To determine why the wild-type Ad vector exhibited inefficient transduction of 3T3-L1 preadipocytes and adipocytes, we examined the expression of CAR, a primary Ad receptor, in 3T3-L1 preadipocytes and adipocytes by RT-PCR analysis (Fig. 2). NIH3T3 and NIH3T3-CAR cells, which are transfectants of the mouse CAR gene, were used as a negative and positive control, respectively. The results showed that CAR mRNA was not present in either 3T3-L1 preadipocytes or adipocytes, suggesting that Ad vectors containing wild-type fiber did not mediate transduction probably due to little expression of CAR. From these results, we concluded that fiber-modification with K7 peptides improved the efficiency of Ad transduction into both 3T3-L1 preadipocytes and adipocytes.

3.2. Suppression of the expression levels of PPARγ in 3T3-L1

Next, we constructed AdK7-H1-PPARγ, which expresses siRNA for PPARγ with K7 peptides-modified fiber knob, and examined whether AdK7-H1-PPARγ inhibited the differentiation of 3T3-L1 preadipocytes into adipocytes. The target

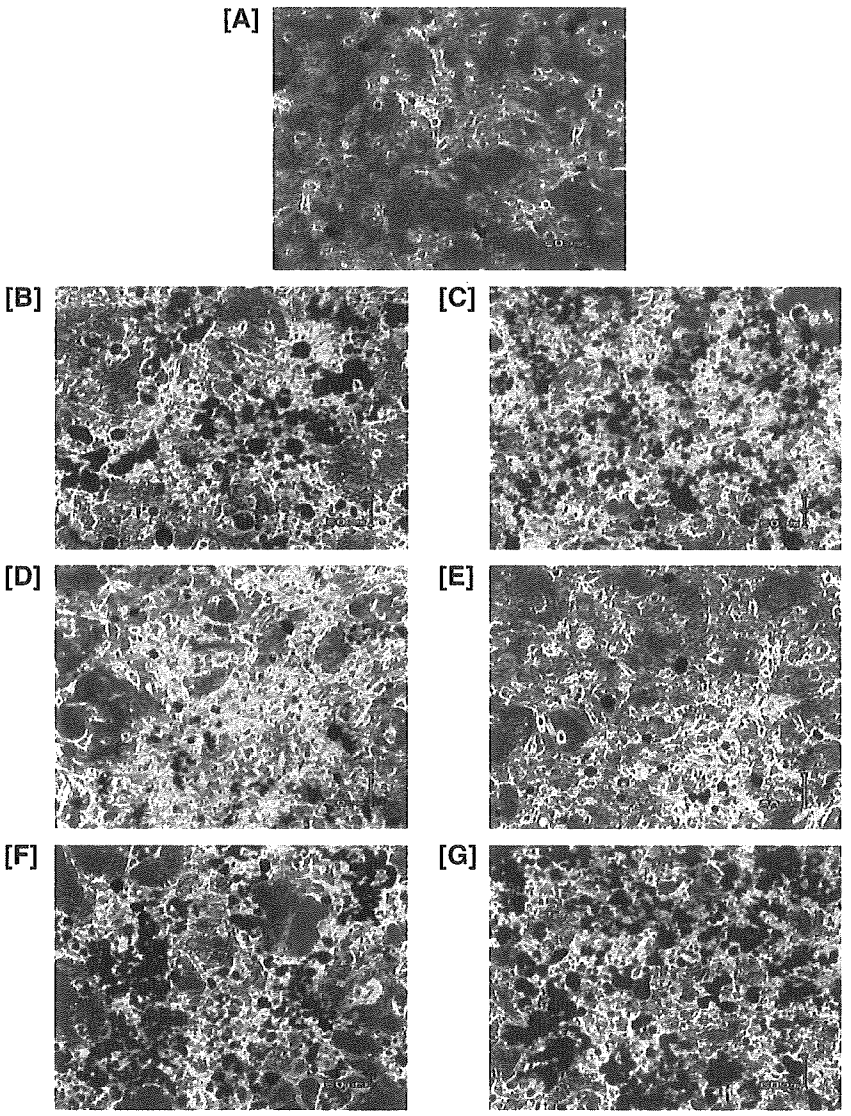

Fig. 4. Suppression of preadipocyte-to-adipocyte differentiation by transduction with AdK7-H1-PPARγ. 3T3-L1 preadipocytes were transduced with each Ad vector for 1.5 h. On the following day, the cells reached confluence. From 3 days after Ad treatment, the cells were cultured with differentiation medium containing pioglitazone, insulin, dexamethasone and 3-isobutyl-1-methylxanthine for 9 days. Then, the intracellular lipid accumulation, which was used as the marker of preadipocyte-to-adipocyte differentiation, was determined by Oil red O staining. (A) 3T3-L1 preadipocytes (3T3-L1 cells cultured with normal medium); (B) 3T3-L1 adipocytes (3T3-L1 cells cultured with differentiation medium without Ad treatment); (C) 3T3-L1 cells cultured with differentiation medium with AdK7-H1 (10,000 VP/cell) treatment; (D) 3T3-L1 cells cultured with differentiation medium with AdK7-H1-PPARγ (3000 VP/cell) treatment; (E) 3T3-L1 cells cultured with differentiation medium with AdK7-H1-PPARγ (10,000 VP/cell) treatment; (F) 3T3-L1 cells cultured with differentiation medium with AdK7-H1-Scramble (10,000 VP/cell) treatment; (G) 3T3-L1 cells cultured with differentiation medium with AdK7-Null (10,000 VP/cell) treatment.

sequence of siRNA for PPARγ was selected to knockdown both PPARγ1 and PPARγ2 (Katayama et al., 2004). We confirmed by Western blotting that AdK7-H1-PPARγ suppresses the expression levels of PPARγ in 3T3-L1 adipocytes (Fig. 3). The levels of PPARγ in the cells treated with 3000 or 10,000 VP/cell of AdK7-H1-PPARγ were decreased to 51% or 16% of the levels in cells treated with AdK7-Null (10,000 VP/cells), respectively. AdK7-H1, AdK7-H1-Scramble and AdK7-Null did not show any effect on the PPARγ expression, compared with non-infected cells. These results indicated that AdK7-H1-PPARγ effectively suppressed the expression of PPARγ in 3T3-L1 cells.

