or somatostatin was detected in livers from wt-PDX1-mice and PDX1-VP16-mice. In addition, in these livers there was no detectable production of amylase, a pancreatic exocrine enzyme (Fig. 2B), which may explain the normal morphogenesis in our experimental animals. On the other hand, pancreatic polypeptide was expressed in livers from PDX1-VP16-mice, and in those from wt-PDX1-mice though to a lesser extent. These results demonstrate that transient expression of PDX1-VP16 alters the character of hepatocytes to preferentially produce insulin and pancreatic polypeptide, but not other endocrine hormones or exocrine enzymes.

Adenoviral gene transfer induced gene expression for 1 week but, after 2 weeks, this expression reportedly disappeared [22]. However, in the present study, the blood glucose lowering effects and hepatic insulin expression persisted for at least 40 days. Therefore, the time course of PDX1 protein expression levels was examined. As shown in Fig. 3A, immunoblotting using anti-VP16 activation domain antibody revealed PDX1-VP16 protein to be expressed on day 3 but expression was markedly decreased on day 7, and undetectable on day 21. Thus, even after disappearance of VP16-PDX1 expression, hepatocytes expressed insulin, resulting in lowering of blood glucose levels. Interestingly, immunoblotting using anti-PDX1 antibody showed that endogenous PDX1 protein, which had the same molecular weight

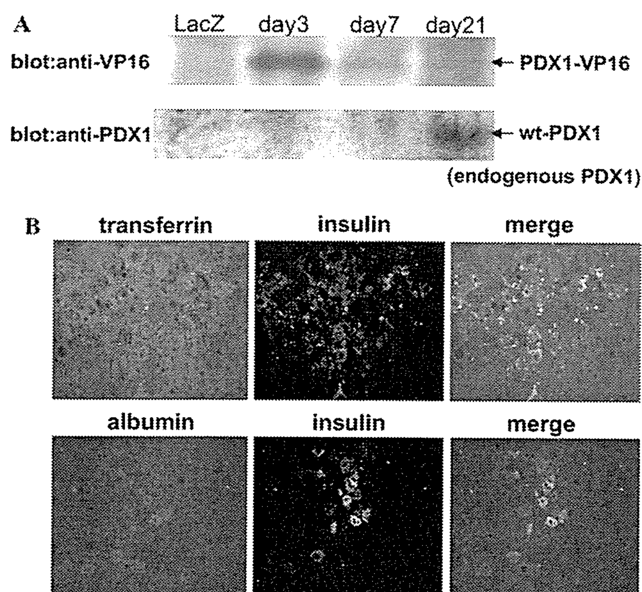

Fig. 3. Treatment with PDX1-VP16 adenovirus induced persistent expression of endogenous PDX1 but albumin and transferrin were co-expressed in insulin-expressing cells. (A) Liver lysates from PDX1-VP16 mice at different time points after adenoviral treatment were immunoblotted with anti-VP16 (upper panel) or anti-PDX1 (lower panel) antibody. (B) Liver sections from PDX1-VP16 mice on day 40 were double-immunostained with insulin (middle panels) and transferrin (upper-left panels) or albumin (lower-left panels) antibodies. Right panels represent the merged images.

as wt-PDX1, came to be expressed on day 21. Thus, transient expression of PDX1-VP16 endowed hepatocytes with certain pancreatic β cell features and endogenous PDX1 expression is likely to maintain the insulin-producing function of these cells.

To determine whether the insulin-producing cells in the liver had completely transdifferentiated and lost their hepatocytic character, liver sections from PDX1-VP16 mice on day 40 were immunostained with insulin and transferrin (upper panels in Fig. 3B) or albumin (lower panels in Fig. 3B). Fluorescence immunohistochemistry revealed that insulin-producing cells in the liver also expressed transferrin and albumin. Expression levels of these liver-specific proteins were not substantially decreased as compared with non-insulin-producing cells around the insulin-producing cells. These findings suggest functional hepatocyte-specific characteristics are maintained in insulin-producing cells in the liver. Thus, these hepatocytes were not completely converted to pancreatic cells.

Discussion

In the present study, administration of recombinant adenovirus containing an activated form of PDX1 efficiently induced insulin production in hepatocytes, resulting in reversal of STZ-induced hyperglycemia. The effects were sustained even when exogenous protein expression was no longer detectable. In turn, endogenous PDX1 protein came to be expressed in hepatocytes, which is likely to be the mechanism underlying the sustained effects. On the other hand, albumin and transferrin expressions were observed in insulin-producing cells, suggesting the maintenance of hepatocyte-specific characteristics.

Ferber et al. [7] reported that administration of wt-PDX1 adenovirus at 2×10^9 pfu/mouse ameliorates STZ-induced hyperglycemia but the observed period was very short (no more than 10 days). The same research group also reported the long-term effects of PDX1 gene transfer but the titer of recombinant adenovirus used was relatively high ($1\text{–}5 \times 10^{10}$ pfu/mouse) [12]. Such high titers may result in liver damage due to adenoviral toxicity. In the present study, to avoid adenoviral toxicity, recombinant adenoviruses were injected at a titer as low as 2×10^8 pfu. With such a small adenoviral delivery, the wt-PDX1 adenovirus exerted very small effects on insulin and glucose levels, whereas PDX1-VP16 adenovirus substantially increased insulin levels and reversed STZ-induced hyperglycemia. These findings suggest that constitutive activation of PDX1 overcomes the inefficiency associated with low expression levels of PDX1 proteins. Thus, adenoviral transfer of the PDX1-VP16 gene into the liver would presumably be safer than wt-PDX1 gene therapy.

HDAD-mediated PDX1 expression in the liver reportedly causes severe hepatitis including marked inflammatory cell infiltration with focal necrosis associated with expression of pancreatic exocrine genes [10]. In addition, conditional transgenic mice generated by crossing CAG-CAT-PDX1 mice with alb-Cre recombinase-mice also displayed functional liver failure with hepatic expression of exocrine enzymes [11]. In these two models, exogenous PDX1 expression is persistent. Transgenes delivered by HDADs are expressed for long periods exceeding several months. In conditional transgenic mice [11], cells, in which the albumin promoter had once been activated, permanently expressed PDX1 driven by the CAG promoter. These findings suggest that high and persistent expression of PDX1 induces exocrine enzyme expression and thereby liver failure. In the present study, exogenous gene expressions of wt-PDX1 and PDX1-VP16 were transient and expression levels were relatively low on day 7 (Fig. 3A). Thus, transient expression appears to be important for endowing hepatocytes with certain features of pancreatic β cells, but not of exocrine cells.

It is noteworthy that exogenous, transient expression of PDX1-VP16 induced prolonged expression of endogenous PDX1 which apparently contributed to persistent insulin production with hepatocytic features. Ber et al. also reported that rat PDX1 gene transduction using first-generation adenovirus induced persistent endogenous (murine) PDX1 expression. Thus, transient expression of wt-PDX1, and more efficiently PDX1-VP16, may induce persistent and low-level expression of endogenous PDX1. In the adult pancreas, persistent but low-level expression of PDX1 is detected only in β cells [3] and PDX1 expression is required for maintaining normal pancreatic β cell function [6]. These observations suggest that persistent, low-level expression of PDX1 is involved in preferential production of insulin and pancreatic popypeptide in hepatocytes.

In transgenic *Xenopus* tadpoles expressing Xlhbox8 (*Xenopus* homolog of PDX1) carrying the VP16 activation domain under a transthyretin promoter, part or all of the liver is reportedly converted to pancreatic tissue without expression of liver-specific gene products, suggesting complete conversion of hepatocytes to pancreatic cells [14]. In contrast, in the present study, insulin-producing cells in the liver in PDX1-VP16 mice also expressed albumin and transferrin, which suggests preservation of hepatocytic functions. This discrepancy may be explained by the differences between amphibian and mammalian cells. Alternatively, the conversion may occur during embryonic differentiation, while, in adult and differentiated hepatocytes, complete transdifferentiation into pancreatic endocrine or exocrine cells would be difficult to achieve even with PDX1-VP16 expression. Although intensive research is necessary to unravel the precise mechanisms underlying transdifferentiation, the partial conversion induced by PDX1-VP16 expression in adult hepatocytes has practical applications, since loss of hepatocytic functions may result in liver failure. Furthermore, incomplete transdifferentiation could prevent the generated insulin-producing cells from being attacked by a destructive autoimmune response in type 1 diabetics.

Acknowledgments

We thank Dr. H. Kanamori (University of Tokyo) for the generous gift of the VP16 gene. We also thank Ms. I. Sato, K. Kawamura, and M. Hoshi for technical support. This work was supported by a Grant-in-Aid for Scientific Research (B2, 15390282), a Grant-in-Aid for Exploratory Research (15659214) to H. Katagiri, and a Grant-in-Aid for Scientific Research (13204062) to Y. Oka from the Ministry of Education, Science, Sports and Culture of Japan. This work was also supported by Tohoku University 21st Century COE Program "CRESCENDO" to J. Imai, J. Gao, and H. Katagiri.

