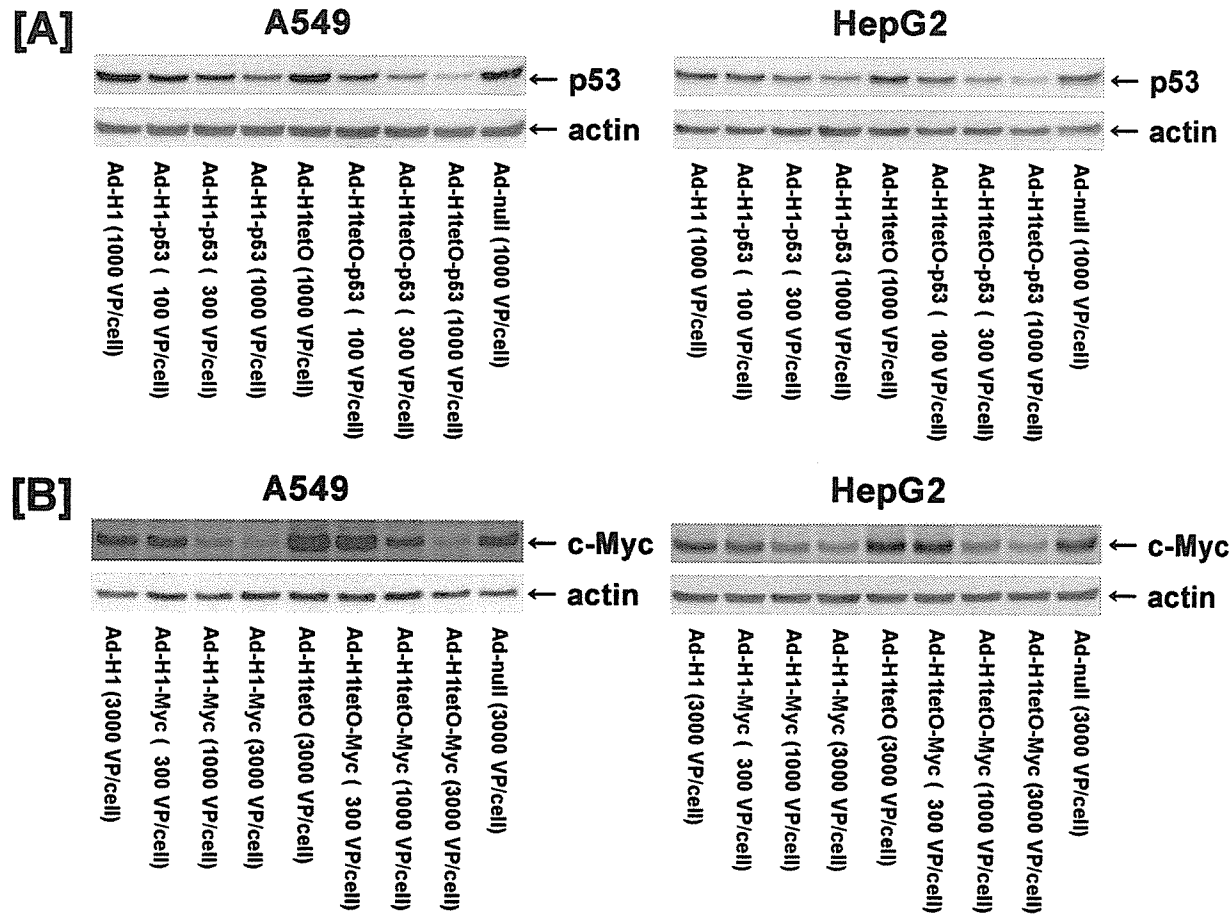

FIG. 2. Dose-dependent suppression of p53 and c-Myc protein expression by Ad vector-delivered siRNA. A549 and HepG2 cells were infected with each Ad vector for 1.5 hr, and then cultured for 3 days. Proteins were then extracted from the cells, and levels of p53 (**A**) and c-Myc (**B**) expression were examined by Western blotting. Actin bands served as an internal control for equal total protein loading.

A549 and HepG2 cells decreased in a dose-dependent manner with Ad vector carrying the siRNA expression cassette for p53 or c-Myc (Ad-H1-p53, Ad-H1-Myc, Ad-H1tetO-p53, or Ad-H1tetO-Myc). In the case of p53, a viral concentration of 1000 virus particles (VP)/cell seemed to be enough to knock down expression. Levels of p53 expression in cells treated with Ad-H1-p53 (1000 VP/cell) or Ad-H1tetO-p53 (1000 VP/cell) were decreased to 35–37 or 14–23%, respectively (Fig. 2A), relative to cells treated with Ad-null (1000 VP/cell), according to Image Gauge software (Fujifilm). In the case of c-Myc, a viral concentration of 3000 VP/cell was required to completely knock down expression, although a viral concentration of 1000 VP/cell enabled a moderate knockdown of expression. c-Myc protein expression in cells treated with Ad-H1-Myc (3000 VP/cell) or Ad-H1tetO-Myc (3000 VP/cell) was decreased to 14–44 or 16–35%, respectively (Fig. 2B), relative to cells treated with Ad-null and Ad-H1 (3000 VP/cell). The difference in degree of gene silencing may reflect the effectiveness of the siRNA sequence against each target gene. Compared with Ad-null, Ad-H1 and Ad-H1tetO did not show any effect on gene expression. These results indicate that the *tetO* sequence, placed between the TATA box and the transcription start site of the H1 promoter, does not interfere with promoter activity, and that Ad vectors containing the mutant H1 promoter, as well as the normal H1 promoter-mediated siRNA expression cassette, efficiently silence target gene expression.

Next, we examined whether regulated gene silencing is obtained by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR, the Ad vector expressing TetR, into A549 cells cultured with or without Dox (10 μg/ml). As shown in Fig. 3, in the presence of Dox the silencing effect on p53 expression decreased in proportion to the dose of Ad-TR. Efficient release of gene silencing was obtained with a 1:6 molar ratio of Ad-H1tetO-p53 to Ad-TR, although more Ad-TR might be required to completely release gene silencing. These results suggest that increased amounts of TetR are required to block transcription from the mutant H1 promoter, which contains the *tetO* sequence, in the presence of Dox (Fig. 3A). In the absence of Dox, p53 expression in cells was silenced by coinfection with Ad-H1tetO-p53 and Ad-TR. Therefore, for the regulated silencing of p53 expression, increased amounts of Ad-TR, compared with Ad-H1tetO-p53, were required. A similar result was observed in experiments on c-Myc expression (Fig. 3B), and also in HepG2 cells (both *p53* and c-*myc* genes; data not shown).

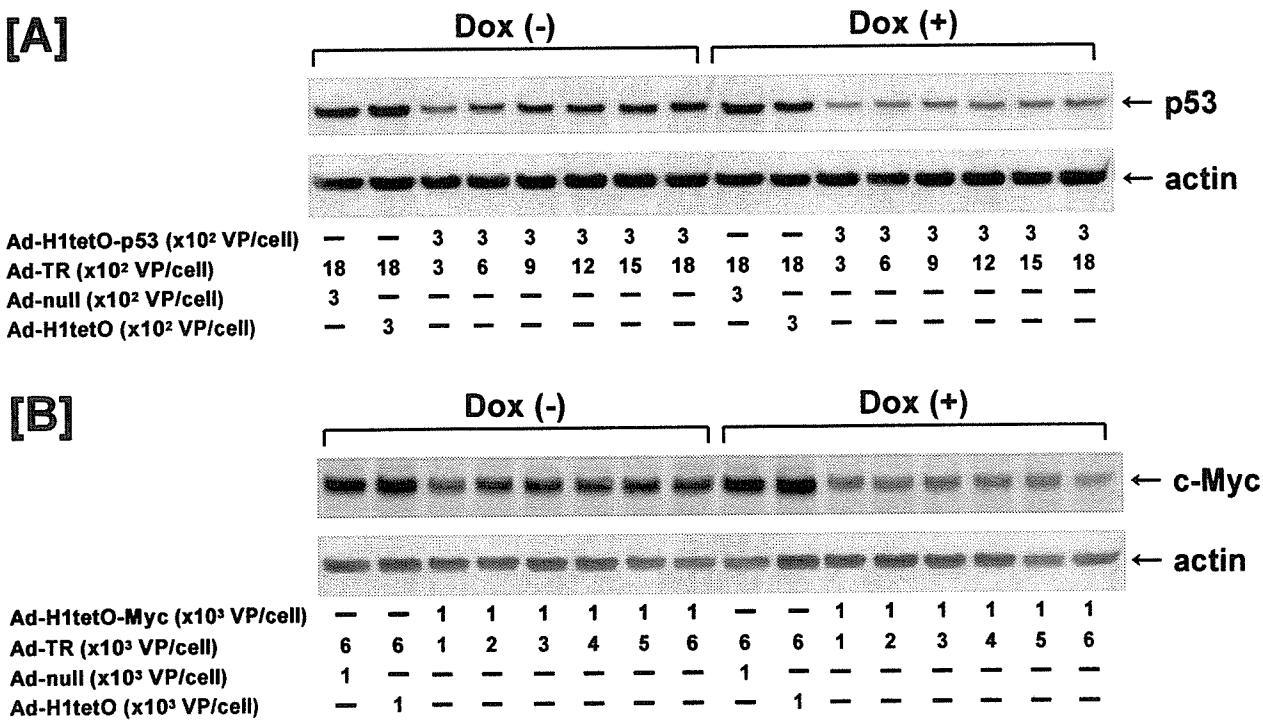

FIG. 3. Regulated suppression of p53 and c-Myc expression by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR. A549 cells were infected with the indicated amounts of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR for 1.5 hr, and then cultured with or without Dox (10 μg/ml) for 3 days. The cells were also infected with Ad-null or Ad-H1tetO plus Ad-TR. Proteins were then extracted from the cells, and levels of p53 (**A**) and c-Myc (**B**) expression were examined by Western blotting. Actin bands served as an internal control for equal total protein loading.

Ad-TR expresses TetR from the conventional cytomegalovirus (CMV) promoter-enhancer (Xu *et al.*, 2003a). Addition of the intron A sequence to the CMV promoter-enhancer, or the use of a stronger promoter such as the hybrid promoter of the β-actin promoter and CMV enhancer (Niwa *et al.*, 1991; Xu *et al.*, 2001, 2003b), would result in higher expression of TetR, thus decreasing the concentration of Ad vector expressing TetR needed to obtain inducible gene silencing efficiently. These modifications for TetR expression would make the system more effective, and would therefore enable more widespread use of this system.

We then examined the Dox concentration responsiveness of Ad vector-mediated RNAi (Fig. 4). A549 cells were coinfected with Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR at a molar ratio of 1:6, and were cultured with medium containing various concentrations of Dox. A Dox concentration of 10^{-1} μg/ml was enough to completely suppress the expression of p53 and c-Myc. At a Dox concentration of 10^{-2} μg/ml, intermediate levels of knockdown of p53 and c-Myc expression were obtained. Ad-H1tetO-p53 plus Ad-TR and Ad-H1tetO-Myc plus Ad-TR in the presence of Dox did not interfere with c-Myc and p53 expression, respectively, suggesting that the suppressive effect was target gene specific. These results suggest that the degree of knockdown of target gene expression can be modulated by Dox concentration.

We next performed a Northern blot analysis of shRNA (siRNA) expression in the presence and absence of Dox (Fig. 5). Levels of shRNA and siRNA expression for *p53* in both A549 and HepG2 cells transduced with Ad-H1tetO-p53 plus Ad-TR in the absence of Dox were significantly reduced compared with those in transduced cells in the presence of Dox (Fig. 5A). The signal of shRNA and siRNA in the absence of Dox was faint. These observations were marked in the case of c-*myc* (Fig. 5B). These results suggested that shRNA expression was tightly regulated in the Ad vector-mediated Dox-inducible RNAi system.

While this work was in progress, a plasmid vector-mediated inducible siRNA expression system using TetR was reported by van de Wetering *et al.* (2003). The mutant H1 promoter in their system contains the *tetO* sequence at a different position (by 1 bp) compared with the position of the *tetO* sequence in the present study. Both positions for insertion of the *tetO* sequence in the H1 promoter seem to be functional for regulated transcription. A similar system using the mutant U6 promoter containing the *tetO* sequence and TetR for inducible RNAi has also been reported (Matsukura *et al.*, 2003). Furthermore, a tetracycline repressor-based system was reported by two groups. In the study by Wiznerowicz and Trono, the *tetO* sequence was placed upstream of the U6 promoter (Chen *et al.*, 2003; Wiznerowicz and Trono, 2003), whereas in the study by Chen *et al.*, the *tetO* sequence was placed in these three regions: (1) upstream of the U6 promoter, (2) between the distal promoter element and the core promoter (PSE) of the U6 promoter, and (3) between the PSE and TATA box of the U6 promoter (Chen *et al.*, 2003; Wiznerowicz and Trono, 2003).