3.3. Suppression of the preadipocyte-to-adipocyte differentiation in 3T3-L1 cells

During the process of preadipocyte-to-adipocyte differentiation, 3T3-L1 preadipocytes initiate the storage of energy in the form of triacylglycerol-rich lipid droplets. The degree of differentiation of 3T3-L1 cells can be evaluated by measuring the accumulation of intracellular lipids, which are stained by Oil red O, and GPDH activity. We next examined whether AdK7-H1-PPARγ suppresses the preadipocyte-to-adipocyte differentiation in 3T3-L1 cells. The 3T3-L1 cells were transduced with Ad vectors and reached confluence on the following day. Two days after reaching confluence, the cells were cultured with differentiation medium for 9 days and stained with Oil red O. Intracellular lipid accumulation was reduced in 3T3-L1 cells transduced with AdK7-H1-PPARγ (Fig. 4). The levels of GPDH activity in the cells treated with 3000 or 10,000 VP/cell of AdK7-H1-PPARγ were decreased to 55% or 33% of the levels in cells treated with AdK7-Null (10,000 VP/cell), respectively (Fig. 5). AdK7-H1, AdK7-H1-Scramble and AdK7-Null did not show any suppressive effect on the accumulation of intracellular lipid and GPDH activity (Fig. 5). These results suggested that AdK7-H1-PPARγ efficiently suppressed the preadipocyte-to-adipocyte differentiation of 3T3-L1 cells.

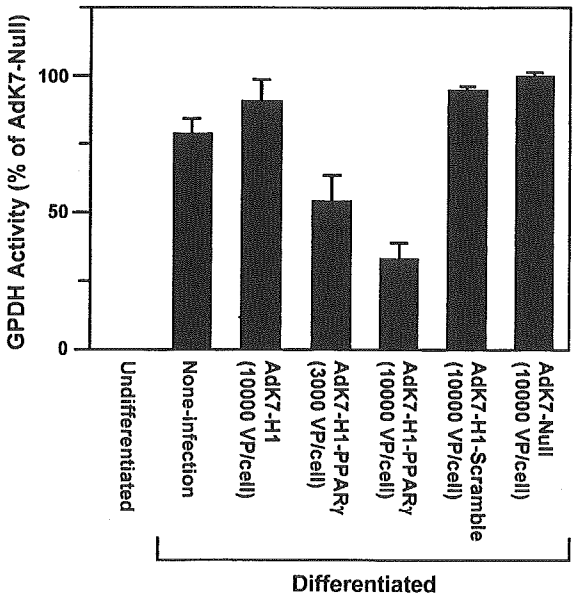

Fig. 5. Suppression of the fatty synthesis on 3T3-L1 cells transduced with AdK7-H1-PPARγ. The cells and virus were treated as described in the legends of Fig. 4. The fatty synthesis was determined by the measurement of GPDH activity in 3T3-L1 cells. Data were expressed as percentage of the GPDH activity of 3T3-L1 cells cultured with differentiation medium with AdK7-Null (10,000 VP/cell) treatment.

4. Discussion

3T3-L1 cell line is widely used for studying adipocyte differentiation and adipose biology. However, this cell line is poorly transduced by the conventional Ad vectors or DNA transfection reagents. In the present study, we successfully transduced nearly 100% of 3T3-L1 preadipocytes by using fiber-modified Ad vectors containing polylysine peptides and showed that Ad vector-mediated RNAi for PPARγ efficiently suppressed the preadipocyte-to-adipocyte differentiation. We also showed that 3T3-L1 adipocytes were efficiently transduced by the fiber-modified Ad vectors containing polylysine peptides. Carlotti et al. reported that Ad vector-mediated gene transfer into 3T3-L1 adipocytes was associated with marked cytopathogenicity (Carlotti et al., 2004). In our results, no cytotoxicity, adipogenicity, or other negative effects on cell function were observed by the Ad vector-mediated gene transfer.

Several strategies have been employed to overcome the poor transduction efficiency of 3T3-L1 cells. Because the low expression of CAR, the primary Ad receptor, in 3T3-L1 cells would the cause of the poor transduction of the conventional Ad vectors (Fig. 2), 3T3-L1 cells stably expressing CAR by the transfection have been developed (Orlicky et al., 2001; Ross et al., 2003). However, CAR is an adhesion molecule which mediates tight junctions and homotypic interactions (Honda et al., 2000; Cohen et al., 2001). Therefore, there might be negative effects of ectopic CAR expression in the process of adipogenesis of 3T3-L1 cells. Another strategy is to use transduction-enhancing agents such as polylysine, lipofectAMINE (Invitrogen Life Technologies), or SuperFect (Qiagen Inc.), which mediate CAR-independent transduction of Ad vectors (Orlicky and Schaack, 2001). These reagents sometimes negatively affect cellular function, e.g., via their cytotoxicity or their inhibition of cell growth and differentiation. Complexes of Ad vectors and transduction-enhancing agents are also non-uniform and are not likely to show reproducible results. Fiber-modified Ad vectors overcome all these problems. Among the vectors tested in the present study, polylysine-modification of the Ad fiber, which is negatively charged, exhibited the most efficient gene transfer to 3T3-L1 preadipocytes and adipocytes. This result correlates well with the report of Orlicky and Schaack that complexes of Ad vectors and polylysine enhanced transduction in 3T3-L1

cells (Orlicky and Schaack, 2001), although the vector in the present study contained fiber that was genetically modified with polylysine (a stretch of seven lysine residues), while their vector is just a complex of Ad and polylysine. 3T3-L1 cells might produce a large number of negatively charged glycosaminoglycans.

PPARγ is a master regulator of adipogenesis and plays an important role in the regulation of insulin sensitivity and glucose homeostasis (Tontonoz et al., 1994b; Wu et al., 1998; Kubota et al., 1999; Berger and Moller, 2002). The inhibition of preadipocyte-to-adipocyte differentiation by the silencing of PPARγ expression in 3T3-L1 cells suggests that the fiber-modified Ad vector-mediated RNAi could be widely used for the basic study of adiposity and diabetes.

Homozygous PPARγ-null mice are embryonically lethal due to placental dysfunction. Heterozygous mice ($PPAR\gamma^{+/-}$) and conditional knockout mice have been used to study the function of PPARγ under in vivo conditions (Kubota et al., 1999). However, generation of these mice is time-consuming. In the heterozygous mice ($PPAR\gamma^{+/-}$), the expression levels of $PPAR\gamma^{+/-}$ cannot be regulated and are half those of the wild-type mice. Since the Ad vectors mediate efficient gene transduction even under in vivo conditions, conditional PPARγ knockdown mice might be generated by the direct in vivo injection of Ad vectors containing the siRNA expression cassette for PPARγ. Knockdown levels of PPARγ expression could be regulated by adjusting the dose of the vector. Koo et al. recently produced PGC-1 (PPARγ coactivator-1) knockdown mice by Ad delivery of PGC-1 RNAi to the liver (Koo et al., 2004).