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Efficient Gene Transfer into Human Trophoblast Cells with Adenovirus Vector Containing Chimeric Type 5 and 35 Fiber Protein

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Received September 8, 2004; accepted September 25, 2004

Recombinant adenovirus (Ad) vectors based on Ad type 5 have been widely used for gene transfer experiments. Conventional Ad type 5 vectors have a narrow range of tropism and are limited by the size of the transgene that can be packaged. To overcome these limitations, we previously developed an Ad vector (Ad5/35 vector) containing a chimeric Ad type 5 and 35 fiber protein. In the current study, we evaluated the ability of the Ad5/35 vector to transfer genes into human trophoblast cell lines (JAR, JEG-3 and BeWo cells), which are used as in vitro models of human placenta. We compared the gene transfer efficiency of Ad5/35 to that of conventional Ad vector. We found that expression of CD46, which are receptors for Ad5/35 vector, are higher than that of coxsackievirus and adenovirus receptor in all 3 trophoblast cell lines, as determined by flow cytometry. Next, we compared the transducing activity of Ad5 vector and Ad5/35 vector that each expressed luciferase as a reporter gene. Ad5/35 vector had greater gene transfer activity than the conventional Ad vector in all 3 trophoblast cell lines (1.82-fold in JAR cells, 5.37-fold in BeWo cells, 6.11-fold in JEG-3 cells). Thus, Ad vector that contains chimeric type 5 and 35 fiber protein can be a powerful tool for gene transfer experiments in human trophoblast cell lines.

Key words adenovirus vector; chimeric fiber; gene therapy; trophoblast

The placenta, which is responsible for the development of the fetus, has broad-ranging functions that include transporting nutrients from maternal fluid into the fetus, secreting hormones, and preventing the transfer of toxic substances into the fetus.[1] The placenta contains a variety of cell types, including trophoblast cells, endothelial cells and epithelial cells. Trophoblast cells are believed to be important for fetal development because they transport nutrients from the mother to the fetus.[1] Human trophoblast cell lines functionally expressed transporters of monocarboxylic acids, folic acid and anti-cancer drugs.[2—5] Gene transfer into trophoblast cells can be a useful tool for clarifying the biology of placenta, but methods to transfer gene into trophoblast cells have never been fully investigated.

Recombinant adenovirus (Ad) vectors can introduce genes of interest into cells and tissues. There are more than 51 serotypes of Ad. Ad type 5 (Ad5) vector has been frequently used in basic research and clinical work.[6] Ad5, which belongs to subgroup C, has been used to prepare recombinant Ad vectors because its genetic and biological characteristics have been extensively studied. There are at least two steps to the infection of cells with Ad5. The first step is the attachment of the virus to coxsackievirus and adenovirus receptor (CAR) on the cell membrane *via* the knob domain of the fiber.[7,8] Then, Ad5 is internalized into the cell through the interaction of RGD (Arg-Gly-Asp) motifs on the penton base of the Ad5 surface with $\alpha v\beta 3$- and $\alpha v\beta 5$-integrins on the cell membrane.[9,10] Transgenes delivered by a conventional Ad5 vector are limited to a size of 8.1—8.2 kb,[11] and Ad5 has poor transduction efficiency in CAR-negative cells.[12] Thus, conventional first-generation Ad vectors have a limited transgene size as well as limited tropism.

To overcome these limitations, we previously developed Ad vectors containing chimeric type 5 and 35 fiber protein.[13,14] Ad type 35 (Ad35), which belongs to subgroup B, was initially isolated from the kidneys and lungs of a renal transplant patient.[15] CD46, which is a receptor for Ad35,[16] is ubiquitously expressed in human cells.[17] The Ad5/35 vector can package 8.8 kb of foreign DNA and can transduce CAR-negative cell lines and various human cell lines more effectively than Ad5 vector.[13,14] Thus, Ad5/35 vector is a promising candidate for mediating efficient gene transfer into human trophoblast cell lines.

In the present study, we examined the expression of CD46 in 3 human trophoblast cell lines (JAR, BeWo and JEG-3), which are used in human trophoblast research. We also evaluated the ability of Ad5/35 vector to transfer genes into the human trophoblast cell lines.

MATERIALS AND METHODS

Cell Culture The BeWo cells (clone b30) were obtained from Dr. Alan Schwarts (Washington University, MO, U.S.A.). BeWo cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% MEM non-essential amino acid solution (Gibco, MD, USA), 1.6 g/l sodium bicarbonate, 0.584 g/l L-glutamine and 3.5 g/l D-glucose. JAR and JEG-3 were obtained from American Type Culture Collection (Manassas, VA, U.S.A.) and were cultured with Minimum essential Eagle's medium (MEM) supplemented with 10% FBS and RPMI-1640 supplemented with 10% FBS, respectively.

Preparation of Ad Vectors Ad-L2, which is the conventional Ad vector derived from Ad type 5, and Ad-F35-L2, which contains chimeric type 5 and 35 fiber protein, were purified as previously described.[13,18,19] Both vectors expressed luciferase. The virus particle titer and infectious (plaque forming unit: PFU) titer were spectrophotometrically deter-

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mined by the methods of Maizel *et al.*[20] and by the method of Kanegae *et al.*,[21] respectively. The PFU to particle ratio was 1 : 14 for Ad-L2 and 1 : 15 for Ad-F35-L2.

Adenovirus-Mediated Gene Transduction into Human Trophoblast Cells Cells (1×10^4 cells) were seeded into a 96-well dish. On the following day, they were transduced with Ad-L2 or Ad-F35-L2 (3000 vector particles per cell) for 1.5 h. After culture for 48 h, luciferase production in the cells was measured using a luciferase assay system (PicaGene LT2.0, Toyo Inki Co., Ltd., Tokyo, Japan).

Flow Cytometry To detect the expression of human CAR on the membrane, cells were labeled with mouse monoclonal antibody RmcB (kindly provided by Dr. J. M. Bergelson, The Children's Hospital of Philadelphia, PA, U.S.A.). The cells were then incubated with fluorescein-labeled secondary antibody (Pharmingen, San Diego, CA, U.S.A.). To detect the expression of human CD46, the cells were labeled with fluorescein-conjugated anti-human CD46 (E4.3; Pharmingen). Labeled cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Tokyo, Japan).

Statistical Analysis The significant difference was calculated using one-way ANOVA followed by Dunnett's test.

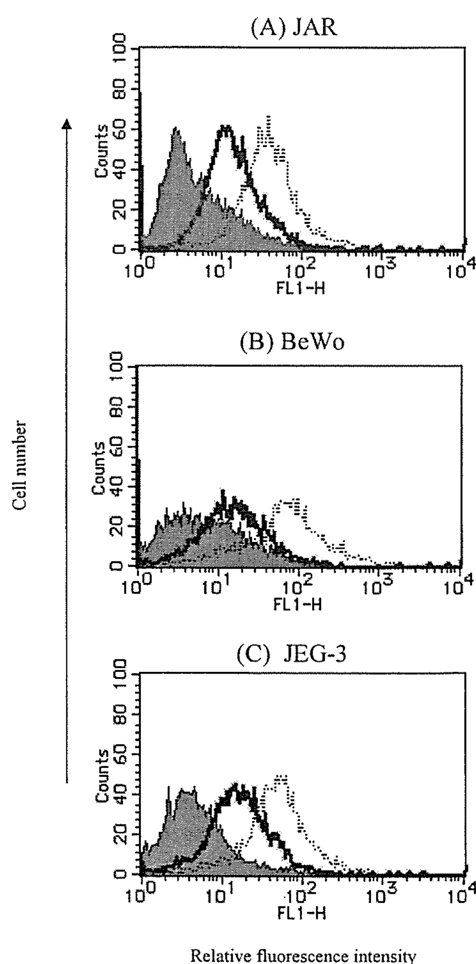

Fig. 1. Flow Cytometric Analysis of CAR and CD46 Expression in Human Trophoblast Cell Lines

Cells were incubated with: (1) anti-CAR antibodies followed by fluorescein-labeled secondary antibody, (2) fluorescein-labeled anti-CD46 antibody or (3) fluorescein-labeled goat IgG. Then, labeled cells were detected by flow cytometry. The dashed regions indicate cells labeled by anti-CD46, the shaded regions indicate cells labeled by goat IgG and the solid regions indicate cells labeled by anti-CAR antibody.

RESULTS AND DISCUSSION

To overcome the limited tropism of conventional Ad5 vectors, we previously developed Ad5/35 vectors that contain chimeric type 5 and type 35 fiber.[13,14] These chimeric Ad vectors can infect cells *via* CD46, a receptor of the type 35 fiber.[14] First, we investigated the expression of CD46 and CAR on the membranes of JAR, JEG-3 and BeWo cells. Flow cytometry analysis showed that CD46 and CAR were expressed on the membranes of all 3 trophoblast cell lines (Fig. 1), suggesting that Ad5/35 vector can infect with the trophoblast cell lines in a CD46-dependent fashion. To compare the transgene activities of the chimeric Ad5/35 vector (Ad-F35-L2) and the conventional Ad5 vector (Ad-L2), we used luciferase as a reporter gene. Ad5/35 mediated greater transgene activity than Ad5 in JAR (1.82-fold), JEG-3 (6.11-fold) and BeWo cells (5.37-fold) (Fig. 2). We previously reported that Ad5/35 vector mediated 100-fold greater expression of reporter gene than Ad5 vector in CAR-negative LN444 cells.[13] Taken together, Ad5/35 may infect with the trophoblast cells *via* different receptors from CAR. Although CD46 is known to be a receptor for Ad type 35,[16] the involvement of unidentified receptors for Ad type 35 in the infection of Ad5/35 vectors is not negligible. Indeed, Erikson *et al.* indicated Ad 35 infected with the cells *via* a trypsin-insensitive receptor.[22] The different transgene activity among JEG-3, JAR and BeWo cells may be caused by different expression profiles of receptors for Ad type 35 among them.