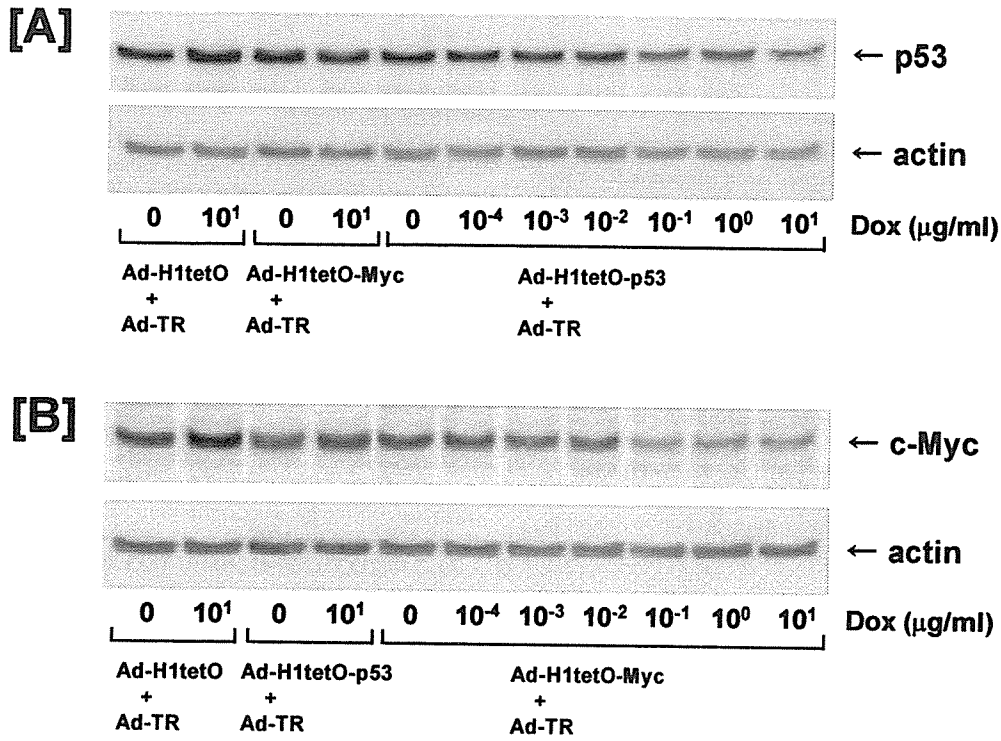

FIG. 4. Dox dose-dependent suppression of p53 and c-Myc expression by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR. A549 cells were infected with Ad-H1tetO-p53 (300 VP/cell) plus Ad-TR (1800 VP/cell) or with Ad-H1tetO-Myc (1000 VP/cell) plus Ad-TR (6000 VP/cell) for 1.5 hr, and then cultured with various concentrations of Dox for 3 days. The cells were also infected with Ad-H1tetO plus Ad-TR. Proteins were then extracted from the cells, and levels of p53 (**A**) and c-Myc (**B**) expression were examined by Western blotting. Actin bands served as an internal control for equal total protein loading.

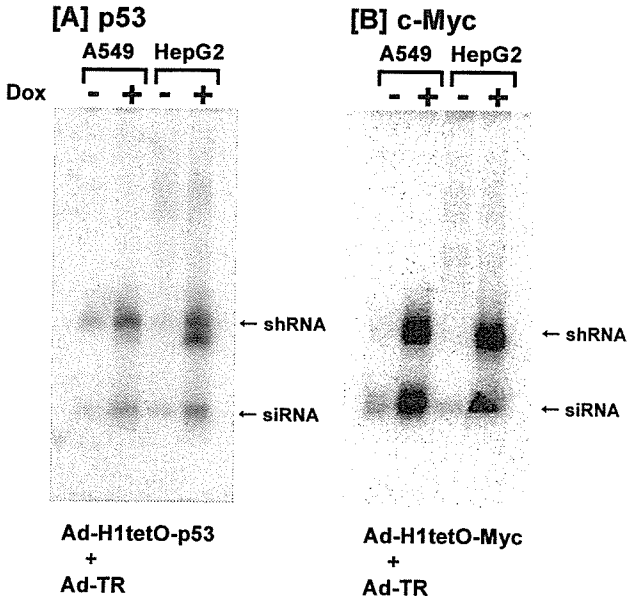

FIG. 5. Dox-inducible *p53* or c-*myc* siRNA expression by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR. A549 and HepG2 cells were infected with Ad-H1tetO-p53 (300 VP/cell) plus Ad-TR (1800 VP/cell) or with Ad-H1tetO-Myc (1000 VP/cell) plus Ad-TR (6000 VP/cell) for 1.5 hr, and then cultured with or without Dox (1 μg/ml) for 3 days. Total RNAs were then extracted from the cells, and levels of *p53* and c-*myc* siRNA expression were examined by Northern blotting.

Ad vector-mediated RNAi represents a new strategy for the study of gene function and therapeutic applications. Ad vector-mediated delivery of siRNA allows efficient transduction into a variety of cell types *in vitro* and *in vivo*. Several studies have reported Ad vector-mediated gene silencing using both the Pol II promoter, in which a mutant CMV promoter was used, and the Pol III (H1) promoter (Xia *et al.*, 2002; Zhao *et al.*, 2003). The combination of Ad vectors and an inducible siRNA expression system offers a superior strategy to researchers. To our knowledge, this study is the first to report on the development of Ad vector-mediated inducible RNAi. Various inducible siRNA expression systems, including a tetracycline repressor-based system, and a capsid-modified Ad vector to change viral tropism, can be easily combined. The system described here has great potential for therapeutic use as well as for a variety of applications, including the study of gene function.

ACKNOWLEDGMENTS

We thank Takashi Fukushima and Tomomi Sasaki for technical assistance. T.H. is the recipient of a fellowship from the Japan Society for the Promotion of Science. Z.L.X. is the recipient of a postdoctoral fellowship from the Japan Health Sciences Foundation. This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan.

REFERENCES

BRUMMELKAMP, T.R., BERNARDS, R., and AGAMI, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science **296,** 550–553.

CHEN, Y., STAMATOYANNOPOULOS, G., and SONG, C.Z. (2003). Down-regulation of CXCR4 by inducible small interfering RNA inhibits breast cancer cell invasion *in vitro*. Cancer Res. **63,** 4801–4804.

CURIEL, D.T. (1999). Strategies to adapt adenoviral vectors for targeted delivery. Ann. N.Y. Acad. Sci. **886,** 158–171.

ELBASHIR, S.M., HARBORTH, J., LENDECKEL, W., YALCIN, A., WEBER, K., and TUSCHL, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411,** 494–498.

KANEGAE, Y., MAKIMURA, M., and SAITO, I. (1994). A simple and efficient method for purification of infectious recombinant adenovirus. Jpn. J. Med. Sci. Biol. **47,** 157–166.

KOIZUMI, N., MIZUGUCHI, H., SAKURAI, F., YAMAGUCHI, T., WATANABE, Y., and HAYAKAWA, T. (2003a). Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and α_v integrin-binding ablation. J. Virol. **77,** 13062–13072.

KOIZUMI, N., MIZUGUCHI, H., UTOGUCHI, N., WATANABE, Y., and HAYAKAWA, T. (2003b). Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. J. Gene Med. **5,** 267–276.

LEE, N.S., DOHJIMA, T., BAUER, G., LI, H., LI, M.J., EHSANI, A., SALVATERRA, P., and ROSSI, J. (2002). Expression of small interfering RNAs targeted against HIV-1 *rev* transcripts in human cells. Nat. Biotechnol. **20,** 500–505.

MAIZEL, J.V., Jr., WHITE, D.O., and SCHARFF, M.D. (1968). The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. Virology **36,** 115–125.

MATSUKURA, S., JONES, P.A., and TAKAI, D. (2003). Establishment of conditional vectors for hairpin siRNA knockdowns. Nucleic Acids Res. **31,** e77.

MCMANUS, M.T., and SHARP, P.A. (2002). Gene silencing in mammals by small interfering RNAs. Nat. Rev. Genet. **3,** 737–747.

MIYAGISHI, M., and TAIRA, K. (2002). U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. Nat. Biotechnol. **20,** 497–500.

MIZUGUCHI, H., and KAY, M.A. (1998). Efficient construction of a recombinant adenovirus vector by an improved *in vitro* ligation method. Hum. Gene Ther. **9,** 2577–2583.

MIZUGUCHI, H., and KAY, M.A. (1999). A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. Hum. Gene Ther. **10,** 2013–2017.

MIZUGUCHI, H., KOIZUMI, N., HOSONO, T., UTOGUCHI, N., WATANABE, Y., KAY, M.A., and HAYAKAWA, T. (2001). A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. Gene Ther. **8,** 730–735.

NIWA, H., YAMAMURA, K., and MIYAZAKI, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108,** 193–199.

PADDISON, P.J., CAUDY, A.A., BERNSTEIN, E., HANNON, G.J., and CONKLIN, D.S. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev. **16,** 948–958.

PAUL, C.P., GOOD, P.D., WINER, I., and ENGELKE, D.R. (2002). Effective expression of small interfering RNA in human cells. Nat. Biotechnol. **20,** 505–508.

SUI, G., SOOHOO, C., AFFAR EL, B., GAY, F., SHI, Y., and FORRESTER, W.C. (2002). A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **99,** 5515–5520.

VAN DE WETERING, M., OVING, I., MUNCAN, V., PON FONG, M.T., BRANTJES, H., VAN LEENEN, D., HOLSTEGE, F.C., BRUMMELKAMP, T.R., AGAMI, R., and CLEVERS, H. (2003). Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. EMBO Rep. **4,** 609–615.

WICKHAM, T.J. (2000). Targeting adenovirus. Gene Ther. **7,** 110–114.

WIZNEROWICZ, M., and TRONO, D. (2003). Conditional suppression of cellular genes: Lentivirus vector-mediated drug-inducible RNA interference. J. Virol. **77,** 8957–8961.

XIA, H., MAO, Q., PAULSON, H.L., and DAVIDSON, B.L. (2002). siRNA-mediated gene silencing *in vitro* and *in vivo*. Nat. Biotechnol. **20,** 1006–1010.

XU, Z.L., MIZUGUCHI, H., ISHII-WATABE, A., UCHIDA, E., MAYUMI, T., and HAYAKAWA, T. (2001). Optimization of transcriptional regulatory elements for constructing plasmid vectors. Gene **272,** 149–156.

XU, Z.L., MIZUGUCHI, H., MAYUMI, T., and HAYAKAWA, T. (2003a). Regulated gene expression from adenovirus vectors: A systematic comparison of various inducible systems. Gene **309,** 145–151.

XU, Z.L., MIZUGUCHI, H., MAYUMI, T., and HAYAKAWA, T. (2003b). Woodchuck hepatitis virus post-transcriptional regulation element enhances transgene expression from adenovirus vectors. Biochim. Biophys. Acta **1621,** 266–271.

YAO, F., SVENSJO, T., WINKLER, T., LU, M., ERIKSSON, C., and ERIKSSON, E. (1998). Tetracycline repressor, TetR, rather than the TetR–mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. Hum. Gene Ther. **9,** 1939–1950.

YU, J.Y., DERUITER, S.L., and TURNER, D.L. (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **99,** 6047–6052.

ZHAO, L.J., JIAN, H., and ZHU, H. (2003). Specific gene inhibition by adenovirus-mediated expression of small interfering RNA. Gene **316,** 137–141.

Address reprint requests to:
Dr. Hiroyuki Mizuguchi
Division of Cellular and Gene Therapy Products
National Institute of Health Sciences
1-18-1 Kamiyoga, Setagaya-ku
Tokyo 158-8501, Japan

E-mail: mizuguch@nihs.go.jp

Received for publication February 5, 2004; accepted after revision June 16, 2004.

Published online: July 16, 2004.