In conclusion, the fiber-modified Ad vectors containing polylysine peptides mediate efficient gene transfer into 3T3-L1 preadipocytes and adipocytes. RNAi of PPARγ by the delivery of modified Ad vectors suppresses the preadipocyte-to-adipocyte differentiation in 3T3-L1 cells. Ad vector-mediated RNAi for PPARγ should be useful for not only studying the biological and physiological mechanism of PPARγ during adipogenesis in adiposity and diabetes, but also in therapeutic application to these and other diseases.

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References

Berger, J., Moller, D.E., 2002. The mechanisms of action of PPARs. Annu. Rev. Med. 53, 409–435.

Brummelkamp, T.R., Bernards, R., Agami, R., 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science 296, 550–553.

Carlotti, F., et al., 2004. Lentiviral vectors efficiently transduce quiescent mature 3T3-L1 adipocytes. Mol. Ther. 9, 209–217.

Cohen, C.J., Shieh, J.T., Pickles, R.J., Okegawa, T., Hsieh, J.T., Bergelson, J.M., 2001. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. Proc. Natl. Acad. Sci. U. S. A. 98, 15191–15196.

Dmitriev, I., et al., 1998. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. J. Virol. 72, 9706–9713.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494–498.

Gaggar, A., Shayakhmetov, D.M., Lieber, A., 2003. CD46 is a cellular receptor for group B adenoviruses. Nat. Med. 9, 1408–1412.

Gregoire, F.M., Smas, C.M., Sul, H.S., 1998. Understanding adipocyte differentiation. Physiol. Rev. 78, 783–809.

Havenga, M.J., et al., 2001. Improved adenovirus vectors for infection of cardiovascular tissues. J. Virol. 75, 3335–3342.

Honda, T., et al., 2000. The coxsackievirus-adenovirus receptor protein as a cell adhesion molecule in the developing mouse brain. Brain Res. Mol. Brain Res. 77, 19–28.

Hosono, T., et al., 2004. Adenovirus vector-mediated doxycycline-inducible RNA interference. Hum. Gene Ther. 15, 813–819.

Katayama, K., et al., 2004. RNA interfering approach for clarifying the PPARgamma pathway using lentiviral vector expressing short hairpin RNA. FEBS Lett. 560, 178–182.

Koizumi, N., Mizuguchi, H., Utoguchi, N., Watanabe, Y., Hayakawa, T., 2003. 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.

Koo, S.H., et al., 2004. PGC-1 promotes insulin resistance in liver through PPAR-alpha-dependent induction of TRB-3. Nat. Med. 10, 530–534.

Krasnykh, V.N., Mikheeva, G.V., Douglas, J.T., Curiel, D.T., 1996. Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. J. Virol. 70, 6839–6846.

Kubota, N., et al., 1999. PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. Mol. Cell 4, 597–609.

Maizel Jr., J.V., White, D.O., 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.

McManus, M.T., Sharp, P.A., 2002. Gene silencing in mammals by small interfering RNAs. Nat. Rev. Genet. 3, 737–747.

Miyagishi, M., 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., Hayakawa, T., 2002. Adenovirus vectors containing chimeric type 5 and type 35 fiber proteins exhibit altered and expanded tropism and increase the size limit of foreign genes. Gene 285, 69–77.

Mizuguchi, H., 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., 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., et al., 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., Miyazaki, J., 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108, 193–199.

Ntambi, J.M., Young-Cheul, K., 2000. Adipocyte differentiation and gene expression. J. Nutr. 130, 3122S–3126S.

Orlicky, D.J., Schaack, J., 2001. Adenovirus transduction of 3T3-L1 cells. J. Lipid Res. 42, 460–466.

Orlicky, D.J., DeGregori, J., Schaack, J., 2001. Construction of stable coxsackievirus and adenovirus receptor-expressing 3T3-L1 cells. J. Lipid Res. 42, 910–915.

Paul, C.P., Good, P.D., Winer, I., Engelke, D.R., 2002. Effective expression of small interfering RNA in human cells. Nat. Biotechnol. 20, 505–508.

Ross, S.A., Song, X., Burney, M.W., Kasai, Y., Orlicky, D.J., 2003. Efficient adenovirus transduction of 3T3-L1 adipocytes stably expressing coxsackie-adenovirus receptor. Biochem. Biophys. Res. Commun. 302, 354–358.

Segerman, A., Atkinson, J.P., Marttila, M., Dennerquist, V., Wadell, G., Arnberg, N., 2003. Adenovirus type 11 uses CD46 as a cellular receptor. J. Virol. 77, 9183–9191.

Shayakhmetov, D.M., Papayannopoulou, T., Stamatoyannopoulos, G., Lieber, A., 2000. Efficient gene transfer into human CD34(+) cells by a retargeted adenovirus vector. J. Virol. 74, 2567–2583.

Shen, C., Buck, A.K., Liu, X., Winkler, M., Reske, S.N., 2003. Gene silencing by adenovirus-delivered siRNA. FEBS Lett. 539, 111–114.

Short, J.J., Pereboev, A.V., Kawakami, Y., Vasu, C., Holterman, M.J., Curiel, D.T., 2004. Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors. Virology 322, 349–359.

Sui, G., Soohoo, C., Affarel, B., Gay, F., Shi, Y., 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.

Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I., Spiegelman, B.M., 1994a. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. 8, 1224–1234.

Tontonoz, P., Hu, E., Spiegelman, B.M., 1994b. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell 79, 1147–1156.

Vidal-Puig, A.J., et al., 1997. Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. J. Clin. Invest. 99, 2416–2422.

Wickham, T.J., et al., 1997. Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. J. Virol. 71, 8221–8229.

Wu, Z., Xie, Y., Morrison, R.F., Bucher, N.L., Farmer, S.R., 1998. PPAR gamma induces the insulin-dependent glucose transporter GLUT4 in the absence of C/EBPalpha during the conversion of 3T3 fibroblasts into adipocytes. J. Clin. Invest. 101, 22–32.

Yu, J.Y., DeRuiter, S.L., 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., Zhu, H., 2003. Specific gene inhibition by adenovirus-mediated expression of small interfering RNA. Gene 316, 137–141.