In summary, this is the first report of efficient gene delivery into JAR, JEG-3 and BeWo cells by Ad vector containing chimeric type 5 and type 35 fiber protein. The chimeric

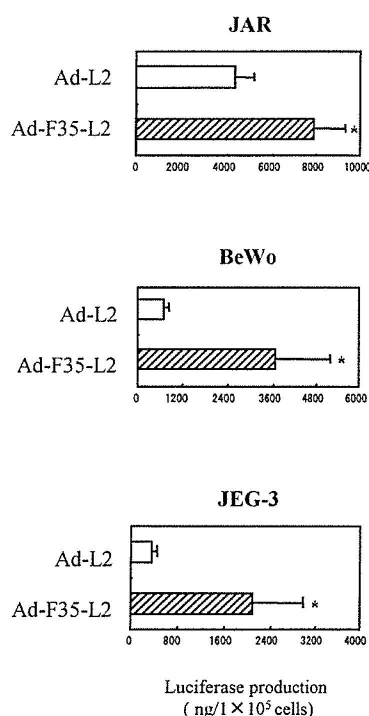

Fig. 2. Comparison of Luciferase Production in Human Trophoblast Cells Transduced by Ad-L2 or Ad-F35-L2

Cells were transduced with 3000 vector particles per cell of Ad-L2 or Ad-F35-L2 for 1.5 h. After culture for 48 h, luciferase production was measured by a luminescent assay. The data are expressed as mean±S.D. ($n=4$). * Significant difference from the Ad-L2-trancduced group ($p<0.01$).

Ad5/35 vector can package a gene of up to 8.8 kb and has broad tropism.[13] Thus, Ad5/35 vectors may be powerful tools for gene transfer experiments in human trophoblast cells.

Acknowledgments We thank Dr. T. Nakanishi (Osaka University, Japan) and Ms. F. Ida for his useful discussion and her technical assistance, respectively. This work was supported by a Grants-in-Aid for Scientific Research (C) (No. 15590139) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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HUMAN GENE THERAPY 15:1034–1044 (November 2004)
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Review

Targeted Adenovirus Vectors

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ABSTRACT

Recombinant adenovirus (Ad) vectors continue to be the preferred vectors for gene therapy and the study of gene function because they are relatively easy to construct, can be produced at high titer, and have high transduction efficiency. However, in some applications gene transfer with Ad vectors is less efficient because the target cells lack expression of the primary receptor, coxsackievirus and adenovirus receptor (CAR). Another problem is the wide biodistribution of vector in tissue following *in vivo* gene transfer because of the relatively broad tissue expression of CAR. To overcome these limitations, various approaches have been developed to modify Ad tropism. In one approach, the capsid proteins of Ad are modified, such as with the addition of foreign ligands or the substitution of the fiber with other types of Ad fiber, in combination with the ablation of native tropism. In other approaches, Ad vectors are conjugated with adaptor molecules, such as antibody and fusion protein containing an anti-Ad single-chain antibody (scFv) or the extracellular domain of CAR with the targeting ligands, or chemically modified with polymers containing the targeting ligands. In this paper, we review advances in the development of targeted Ad vectors.

INTRODUCTION

ADENOVIRUS VECTORS have been expected to play a prominent role in gene therapy because of their extremely high transduction efficiency. However, one of the hurdles confronting gene transfer by adenovirus (Ad) vectors is their inefficient transduction to target cells lacking sufficient expression of the coxsackievirus and adenovirus receptor (CAR), the primary receptor; such cells include many advanced tumor cells, skeletal muscle cells, smooth muscle cells, peripheral blood cells, hematopoietic stem cells, dendritic cells, and so on. A high dose of vector is required to achieve efficient gene transfer to these cell types. This in turn increases unwanted side effects, such as vector-associated immunogenic toxicities.

Another hurdle confronting Ad vector-mediated gene transfer is their nonspecific distribution in tissue after *in vivo* gene transfer because of the relatively broad expression of CAR, α_v integrin (the secondary receptor), and heparan sulfate (the third receptor). This property imposes an increased risk of toxicity due to vector dissemination to nontargeted cells, such as antigen-presenting cells (e.g., macrophages and dendritic cells). This occurs even when Ad vectors are locally administered to the tissue of interest. Vector targeting to a specific tissue or cell type would enhance gene therapy efficacy and permit the delivery of lower doses, which should result in reduced toxicity.

Several approaches have been developed to overcome these hurdles, including genetic modification of Ad capsid proteins, such as fiber, penton base, hexon, and protein IX (pIX), and conjugation-based modification of virus such as antibody or bispecific fusion protein, and chemical modification by polymers containing the targeting ligands (Fig. 1). To improve gene transfer efficiency, modification of tropism is required. To target gene transfer, both the ablation of natural tropism and introduction of cell-specific tropism are required. In this paper we review approaches to developing targeted Ad vectors.

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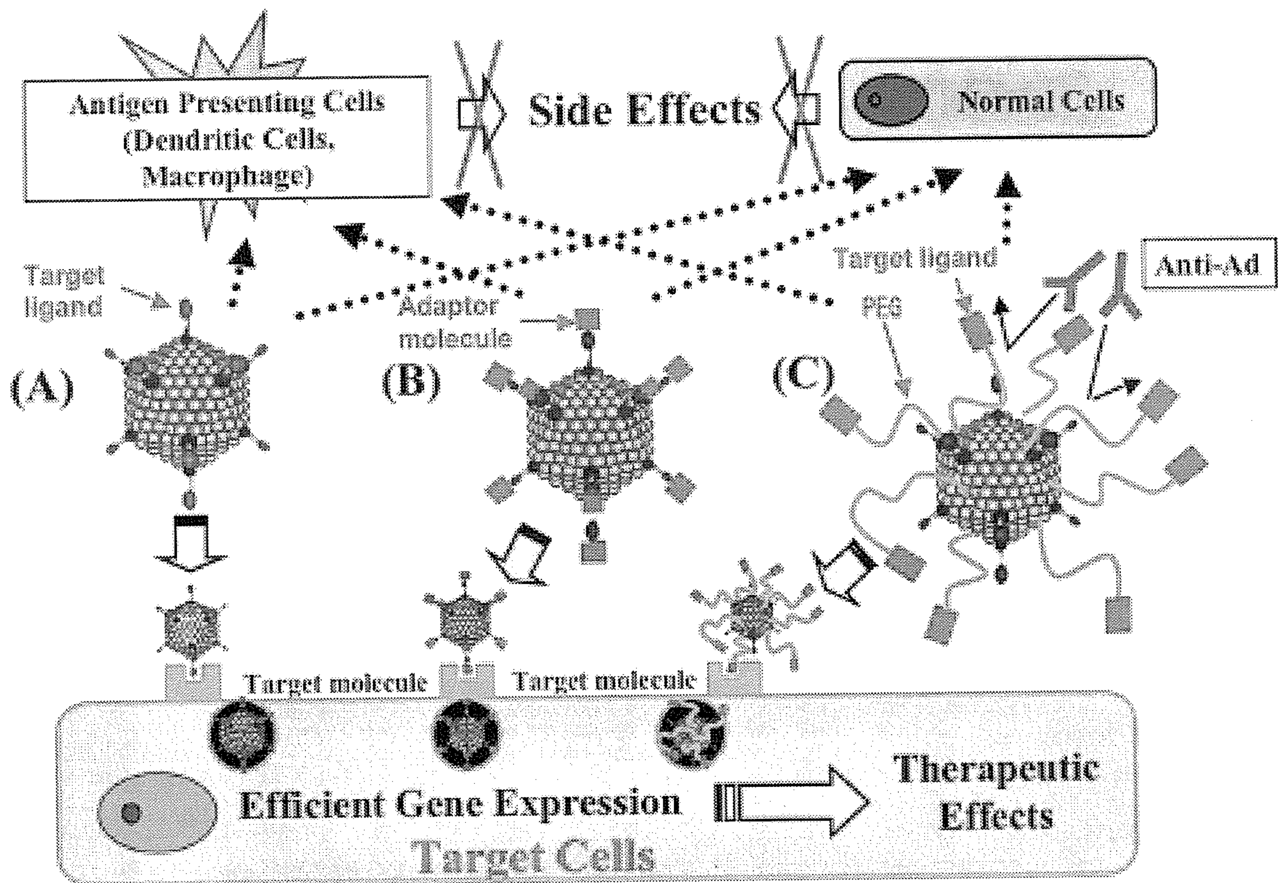

FIG. 1. Three approaches to developing targeted Ad vectors. (**A**) Genetic modification of virus capsid. (**B**) Modification by the use of adaptor molecules. (**C**) Chemical modification by polymers with ligands.

KINETICS OF ADENOVIRUS VECTOR-MEDIATED GENE TRANSFER *IN VIVO*

Important determinants of virus clearance from the bloodstream include interactions between viral components and cellular receptors, virion size, net charge of the viral particle, and anatomical barriers, such as tightness of the basal membrane of endothelial cells. Understanding factors that impact on the kinetics of blood clearance and the biodistribution of Ad vectors would be beneficial to advancing their application as therapeutic agents.

Systemically administered Ad vectors are rapidly cleared from the blood of mice, with a half-life of less than 3 min (Alemany *et al.*, 2000; Koizumi *et al.*, 2003a; Sakurai *et al.*, 2003b). Liver Kupffer cells play a central role in clearing Ad genomes from the bloodstream (Lieber *et al.*, 1997; Wolff *et al.*, 1997; Worgall *et al.*, 1997). Activated Kupffer cells (and monocytes and resident macrophages) release proinflammatory cytokines/chemokines such as interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), interferon γ-inducible protein 10 (IP-10), and RANTES (regulated on activation, normal T cell expressed and secreted), causing the activation of an innate immune response (Liu and Muruve, 2003). It has been proposed that a low dose of Ad vectors ($\sim 10^{10}$ vector particles) is rapidly sequestered by Kupffer cells (nonparenchymal cells), whereas higher doses of Ad vectors are delivered into both Kupffer cells and parenchymal cells, leading to a nonlinear dose response in hepatic transgene expression (Tao *et al.*, 2001). At a dose of 3.0×10^{10} vector particles, Ad vectors are likely to be equally distributed to Kupffer and parenchymal cells (Koizumi *et al.*, 2003a).