Department of Biopharmaceutics[1], Graduate School of Pharmaceutical Sciences, Osaka University, Japan; Department of Pharmaceutics[2], School of Pharmaceutical Sciences, Zhejiang University, P.R. China; Division of Cellular and Gene Therapy Products[3], National Institute of Health Sciences, Japan

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

J.Q. GAO[1,2], S. INOUE[1], Y. TSUKADA[1], K. KATAYAMA[1], Y. ETO[1], S. KURACHI[1], H. MIZUGUCHI[3], T. HAYAKAWA[3], Y. TSUTSUMI[1], T. MAYUMI[1], S. NAKAGAWA[1]

Received December 9, 2003, accepted February 25, 2004

Shinsaku Nakagawa, Ph.D Yamadaoka 1–6, 565–0871 Suita city, Osaka, Japan
hakagawa@phs.osaka-u.ac.jp

Pharmazie 59: 571–572 (2004)

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.

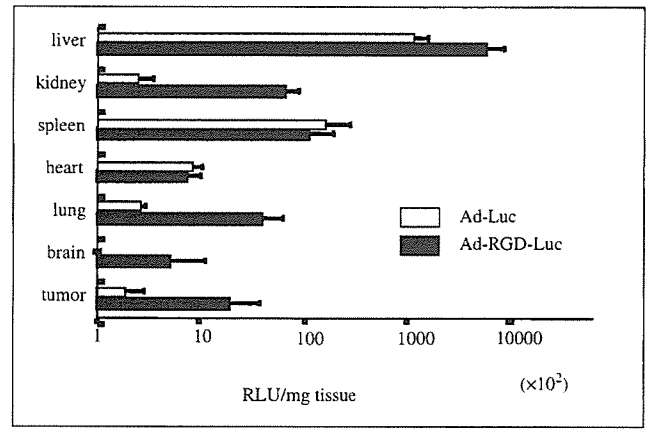

Fig. 2: Luciferase activity in organs after i.v. injection of Ad-Luc and Ad-RGD-Luc. The C57BL/6 mice were intradermally inoculated with 2×10^5 B16BL6 melanoma cells. After six days, 2×10^9 viral particles/mouse of Ad-Luc or Ad-RGD-Luc were injected into tail vein, respectively and the organs were harvested 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-

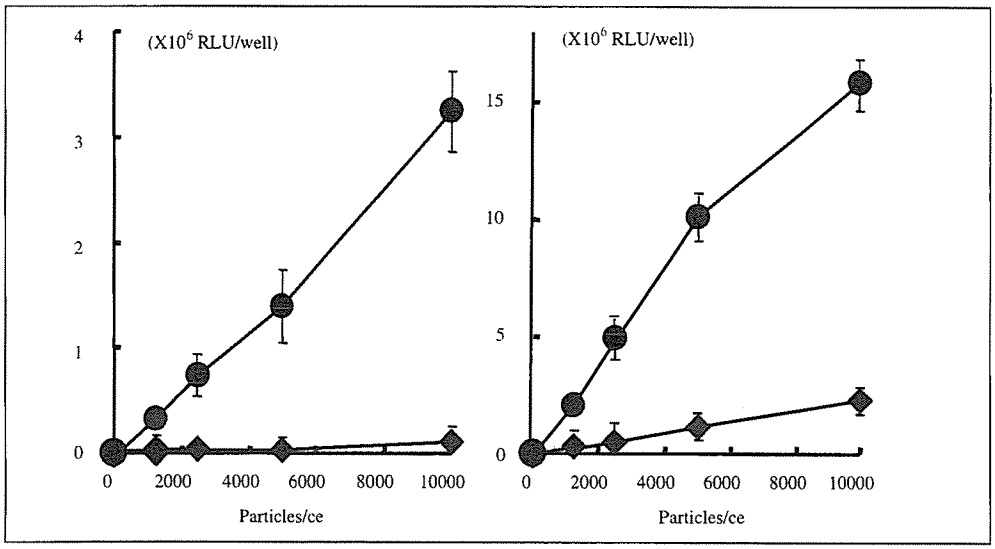

Fig. 1:
Luciferase expression of Ad-Luc or Ad-RGD-Luc infected A2058 and B16BL6 melanoma cells. A2058 cells (right) and B16BL6 cells (left) were transduced with Ad-Luc (◆) or Ad-RGD-Luc (●) respectively at the indicated viral particles/cell for 48 h. Subsequently, the cells were washed, collected, and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

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.

References

Asaoka K, Tada M, Sawamura Y et al. (2000) Dependence of efficient adenoviral gene delivery in malignant glioma cells on the expression levels of the Coxsackievirus and adenovirus receptor. J Neurosurg 92: 1002–1008.

Bergelson JM, Cunningham JA, Droguett G et al. (1997) Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science. 275: 1320–1323.

Gao JQ, Tsuda Y, Katayama K et al. (2003) Anti-tumor effect by interleukin-11 receptor alpha-locus chemokine/CCL27, introduced into tumor cells through a recombinant adenovirus vector. Cancer Res 63: 4420–4425.

Gao JQ, Alexandre LS, Tsuda Y et al. (2004) Tumor-suppressive activities by chemokines introduced into OV-HM cells using fiber-mutant adenovirus vectors. Pharmazie 59: 238–239.

Mizuguchi H, Koizumi N, Hosono T et al. (2001a) A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. Gene Ther 8: 730–735.

Mizuguchi H, Kay MA, Hayakawa T (2001b) *In vitro* ligation-based cloning of foreign DNAs into the E3 and E1 deletion regions for generation of recombinant adenovirus vectors. Biotechniques 30: 1112–1114.

Mizuguchi H, Kay MA (1998) Efficient construction of a recombinant adenovirus vector by an improved *in vitro* ligation method. Hum Gene Ther 9: 2577–2583.

Tomko RP, Xu R Philipson L (1997) HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc Natl Acad Sci USA 94: 3352–3356.

Wickham TJ, Mathias P, Cheresh DA et al. (1993) Integrins αvβ3 and αvβ5 promote adenovirus internalization but not virus attachment. Cell 73: 309–319.

Wickham TJ, Filardo EJ, Cheresh DA et al. (1994) Integrin αvβ5 selectively promotes adenovirus mediated cell membrane permeabilization. J Cell Biol 127: 257–264.

Neutralizing antibody evasion ability of adenovirus vector induced by the bioconjugation of MPEG-SPA

Yusuke ETO,[#,a] Jian-Qing GAO,[#,a,b] Fumiko SEKIGUCHI,[a] Shinnosuke KURACHI,[a] Kazufumi KATAYAMA,[a] Hiroyuki MIZUGUCHI,[c] Takao HAYAKAWA,[d] Yasuo TSUTSUMI,[a] Tadanori MAYUMI,[a] and Shinsaku NAKAGAWA,[*,a]

[a]*Graduate School of Pharmaceutical Sciences, Osaka University; Yamadaoka 1-6, Suita city, Osaka 565-0871, Japan:* [b]*Department of Pharmaceutics, College of Pharmaceutical Sciences, Zhejiang University, 353 Yanan Road, Hangzhou, Zhejiang 310031, P.R.China.:* [c]*Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, Tokyo 158-8531, Japan:* [d]*National Institute of Health Sciences, Tokyo 158-8531, Japan.*

*To whom correspondence should be addressed. e-mail: nakagawa@phs.osaka-u.ac.jp

# These authors contributed equally to the work.

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

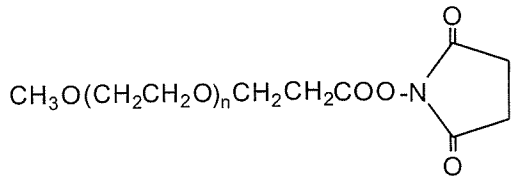

Fig.1. mPEG-SPA methoxypolyethylene glycol succinimidyl propionate, M.W. 5,000

Table 1. Viral particle sizes of PEGylated Ad and unmodified-Ad

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

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

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 ℃. 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 5,000, 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℃ for 45 min with gentle stirring (300 rpm). The particle size of PEG-Ad was measured by ZETASIZER 3000HS (Malvern, UK).

SDS-PAGE analysis Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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. USA) and 5% (v/v) 2-mercaptothanol was added to the final solution. After heating at 95℃ 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 RainbowTM molecular weight marker (Amersham Life Science, USA) 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 ℃.

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, USA) and Microlumat Plus LB 96

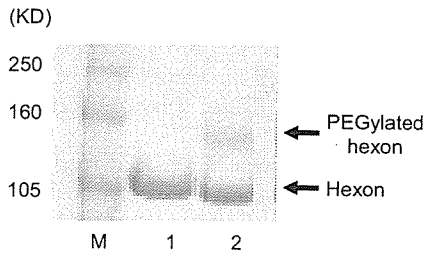

Fig. 2. SDS-PAGE analysis of PEGylated hexon Lane M, protein marker ; lane 1, unmodified-Ad ; lane 2, PEG-Ad. Further details are described in the methods.

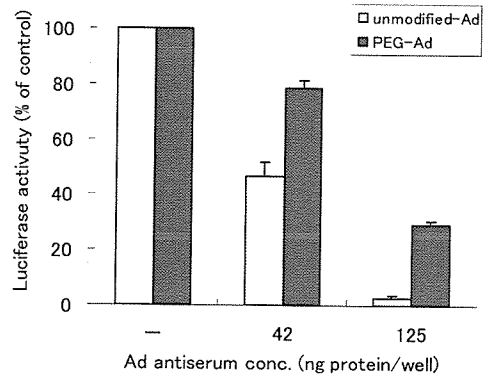

Fig. 3. Transduction efficiency of unmodified-Ad and PEG-Ad in the presence or absence of Ad antiserum A549 cells ($1x10^4$ cells) were transduced with 1000 particles/cell of unmodified-Ad and PEG-Ad in the presence or absence of Ad antiserum respectively. Luciferase expression was measured after 24 hr. Each point was represented as mean ± S.D. (n=3).

(Perkin Elmer, USA) after cells were lysed with Luciferase Cell Culture Lysis Reagent (Promega, USA) 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 coxackie-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 required 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.

ACKNOWLEDGEMENTS

This study was supported in part by the Research on Health Sciences focusing on Drug Innovation from The Japan Health Sciences Foundation; by Grants-in-Aid for Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and by grants from the Ministry of Health and Welfare in Japan.

REFERENCES

1) Gao J.Q., Tsuda Y., Katayama K., Nakayama T., Hatanaka Y., Tani Y., Mizuguchi H., Hayakawa T., Yoshie O., Tsutsumi Y., Mayumi T., Nakagawa S., *Cancer Res.*, **63**, 4420−5 (2003).
2) Crystal, R.G., *Science*, **270**, 404−10 (1995).
3) Wilson J.M., *N. Engl. J. Med.*, **334**, 1185−7 (1996).
4) Smith T.A., Mehaffey M.G., Kayda D.B., Saunders J.M., Yei S., Trapnell B.C., McClelland A., Kaleko M., *Nat. Genet.*, **5**, 397−402 (1993)
5) Hitt M., Addison C., Graham F., *Adv. Pharmacol.*, **40**, 137−206 (1997).
6) Connelly S., Andrews J.L., Gallo A.M., Kayda D.B., Qian J., Hoyer L., Kadan M.J., Gorziglia M.I., Trapnell B.C., McClelland A., Kaleko M., *Blood.*, **91**, 3273−3281 (1998).
7) Zimmer K.P., Bendiks M., Mori M., Kominami E., Robinson M.B., Ye X., Wilson J.M., *Mol. Med.*, **5**, 244−253 (1999).
8) Wohlfart C., *J. Virol.*, **62**, 2321−8 (1988).
9) Mastrangeli A., Harvey B.G., Yao J., Wolff G., Kovesdi I., Crystal R.G., Falck-Pedersen E., *Hum. Gene Ther.*, **7**, 79−87 (1996).
10) Yei S., Mittereder N., Tang K., O'Sullivan C., Trapnell B.C., *Gene Ther.*, **1**, 192 − 200 (1994).
11) Inada Y., Furukawa M., Sasaki H., Kodera Y., Hiroto M., Nishimura H., Matsushima A., *Trends Biotechnol*, **13**, 86−91 (1995).
12) Tsutsumi Y., Tsunoda S., Kamada H., Kihira T., Nakagawa S., Kaneda Y., Kanamori T., Mayumi T., *Br. J. Cancer*, **74**, 1090 − 5 (1996).
13) Von Seggern D.J., Chiu C.Y., Fleck S.K., Stewart P.L., Nemerow G.R., *J. Virol.*, **73**, 1601−8 (1999).
14) Koizumi N., Mizuguchi H., Sakurai F., Yamaguchi T., Watanabe Y., Hayakawa T., *J. Virol.*, **77**, 13062−72 (2003).
15) Mizuguchi H., Kay M.A., *Hum. Gene Ther.* **9**, 2577−83 (1998).
16) Maizel J.V. Jr., White D.O., Scharff M.D., *Virology*, **36**, 115−25 (1968).
17) Li W.M., Mayer L.D., Bally M.B., *J. Pharmacol. Exp. Ther.*, **300**, 976−83 (2002).
18) Chillon M., Lee J.H., Fasbender A., Welsh M.J., *Gene Ther.*, **5**, 995−1002 (1998).
19) Tsutsumi Y., Kihira T., Tsunoda S., Kamada H., Nakagawa S., Kaneda Y., Kanamori T., Mayumi T., *J. Pharmacol. Exp. Ther.*, **278**, 1006−11 (1996).