〔The 2nd Annual Meeting of JHUPO〕

Two-dimensional electrophoretic analysis of disease-associated proteins in human cerebrospinal fluid from patients with rheumatoid arthritis

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Two-dimensional electrophoretic analysis of disease-associated proteins in human cerebrospinal fluid from patients with rheumatoid arthritis

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SUMMARY

Comparing protein expression in the cerebrospinal fluid (CSF) of rheumatoid arthritis (RA) patients with that of controls, makes possible the uncovering of proteins that affect disease progression and regulate responsiveness to drugs. Two-dimensional gel electrophoresis (2-DE) and silver staining were used for identifying disease-associated CSF proteins in RA patients. First, to enhance the detection of CSF proteins and to improve the separation of their isoforms by 2-DE, CSF samples were pre-treated with an albumin and IgG removal kit, then by acetone precipitation. The 2-DE analysis revealed more than 1600 spots by the removal of albumin and immunoglobulin from CSF. The expression of the protein spots was not greatly changed in either group, but some notable changes in protein spots were observed in two RA samples. In particular, the expression of an approximately 50 kD protein increased markedly, whereas that of two sequential protein spots of 10–15 kD and with neutral pI decreased in the RA samples. These preliminary results suggest that the proteomic method is conducive to clarifying the mechanism of RA crises, and that some of the expression-changed proteins may be new candidates for disease-associated proteins of RA.

Key words: rheumatoid arthritis, cerebrospinal fluid, two-dimensional gel electrophoresis proteomics.

INTRODUCTION

Proteomics is a powerful tool in the search for potential proteins that function as biomarkers of various diseases. Identification of disease-associated proteins which are induced to change their expression compared with controls of particular diseases, and clarification of pathogenesis by two-dimensional gel electrophoresis(2-DE) and mass spectrometry have been reported[1–5]. Rheumatoid arthritis (RA) is a disease characterized by chronic polyarticular synovial inflammation and progressive destruction of cartilage and bone. A number of proteolytic enzymes (matrix metalloproteinases (MMP), cathepsins and peptidases) that degrade cartilage proteoglycans and collagen have demonstrated elevated levels in such tissues[6, 7]. Furthermore, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), various cytokines and inhibitors of enzymes also play significant roles in the pathogenesis of RA[8, 9]. These proteins, intricately associated with the knee and joint sites, turn malignant, leading to chronic inflammation and finally to the destruction of joints. Effective treatment is provided by several kinds of medication such as nonsteroidal anti-inflammatory drugs

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Abbreviations: RA, rheumatoid arthritis; CSF, cerebrospinal fluid; 2-DE, two-dimensional gel electrophoresis.
JHUPO: Japan Human Proteome Organization
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(NSAID), anti-rheumatoid drugs (DMARD) and biological reagents (anti-TNF receptor and IL-1 antagonist)[10–12]. Despite active treatment, however, numerous patients do not recover and continue to experience pain, sustain bone destruction and reach a chronic state.

Pain in RA, considered chronic and nociceptive, is stimulated in various nociceptors at peripheral sites (knee and joints), is a signal transduced through the spinal cord to the cerebrum and is conscious. A neuropeptide called spinorphin (LVVYPWT), an endogenous peptide derived from bovine spinal cord, which plays a role in anti-inflammatory and anti-nociceptive activity has been characterized[13, 14]. Furthermore to clarify the roles of spinorphin in inflammation and pain control, we focused on the changes in the activities of spinorphin and its metabolic enzymes in cerebrospinal fluids (CSF) of RA patients with chronic pain and inflammatory states. It is considered that changes in the protein composition of CSF may be reflected in alterations of the expressional pattern which is caused by the deterioration of disease in the central nervous system. The final goal of our study is to identify disease-associated proteins in the CSF of RA patients. In this study, the protein compositions in the CSFs from RA patients were analyzed by 2-dimensional gel electrophoresis (2-DE) and compared with those of controls.

MATERIALS AND METHODS

Materials

Tris(hydroxymethyl)aminomethane, tricine, iodoacetamide, thiourea, CHAPS and glycerol were purchased from Sigma-Aldrich Co. St Louise, MO, USA. Urea, 2-mercaptoethanol, dithiothreitol, methylenebisacrylamide and ampholine (pH 3.5–9.5) were purchased from Amersham Biosciences, Uppsala, Sweden. Sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylethylenediamine, glycerin, methanol and acetic acid were from Wako Chemical Ind., Ltd., Osaka, Japan. Silicone oil was from Shin-Etsu Silicone Chemical Co. Ltd., Tokyo, Japan. All other reagents were of electrophoresis grade.

Human cerebrospinal fluid

Cerebrospinal fluid (CSF) obtained from candidates for surgery under spinal anesthesia was studied. The diagnosis of RA was based on clinical criteria described in International Diagnostic Criteria[15]. The patients with RA (one man and one woman, 71 and 84 years old; mean, 77.5 years) had been treated with medication including anti-inflammatory drugs, gold, methotrexate, sulfasalazine, corticosteroids, bucillamine and D-penicillamine. Patients scheduled to undergo herniorrhaphy, ovariectomy or transurethral resection were designated as the control group (two women and three men; 37–87 years old; mean 59.8± 19.9 years); none of them had been treated with high doses of corticosteroids or intraarticular steroids. The study was approved by the Human Studies Committee, and informed consent was obtained from each patient.

Pre-treatment

Human CSF was pre-treated with an Albumin and IgG Removal kit (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) and with acetone precipitation according to the manufacturer's procedure. Briefly, 0.75 ml of the slurry included in the resin coated with specific antibodies was added to a tube containing 1 ml CSF and mixed. The mixture was rotated on a rotatory shaker for 60 min at room temperature, then centrifuged for 5 min at 6,500×g. The filtrate was collected and mixed with 4 volumes of ice-cold acetone. The proteins in the solution were allowed to precipitate at –20°C for at least 2 hrs. The solution was centrifuged at 13,000×g for 10 min; the protein pellets were then harvested, air-dried (typically 5–10 min at room temperature) and dissolved in lysis buffer (8 *M* urea, 2% ampholine (pH 3.5–9.5), 3% CHAPS, 4% glycerol and 4.5% 2-mercaptoethnol) for isoelectric focusing. Protein content was measured according to Bradford's method[16].

Isoelectric focusing (IEF)

IEF in the first dimension to separate the proteins according to their charge and strips (13 cm long; 3–10 pH non-linear range) were used. First-dimensional electrophoresis was conducted on a Multiphore II (Amersham Biosciences, Sweden) IEF system. Briefly, the lysis buffer was added to 50 μg pretreated CSF protein to a total volume of up to 250 μl. After direct rehydration of the IPG dry strip with the mixture, IEF was carried out on a stepwise program: 300 V for 6 h, 500 V for 1 h, 1000 V for 1 h, 1500 V for 1 h, 2000 V for 1 h, 2500 V for 1 h, 3000 V for 1 h, 3500 V for 36 h. After 1-D electrophoresis, the strips were stored at –80°C until 2-DE.