The liver directivity of the systematically administered Ad vectors can also be applied when local administration of the vectors is performed. Even if the Ad vector is injected into local tissues such as tumors, large amounts of vector are distributed into the bloodstream and targeted into the liver, causing unwanted side effects (Mizuguchi and Hayakawa, 2002b; Okada *et al.*, 2003). The process of Ad vector-mediated liver transduction is influenced by interactions between viral components and cellular receptors (discussed in Truly Targeted Adenovirus Vectors, below), the size of the sinusoidal fenestrae (Fechner *et al.*, 1999; Lievens *et al.*, 2004), and the complement system (Zinn *et al.*, 2004). Lievens *et al.* showed that Ad vector-mediated liver transduction in Dutch Belt rabbits, with 124-nm sinusoidal fenestrae, is significantly higher than that in New Zealand White rabbits, which have 108-nm sinusoidal fenestrae, and Fauve de Bourgogne rabbits with 105-nm sinusoidal fenestrae (Lievens *et al.*, 2004). The increase in sinusoidal fenestrae to 123 nm in New Zealand White rabbits by the intraportal injection of sodium decanoate enhances Ad vector-mediated liver transduction, confirming that the size of the sinusoidal

fenestrae is an important determinant for liver transduction (Lievens *et al.*, 2004). For targeting Ad vector to extrahepatic tissues, it is important to avoid distribution into parenchymal and nonparenchymal (Kupffer) cells of the liver as well as other tissues, such as spleen.

APPROACHES TO DEVELOPING TARGETED ADENOVIRUS VECTORS

Genetic modification of the virus capsid

Modification of virus tropism. Modification of the fiber proteins has been used to successfully overcome barriers to transduction due to a paucity of CAR. Two approaches have been used for this purpose. One is the addition of foreign peptides to the HI loop or C terminus of the fiber knob (Wickham *et al.*, 1997; Dmitriev *et al.*, 1998; Krasnykh *et al.*, 1998; Mizuguchi *et al.*, 2001; Koizumi *et al.*, 2003b). Another is the substitution of fibers derived from other Ad serotypes, which bind to receptor molecules other than CAR (Gall *et al.*, 1996; Stevenson *et al.*, 1997; Chillon *et al.*, 1999; Shayakhmetov *et al.*, 2000; Mizuguchi and Hayakawa, 2002a). Both approaches allow Ad tropism to be expanded (or changed) via binding of the modified fiber protein with a different cellular receptor.

Expanded and higher rates of gene transfer have been reported on the basis of the use of mutant fiber proteins containing an Arg-Gly-Asp (RGD) peptide (Wickham *et al.*, 1997; Dmitriev *et al.*, 1998; Hidaka *et al.*, 1999; Mizuguchi *et al.*, 2001) or a stretch of lysine residues (KKKKKKK [K_7] peptide) (Wickham *et al.*, 1997; Hidaka *et al.*, 1999), which target α_v integrins or heparan sulfates to the cellular surface, respectively. The RGD peptide has been displayed in the HI loop or C terminus of the fiber knob, whereas the K_7 peptide has been displayed at the C terminus of the fiber knob. There have also been reports of inserting the peptides into the HI loop of the fiber knob, including those discovered by phage display library to show high affinity for vascular endothelial cells (Nicklin *et al.*, 2000), cancer cells (Nicklin *et al.*, 2003), transferrin receptor (Xia *et al.*, 2000), and vascular smooth muscle cells (Work *et al.*, 2004).

Altered vector tropism was reported by substitution of the Ad type 5 (Ad5) fiber protein into that of Ad3, Ad7, Ad11, Ad16, Ad17, Ad35, and others (Gall *et al.*, 1996; Stevenson *et al.*, 1997; Chillon *et al.*, 1999; Shayakhmetov *et al.*, 2000; Goossens *et al.*, 2001; Havenga *et al.*, 2001; Rea *et al.*, 2001; Stecher *et al.*, 2001; Mizuguchi and Hayakawa, 2002a). Most Ad serotypes belonging to the subgroups A, C, D, E, and F use CAR as the initial receptor for the virion (Roelvink *et al.*, 1998), whereas Ad serotype B uses other molecules for infection (Roelvink *et al.*, 1998; Arnberg *et al.*, 2000a,b; Law and Davidson, 2002; Burmeister *et al.*, 2004). Ad8, Ad19, and Ad37, which belong to serotype D, use sialic acids as the primary receptor (Arnberg *et al.*, 2000a,b; Burmeister *et al.*, 2004). CD46, CD80, and CD86 were identified as cellular receptor(s) of Ad belonging to subgroup B, including Ad3, Ad11, Ad14, Ad16, Ad21, Ad35, and Ad50 (Gaggar *et al.*, 2003; Segerman *et al.*, 2003; Short *et al.*, 2004). Human CD34-positive cells, dendritic cells, synoviocytes, vascular endothelial cells (ECs), and smooth muscle cells (SMCs), which were poorly transfectable by conventional Ad vectors, were efficiently transfected by fiber-substituted Ad vectors (Shayakhmetov *et al.*, 2000; Goossens *et al.*, 2001; Havenga *et al.*, 2001; Okada *et al.*, 2001; Rea *et al.*, 2001). Mercier *et al.* described the creation of a chimeric Ad vector encoding the reovirus attachment protein $\sigma 1$, which targets cells expressing the junctional adhesion molecule 1 (JAM1) (Mercier *et al.*, 2004).

When modified Ad vectors are injected locally into target tissue expressing corresponding receptors, the affinity of the vector for the cells increases, thereby resulting not only in higher transduction efficiency, but also in decreased vector dissemination. We reported that the intratumoral administration of luciferase-expressing Ad vectors containing the RGD peptide in the HI loop of the fiber knob resulted in nearly 40 times more transgene production in tumor, but 8 times less transgene expression in liver in the B16 mouse melanoma model as compared with conventional Ad vectors (Mizuguchi and Hayakawa, 2002b).

Other candidate locations for insertion of foreign ligands into the Ad capsid are the pIX, the penton base, and the hypervariable region (HVR) 5 of hexon loop L1 (Wickham *et al.*, 1995; Vigne *et al.*, 1999; Dmitriev *et al.*, 2002; Vellinga *et al.*, 2004). Among them, pIX seems to be the most promising. pIX is a minor structural protein that is contained in the Ad virion, and enhances the structural integrity of the particles by stabilizing hexon–hexon interaction (Ghosh-Choudhury *et al.*, 1987; Furcinitti *et al.*, 1989). It also plays a role in transcriptional activity and nuclear reorganization (Rosa-Calatrava *et al.*, 2001). Foreign ligands are displayed at the C terminus of the pIX of Ad (Dmitriev *et al.*, 2002). The attractive characteristics of ligand insertion into the pIX region is that the C terminus of pIX tolerates the insertion of large peptides. By incorporation of the pIX–green fluorescent protein (GFP) fusion protein, a fluorescent Ad was generated (Le *et al.*, 2004; Meulenbroek *et al.*, 2004). The insertion of higher affinity ligands such as single-chain antibodies (scFv) would be ideal, although generating such Ad vectors might be difficult because of impaired assembly of complex scFv–pIX fusion proteins in the nucleus. One problem with pIX fusions is that Ad pIX resides below the top of the hexon capsomer, within the core of the virus. This problem was circumvented by incorporating an α-helical spacer into the ligand–pIX fusion protein so as to lift the ligand and expose it to the surface of the capsid (Vellinga *et al.*, 2004). However, Ad vectors containing the RGD motif in the C terminus of pIX with α-helical spacers are likely to be less efficient than Ad vectors containing the RGD motif in the HI loop of the fiber knob (Vellinga *et al.*, 2004). Additional modification may be required for improved efficacy and specificity of retargeting.

Several groups have developed an Ad vector from an entire Ad35, and have demonstrated higher transduction efficiency for the Ad35 vector into human CD34-positive cells and dendritic cells compared with the conventional Ad5 vector (Gao *et al.*, 2003; Sakurai *et al.*, 2003a,b; Seshidhar Reddy *et al.*, 2003; Vogels *et al.*, 2003). In addition, Ad35 vectors have the advantage of evading humoral immune responses against Ad5. However, fiber-substituted Ad5 vectors containing fiber proteins of another serotype do not circumvent the immune response against Ad5 (Gall *et al.*, 1996; Ophorst *et al.*, 2004), because hexon is the major target of host-neutralizing antibodies in Ad5 infec-

tion (Gall *et al.*, 1996, 1998; Roy *et al.*, 1998). The Ad35 vector would be an effective alternative for use in persons with neutralizing antibodies in Ad5, and in the second injection when the Ad5 vector is used in the first injection of *in vivo* gene therapy.

Truly targeted adenovirus vectors. Although modifications described above yield Ad vectors with greatly improved transduction to many cells lacking in CAR expression, when systemically administered, vector dissemination, resulting in accumulation in liver, is unavoidable. To create a strictly targeted Ad vector, two basic requirements must be met. The first is construction of vectors that abolish natural viral tropism. The second is identification and incorporation of a foreign ligand with high affinity for a specific cellular receptor into the capsid of Ad vectors.