nature biotechnology

Antibody-targeted cell fusion

Takafumi Nakamura[1], Kah-Whye Peng[1], Sompong Vongpunsawad[1], Mary Harvey[1], Hiroyuki Mizuguchi[2], Takao Hayakawa[3], Roberto Cattaneo[1] & Stephen J Russell[1]

Membrane fusion has many potential applications in biotechnology. Here we show that antibody-targeted cell fusion can be achieved by engineering a fusogenic viral membrane glycoprotein complex. Three different single-chain antibodies were displayed at the extracellular C terminus of the measles hemagglutinin (H) protein, and combinations of point mutations were introduced to ablate its ability to trigger fusion through the native viral receptors CD46 and SLAM. When coexpressed with the measles fusion (F) protein, using plasmid cotransfection or bicistronic adenoviral vectors, the retargeted H proteins could mediate antibody-targeted cell fusion of receptor-negative or receptor-positive index cells with receptor-positive target cells. Adenoviral expression vectors mediating human epidermal growth factor receptor (EGFR)-targeted cell fusion were potently cytotoxic against EGFR-positive tumor cell lines and showed superior antitumor potency against EGFR-positive tumor xenografts as compared with control adenoviruses expressing native (untargeted) or CD38-targeted H proteins.

Cell fusion is essential for fertilization and for the development of placenta, muscle and bone[1]. It provides a basis for stem cell plasticity[2] and is central to the pathogenesis of numerous viral infections[3–5]. Cell fusion is also a scientific tool, used in the production of monoclonal antibodies[6], the identification of oncogenes and tumor suppressor genes[7] and the elucidation of chromosomal functions[8]. Cell fusion has therapeutic applications in cancer gene therapy[9], virotherapy[10] and the generation of novel cancer vaccines[11,12]. We are therefore developing technology to control cell fusion, restricting and redirecting it to achieve target specificity.

Fusion of measles-infected cells is mediated by the viral H and F proteins, which together form a fusogenic membrane glycoprotein complex[3]. The H protein mediates attachment to either one of the viral receptors CD46[13] or SLAM[14] on the cell surface, and signals to the F protein to trigger cell fusion[15]. The steps required to retarget this cell fusion reaction are ablation of H protein–mediated CD46 and SLAM receptor recognition and introduction of a new binding specificity in the H glycoprotein, while preserving its ability to trigger conformational changes in the F protein that lead to membrane fusion. We have demonstrated that single-chain antibodies (scFvs) against carcinoembryonic antigen (CEA) and CD38 (a myeloma cell marker) could be displayed at the C terminus of hybrid H proteins where they triggered F protein–mediated fusion upon binding via the antibody to the targeted receptor[16,17]. However, fusion was also triggered via the natural measles receptors, CD46 and SLAM, which are widely expressed on normal tissues.

We sought to improve target specificity by introducing mutations in the H protein that would block its interactions with CD46 and SLAM. It is known that amino acids 451 and 481 in the H protein play an important role in the interaction with CD46[18]. In addition, by alanine scanning mutagenesis, we recently identified mutations at positions 529 and 533 that ablate fusion through SLAM[19]. We fused an anti-CD38 scFv to the C terminus of the H protein and mutated residues involved in binding to CD46 (451,481) and SLAM (529, 533) (Fig. 1a). Receptor-specific fusion support by the chimeric H protein expression plasmids was determined after F protein–plasmid cotransfection in cells expressing either CD46, SLAM or CD38 (Fig. 1b). Syncytial cytopathic effect was scored by counting syncytia. Paired mutations at positions 451 and 529, or 481 and 533, supported fusion via the targeted CD38 receptor but not via CD46 or SLAM. These data proved conclusively that antibody-targeted cell fusion can be achieved. However, the fusion support activity of the fully retargeted H chimeras on Chinese hamster ovary (CHO)-CD38 cells was considerably lower than that of the original nonablated chimeric protein, H-CD38.

To address the issue of suboptimal fusion support by fully retargeted chimeric H glycoproteins, we focused on residue 481, as amino acid substitutions at this position can have a strong effect on fusion triggering activity[20]. We therefore generated additional H protein chimeras mutated as before at residue 533 (R533A) but with different substitutions at position 481 (Y481M, Y481Q, Y481A) in place of Y481N. Interestingly, all of the new 481-substituted H protein chimeras retained the fully retargeted phenotype but showed higher fusion support activity than the original Y481N mutant on CHO-CD38 cells. In particular, the CD38-targeted chimera carrying mutation Y481A in addition to R533A (H_{AA}-CD38) was slightly more fusogenic on CHO-CD38 cells than was the nonablated chimera H-CD38 (Fig. 1b,c).

Because fusion support activity has been reported to depend on efficient transport and cell surface expression of the H protein[21], we determined total quantities of several chimeric H proteins both in whole cell lysates of transfected cells and at the cell surface (Fig. 1d,e). In CHO-CD38 cells, total cellular H protein expression from the

[1]Molecular Medicine Program, Mayo Foundation, 200 First St. SW, Rochester, Minnesota 55905, USA. [2]Division of Cellular and Gene Therapy Products, [3]National Institute of Health Sciences, Tokyo 158-8501, Japan. Correspondence should be addressed to S.J.R. (sjr@mayo.edu).

Published online 15 February 2004; doi:10.1038/nbt942

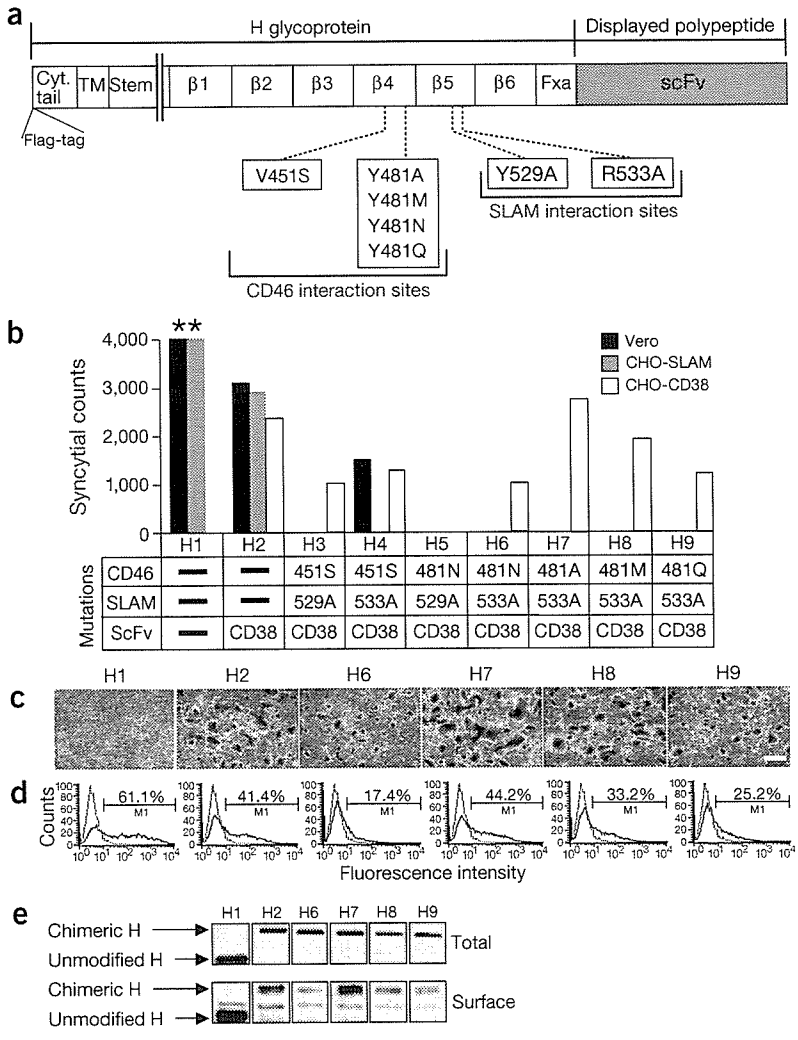

Figure 1 Fusogenic properties of mutant H glycoproteins displaying anti-CD38 antibody. (**a**) Schematic representation of MV-Edm H protein[37] showing H residues mediating CD46 or SLAM interactions. The single-chain antibody is displayed as a C-terminal extension of H glycoprotein and an N-terminal FLAG tag (DYKDDDK) is included to facilitate immunoblot detection. cyt., cytoplasmic; TM, transmembrane; Fxa, Factor Xa cleavage site (IEGR). Standard one-letter abbreviations are used to denote amino acid residues. (**b**) Receptor-specific fusion support by chimeric H expression plasmids after cotransfection with measles F plasmid. Syncytia in five representative fields were counted, and the number of syncytia per well was calculated. Asterisks indicate that the syncytia were not countable because >90% of the cells were in syncytia. (**c**) CHO-CD38 cells were cotransfected with indicated H expression plasmids and measles F, and syncytia were photographed 24 h later. Scale bar, 200 μm. (**d**) Cell surface expression of chimeric H proteins (bold lines), relative to mock transfected cells (thin lines) was determined by FACS analysis after labeling with anti-H antibody. See **b** for H-construct designations. (**e**) Total and cell surface H protein expression levels were also estimated by immunoblotting of cell lysates or of surface biotinylated proteins immunoprecipitated with anti-Flag antibody. See **b** for H-construct designations. The fusogenic activities of the CD38-displaying H chimeras correlate closely with their levels of surface expression.

different chimeric H protein expression plasmids was similar (**Fig. 1e**). However, cell surface expression, determined by immunoprecipitation of surface biotinylated protein (**Fig. 1e**) and by fluorescence-activated cell sorting (FACS) analysis (**Fig. 1d**), differed substantially among the various chimeras. Cell surface expression of every chimeric H protein tested was consistently in accord with the intensity of its fusion support activity on CHO-CD38 cells. In particular, the slightly higher surface expression of the doubly ablated H_{AA}-CD38 mutant in comparison to the nonablated H-CD38 chimera showed that the Y481A and R533A mutations provide the optimal platform for generation of fully retargeted H proteins by scFv display. Indeed, this conclusion was further confirmed by comparing panels of chimeric H proteins displaying alternative scFvs (data not shown).

To determine whether our findings could be generalized to scFvs targeting human cellular receptors other than CD38, we generated doubly ablated (Y481A, R533A) H-protein chimeras displaying C-terminal scFvs recognizing H_{AA}-CEA[16,22] or H_{AA}-EGFR[23]. Each of the doubly ablated, scFv-displaying chimeric H proteins supported fusion, leading to cell death exclusively in cells expressing the relevant targeted receptor (**Fig. 2a,b**). Background fusion via CD46 or SLAM was not observed. In contrast to these targeted H proteins, the unmodified H protein led to extensive syncytium formation and cytotoxicity in CHO-CD46 and CHO-SLAM cells. Thus, F protein–mediated cell fusion could be fully and accurately redirected through different antibody-receptor interactions by displaying scFvs at the C terminus of a doubly ablated, receptor-blind H protein.