SDS-PAGE

The strip was first equilibrated twice for 15 min in a reducing equilibration buffer (6 *M* urea, 0.5 *M* Tris/HCl, pH 6.8, 25% glycerol, 2% SDS, a trace of bromophenol blue) containing 65 m*M* dithiothreitol and equilibrated again for 15 min with the equilibration buffer containing 135 m*M* iodoacetamide. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was then used in the second-dimensional electrophoresis at 20 mA for about 4–5 h, as described[17].

Silver staining

Because of its high sensitivity, silver staining was carried out for the detection of proteins with 2D-silver stain II-Daiichi (Daiichi Pure Chemicals Co. Ltd, Tokyo) according to the manufacturer's procedure.

RESULTS AND DISCUSSION

Effect of pretreatment

To analyze proteins associated with rheumatoid arthritis (RA), a disease characterized by chronic pain and immunological disorders, 2-DE was conducted on CSF samples. First, to obtain a high quality 2D pattern and reproducibility, CSF samples were pre-treated under several procedures. Albumins (constituting >50% of total protein content) and immunoglobulins (constituting >15% of total protein content)[18] were removed from CSF with an Albumin and IgG Removal kit, because the amounts of both major proteins varied among the samples and therefore the detection of minor components was difficult. The 2D profiles of the treated samples were then compared with those of the non-treated samples. Additional spots in 2-DE were visualized by this procedure (Fig. 1), although some spots which might be associated with albumin, were excluded. In particular, minor spots of 60–100 kD at neutral pI which were covered with albumin became clear and visible. Heavy and light chains of the IgG were also removed. The effect of another albumin removal kit (Montage Albumin Deplete kit, Millipore) for generating 2-D profiles was not as efficient as that of the above kit (the former removed only 50–60% of the albumin). We also examined the depletion of salts by acetone precipitation, TCA/acetone precipitation and a Clean-up kit (Amersham Bioscience (SF) Corp., CA, USA). The recovery of protein content was greater (about 1.5–2 fold) by acetone precipitation and the Clean-up kit than by the TCA/acetone precipitation. Effective procedures for removing salts are essential because the protein content in CSF is less than that in serum. We selected the acetone precipitation method for its higher yield and simpler application.

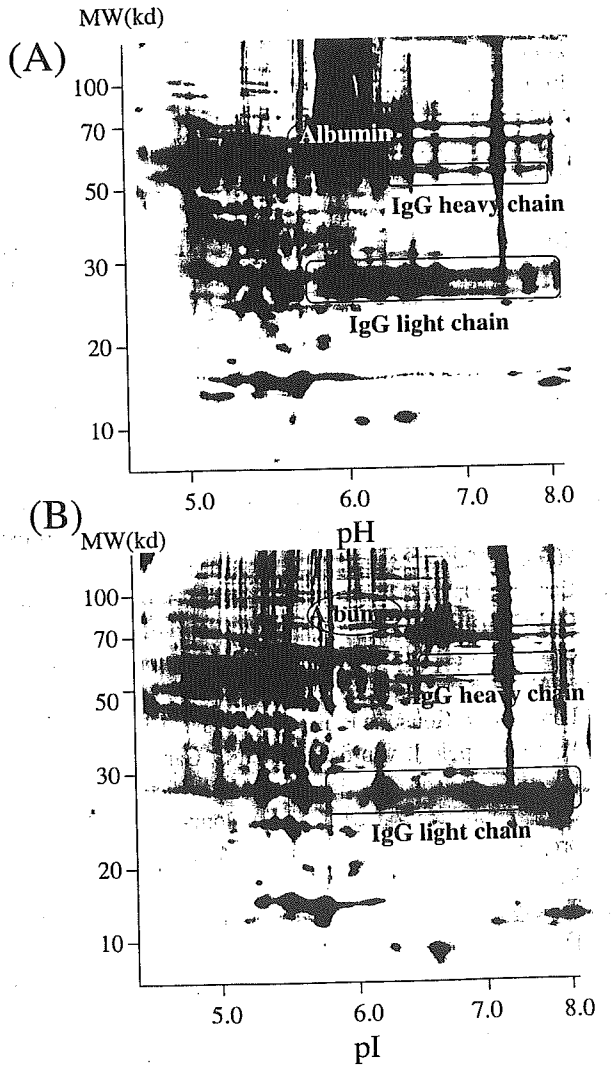

Fig. 1. Effect of pretreatment for albumin and IgG removal from cerebrospinal fluids.

2-DE profiles of (A) non-treated sample and (B) removal-kit-treated sample.

2-DE profile of CSF samples from patients with RA

To uncover disease-associated proteins in RA samples, 2D-profiles in the two RA and five controls were analyzed and compared. Serotransferrin (STF), prostaglandin-D synthase (PDS) and Transthyretin (TTR), which have specific expression in CSF and are used as reference markers[19] of CSF, were consistently detectable in both groups (Fig. 2 and Table 1). Macroscopic comparison of the 2D-profiles revealed several expression-changed proteins in the RA samples. Significantly, the expression of spots A and B decreased, whereas that of spot C increased in the RA samples compared with those in controls, except in one of five cases. The result was similar in both RA samples. Spots A and B consisted of several sequential components, which might be isoforms with several different charges modified by post-translational changes.

Table 1. Summary of characteristic proteins in CSF of patients with rheumatoid arthritis

	CSF Marker			A	B	C
	STF	TTY	PDS			
Con 1	+++	+++	+++	+	±	−
Con 2	+++	+++	+++	+++	−	−
Con 3	+++	+++	+++	++	±	++
Con 4	+++	+++	+++	+	±	−
Con 5	+++	+++	+++	+++	+++	−
RA 1	+++	+++	+++	−	−	++
RA 2	+++	+++	+++	−	−	++

The intensity of each spot was estimated by Phoretix 2D Advanced software and was classified to five-grade system as described below; − negative, ± faint, + weak, ++ moderate and +++ strong. STF is Serotransferrn, TTY is Transthyretin and PDS is Prostagrandin-D synthase.