The capsid proteins determine the tropism of Ad. Because the fiber knob binds with CAR, this interaction first must be abolished. Mutation of the AB, DE, or FG loop of the fiber knob has been reported to abolish the fiber–CAR interaction (Bewley *et al.*, 1999; Kirby *et al.*, 1999; Roelvink *et al.*, 1999). These mutations of the fiber knob greatly reduce the transduction efficiency of Ad vectors to CAR-positive cells *in vitro*. In another strategy, Nakamura *et al.* replaced the tail, shaft, and knob domains of the Ad5 fiber with those of the Ad40 short fiber, which is hypothesized not to bind to any receptors (Nakamura *et al.*, 2003). In addition, interaction of the RGD motif of the penton base with α_v integrin must be abolished, although this interaction might be minor, at least *in vitro* (Mizuguchi *et al.*, 2002). The ablation of α_v integrin binding was accomplished by deletion of the RGD motif of the penton bases. Several articles reported that a single mutation of either the fiber knob or penton base does not change the biodistribution of Ad vectors in mice after *in vivo* injection (Alemany and Curiel, 2001; Leissner *et al.*, 2001; Mizuguchi *et al.*, 2002), whereas double mutation reduces liver transduction (Einfeld *et al.*, 2001; Koizumi *et al.*, 2003a), although two groups showed that double mutation also does not reduce liver transduction (Martin *et al.*, 2003; Smith *et al.*, 2003b). The reason for this discrepancy is unclear. However, Nicol *et al.* reported that combining fiber knob and penton base mutations reduces liver transduction by 509-fold in rats, an effect not observed in parallel experiments in mice (Nicol *et al.*, 2004). Subtle differences among the vectors, such as differences in mutated amino acids, experimental animal strains used, or injected doses, might have caused these discrepancies. Furthermore, the fiber shaft domain of Ad5 was reported to be involved in accumulation in the mouse liver of systemically administrated Ad vectors (Nakamura *et al.*, 2003; Smith *et al.*, 2003b), possibly because of the interaction of the KKTK (Lys-Lys-Thr-Lys) motif on the fiber shaft of Ad5 with heparan sulfate (Smith *et al.*, 2003b). This effect was also observed in nonhuman primate (cynomolgus monkey) models (Smith *et al.*, 2003a). According to our data, triple mutation of

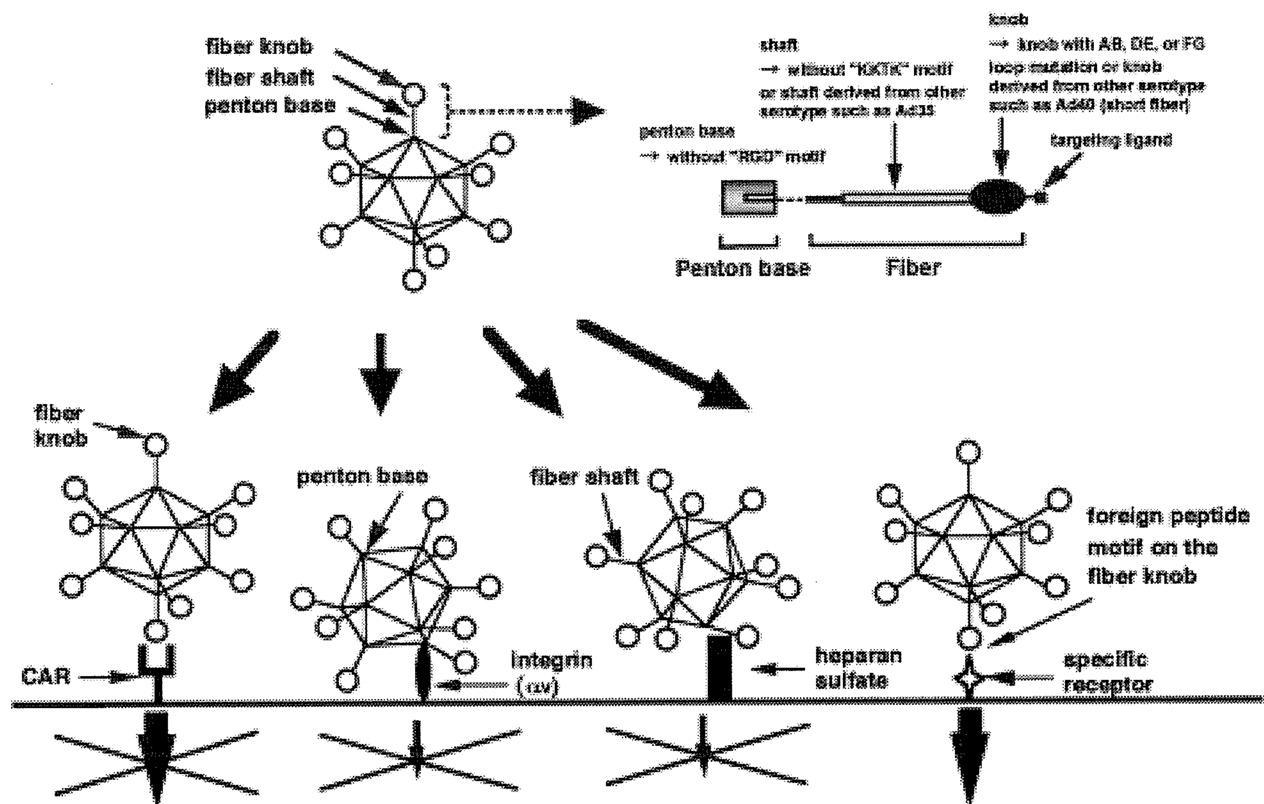

FIG. 2. Schematic diagram of Ad vectors targeted by the genetic approach. The CAR-, α_v integrin-, and heparan sulfate-binding activities of the Ad capsid are completely ablated by mutations in the fiber knob, the fiber shaft, and the penton base, respectively (Koizumi *et al.*, 2003a; Nicol *et al.*, 2004). Targeting ligands should be incorporated in the virus capsid, such as the fiber, the penton base, the hexon, or pIX. The resulting targeted Ad vectors transduce cells via the incorporated foreign ligand-dependent, CAR-, α_v integrin-, and heparan sulfate-independent pathway.

the fiber knob, shaft, and penton base mediated levels of liver transduction more than 30,000-fold lower than that of conventional Ad vectors (Koizumi *et al.*, 2003a). This vector contains a CAR-binding ablated mutant fiber knob derived from Ad5, a fiber shaft derived from Ad35 (the fiber shaft of Ad35 does not have the KKTK motif and is shorter than that of Ad5 [Ad5 fiber shaft, 6 β repeats; Ad5 fiber shaft, 22 β repeats]), a fiber tail derived from Ad5, and a mutant penton base of Ad5 without the RGD motif. Ad vectors, with mutations in two domains of the fiber knob, the fiber shaft, and the penton base, showed a level of liver transduction intermediate between that of conventional Ad vectors and the triple-mutant Ad vectors (Koizumi *et al.*, 2003a). Nicol *et al.* reached a similar conclusion in a rat model (Nicol *et al.*, 2004). Thus, Ad tropism would be determined by at least three factors: the fiber knob, the fiber shaft, and the RGD motif of the penton base (Figs. 1A and 2). Triple mutations, including the fiber knob, the fiber shaft, and the RGD motif of the penton base, should be preferable for the platform of targeted Ad vectors.

A detailed study on vector distribution to the liver, however, suggested that triple-mutant Ad vectors distribute to nonparenchymal cells to a similar extent as conventional vectors, and that both vectors are cleared rapidly from the bloodstream, having a half-life of less than 2 min (Koizumi *et al.*, 2003a). This nonparenchymal cell-mediated clearance might present an obstacle to the development of targeted Ad vectors that incorporate a foreign ligand into the viral capsid. One promising strategy to overcome this problem might be intraperitoneal, not intravenous, injection of the vector. Akiyama *et al.* reported that the intraperitoneal administration of CAR and integrin binding-ablated Ad vectors increases their persistence in the bloodstream, although the mechanism by which this occurs is unknown (Akiyama *et al.*, 2004). Extended release of the vector from the cavity might change its pharmacokinetics. More detailed study is needed to clarify nonparenchymal cell-mediated vector clearance. Lower clearance from the bloodstream may lead to increased delivery of the vector to the tissue of interest, if an appropriate targeting ligand is incorporated into the vector.

The identification of targeting ligands that are displayed in the capsid, such as fiber and pIX, is another challenge. A display library using filamentous phage is widely used for the identification of functional peptides for targeting. Although some success in identifying peptide ligands for the targeted Ad vectors was reported (Nicklin *et al.*, 2000; Xia *et al.*, 2000; Work *et al.*, 2004), most peptides that are identified by phage display libraries are not functional when they are displayed in the fiber knob of Ad vectors. Foreign peptides inserted into the HI loop of the fiber knob are constrained at both the N and C termini, whereas peptides inserted at the C terminus of the fiber knob are constrained only at the N terminus. In contrast, peptides identified by filamentous phage display library are constrained only at the C terminus, when the peptides are displayed as a fusion protein with the product of gene III of the phage. The lack of efficacy of peptides inserted in the fiber knob could be due to this difference when the peptides are identified. Furthermore, the lack of efficacy would be dependent on conformational changes after ligation of the peptide to the fiber knob. To overcome these limitations, Pereboev *et al.* employed a modified filamentous phage-displayed system, pJuFo, which was originally designed to display C-terminal protein fragments (Pereboev *et al.*, 2001). They developed a system for displaying peptides in the context of the fiber knob on the surface of the phage. A display system based on phage λ, which expresses a functional Ad fiber knob on the surface, was also developed (Fontana *et al.*, 2003). By using these systems, Ad vectors containing novel peptide ligands were generated, transducing NIH3T3 and dendritic cells at 100- to 1000-fold higher efficiency than conventional vectors (Fontana *et al.*, 2003). The development and evaluation of the next generation of targeted vectors by incorporating the novel peptides into native tropism-ablated Ad vectors is expected. In the case of the adeno-associated virus (AAV) vector, a method for incorporating random small peptides in the viral capsid has been developed (Muller *et al.*, 2003). This type of screening for ligands might be useful for targeted Ad vector, although the creation of an Ad library with wide diversity is a challenge.