Genes coding for fusogenic membrane glycoproteins have recently been exploited for cytoreductive gene therapy for cancer, whereby transduced cancer cells fuse with neighboring nontransduced cells, leading to tumor regression[9]. To demonstrate the potential of targeted cell fusion for cytoreductive gene therapy of human cancer, we generated bicistronic adenovirus vectors expressing measles F protein with EGFR-targeted, CD38-targeted or untargeted H proteins, and compared their specificity and potency against human ovarian SKOV3ip.1 tumor cells as a treatment model. SKOV3ip.1 cells express low levels of primary coxsackievirus-adenovirus receptor (CAR) and are therefore relatively resistant to adenovirus transduction (**Fig. 3a**). Adenoviral vectors were therefore used at relatively high multiplicity of infection, standardized to particle counts for all three vectors, to ensure a reasonable efficiency of transduction of ~1% of the tumor cells (**Fig. 3b**, first panel). The SKOV3ip.1 cells express abundant CD46 and abundant EGFR, but minimal CD38 (**Fig. 3a**). Surprisingly, in contrast to adenoviruses expressing untargeted measles H protein, the EGFR-targeted adenoviruses could mediate very efficient targeted fusion and killing of SKOV3ip.1 cells. One possible explanation for this difference is that there is a higher absolute density of EGFR compared with CD46 on the SKOV3ip.1 cells. Alternatively, it is possible that the efficiency of F protein triggering by receptor-bound H protein is intrinsically higher

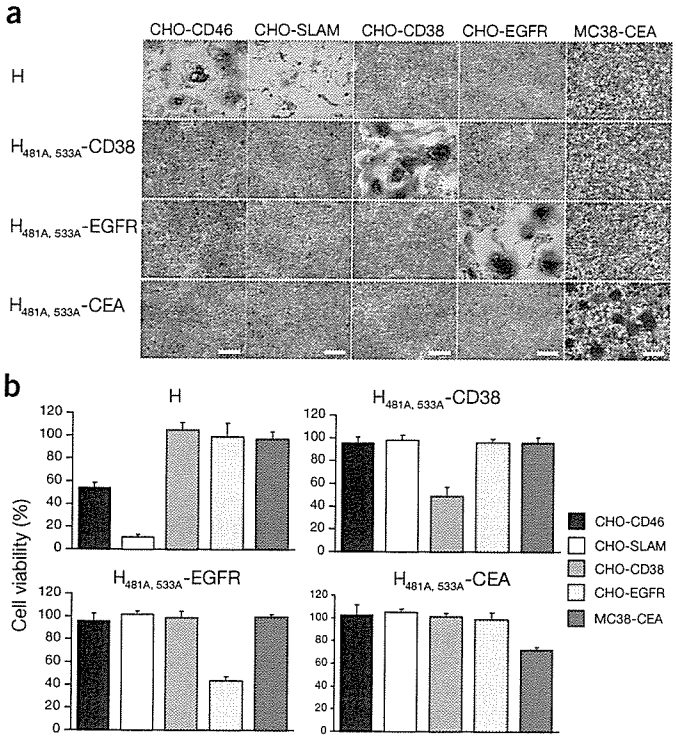

Figure 2 Antibody-targeted cell fusion and cell killing. (**a**) Target cells expressing indicated receptors were cotransfected with indicated H-expression plasmids plus measles F protein, and syncytia were photographed 24 h later. Scale bar, 200 μm. (**b**) Cell viability was determined 36 h after transfection and is shown as the percentage cell survival compared to control mock-transfected cultures. H proteins carrying Y481A and R533A mutations, and displaying cell-targeting scFvs to CD38, EGFR or CEA caused no fusion through CD46 or SLAM receptors, but efficiently mediated targeted cell fusion via CD38, EGFR or CEA, respectively.

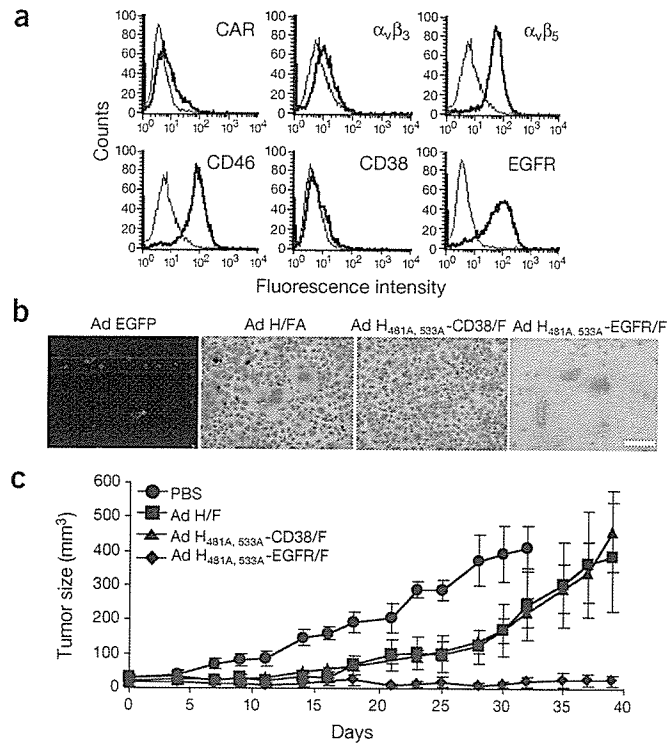

Figure 3 Targeted cytoreductive gene therapy using homologous targeted cell fusion. (**a**) Expression of relevant receptors by human SKOV3ip.1 ovarian tumor cells was determined by FACS analysis. (**b**) Cells were infected with adenoviral vectors expressing EGFP, measles F and H proteins, F protein with CD38-targeted H or F proteins with EGFR-targeted H protein at an MOI of 300 particles/cell. Cells were photographed 48 h after infection. Transduction with adenovirus vectors encoding EGFP is low due to deficiency of CAR. Transduction with the H/F vector led to moderate H/F-induced cell fusion. In contrast, the EGFR-targeted H/F vector caused massive cell fusion and cytotoxicity, whereas the CD38-targeted H/F vector had no effect on these CD38-negative SKOV3ip.1 cells. Scale bar, 200 μm. (**c**) Intratumoral injection of the EGFR-targeted H/F vector elicited potent antitumor effects in contrast to PBS, untargeted H/F and CD38-targeted H/F vectors, and three of six mice in the group showed complete regression.

after binding to EGFR. As expected, control vectors expressing the CD38-targeted H protein showed no fusion activity in SKOV3ip.1 cells.

We next evaluated the *in vivo* effects of these adenoviral vectors against well-established SKOV3ip.1 xenografts implanted subcutaneously in athymic mice. Adenoviral vectors mediating EGFR-targeted fusion showed much greater therapeutic potency in this intratumoral therapy model than control vectors mediating fusion through CD46 (untargeted) or CD38 ($P = 0.0013$ compared to PBS, or $P \leq 0.05$ compared to Ad H/F and Ad $H_{481A, 533A}$-CD38/F; comparisons made on day 32) (Fig. 3c). Histological analysis of explanted tumors 3 d after they were injected with the different adenoviral vectors showed that cell fusion was considerably more prominent in tumors inoculated with vector expressing the EGFR-targeted H protein (data not shown). Taken together, these data demonstrate the superior specificity and potency of vectors mediating antibody-targeted cell fusion in a clinically relevant, cytoreductive, gene therapy model.

Increasingly, cells are exploited as therapeutic agents and antibody-targeted fusion has considerable potential to enhance the therapeutic outcome; stem cells are used for tissue repair, immune effector cells for tumor therapy and vector-modified cells for delivery of diverse genetic payloads[2,24,25]. Recent evidence indicates that stem cell plasticity may be a direct consequence of cell fusion[25] and might therefore be greatly enhanced by directing the stem cells to fuse efficiently with a desired target tissue. Also, heterokaryons obtained by fusing tumor cells with professional antigen-presenting cells (APCs) are known to be potent stimulators of antitumor immunity[11,12]. Antibody-targeted fusion could be used to generate these hybrid cells *in situ* by directing APCs to fuse specifically with tumor cells. Finally, targeted cell fusion provides an appealing strategy to mediate the irreversible trapping of cellular gene delivery vehicles at predetermined target sites.

To demonstrate that heterologous cell fusion between an immune effector cell and an epithelial tumor could be accurately targeted, we infected K562 human erythroleukemic cells with adenoviral vectors expressing nontargeted, EGFR-targeted or CD38-targeted H proteins. K562 cells transduced with the EGFR-targeted or CD38-targeted vector did not fuse with each other but underwent heterologous fusion with EGFR-positive epithelial tumor cells (A431) or with CD38-expressing suspension Jurkat T cells, respectively (Fig. 4a,b). In addition, the EGFR-targeted heterologous fusion was blocked by the presence of anti-EGFR antibody whereas the nontargeted heterologous fusion between K562 and A431 was not (Fig. 4c). An important question arising from these studies relates to the stability of the hybrid cells generated by this method. This is currently under investigation as it is likely to be a key parameter for some of the suggested applications. Our preliminary observation is that the stability of the cell hybrids

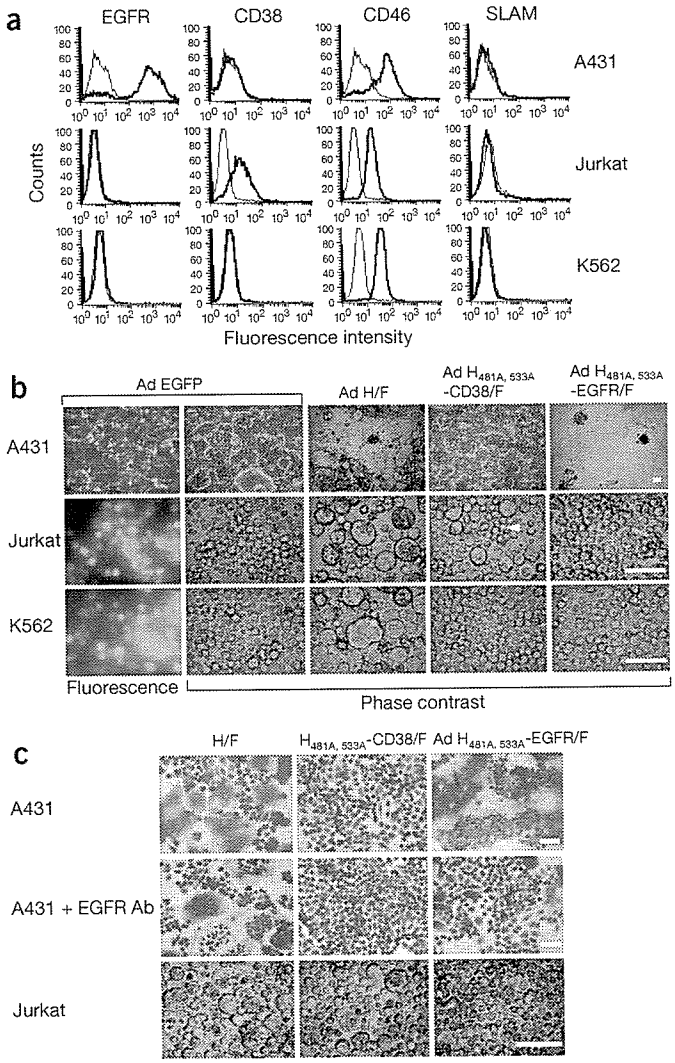

Figure 4 Adenoviral vectors mediating homologous or heterologous targeted cell fusion. (**a**) Expression of relevant receptors by human tumor cell lines was determined by FACS analysis. (**b**) Cell lines were infected with adenoviral vectors expressing EGFP, measles F and H proteins, F with CD38-targeted H or F proteins with EGFR-targeted H protein at an MOI of 1,000 particles/cell (for A431) or 10,000 particles/cell (for Jurkat T and K562 cells). Cells were photographed 48 h after infection. Adenovirus vectors encoding CD38 and EGFR-targeted H proteins could mediate targeted fusion and killing of CD38-positive Jurkat T cells or EGFR-positive A431 cells, respectively, but not of receptor-negative K562 cells. The red arrow indicates a syncytium and the white arrow indicates a single cell. (**c**) However, washed K562 cells expressing CD38 or EGFR-targeted H proteins readily underwent heterologous antibody-targeted cell fusion within 12 h when added to CD38-positive Jurkat T or EGFR-positive A431 cells, respectively. In addition, heterologous EGFR-targeted fusion of adenovirus-infected K562 cells was selectively blocked in the presence of anti-EGFR 528 antibody (EGFR Ab; final concentration 200 ng/ml). Scale bar, 80 μm (**b**,**c**).

varies greatly depending upon cell lineage, culture conditions and the number of cell nuclei in each syncytium.