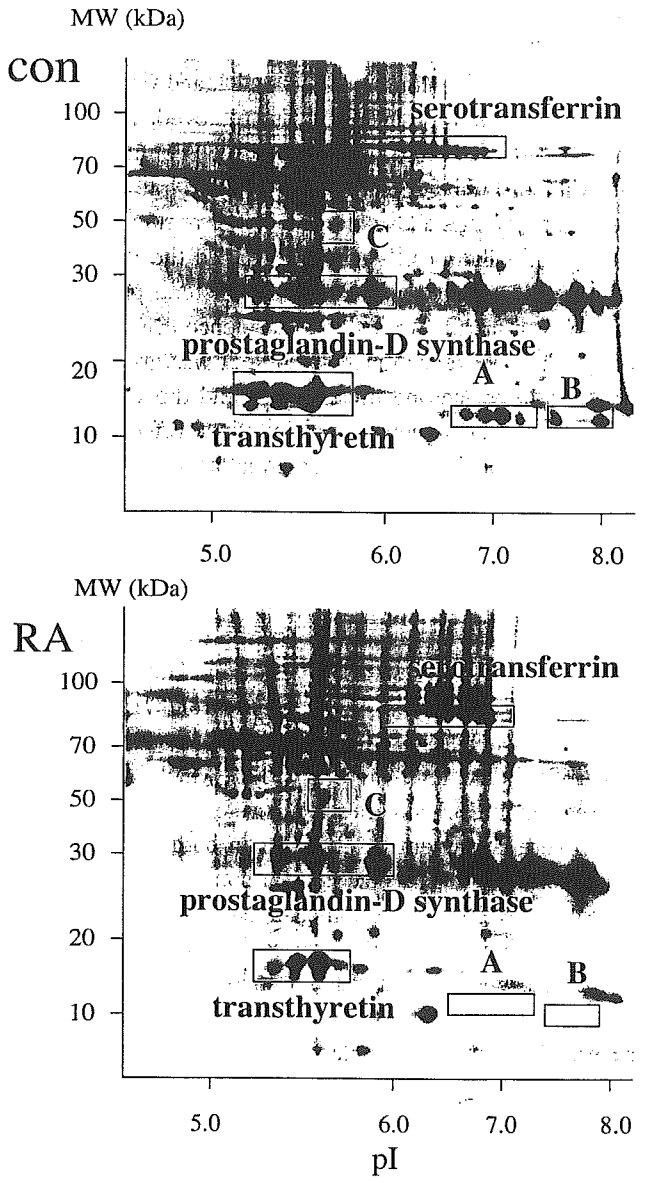

Fig. 2. 2-DE profile of proteins in cerebrospinal fluid (CSF) of patients with rheumatoid arthritis.

Serotransferrin, prostaglandin-D synthase and transthyretin are reference markers of human CSF. Rectangles represent spots of markedly expression-changed proteins. Con: CSF from control; RA: CSF from patients with RA.

It is reported that in the process of pathological and inflammatory states of RA, the levels of enzymatic activity of various proteinases and cytokine expression increase in the synovial fluid[6–9]. In this study, we have shown the changes in the several proteins in CSF of RA patients. Our results suggested that in the CSF besides in the synovial fluid, changes in the protein components which control pathophysiological change in the process of disease-deterioration. These proteins (spots A, B and C) have not been identified to date,but it would be interesting to clarify their relation to the pathology of disease. Spinorphin plays a role in the control of pain and inflammation in the body and changes have been observed in spinorphin levels and in the enzymatic activity of a spinorphin-processing enzyme, dipeptidyl peptidase III (DPPIII), in human cerebrospinal fluids[20]. When future studies clarify the relation between the pain-controlling system and the expression-changed proteins, the role of spinorphin as a new inhibitor in RA crises may be within reach.

In conclusion, our results suggest that this proteomic study is conducive to uncovering disease-associated proteins and to clarifying the mechanism of RA crises, and that the expression-changed proteins described in this study, may be new candidates in disease-associated proteins of RA. For more specific diagnostic and prognostic markers than those presently in use, additional data by comparisons between diseases and controls would be valuable.

ACKNOWLEDGEMENT

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REFERENCES

1) Zheng PP, Luider TM, Pieters R, Avezaat CJ, van den Bent MJ, Sillevis Smitt PA, Kros JM. Identification of tumor-related proteins by proteomic analysis of cerebrospinal fluid from patients with primary brain tumors. J Neuropathol Exp Neurol 2003;62:855–862.
2) Davidsson P, Sjogren M, Andreasen N, Lindbjer M, Nilsson CL, Westman-Brinkmalm A, Blennow K. Studies of the pathophysiological mechanisms in frontotemporal dementia by proteome analysis of CSF proteins. Brain Res Mol Brain Res 2002;109:128–133.
3) Guillaume E, Zimmermann C, Burkhard PR, Hochstrasser DF, Sanchez JC. A potential cerebrospinal fluid and plasmatic marker for the diagnosis of Creutzfeldt-Jakob disease. Proteomics 2003;3:1495–1499.
4) Rohiff C. Proteomics in molecular medicine: Applications in central nervous systems disorders. Electrophoresis 2000;21:1227–1234.
5) Carrette O, Demalte I, Scherl A, Yalkinoglu O, Corthals G, Burkhard P. Hochstrasser DF, Sanchez JC. A panel of cerebrospinal fluid potential biomakers for the diagnosis of Alzheimer's disease. Proteomics 2003;3:1486–1494.
6) Landewe R, Geusens P, Boers M, van der Heijde D, Lems W, te Koppele J, van der Linden S, Garnero P. Markers for type II collagen breakdown predict the effect of disease-modifying treatment on long-term radiographic progression in patients with rheumatoid arthritis. Arthritis Rheum 2004;50:1390–1399.
7) Tchetverikov I, Lard LR, DeGroot J, Verzijl N, TeKoppele JM, Breedveld FC, Huizinga TW, Hanemaaijer R. Matrix metalloproteinases-3, -8, -9 as markers of disease activity and joint damage progression in early rheumatoid arthritis. Ann Rheum Dis 2003;62:1094–1099.
8) Cross A, Edwards SW, Bucknall RC, Moots RJ. Secretion of oncostatin M by neutrophils in rheumatoid arthritis. Arthritis Rheum 2004;50:1430–1436.
9) Raap T, Justen H-P, Miller LE, Cutoro M, Scholmerich J, Straub RH. Neurotransmitter modulation of interleukin 6