Propagation of modified Ad vectors that no longer bind with cellular receptors (CAR, α_v integrin, and heparan sulfate) requires a special packaging cell line. Two types of packaging cell lines have been reported. One utilizes 293 cells modified to express an artificial receptor molecule (Douglas *et al.*, 1999; Roelvink *et al.*, 1999) that should not have any natural analogs, such as the anti-His single-chain antibody (scFv) and anti-hemagglutinin (HA) scFv. The other approach is to use 293 cells expressing Ad5 fiber protein (Fiber-293 cells) (Von Seggern *et al.*, 1998; Legrand *et al.*, 1999; Koizumi *et al.*, 2003a). In the case of cell lines expressing anti-His scFv, a His tag sequence has been introduced into the C-terminal region of the fiber knob in Ad vectors (Douglas *et al.*, 1999), whereas in the case of cell lines expressing anti-HA scFv, an HA tag sequence has been introduced into the HI loop of the fiber knob or the penton base instead of the RGD motif (Roelvink *et al.*, 1999). Modified Ad vectors are generated by interaction of the tag sequence in the virus with the scFv against the tag sequence on the cells. When the modified Ad vectors are propagated in Fiber-293 cells, wild-type fibers are incorporated in the virus during amplification, resulting in the virus containing both wild-type fibers and mutated fibers. This virus infects 293 (Fiber-293) cells via the wild-type fiber. At the final stage of viral amplification, mutated Ad vectors are allowed to infect normal 293 cells. The recovered viruses should contain only mutant fiber proteins. When Fiber-293 cells have been used as packaging cell lines, either the HI loop or the C-terminal region of the fiber knob as well as the penton base can be used to display a foreign ligand on the vectors. This makes these cells advantageous over cell lines expressing anti-His scFv or anti-HA scFv. In both methods, modified vectors were generated to particle titers similar to that of conventional Ad vectors (Douglas *et al.*, 1999; Roelvink *et al.*, 1999; Koizumi *et al.*, 2003a).

Another strategy to ablate CAR binding by Ad vectors is to proteolytically remove the knob domain of Ad fibers via the insertion of a single factor Xa cleavage site in the fiber shaft, between the cellular ligand and knob domain (Magnusson *et al.*, 2001; Hong *et al.*, 2003; Gaden *et al.*, 2004). As cellular ligands, the RGD peptide and a 58-residue oligopeptide termed the affibody, which binds specifically to the human IgG1 Fc domain, were introduced and ligand-mediated gene transfer was reported (Magnusson *et al.*, 2001; Hong *et al.*, 2003; Gaden *et al.*, 2004).

Ad vectors in which the fiber protein was replaced with phage T4 fibritin were also developed (Krasnykh *et al.*, 2001;

Belousova *et al.*, 2003; Papanikolopoulou *et al.*, 2004). In these vectors, structural similarity between the Ad fiber and bacteriophage T4 fibritin proteins was used, and the fiber shaft and knob domains were replaced with T4 fibritin and a receptor-binding ligand. The human CD40 ligand was functionally displayed in the chimeric fiber of the Ad vectors (Belousova *et al.*, 2003). This approach seems to overcome structural conflicts between the fiber and the targeting ligand.

As described above, several types of vector systems have been developed, using a genetic strategy. These vectors would provide a platform for future targeted Ad vector development. Future efforts should be directed toward novel ligands for specific tissue targeting.

Modification by the use of adaptor molecules

Retargeting of Ad infection can also be achieved through the use of bispecific or bifunctional adaptor molecules composed of an anti-fiber antibody fragment and a cell-binding component. Douglas *et al.* conjugated folate to the neutralizing Fab fragment of an anti-fiber monoclonal antibody (mAb). This Fab–folate conjugate was complexed with an Ad vector and shown to redirect, at high efficiency, the Ad infection of target cells via the folate receptor (Douglas *et al.*, 1999). The Fab fragment of the anti-fiber mAb has been utilized to conjugate with several other ligands including fibroblast growth factor 2 (FGF-2) (Goldman *et al.*, 1997; Sosnowski *et al.*, 1999), epidermal growth factor receptor (EGFR) (Miller *et al.*, 1998), and an anti-CD40 mAb fragment (Tillman *et al.*, 1999). Reynolds *et al.* succeeded in targeting pulmonary endothelial cells *in vivo* by the intravenous injection of Ad vectors complexed with bispecific antibody against the Ad fiber knob and angiotensin-converting enzyme (Reynolds *et al.*, 2000). In a similar strategy, the anti-Ad fiber knob scFv (Watkins *et al.*, 1997; Haisma *et al.*, 2000; Nettelbeck *et al.*, 2001) or the extracellular domain of CAR (Dmitriev *et al.*, 2000; Itoh *et al.*, 2003) was used as the attachment molecule with the virus. Fusion proteins or complexes of ligands with the anti-Ad fiber knob scFv or CAR were used as adaptor molecules (Fig. 1B).

Combination of the adaptor molecule and genetically modified capsids of the Ad vector has also been reported. The Fc-binding domain of staphylococcal protein A was genetically incorporated into the Ad fiber protein (Henning *et al.*, 2002; Korokhov *et al.*, 2003; Volpers *et al.*, 2003). Two studies incorporated the Fc-binding domain into either the HI loop or C terminus of the fiber knob (Korokhov *et al.*, 2003; Volpers *et al.*, 2003), whereas one study incorporated the Fc-binding domain into a knob-deleted fiber containing seven shaft repeats and an external trimerization motif (Henning *et al.*, 2002). Targeting components such as the antibody and fusion protein of the ligand with the Fc domain of immunoglobulin effectively bind to the modified Ad vectors, resulting in specific gene delivery. Because the target-specific ligands such as antibodies are simply changed in this system, these types of Ad vectors should be useful for systematic screening and detection of the target-specific ligands, as well as for therapeutic applications.

Metabolically biotinylated Ad vectors have been developed as another type of vector with adaptor molecule and genetically modified capsid. Barry and colleagues designed a system based on the fusion of a truncated form of the *Propionibacterium shermanii* 1.3S transcarboxylase domain (PSTCD), which functions as a biotin acceptor peptide (BAP) and is efficiently biotinylated by human holocarboxylase synthetase, to the C terminus of the Ad fiber protein (Parrott *et al.*, 2003) or the C terminus of the Ad pIX protein (Campos *et al.*, 2004). In this system, Ad vectors containing BAP are metabolically biotinylated during vector production by the endogenous biotin ligase in 293 cells, resulting in covalently biotinylated virions. Biotinylated Ad vectors are useful as a platform for avidin-based ligand screening and vector targeting by conjugating biotinylated ligands to the virus, using high-affinity tetrameric avidin. Their group performed ligand screening for dendritic cells, using biotinylated Ad vectors (Parrott *et al.*, 2003).

Theoretically, in all the approaches discussed above, any conjugates with one component directed against the Ad capsid (or modified capsid) and the second component directed against the cell surface protein can be applied to increase transduction of target cells. The advantage is that the natural tropism of the fiber knob is usually ablated, possibly as a result of steric hindrance by adaptor molecules. One limitation is that complexes of Ad vectors and adaptor molecules are nonuniform, and batch-to-batch difference of the vectors might occur.

Chemical modification by polymers

Chemical modification with polyethylene glycol (PEG; PEGylation) is frequently used in pharmaceutic preparations to provide a hydrophilic coat and to increase the blood persistence of therapeutic peptides and proteins (Harris and Chess, 2003). Modification of Ad vectors with PEG, in which the activated PEG reacts preferentially with the ϵ-amino terminal of lysine residues on the capsid, including the hexon, fiber, and penton base, prolongs persistence in the blood and circumvents neutralization of the Ad vectors by antibodies (O'Riordan *et al.*, 1999; Romanczuk *et al.*, 1999; Alemany *et al.*, 2000; Croyle *et al.*, 2000, 2001, 2002; Lanciotti *et al.*, 2003; Eto *et al.*, 2004; Ogawara *et al.*, 2004) (Fig. 1C). Furthermore, PEGylated Ad vectors attenuate the ability of the vector to be taken up by antigen-presenting cells, thereby reducing inflammatory responses. Animals administered PEGylated Ad vectors exhibited reduced levels of both cell-mediated and humoral immune responses, resulting in significant gene expression on readministration of unmodified Ad vectors in the lung (O'Riordan *et al.*, 1999; Croyle *et al.*, 2001). However, the PEGylation of Ad vectors leads to loss of infectivity due to steric hindrance by PEG chains (O'Riordan *et al.*, 1999; Alemany *et al.*, 2000; Croyle *et al.*, 2000, 2001, 2002; Lanciotti *et al.*, 2003; Eto *et al.*, 2004; Ogawara *et al.*, 2004). The extent of loss of infectivity and extension of blood retention half-time are dependent on the degree of PEG modification (Eto *et al.*, 2004). The efficiency of transduction of 34% modified PEGylated Ad vectors was approximately 200-fold lower than that of unmodified Ad (Eto *et al.*, 2004).

