5A) and 10^{10} Ad-RGD-LacZ-injected (Fig. 5B) tumors, we observed a number of microvessels at the periphery and necrosis depending on innutrition due to acute tumor growth at the basal zone. On day 2 post-treatment, all tumors treated with Ad, including Ad-RGD-LacZ, showed a swelling that was not present in PBS-treated tumors. On day 2 post-treatment, tumors injected with 10^9 Ad-TNFα exhibited few vessels and broad necrosis (Fig. 5C), and on day 3 post-treatment, further vessel reduction and enlargement of the necrotic region at the tumor center were induced (Fig. 5E). In contrast, on day 2 after administration of 10^9 Ad-RGD-TNFα, vessels were remarkably dilated and severe hemolytic necrosis was induced in the upper region (Fig. 5D). Moreover, by day 3, tumors treated with 10^9 Ad-RGD-TNFα were dramatically regressed due to hemolytic necrosis (Fig. 5F). These finding suggested that the anti-tumor effects of Ad-RGD-TNFα or Ad-TNFα result from vessel destruction by TNFα that was expressed in the tumors.

Tumor volumes on day 21 after tumor challenge were reduced by Ad-RGD-TNFα- and Ad-TNFα-injection in an Ad-dose-dependent manner. Growth inhibition upon administration of 10^8 Ad-RGD-TNFα was comparable to that of 10^9 Ad-TNFα-treatment (Table III). We, therefore, determined that the RGD fiber mutant Ad system could reduce the dosage of Ad carrying the TNFα gene to about one-tenth of that in the case of conventional Ad, while maintaining an equal therapeutic effect against melanoma. In addition, two of six mice and one of six mice in the 10^{10} Ad-RGD-TNFα and 10^{10} Ad-TNFα-injected groups, respectively, showed complete regression on day 90 after B16 BL6 tumor challenge. However, an extreme reduction in body weight was observed in both groups, and two of six mice injected with 10^{10} Ad-TNFα suddenly died on day 9 post-Ad-treatment. Although the cause of body weight reduction and sudden death was not traced completely, we confirmed that about 1% of Ad injected into the tumor shifted into the systemic circulation, and that both a very high concentration of TNFα in the blood and extreme inflammation in liver and spleen on day 2 after Ad-treatment were apparent in the mice which showed a remarkable weight reduction (unpublished data). Therefore, we speculated that excessive TNFα, which was produced in tumor administered with high doses of Ad-TNFα or Ad-RGD-TNFα and thus leaked from the tumor, may have been distributed through the blood flow to other tissues, such as liver and spleen, and caused side-effects due to extreme inflammatory reaction. Because this knowledge is very important and a potentially very serious problem for TNFα-gene therapy, further analysis of the tissue distribution of Ad, the changes of the TNFα levels in blood, and the tissue pathology after intratumoral injection of high doses of Ad-RGD-TNFα is in progress.

In conclusion, the RGD fiber-mutant Ad system may be a very useful and powerful tool for gene therapy against melanoma due to the significant reduction in Ad-dosage required for effective therapy, a consequence of more efficient gene transduction and expression. Because Ad is cytopathic at high doses, a lower Ad-dosage is preferred in

in vivo gene therapy to prevent adenoviral toxicity. Several approaches using adjunct materials for targeting, such as bispecific antibodies,[36, 37] molecular adaptors,[38, 39] or cationic liposomes,[40, 41] have been developed to improve Ad-mediated gene transfer into target cells that express little or no CAR. However, application of these methods for *in vivo* gene therapy is limited due to the uncertainty about the *in vivo* stability of complexes between Ad and the adjunct materials. Likewise, limitations also arise due to the difficulties associated with constructing new vectors. In contrast, upon insertion of oligonucleotides corresponding to arbitrary peptides, the fiber-mutant Ad not only targets molecules abundantly expressed on cells, but also requires only a simple *in vitro* ligation using standard molecular biology reagents for construction of the vector, which does not generate replication-competent or null vectors.[18–22] We believe that this fiber-mutant Ad system will contribute considerably to the advance of gene therapy for melanoma.

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