The three cellular receptors targeted in this study belong to widely differing receptor families. CD38 is a 45 kDa type II transmembrane glycoprotein with NAD(P)$^+$glycohydrolase and cell signaling activity[26]; CEA is a heavily glycosylated type I membrane glycoprotein involved in cell adhesion[27]; and EGFR is a type I membrane glycoprotein that undergoes dimerization and rapid endocytosis upon binding EGF[28]. Thus, our data suggest that receptor choice is not a limitation for cell fusion and that it should be possible to target the process through a broad array of cell surface antigens, irrespective of their particular structure. Besides giving insight into the remarkable plasticity of cell fusion triggering mechanisms, antibody-targeted fusion has great potential as a research tool and provides a versatile platform for novel targeted therapies.

METHODS

Cell culture. Vero African green monkey kidney cells (#CCL-81), A431 human epidermoid carcinoma cells (#CRL-1555), Jurkat T-cell leukemia cells (#TIB-152) and K562 human erythroleukemic cells (#CCL-243) were purchased from American Type Culture Collection (ATCC). All cell lines were grown at 37 °C in media recommended by the suppliers in a humidified atmosphere of 5% CO_2. The SKOV3ip.1 ovarian tumor cells were maintained in alpha-MEM (Irvine Scientific) supplemented with 20% (v/v) FBS (Gibco). CHO-CD46 cells were generated by stable transfection of the parental CHO cells using a CD46-C1 isoform expression plasmid[29]. CHO clones stably expressing CD46 were selected using 1.2 mg/ml G418 (Gibco-BRL). Clones expressing high levels of CD46 were identified by flow cytometry using a fluorescein isothiocyanate (FITC)-conjugated, anti-CD46 antibody (Pharmingen). The CHO-CD46 and CHO-CD38[17] cells were grown and maintained in DMEM (Gibco) containing 10% (v/v) FBS, penicillin and streptomycin (DMEM-10) at 37 °C in an atmosphere of 5% CO_2 with 1 mg of G418/ml. CHO-EGFR cells[30] and MC38-cells[31] were grown and maintained in DMEM-10 with 0.5 mg/ml of G418 at 37 °C in an atmosphere of 5% CO_2. CHO-SLAM cells[14] were grown in RPMI 1640 (Gibco) containing 10% FBS, penicillin and streptomycin with 0.5 mg/ml of G418 at 37 °C in an atmosphere of 5% CO_2.

H expression plasmids, transfections and cell fusion assay. Site-directed mutagenesis of pCGHX α-CD38, a measles H glycoprotein expression construct displaying the CD38 scFv[17], was done using the Quick-Change system (Stratagene). Constructs encoding chimeric H proteins with C-terminal scFVs recognizing CEA[22] or EGFR[23] were easily made by exchanging the CD38 scFv fragment via flanking *Sfi*I and *Not*I cloning sites. Cells (8×10^4/well in 24-well plates) were cotransfected with 0.5 μg pCGF, a measles F expression plasmid[32] and 0.5 μg of the appropriate H mutant expression plasmid using Superfect (Qiagen). At 24 h after transfection, the cells were fixed in 0.5% (v/v) glutaraldehyde and stained with 0.1% (w/v) crystal violet, and the syncytia were scored and photographed. For cytotoxicity studies, cells (2×10^4/well in 96-well plates) were transfected with 0.25 μg of pCGF plasmid and 0.25 μg of the appropriate H-protein plasmid, and cell viability was assessed 36 h after transfection using the CellTiter96R Aqueous Non-Radioactive Cell Proliferation Assay (Promega). Results represent means ± s.d. of triplicate determinations.

Surface biotinylation, western blotting and FACS analysis. Cells (4×10^5/well in 6-well plates) were transfected with the appropriate H-protein mutant expression plasmids. After 24 h, the transfected cells were washed two times with 1 ml of ice-cold PBS, and surface proteins were labeled with biotin-7-*N*-hydroxysuccinimide ester for 15 min at 20 °C using a Cellular Labeling kit (Roche). The reaction was stopped by adding NH_4Cl (final concentration, 50 mM) for 15 min at 4 °C. The cells were then washed once, and treated with 500 μl of lysis buffer (50 mM Tris, pH 7.5, 1% (v/v) Igepal CA-630 (Sigma), 1 mM EDTA, 150 mM NaCl, protease inhibitor cocktail (Sigma)) for 15 min at 4 °C, and the lysates were subjected to centrifugation at 4 °C for 15 min at 12,000*g*. Then 20 μl of the resulting postnuclear fraction was directly mixed with an equal volume of SDS loading buffer (130 mM Tris, pH 6.8, 20% (v/v) glycerol, 10% (v/v) SDS, 0.02% (w/v) bromophenol blue, 100 mM dithiothreitol). These samples (40 μl) were denatured for 5 min at 95 °C, fractionated on a 7.5% SDS-polyacrylamide gel, blotted to polyvinylidene difluoride membranes (Bio-Rad), immunoblotted with anti-Flag M2 antibody conjugated to horseradish peroxidase (Sigma) and developed using an enhanced chemiluminescence kit (Pierce) for detection of total H protein. The biotinylated H proteins were immunoprecipitated with anti-Flag antibodies using an immunoprecipitation kit (Roche). We mixed 50 μl of protein A–coated agarose beads with

350 µl of the postnuclear supernatant and 1 µl of anti-Flag M2 antibody (Sigma), followed by overnight incubation at 4 °C under rotation. The agarose beads were then washed three times before resuspension in 50 µl of loading buffer and boiled for 2 min at 100 °C to elute bound proteins. As described above, the samples (40 µl) were fractionated on an SDS-polyacrylamide gel, blotted to polyvinylidene difluoride membranes, and probed with peroxidase-coupled streptavidin (Roche), followed by detection of surface H protein using an enhanced chemiluminescence kit. Alternatively, the surface expression level of H protein was detected by FACS analysis. Twenty-four hours after transfection of the appropriate H mutant or mock plasmid, cells were washed twice with PBS and resuspended in ice-cold PBS containing 2% (v/v) FBS at a concentration of 10^5 cells/ml. The cells were then incubated for 60 min on ice with 1:150 final dilution of the primary mouse monoclonal ascites antibody recognizing measles H protein (Chemicon). Subsequently, the cells were washed with 2% (v/v) FBS/PBS and incubated for an additional 30 min with 1:150 final dilution of FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). After washing with 2% (v/v) FBS/PBS, the cells were analyzed by flow cytometry using a FACScan system with CELLQuest software (Becton Dickinson). Expression of relevant receptors in A431, Jurkat T cells and K562 human tumor cells was similarly detected by FACS analysis using anti-EGFR 528 (Santa Cruz Biotechnology), anti-CD38, anti-CD46, anti-SLAM antibodies (Pharmingen), anti-CAR antibody RmcB (ATCC), anti-$\alpha_v\beta_3$ antibody (Chemicon) or anti-$\alpha_v\beta_5$ antibody (Gibco).

Adenoviral vectors. All recombinant adenoviruses were constructed using an *in vitro* ligation method as described previously[33]. H, H_{AA}-CD38, H_{AA}-EGFR or EGFP (Clontech) coding sequences were cloned downstream of a human cytomegalovirus immediate early promoter/enhancer ($P_{CMV\ IE}$) in the pHM5 shuttle vector. The F gene was cloned downstream of the $P_{CMV\ IE}$ in the pHM11 shuttle vector. Expression cassettes were transferred from the pHM5 or pHM11 shuttle vectors into the E1 or E3-deleted regions, respectively, of the adenoviral vector plasmid pAdHM48. Genes encoding H protein and EGFP were cloned into the E1 site and the gene encoding F was cloned into E3. The resulting recombinant adenovirus genomes were transfected into human embryonic kidney (HEK)-293 cells. Because expression of measles F and H proteins causes cell fusion and is toxic to HEK-293 cells, viruses were rescued in the presence of a fusion inhibitory peptide (FIP; Bachem), which blocks F/H-protein mediated fusion[34]. The resulting recombinant adenoviruses were propagated in HEK-293 cells in the presence of FIP peptide, and were purified by CsCl-equilibrium centrifugation as described previously[35]. Purified virion preparations were dialyzed against 10 mM PBS, 10% (v/v) glycerol, and finally stored at –80 °C. Viral particle numbers (particles/ml) were calculated from optical density measurements at 260 nm (OD_{260})[36]. All viruses showed similar physical particle titers of approximately 10^{11} particles/ml: Ad EGFP, 1.41×10^{11}; d H/F, 5.75×10^{11}; Ad $H_{481A,533A}$-CD38/F, 2.59×10^{11}; Ad $H_{481A,533A}$-EGFR/F, 3.20×10^{11}.

***In vivo* experiments.** All experimental protocols are approved by the Mayo Foundation Institutional Review Board and Institutional Animal Care and Use Committee. To establish subcutaneous tumors, 6-week-old athymic nu/nu female mice (Harlan Sprague Dawley) were injected with 5×10^6 tumor cells. When the tumors measured 0.3–0.4 cm in diameter, mice received four intratumoral injections of Ad H/F ($n = 6$), Ad $H_{481A,533A}$-CD38/F ($n = 5$), or Ad $H_{481A,533A}$-EGFR/F ($n = 6$) at 7×10^9 viral particles (total 2.8×10^{10}), on days 0, 1, 3 and 4. Control tumors were injected with an equal volume of PBS only ($n = 5$). Animals were killed at the end of the experiment, when tumor burden reached 10% of body weight or when ulcer was seen in tumor. The tumor diameter was measured three times per week and the volume (product of 0.5 × length × length × width) was calculated as mean ± s.e.m. Statistical analysis was performed by analysis of variance followed by Fisher's test, and $P < 0.05$ was considered to be statistically significant.

ACKNOWLEDGMENTS

We thank C.D. James for CHO-EGFR cells, Y. Yanagi for CHO-SLAM cells, J. Schlom for MC38-CEA cells, E. Vitetta for SKOV3ip.1 cells, J.P. Atkinson for CD46 plasmid, J.A. Lust for CD38 scFv, R. Hawkins for CEA scFv and G. Winter for EGFR scFv. We also thank M.J. Federspiel and R.G. Vile for critical reading of the manuscript. This study is supported by the Mayo Foundation, Harold W. Siebens Foundation and NIH grants CA100634-01 and CA90636-01.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 17 October; accepted 11 December 2003
Published online at http://www.nature.com/naturebiotechnology/