To overcome the decreased efficiency of infection of PEGylated Ad vectors, vectors containing functional molecules on the tip of PEG have been developed (Lanciotti *et al.*, 2003; Eto *et al.*, 2004; Ogawara *et al.*, 2004). Lanciotti *et al.* reported targeted Ad vectors, using heterofunctional PEG and FGF-2 (Lanciotti *et al.*, 2003). The transduction of Ad/PEG/FGF2 is dependent on the FGF-2 receptor, and is independent of CAR. In

an intraperitoneal model of ovarian cancer, Ad/PEG/FGF2 mediated increased transgene expression in tumor tissue and reduced localization of the vectors to nontarget tissues compared with unmodified Ad vectors (Lanciotti *et al.*, 2003). Ogawara *et al.* reported PEGylated Ad vectors containing E-selectin-specific antibody at the tip of PEG, which target activated endothelial cells (Ogawara *et al.*, 2004). They showed that the systemic administration of PEGylated Ad vectors with anti-E-selectin antibody selectively targeted inflamed skin and mediated local transgene expression in mice with a delayed-type hypersensitivity (DTH) inflammation. As for modification with peptides, α_v integrin-specific RGD peptide-modified PEGylated Ad vectors have been developed (Eto *et al.*, 2004; Ogawara *et al.*, 2004).

Ad vectors coated with polymers other than PEG have also been developed. Seymour's group used a multivalent hydrophilic polymer based on poly[*N*-(2-hydroxypropyl)methacrylamide] to modify Ad vectors (Fisher *et al.*, 2001; Green *et al.*, 2004). Their vector showed an extended plasma circulation time and decreased toxicity, and evaded neutralizing antibodies.

Approaches by chemical modification with polymers are advantageous, in that many ligands, such as peptides, antibodies, and antigens, may be applied to the tips of the polymers. A great deal of knowledge and techniques about chemical modification have been acquired in the study of pharmaceutic preparations for drug delivery systems. Although improved pharmacokinetic properties of polymer (including PEG)-coated Ad vectors without ligands have been reported, those of polymer-coated Ad vectors with ligands have not been reported in detail. The exact nature of those vectors must be characterized further.

CONCLUSIONS

In this review, we have focused on the development of targeted Ad vectors based on specific virus entry mechanisms. These approaches are easily combined with transcriptional targeting, using tissue/cell-specific promoters. Ideally, combining a better targeted vector containing a modified capsid with a fully deleted Ad genome (i.e., helper-dependent Ad vectors) is desirable to reduce the innate and acquired immunogenicity of the vectors. These combined vectors should be carefully evaluated in terms of transgene expression profile, distribution of the vectors, and pharmacokinetics, including circulation half-life, interaction with blood components, and so on. Anatomical barriers, such as the tightness of endothelial cells, should also be taken into account, because the vectors must pass endothelial barriers to reach target tissues. Although progress still needs to be made in perfecting targeted Ad vectors, steady improvements have been achieved through comprehensive approaches. Targeted Ad vectors are a source of great promise for gene therapy in future, because they enhance gene therapy efficacy and permit the delivery of lower doses, which should result in reduced toxicity.

ACKNOWLEDGMENTS

We thank Z.-L. Xu and F. Sakurai for their discussions with us. H.M. is supported by grants from the Ministry of Health, Labor, and Welfare of Japan and by a Grant-in-Aid for Scientific Research on Priority Areas of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

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Received for publication September 14, 2004; accepted after revision October 7, 2004.

Published online: October 28, 2004.

HUMAN GENE THERAPY 15:813–819 (August 2004)
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Brief Report

Adenovirus Vector-Mediated Doxycycline-Inducible RNA Interference

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ABSTRACT

RNA interference (RNAi) is a powerful tool for the knockdown of gene expression. Here, we report on the development of an adenovirus (Ad) vector-mediated doxycycline (Dox)-inducible small interfering RNA (siRNA) expression system. We used this siRNA system to control the expression of p53 and c-Myc in human cancer cells. Coinfection of Ad vectors containing the siRNA expression system under the control of the Dox-inducible H1 promoter and Ad vectors expressing a tetracycline repressor inhibited the expression levels of p53 and c-Myc in a dose-dependent manner with both Dox and viral dose. Regulated silencing of p53 and c-Myc expression was obtained. Because an Ad vector-mediated inducible RNAi system can efficiently transduce a variety of cell types *in vitro* and *in vivo*, and the degree of loss of gene expression can be modulated according to the dose of Dox, this expression system should be a useful tool for both basic research on the analysis of gene function and therapeutic applications of RNAi.

INTRODUCTION

RNA INTERFERENCE (RNAi) mediates the sequence-specific suppression of gene expression in a wide variety of eukaryotes by double-stranded RNA homologies to the target gene (McManus and Sharp, 2002). In mammalian cells, small interfering RNA (siRNA; 19- to 29-nucleotide RNA) leads to the inhibition of target gene expression in a sequence-specific manner (Elbashir *et al.*, 2001). Vector-based siRNA systems have also been developed with RNA polymerase III (Pol III) promoters, such as the small nuclear RNA U6 promoter or the human RNase P RNA H1 promoter, to express siRNA (Brummelkamp *et al.*, 2002; Lee *et al.*, 2002; Miyagishi and Taira, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002). Because Pol III promoters, however, are constitutive and ubiquitous, knockdown of the target gene in an inducible or cell-specific manner is more difficult than with the RNA polymerase II (Pol II) promoters. An inducible RNAi system becomes a more powerful tool for the analysis of gene function, because the loss-of-function or phenotype change can be analyzed according to the degree of loss of gene expression.

In the present study, we developed a doxycycline (Dox)-inducible siRNA expression system utilizing the H1 promoter containing a tetracycline operator (*tetO*) sequence. This vector system was constructed by modifying the Pol II promoter-based gene regulation system, using the tetracycline repressor (TetR), which was developed by Yao *et al.* (1998). In the absence of Dox, TetR binds the *tetO* sequence in the modified H1 promoter, thus preventing transcription. In contrast, TetR does not bind the *tetO* sequence in the presence of Dox, thus allowing transcription. Therefore, target gene expression is turned off in the absence of Dox, but is turned on in its presence.

As a delivery system for the inducible-siRNA expression cassette, the adenovirus (Ad) vector was employed because of its

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numerous attractive characteristics. Recombinant Ad vector has been extensively used to deliver foreign genes to a variety of cell types and tissues both *in vitro* and *in vivo*. Ad vector can be easily grown to high titer, and can efficiently transfer genes into both dividing and nondividing cells. Furthermore, several types of improved Ad vector systems, such as tropism-modified vectors, have been developed (Curiel, 1999; Wickham, 2000; Koizumi *et al.*, 2003a,b). An Ad vector-mediated inducible siRNA expression system would be an effective strategy to use for the basic analysis of gene function and have potential for therapeutic use. In the present study, we demonstrate the efficiency of Ad vector-mediated inducible RNAi against two endogenous genes: *p53* and c-*myc*.

MATERIALS AND METHODS

Cells

293 cells were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). A549 cells were cultured with F12-K nutrient mixture (Kaighn's modification) medium supplemented with 10% FCS. HepG2 cells were cultured with minimum essential medium supplemented with 10% FCS.

Plasmid and virus

H1 promoter was amplified from human genomic DNA (BD Biosciences Clontech, Palo Alto, CA), using the following primers: 5′-ccatggaattcgaacgctgacgtc-3′ and 5′-gcaagcttagatctgtgtctcatacagaacttataaattccc-3′. The amplified polymerase chain reaction (PCR) product was inserted into the *Eco*RI–*Bgl*II site of pHM5 (Mizuguchi and Kay, 1999), generating pHM5-H1. H1 promoter containing the *tetO* sequence was amplified from pHM5-H1, using the following primers: 5′-tttgccagaattcgaacgctgacgtcatcaacccg-3′ and 5′-ttggaaga<u>tctctatcactgataggga</u>cttataagattcccaaatccaaagacatttcacgtttatg-3′ (*tetO* sequence is underlined). The amplified PCR product was inserted into the *Eco*RI–*Bgl*II site of pHM5-H1, generating pHM5-H1tetO. pHM5-H1 and pHM5-H1tetO are designed to express short hairpin RNA (shRNA) on the insertion of an appropriate sequence into the *Bgl*II–*Xba*I site. To insert the target sequence that encodes *p53* and c-*myc* shRNA, oligonucleotides for *p53* (5′-gatccccgactccagtggtaatctac<u>ttcaagaga</u>gtagattaccactggagtctttttggaaat-3′ and 5′-ctagatttccaaaaagactccagtggtaatctac<u>tctcttgaa</u>gtagattaccactggagtcggg-3′) (Brummelkamp *et al.*, 2002) and c-*myc* (5′-gatccccgatgaggaagaaatcgat<u>gttcaagaga</u>catcgatttcttcctcatctttttggaaat-3′ and 5′-ctagatttccaaaaagatgaggaagaaatcgat<u>gtctcttgaa</u>catcgatttcttcctcatcggg-3′) (loop sequences are underlined) (van de Wetering *et al.*, 2003) were synthesized, annealed, and cloned into the *Bgl*II and *Xba*I sites of pHM5-H1 and pHM5-H1tetO, generating pHM5-H1-p53, pHM5-H1-Myc, pHM5-H1tetO-p53, and pHM5-H1tetO-Myc, respectively. The sequence was verified with a DNA sequencer (ABI PRISM 310; Applied Biosystems, Foster City, CA).