(IL-6) and IL-8 (IL-8) secretion of synovial fibroblasts in patients with rheumatoid arthritis compared to osteoarthritiss. J Rhumatol 2000;27:2558–2565.
10) Klimiuk PA, Sierakowski S, Domyslawska I, Chwiecko J. Effect of repeated infliximab therapy on serum matrix metalloproteinases and tissue inhibitors of metalloproteinases in patients with rheumatoid arthritis. J Rheumatol 2004;31:238–242.
11) Dougados M, Emery P, Lemmel EM, de la Serna R, Zerbini CA, Brin S, van Riel P. Efficacy and safety of leflunomide and predisposing factors for treatment response in patients with active rheumatoid arthritis: RELIEF 6-month data. J Rheumatol 2003;30:2572–2579.
12) De A, Blotta HM, Mamoni RL, Louzada P, Bertolo MB, Foss NT, Moreira AC, Castro M. Effects of dexamethasone on lymphocyte proliferation and cytokine production in rheumatoid arthritis. J Rheumatol 2002;29:46–51.
13) Yamamoto Y, Kanazawa T, Shimamura M, Ueki M, Hazato T. Inhibitory action of spinorphin, an endogenous regulator of enkephalin degrading enzymes, on carrageenan-induced polymorphonuclear neutrophil accumulation in mouse air-pouches. Life Sciences 1998;62:1767–1773.
14) Yamamoto Y, Ono H, Ueda H, Shimamura M, Nishimura K, Hazato T. Spinorphin as an endogenous inhibitor of enkephalin-degrading enzymes: Roles in Pain and Inflammation. Current Protein and Peptide Science 2002;3:587–599.
15) Arnett FC, Edworthy SM, Bloch DA. The American Rheumatism Association 1987 received criteria for the classificqation of rheumatoid arthritis. Arthritis Rheum 1988;31:315–324.
16) Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72: 248–254.
17) Toda T. Standardization of protocol for Immobiline 2-D PAGE and construction of 2-D PAGE protein database on World Wide Web home page. Jpn J Electrophoresis 1997; 41:13–20.
18) Yuan X, Russel T, Wood G, Desiderio DM. Analysis of the human lumbar cerebrospinal fluid proteome. Electrophoresis 2002;23:1185–1196.
19) Burkhard PR, Rodrigo N, May D, Sztajzel R, Sanchez J-C, Hochstrasser DF, Schiffer E, Reverdin A, Lacroix JS. Assessing cerebrospinal fluid rhinorrhea: A two-dimensional electrophoresis approach. Electrophoresis 2001;22: 1826–1833.
20) Yamamoto Y, Sato H, Fukasaku S, Hiramatsu K, Tai S, Shimamura M, Hazato T. Levels of spinorphin in cerebrospinal fluid derived from patients with pain increase with decreasing dipeptidyl peptidase III. Pain Research in press.

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Approaches to improving the kinetics of adenovirus-delivered genes and gene products

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Abstract

Adenovirus (Ad) vectors have been expected to play a great role in gene therapy because of their extremely high transduction efficiency and wide tropism. However, due to the intrinsic deficiency of their immunogenic toxicities, Ad vectors are rapidly cleared from the host, transgene expression is transient, and readministration of the same serotype Ad vectors is problematic. As a result, Ad vectors are continually undergoing refinement to realize their potential for gene therapy application. Even after 1999, when a patient fatally succumbed to the toxicity associated with Ad vector administration at a University of Pennsylvania (U.S.) experimental clinic, enthusiasm of gene therapists for Ad vectors has not waned. With great efforts from various research groups, significant advances have been achieved through comprehensive approaches to improving the kinetics of Ad vector-delivered genes and gene products.

Keywords: Immunogenic toxicities; Biodistribution; Cationic liposome; PEGylation; Helper-dependent; Targeting; In-cis acting element; Integration; Regulatable expression

Contents

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1. Introduction

Viral vectors show great promise for gene delivery in both basic research and therapeutic applications. It is vital to select the most appropriate viral vector for each specific application, and a number of factors must be taken into consideration when making such a selection. These include the efficiency and specificity with which the vector infects the target cells, the transgene size, the level and duration of the transgene expression, the question of whether regulation of the transgene is needed, and the level of toxicity that can be tolerated. There are now more than 10 viral vector types in use, derived from common human or mammalian viral pathogens including retrovirus, adenovirus, adeno-associated virus, lentivirus, herpes simplex virus, and poxvirus. However, there is no single viral vector type meeting all the requirements, and the methods for using viral vectors to deliver genes are continually being refined.

Adenoviruses (Ad) are nonenveloped viruses containing an icosahedral protein capsid with a diameter of approximately 80 nm. There are at least 51 serotypes of human Ad identified and classified into six different subgroups (A–F), many of which are associated with respiratory, gastrointestinal, or ocular diseases. Of them, Ad serotype 5 (Ad5) along with Ad serotype 2, both belonging to subgroup C, have been the most extensively studied for use as vectors in gene therapy applications. Ad capsids consist of three major protein components: the hexon, penton base, and fiber. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices (Fig. 1). The two components of the penton capsomer, the fiber and penton base, interact with distinct cell surface receptors during the entry of Ad into susceptible cells. Fiber proteins consist of three distinct domains: tail, shaft, and knob. Each domain has distinct functions in host cell infection. The amino-terminal tail anchors the fiber

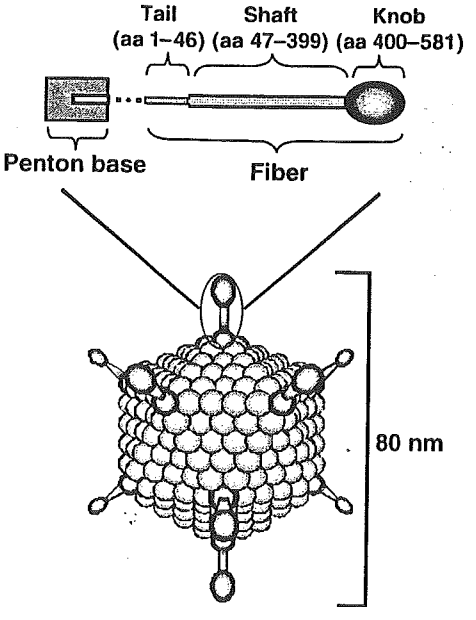

Fig. 1. Schematic diagram of a human Ad serotype 5 virion. The double stranded virus genome is packaged within an icosahedral protein capsid. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices.

to the Ad capsid through association with the penton base [1]. The shaft extends away from the virion surface and, in Ad5, is composed of 22 pseudorepeats of 15 amino acids in a triple-β-spiral conformation [2]. By extending the knob away from the virion, the shaft facilitates its interaction with host receptor [1]. The trimeric subunits of the carboxyl (C)-terminal knob domain are responsible for binding to the host's primary cellular receptor [3,4].