1. Shemer, G. & Podbilewicz, B. Fusomorphogenesis: cell fusion in organ formation. *Dev. Dyn.* **218**, 30–51 (2000).
2. Holden, C. & Vogel, G. Stem cells. Plasticity: time for a reappraisal. *Science* **296**, 2126–2129 (2002).
3. Griffin, D.E. Measles Virus. in *Fields Virology* (eds. Knipe, D.M. & Howley, P.M.) 1402–1442 (Lippincott Williams & Wilkins, Philadelphia, 2001).
4. Freed, E.O. & Martin, M.A. HIVs and their replication. in *Fields Virology* (eds. Knipe, D.M. & Howley, P.M.) 1971–2042 (Lippincott Williams & Wilkins, Philadelphia, 2001).
5. Cohen, J.I. & Straus, S.E. Varicella-Zoster virus and its replication. in *Fields Virology* (eds. Knipe, D.M. & Howley, P.M.) 2707–2730 (Lippincott Williams & Wilkins, Philadelphia, 2001).
6. Kohler, G. & Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495–497 (1975).
7. Dorssers, L.C. & Veldscholte, J. Identification of a novel breast-cancer-anti-estrogen-resistance (BCAR2) locus by cell-fusion-mediated gene transfer in human breast-cancer cells. *Int. J. Cancer* **72**, 700–7005 (1997).
8. Dieken, E.S., Epner, E.M., Fiering, S., Fournier, R.E. & Groudine, M. Efficient modification of human chromosomal alleles using recombination-proficient chicken/human microcell hybrids. *Nat. Genet.* **12**, 174–182 (1996).
9. Galanis, E. *et al.* Use of viral fusogenic membrane glycoproteins as novel therapeutic transgenes in gliomas. *Hum. Gene Ther.* **12**, 811–821 (2001).
10. Peng, K.W. *et al.* Intraperitoneal therapy of ovarian cancer using an engineered measles virus. *Cancer Res.* **62**, 4656–4662 (2002).
11. Guo, Y. *et al.* Effective tumor vaccine generated by fusion of hepatoma cells with activated B cells. *Science* **263**, 518–520 (1994).
12. Bateman, A. *et al.* Fusogenic membrane glycoproteins as a novel class of genes for the local and immune-mediated control of tumor growth. *Cancer Res.* **60**, 1492–1497 (2000).
13. Dorig, R.E., Marcil, A., Chopra, A. & Richardson, C.D. The human CD46 molecule is a receptor for measles virus (Edmonston strain). *Cell* **75**, 295–305 (1993).
14. Tatsuo, H., Ono, N., Tanaka, K. & Yanagi, Y. SLAM (CDw150) is a cellular receptor for measles virus. *Nature* **406**, 893–897 (2000).
15. von Messling, V., Zimmer, G., Herrler, G., Haas, L. & Cattaneo, R. The hemagglutinin of canine distemper virus determines tropism and cytopathogenicity. *J. Virol.* **75**, 6418–6427 (2001).
16. Hammond, A.L. *et al.* Single-chain antibody displayed on a recombinant measles virus confers entry through the tumor-associated carcinoembryonic antigen. *J. Virol.* **75**, 2087–2097 (2001).
17. Peng, K.W. *et al.* Oncolytic measles viruses displaying a single-chain antibody against CD38, a myeloma cell marker. *Blood* **101**, 2557–2562 (2003).
18. Lecouturier, V. *et al.* Identification of two amino acids in the hemagglutinin glycoprotein of measles virus (MV) that govern hemadsorption, HeLa cell fusion, and CD46 downregulation: phenotypic markers that differentiate vaccine and wild-type MV strains. *J. Virol.* **70**, 4200–4204 (1996).
19. Vongpunsawad, S., Oezgum, N., Braun, W. & Cattaneo, R. Selectively receptor-bind measles viruses: identification of the SLAM-and CD46-interacting residues and their localization on a new hemagglutinin structural model. *J. Virol.* **78**, 302–313 (2004).
20. Xie, M., Tanaka, K., Ono, N., Minagawa, H. & Yanagi, Y. Amino acid substitutions at position 481 differently affect the ability of the measles virus hemagglutinin to induce cell fusion in monkey and marmoset cells co-expressing the fusion protein. *Arch. Virol.* **144**, 1689–1699 (1999).
21. Cattaneo, R. & Rose, J.K. Cell fusion by the envelope glycoproteins of persistent measles viruses which caused lethal human brain disease. *J. Virol.* **67**, 1493–1502 (1993).
22. Chester K.A. *et al.* Phage libraries for generation of clinically useful antibodies. *Lancet* **343**, 455–456 (1994).
23. Kettleborough, C.A., Saldanha, J., Heath, V.J., Morrison, C.J. & Bendig, M.M. Humanization of a mouse monoclonal antibody by CDR-grafting: the importance of framework residues on loop conformation. *Protein Eng.* **4**, 773–783 (1991).
24. Wang, G. *et al.* A T cell-independent antitumor response in mice with bone marrow cells retrovirally transduced with an antibody/Fc-gamma chain chimeric receptor gene recognizing a human ovarian cancer antigen. *Nat. Med.* **4**, 168–172 (1998).
25. Wang, X. *et al.* Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* **422**, 897–901 (2003).
26. Mehta, K., Shahid, U. & Malavasi, F. Human CD38, a cell-surface protein with multiple functions. *FASEB J.* **10**, 1408–1417 (1996).
27. Obrink, B. CEA adhesion molecules: multifunctional proteins with signal-regulatory properties. *Curr. Opin. Cell Biol.* **9**, 616–626 (1997).
28. Carpenter, G. Receptor tyrosine kinase substrates: src homology domains and signal transduction. *FASEB J.* **6**, 3283–3289 (1992).
29. Kemper, C. *et al.* Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature* **421**, 388–392 (2003).
30. Schneider, U., Bullough, F., Vongpunsawad, S., Russell, S.J. & Cattaneo, R.

Recombinant measles viruses efficiently entering cells through targeted receptors. *J. Virol.* **74**, 9928–9936 (2000).
31. Robbins, P.F. *et al.* Transduction and expression of the human carcinoembryonic antigen gene in a murine colon carcinoma cell line. *Cancer Res.* **51**, 3657–3662 (1991).
32. Cathomen, T., Buchholz, C.J., Spielhofer, P. & Cattaneo, R. Preferential initiation at the second AUG of the measles virus F mRNA: a role for the long untranslated region. *Virology* **214**, 628–632 (1995).
33. Mizuguchi, H., Xu, Z.L., Sakurai, F., Mayumi, T. & Hayakawa, T. Tight positive regulation of transgene expression by a single adenovirus vector containing the rtTA and tTS expression cassettes in separate genome regions. *Hum. Gene Ther.* **14**, 1265–1277 (2003).
34. Firsching, R. *et al.* Measles virus spread by cell-cell contacts: uncoupling of contact-mediated receptor (CD46) downregulation from virus uptake. *J Virol.* **73**, 5265–5273 (1999).
35. Kanegae, Y. *et al.* Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucl. Acids Res.* **23**, 3816–3821 (1995).
36. Mittereder, N., March, K.L. & Trapnell, B.C. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J. Virol.* **70**, 7498–7509 (1996).
37. Langedijk, J.P., Daus, F.J., & van Oirschot, J.T. Sequence and structure alignment of Paramyxoviridae attachment proteins and discovery of enzymatic activity for a morbillivirus hemagglutinin. *J. Virol.* **71**, 6155–6167 (1997).

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1670 (2004) 172–180
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Optimization of antitumor efficacy and safety of in vivo cytokine gene therapy using RGD fiber-mutant adenovirus vector for preexisting murine melanoma

Yuka Okada[a], Naoki Okada[b,*], Hiroyuki Mizuguchi[c], Koichi Takahashi[a], Takao Hayakawa[d], Tadanori Mayumi[e], Nobuyasu Mizuno[a]

[a] *Department of Pharmaceutics, School of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Kyuban-cho, Koshien, Nishinomiya, Hyogo 663-8179, Japan*
[b] *Department of Biopharmaceutics, Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina, Kyoto 607-8414, Japan*
[c] *Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan*
[d] *National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan*
[e] *Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan*

Received 16 September 2003; received in revised form 5 December 2003; accepted 5 December 2003

Abstract

We previously reported that RGD fiber-mutant adenovirus vector (AdRGD) was a very useful vector system for in vivo cytokine gene therapy for established murine B16BL6 melanoma. However, intratumoral administration of AdRGD expressing tumor necrosis factor α (AdRGD-TNFα) at high dose revealed not only the dramatic reinforcement of anti-tumor effect but also serious adverse effects, such as body weight reduction and sudden death, caused by high-level TNF-α leakage from the tumor into circulation. These results strongly suggested that the determination of 'limiting dose', which demonstrated therapeutic effectiveness without adverse effect, against each vector was important for the development of appropriate cytokine gene therapy. In the present study, we investigated the efficacy and the safety of AdRGD expressing interleukin-12 (AdRGD-IL12) in murine melanoma model, and determined its limiting dose. Moreover, we demonstrated that combination therapy using AdRGD-IL12 and AdRGD-TNFα at limiting doses or less could achieve more effective tumor regression without adverse effects. Therefore, we conclude that a combination of multiple AdRGD expressing cytokines having distinct anti-tumor mechanisms can contribute to the establishment of in vivo cytokine gene therapy for melanoma, which possesses both excellent efficacy and high safety.

Keywords: Adenovirus vector; Melanoma; Gene therapy; Fiber-mutant; IL-12; TNF-α

1. Introduction

Because the prognosis of patients with melanoma is generally poor and the tumors commonly metastasize [1,2], the development of innovative approaches including gene therapy for treatment of malignant melanoma is imperative for improving the cure rate. To realize the effective gene therapy for melanoma, we have focused on the development of a novel vector system that can improve the gene transduction efficiency against melanoma cells.

We previously demonstrated that RGD fiber-mutant adenovirus vector (AdRGD), which contains an α_v-integrin tropism due to an RGD peptide inserted into the HI loop of the fiber knob, was superior to conventional adenovirus vector (Ad) in gene transduction efficiency to melanoma both in vitro and in vivo [3]. In addition, the intratumoral injection of human tumor necrosis factor α (TNF-α)-expressing AdRGD (AdRGD-TNFα) could more effectively induce hemorrhagic necrosis and regression in established murine B16BL6 melanoma as compared with administration of conventional Ad-TNFα. Although these results suggested that AdRGD was useful for in vivo cytokine gene therapy for melanoma, leakage of TNF-α into systemic circulation from the tumor resulted in serious

Abbreviations: Ad, adenovirus vector; AdRGD, RGD fiber-mutant Ad; CAR, coxsackie-adenovirus receptor; CTL, cytotoxic T lymphocyte; FBS, fetal bovine serum; IFN-γ, interferon γ; IL-12, interleukin 12; NK, natural killer; PBS, phosphate-buffered saline; Th, helper T; TNF-α, tumor necrosis factor α

* Corresponding author. Tel.: +81-75-595-4662; fax: +81-75-595-4761.
E-mail address: okada@mb.kyoto-phu.ac.jp (N. Okada).

0304-4165/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagen.2003.12.002

adverse effects, including extreme reduction in body weight, in mice that received intratumoral administration of AdRGD-TNFα at high doses [4]. Therefore, further optimization of the in vivo cytokine gene therapy using AdRGD is required to improve efficacy and safety. Our alternative approaches, which can repress the adverse effects by improving the effectiveness per vector dosage, may be the combination of two or more AdRGDs that express distinct cytokines such as TNF-α and interleukin 12 (IL-12).

IL-12 is a 70 kDa (p70) heterodimer protein in which the 40 kDa (p40) and 35 kDa (p35) subunits are connected by one S-S bond [5,6], and plays a key role in the induction of cellular immune responses, such as enhancement of proliferation and cytotoxic activity in natural killer (NK) cells and cytotoxic T lymphocytes (CTL) [7,8], production of interferon-γ (IFN-γ) from activated cells [8–10], and promotion of differentiation of helper T type 1 (Th1) cells from Th0 cells [8,11,12]. IFN-γ is involved in IL-12-mediated tumor regression [13], and IL-12 also exhibits an anti-angiogenic effect that can account for some anti-tumor activity [14]. Because systemic administration of recombinant IL-12 at high doses induces adverse effects associated with high systemic peak concentrations [15,16], the intratumoral administration of a vector carrying the IL-12 gene is a promising approach for efficient induction of antitumor effects and repression of adverse systemic effects due to the persistent local expression of cytokines in the tumor.

In the current study, we investigated the therapeutic efficacy and adverse effects of in vivo gene therapy using IL-12-expressing AdRGD (AdRGD-IL12) in the murine B16BL6 melanoma model and compared the response to that of conventional Ad-IL12. We also attempted to optimize the in vivo cytokine gene therapy protocol for melanoma by combining AdRGD-IL12 and AdRGD-TNFα due to the distinct mechanisms of their antitumor effects.

2. Materials and methods

2.1. Cell lines and mice

Murine melanoma B16BL6 cells were obtained from JCRB cell bank (Tokyo, Japan) and cultured in minimum essential medium supplemented with 5% fetal bovine serum (FBS) and antibiotics. HEK293 cells (JCRB cell bank) were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics. EL4 cells and YAC-1 cells were purchased from ATCC (Manassas, VA, USA) and cultured in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol, and antibiotics. Female C57BL/6 mice, aged 6–7 weeks, were purchased from SLC Inc. (Hamamatsu, Japan) and held under specified pathogen-free conditions. Animal experimental procedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

2.2. Vectors

The replication-deficient AdRGD-IL12 and conventional Ad-IL12, which carried the murine IL-12 gene derived from mIL-12 BIA/pBluescript II KS(-) [17] (kindly provided by Dr. Hiroshi Yamamoto; Department of Immunology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Japan), were constructed by an improved in vitro ligation method using pAdHM15-RGD and pAdHM4, respectively [18–20]. The expression cassette, which was designed to be transcribed in order from IL-12 p35 cDNA to the internal ribosome entry site sequence to IL-12 p40 cDNA under the control of the cytomegalovirus promoter, was inserted into the E1-deletion region of each E1/E3-deleted Ad vector. The construction of AdRGD-TNFα was described previously [3]. All vectors were propagated in HEK293 cells, purified by two rounds of CsCl gradient centrifugation, dialyzed with phosphate-buffered saline (PBS) containing 10% glycerol, and stored at −80 °C. The number of vector particles (VP) in vector stock was spectrophotometrically determined by the method of Maizel et al. [21].

2.3. Analysis of gene transduction in vitro

B16BL6 cells cultured on 24-well plates were infected with Ad-IL12 or AdRGD-IL12 for 2 h at various VP/cell in 100 μl of FBS-free medium. After washing twice with PBS, a 500-μl aliquot of culture medium was added to each well. Two days later, the supernatants were collected and the IL-12 p70 level was measured using a murine IL-12 p70 ELISA KIT (Biosource International, Camarillo, CA, USA).