Ad vectors were constructed by an improved *in vitro* ligation method (Mizuguchi and Kay, 1998, 1999). Briefly, pHM5-H1-p53, pHM5-H1-Myc, pHM5-H1tetO-p53, and pHM5-H1tetO-Myc were digested with I-*Ceu*I and PI-*Sce*I, and then ligated with I-*Ceu*I- and PI-*Sce*I-digested pAdHM15-RGD (Mizuguchi *et al.*, 2001). The resulting plasmids were digested with *Pac*I and transfected into 293 cells plated in a 60-mm dish with SuperFect (Qiagen, Valencia, CA), according to the manufacturer's instructions. Viruses (Ad-H1-p53, Ad-H1-Myc, Ad-H1tetO-p53, and Ad-H1tetO-Myc) were prepared as described previously (Mizuguchi and Kay, 1998). Ad vectors containing only the H1 promoter sequence (without a target sequence) (Ad-H1 and Ad-H1tetO) were similarly prepared. Ad-TR, the Ad vector expressing TetR, had been previously prepared (Xu *et al.*, 2003a). Ad-null contains no transgene in the E1 deletion region. Virus was purified by $CsCl_2$ gradient centrifugation, dialyzed with a solution containing 10 m*M* Tris (pH 7.5), 1 m*M* $MgCl_2$, and 10% glycerol, and stored in aliquots at −70°C. Determination of virus particle titer and infectious titer (plaque-forming units; PFU) was accomplished spectrophotometrically by the method of Maizel *et al.* (1968) and by the method of Kanegae *et al.* (1994), respectively. The PFU-to-particle ratio was 1:56 for Ad-H1-p53, 1:58 for Ad-H1-Myc, 1:56 for Ad-H1tetO-p53, 1:65 for Ad-H1tetO-Myc, 1:36 for Ad-H1, 1:50 for Ad-H1tetO, 1:24 for Ad-TR, and 1:57 for Ad-null.

Adenovirus-mediated gene transduction

A549 and HepG2 cells (2×10^5 cells) were seeded into a 12-well dish. The next day, they were transduced with the Ad vectors for 1.5 hr. The cells were cultured with medium containing various concentrations of Dox (BD Biosciences Clontech), a derivative of tetracycline. Tet system-approved FCS (BD Biosciences Clontech), a tetracycline-free serum that has been determined to be optimal for the tetracycline-controllable expression system, was used as the FCS.

Western blotting for p53 and c-Myc proteins

Cell extracts were prepared in lysis buffer (25 m*M* Tris [pH 7.5], 1% Triton X-100, 0.5% sodium deoxycholate, 5 m*M* EDTA, 150 m*M* NaCl) containing a cocktail of protease inhibitors (Sigma, St. Louis, MO). The protein content was measured with an assay kit from Bio-Rad (Hercules, CA), using bovine serum albumin as the standard. Protein samples (10 μg) were electrophoresed on 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gels under reducing conditions, followed by electrotransfer to Immobilon-P membranes (Millipore, Bedford, MA). After blocking in Block Ace (Dainippon Pharmaceuticals, Osaka, Japan), the filters were incubated with antibodies against p53 (Santa Cruz Biotechnology, Santa Cruz, CA), c-Myc (Santa Cruz Biotechnology), and actin (Oncogene Research Products, San Diego, CA), followed by incubation in the presence of peroxidase-labeled goat anti-mouse IgG antibody (American Qualex Antibodies, San Clemente, CA) or peroxidase-labeled goat anti-mouse IgM antibody (Oncogene Research Products). The filters were developed using chemiluminescence (ECL Western blotting detection system; Amersham Biosciences, Piscataway, NJ). Signals were read with an LAS-3000 (Fujifilm, Tokyo, Japan), and quantified by Image Gauge software (Fujifilm).

Northern blot for p53 *and* c-myc *siRNAs*

Total RNA was isolated with ISOGEN reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruc-

tions. To determine the levels of *p53* and c-*myc* siRNAs, 20 μg of total RNA, which was denatured with formamide, was separated on 15% polyacrylamide gels containing 7 *M* urea and electrotransferred to Hybond-N+ membrane (Amersham Biosciences). Loading was checked by ethidium bromide staining. Hybridization was performed with Rapid-Hyb buffer (Amersham Biosciences). Probes which were antisense oligonucleotide (19 bp) of target sequence, were labeled with a MEGALABEL DNA 5′-end labeling kit (TaKaRa Bio, Shiga, Japan). Signals were read with a BAS-2500 (Fujifilm).

RESULTS AND DISCUSSION

Using a combination of Ad vectors and an siRNA expression system is clearly an advantage in gene transfer experiments and therapeutic applications. An inducible siRNA expression system is more desirable, because the degree of gene silencing can be controlled by adjusting the dose or concentration of the inducer. In this study, we developed Ad vectors containing a Dox-inducible siRNA expression system. For inducible siRNA expression, the *tetO* sequence was placed between the TATA box and transcription start site of the H1 promoter (the sequence is described in Materials and Methods). Various Ad vectors, in which target sequences were inserted to express shRNA under the control of the H1 promoter and a mutant H1 promoter containing the *tetO* sequence, were constructed and are shown in Fig. 1. For proof of concept, the expression of endogenous genes *p53* and c-*myc* was silenced.

First, to examine the feasibility of the Ad vector-mediated siRNA expression system, p53 and c-Myc expression was constitutively knocked down by infection with Ad vectors containing the normal H1 promoter or a mutant H1 promoter containing the *tetO* sequence. A549 and HepG2 cells were infected with various concentrations of Ad vector (Ad-H1-p53, Ad-H1-Myc, Ad-H1tetO-p53, Ad-H1tetO-Myc, Ad-H1, Ad-H1tetO, or Ad-null), and cultured without Dox for 3 days. Levels of p53 and c-Myc protein expression were examined by Western blotting (Fig. 2). Expression of actin was also measured as an internal control. Expression of p53 and c-Myc in

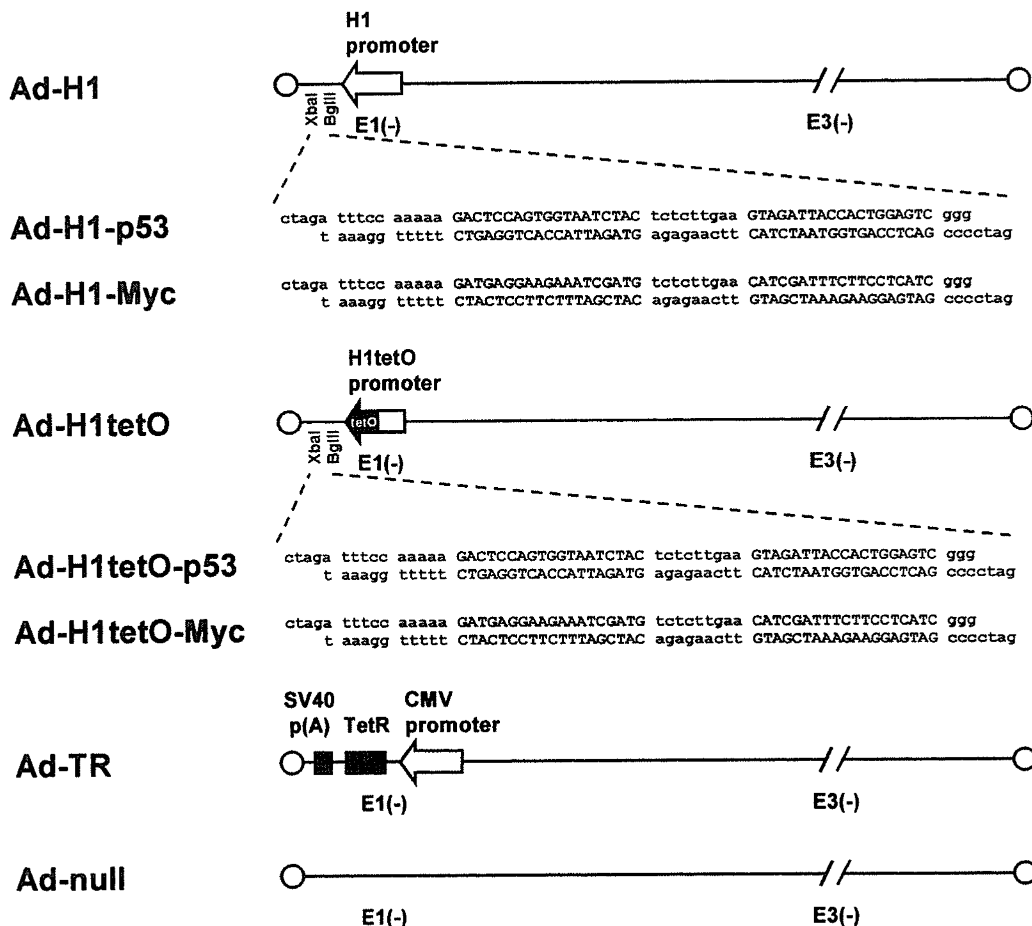

FIG. 1. Structure of Ad vectors used in the present study. The H1 promoter-based siRNA expression cassette was inserted into the E1 deletion region of the Ad genome. For inducible siRNA expression, a tetracycline operator (*tetO*) sequence was introduced downstream of the TATA box in the H1 promoter, as described in Materials and Methods. Target sequences against *p53* and c-*myc* genes are shown in upper case letters. Ad-TR is Ad vector containing a tetracycline repressor sequence under the control of the CMV promoter-enhancer. Ad-null is Ad vector without foreign genes in the E1 deletion region.