2.4. Tumor inoculation and intratumoral administration of vectors in animal experiments

B16BL6 cells were intradermally inoculated into the abdomen of C57BL/6 mice at 2×10^5 cells/mouse. Six days later, established tumors with diameters of 5–7 mm were injected with each vector at various VP in 50-μl PBS.

2.5. In vivo gene transduction analysis

On day 2 after intratumoral Ad-IL12- or AdRGD-IL12-injection at 10^8 or 10^9 VP, B16BL6 tumors were removed, weighed, and homogenized in PBS containing 10 μg/ml aprotinin and 100 μM phenylmethylsulfonyl fluoride. After centrifugation at 15,000 rpm for 30 min, IL-12 p70 levels in the supernatants were measured using ELISA.

2.6. In vivo cytokine gene therapy model

B16BL6 tumor size and body weight in mice intratumorally injected with each vector were measured three times a week, and the tumor volume and the relative body weight were calculated by the following formulas, respectively:

tumor volume (mm^3)=(major axis) × (minor axis)^2 × 0.5236 [22], relative body weight=(body weight after Ad-treatment)/(body weight before Ad-treatment). Mice containing tumors >20 mm were euthanized. On day 90 after tumor challenge, all survivors were euthanized.

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

Established B16BL6 tumors were injected with Ad-IL12 or AdRGD-IL12 at 10^9 VP. At 1 week after Ad-injection, non-adherent splenocytes were prepared from these mice and directly used as NK effector cells. A surplus of splenocytes was restimulated in vitro using B16BL6 cells, which were cultured in media containing 100 U/ml recombinant murine IFN-γ (PeproTech EC Ltd., London, England) for 24 h and inactivated with 50 μg/ml mitomycin C at 37 °C for 30 min, at an effector/stimulator ratio of 10:1 in RPMI 1640 supplemented with 10% FBS, 50 μM 2-mercaptoethanol, and antibiotics. Five days later, the splenocytes were collected and used as CTL effector cells. Target cells (YAC-1 cells, B16BL6 cells, and EL4 cells) were Eu-labeled and an Eu-release assay was performed as previously described [23]. Cytolytic activity was determined using the following formula: % lysis=[(experimental Eu-release − spontaneous Eu-release)/(maximum Eu-release − spontaneous Eu-release)] × 100. Spontaneous Eu-release of the target cells was <10% of maximum Eu-release by detergent in all assays.

2.8. In vivo depletion analysis

Mice bearing B16BL6 tumor were intratumorally injected with AdRGD-IL12 at 10^9 VP, and then intraperitoneally injected five times with 100-μl ascites from ICR-nu/nu mice intraperitoneally injected with GK1.5 hybridoma (anti-CD4) [24] or 53-6.72 hybridoma (anti-CD8) [25] (kindly provided by Dr. Hiroshi Yamamoto, Department of Immunology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Japan), 40-μl rabbit anti-asialoGM1 antiserum (Wako Chemical, Osaka, Japan), or a mixture of these three antibody reagents at 2-day intervals. Depletion of T cell subsets and NK cells was monitored by flow cytometry, which showed >90% specific depletion in splenocytes at day 4 after the first antibody administration in each protocol.

2.9. Determination of IL-12 and IFN-γ levels in sera from mice intratumorally injected with AdRGD-IL12 or Ad-IL12 at high dose

Blood samples were collected from the tail veins of B16BL6 tumor-bearing mice intratumorally injected with AdRGD-IL12 or Ad-IL12 at 10^{10} or 10^{11} VP. Sera were immediately separated by centrifugation, and combined with 1/9 volume of PBS containing 10 mM EDTA and 100 μg/ml aprotinin. IL-12 and IFN-γ levels in sera were measured using the murine IL-12 p70 ELISA KIT and the murine IFN-γ ELISA KIT (Biosource International), respectively.

3. Results and discussion

Of the existing vector systems for gene therapy, Ad provides the highest transduction efficiency to a wide variety of cell types and tissues, regardless of the mitotic status of the cells [26–29]. Ad can also be easily amplified at high titers and concentrated by centrifugation, thereby facilitating application to gene therapy based on efficient direct in vivo gene delivery for various kinds of diseases [30]. Because of these advantages, Ad is widely used in clinical research of gene therapy as well as retrovirus vector. However, melanoma, which is an important target for gene therapy, requires high Ad dosage for sufficient gene expression. We previously reported that the resistance of melanoma cells to Ad-mediated gene transduction was caused by extremely poor expression of coxsackie-adenovirus receptor (CAR), the primary Ad receptor, on the cell surface, and that AdRGD, which targets α_v-integrins during attachment to cells, exhibited highly efficient foreign gene transduction in melanoma cells as compared with conventional Ad [3]. In the present report, we therefore investigated the efficacy and safety of IL-12 gene therapy using AdRGD on the murine B16BL6 melanoma model, and also tested combination therapy using AdRGD-IL12 and AdRGD-TNFα to optimize in vivo cytokine gene therapy for melanoma.

3.1. Transduction efficiency of IL-12 genes to B16BL6 cells or tumors by AdRGD or conventional Ad

To assess efficiency of productive gene transfer into melanoma cells, we compared the level of IL-12 secretion in supernatants from a 2-day culture of AdRGD-IL12- or Ad-IL12-infected B16BL6 cells (Fig. 1A). The IL-12 levels increased in a VP-dependent manner, and IL-12 secretion from melanoma cells infected with AdRGD-IL12 was about 70-fold and 90-fold higher at 5000 VP/cell and 20,000 VP/cell, respectively, than that from Ad-IL12-infected cells. The viability of B16BL6 cells infected at the highest dose, 20,000 VP/cell, did not change from that of uninfected cells (data not shown), suggesting that IL-12 accumulated in culture media but did not directly injure B16BL6 cells.

Likewise, we compared in vivo gene transduction efficiency of AdRGD-IL12 and Ad-IL12 to B16BL6 tumors established in C57BL/6 mice (Fig. 1B). On day 2 after Ad-treatment, the IL-12 levels in B16BL6 tumors injected with AdRGD-IL12 were 7-fold higher at 10^9 VP/tumor than in Ad-IL12-injected tumors. In addition, AdRGD-IL12 injection at 10^8 VP/tumor attained equal expression of IL-12 in B16BL6 tumors as obtained with Ad-IL12 injection at a 10-fold higher dose, 10^9 VP/tumor. Taken together, these results clearly suggest that AdRGD is a very potent vector

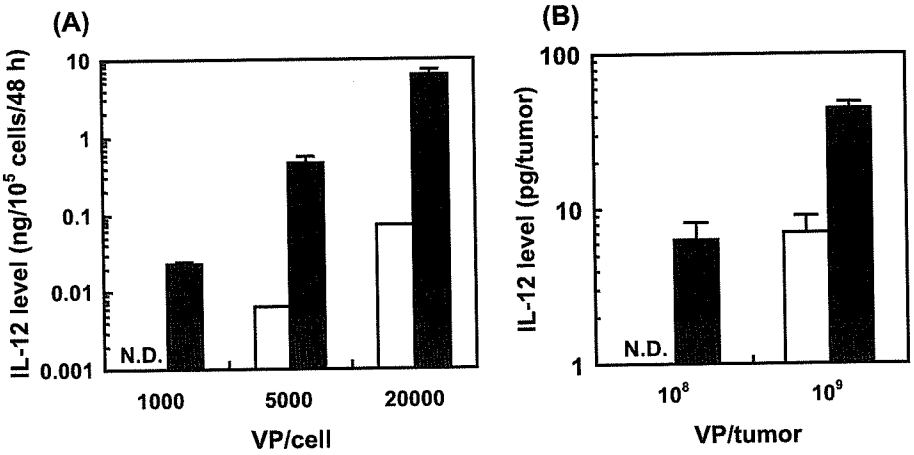

Fig. 1. IL-12 production levels in B16BL6 cells or tumors infected with AdRGD-IL12 or Ad-IL12. (A) B16BL6 cells were infected with Ad-IL12 (open column) or AdRGD-IL12 (closed column) at indicated VP/cell for 2 h. Two days later, the level of IL-12 secreted into culture medium was measured by ELISA. The data represent the mean ± S.D. of three independent cultures. (B) Established B16BL6 tumors with diameters of 5–7 mm in C57BL/6 mice were injected with Ad-IL12 (open column) or AdRGD-IL12 (closed column) at indicated VP/tumor. Two days later, tumors were removed and homogenized in PBS containing 10 μg/ml aprotinin and 100 μM phenylmethylsulfonyl fluoride. The homogenates were centrifuged and the IL-12 level in the supernatants was determined by ELISA. Data are presented as mean ± S.D. of three mice. N.D.: IL-12 level was not detectable.

system for foreign gene transduction in melanoma both in vitro and in vivo, as we showed in our previous studies [3,4,31]. Furthermore, since the majority of tumors is positive for α_v-integrin [32,33] and some tumors exhibit a decline in CAR expression with progressing malignancy [34,35], AdRGD is promising for gene therapy for many advanced cancers as well as melanoma.

3.2. Tumor regression and body weight change in mice intratumorally injected with AdRGD-IL12 or Ad-IL12

We next evaluated the anti-tumor effects following the intratumoral injection of AdRGD-IL12 or Ad-IL12 in the murine B16BL6 melanoma model. As shown in Fig. 2A, tumor growth was inhibited by intratumoral administration of Ad-IL12 or AdRGD-IL12 in a VP-dependent manner, and growth inhibition upon administration of 10^8 AdRGD-IL12 was comparable to that after treatment with 10^9 Ad-IL12. This outcome was positively correlated with IL-12 expression in B16BL6 tumors injected with each Ad type, as shown in Fig. 1B. We therefore determined that AdRGD could reduce the vector dosage in in vivo IL-12 gene therapy to about one-tenth that of conventional Ad while maintaining an equal therapeutic effect against melanoma. The body weight of B16BL6 tumor-bearing mice intratumorally injected with AdRGD-IL12 or Ad-IL12 at a higher dose, 10^{10} or 10^{11} VP/tumor, decreased remarkably for several days after vector administration, although we observed a very strong antitumor effect (Fig. 2B). Based on these results, we determined that the 'limiting dose' of

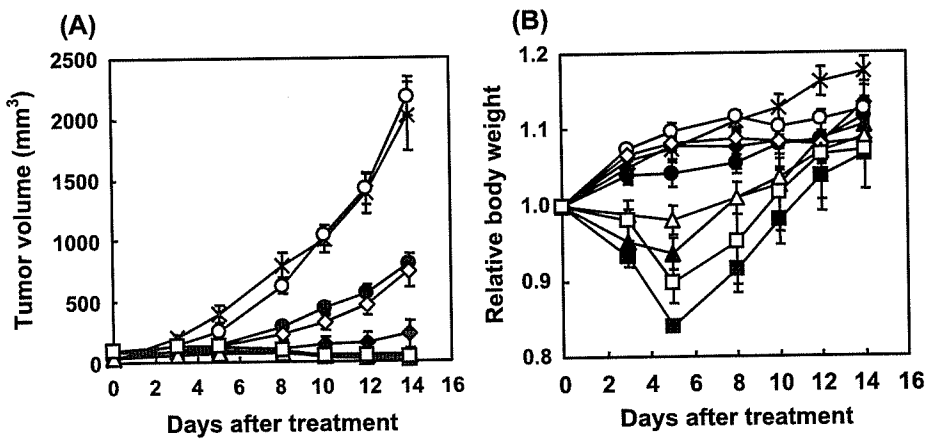

Fig. 2. Anti-B16BL6 tumor effect and body weight change upon an intratumoral administration of Ad-IL12 or AdRGD-IL12. B16BL6 cells were intradermally inoculated into C57BL/6 mice at 2×10^5 cells/mouse. Six days later, the tumors were injected with Ad-IL12 [(○) 10^8, (◇) 10^9, (△) 10^{10}, or (□) 10^{11} VP/tumor], AdRGD-IL12 [(●) 10^8, (◆) 10^9, (▲) 10^{10}, or (■) 10^{11} VP/tumor], or PBS (×). The tumor size and body weight were determined three times a week, and the tumor volume (A) and the relative body weight (B) were calculated according to the formula described in Materials and methods. The data are representative of two independent experiments. Each point represents the mean ± S.E. of four to five mice.