Human Ad5 contains a linear, approximately 36 kb, double-stranded DNA genome encoding over 70 gene products. The viral genome contains five early transcription units (E1A, E1B, E2, E3 and E4), two early delayed (intermediate) transcription units (pIX and IVa2) and five late units (L1–L5), which mostly encode structural proteins for the capsid and internal core. Inverted terminal repeats (ITR) at the end of the viral genome function as replication origins (Fig. 2). The E1A gene is the first transcription unit to be activated shortly after infection, and is essential to the activation of other promoters and the replication of the viral genome. In the first-generation Ad vectors, the E1 (E1A and E1B) gene is deleted and the virus is propagated in E1-transcomplementing cell lines, such as 293 [5], 911 [6], or PER.C6 cells [7]. The E3 region-encoded proteins modulate the host defense, but are not required for viral replication in vitro; thus, the E3 region is often deleted to enlarge the packagable size limit for foreign genes. Since up to 3.2 and 3.1 kb of the E1 and E3 regions, respectively, can be deleted [8], and approximately 105% of the wild-type genome can be packaged into the virus without affecting the viral growth rate and titer [9], E1/E3-deleted Ad vectors allow the packaging of approximately 8.1–8.2 kb of foreign genes [8].

The coxsackievirus and adenovirus receptor (CAR), a broadly distributed type I membrane protein, has been identified as the primary receptor for Ad of subgroups A, C, D, E and F [10–12]. Entry of Ad5 into cells is initiated by the attachment of fiber on the surface of the capsid to the CAR on the cell surface. The affinity of the RGD (Arg-Gly-Asp) peptide at the penton base of the Ad5 capsid to the cell surface molecules of the integrin family, such as $\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_1$, helps mediate the internalization of Ad5 into the cell [13–15]. Furthermore, heparan sulfate glycosamino-glycans have also been reported to serve as primary attachment sites for Ad2 and Ad5 [16]. The abundant expression of these receptors in various cells determines the wide tropism of Ad vectors. Internalized Ad reaches the endosomal pathway and avoids lysosomal degradation. Inside the endosome, a stepwise disassembly program takes place, allowing the Ad to release its genome into the nucleus. During this process, the pH of the endosome decreases, leading to the release of the fiber from the virion and the dissociation of the penton base [17]. The resulting endosome rupture allows viral DNA to escape from inside the degraded capsid and to enter the nucleus. This uncoating process of the Ad starts immediately after internalization and ends 40 min after infection with translocation of the Ad into the nucleus. As early as 60 min after infection, the Ad begins to transcribe its genome in the host cell [18].

Ad vectors are the most efficient class of vector in terms of delivering genes into both dividing and non-dividing cells. They have large packaging ability for foreign genes and can be easily grown to high titers and purified for clinical applications. Furthermore, Ad is nononcogenic, and Ad-related pathology is mostly limited to mild upper respiratory tract infections. All these advantageous features lead to increasing number

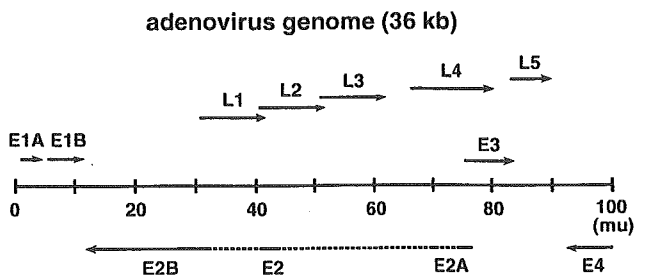

Fig. 2. Genome structure of human Ad serotype 5. The early transcription units E1 (and E3) are deleted for the first-generation Ad vectors.

of clinical protocols employing Ad vectors. As of October of 2003, the percentage of gene therapy protocols utilizing Ad vectors was at 27% (636 protocols) and the percentage of patients treated with Ad vectors at 18% (3496) (Journal of Gene Medicine Website, www.wiley.co.uk/genmed/clinical). This proportion is second only to retroviral vectors.

However, the immune response to the Ad vector-transduced cells dramatically affects the kinetics of Ad vector-delivered genes and the gene products. The potent immunogenic toxicities and consequent short-lived transgene expression of Ad vectors are undesirable properties if Ad vectors are to be more broadly applied. Though Ad vectors find niches in the treatment of degenerative diseases like vascular and coronary artery diseases in which transient transgene expression is advantageous [19,20], and for cancer, in which cellular toxicity and immunogenicity might enhance antitumor effects [21], less antigenic Ad vectors with long-term transgene expression are preferable in most cases. Here, we would like to highlight various approaches to overcoming the hurdle of Ad vector immunogenic toxicities to improve the kinetics of Ad vector-delivered genes and gene products.

2. Immunogenic toxicities of Ad vectors

The greatest obstacle for gene therapists wanting to exploit Ad vectors is the issue of their viral immunogenic toxicities. With the extensive efforts of various research groups, more and more data concerning Ad vector immunogenic toxicities have been obtained. However, it is apparent that the immunogenic toxicities associated with the use of Ad vectors are extremely complex, involving both innate and adaptive immune responses, along with apoptosis, and we are still far from a thorough understanding of all the aspects of the toxic interaction between Ad vectors and their hosts.

The complexity of Ad vector immunogenic toxicities lie in the fact that they induce multiple components of the immune response [22–24]. The cytotoxic T-lymphocyte (CTL) response can be elicited against viral gene products and/or transgene products expressed by transduced cells, resulting in a host immune attack against the transduced cells and the elimination of transgene-expressing cells. The Ad capsid itself can induce humoral virus-neutralizing antibody responses, which prevent transgene expression on subsequent administration of vectors of the same serotype, and which also provoke potent cytokine-mediated inflammatory responses during which NF κB activation might play a central role [25,26]. It is thought that among the inflammatory cytokines, TNF-α plays a dominant role in Ad vector clearance [26,27]. Wilson's group demonstrated that systemically administered Ad vectors preferentially activated dentritic cells and macrophages in the spleen to release inflammatory cytokines, independent of transgene expression [28,29]. This suggests in designing targeted Ad vectors, it should be taken into consideration to reduce transduction of or sequestration by antigen presenting cells.

Like all drug-associated toxicities, the degree to which Ad vectors induce harmful immune-mediated and inflammatory responses and other toxic side effects is dose-dependent [30–32]. Highly dangerous inflammatory responses might be inappropriately activated, especially at high doses of Ad vectors. During the tragic 1999 gene therapy trial for deficiency of ornithine transcarbamylase (OTC) at the University of Pennsylvania (U.S.), an escalated dose of Ad vector (3.8×10^{13} particles) systemically administered through the hepatic artery induced a massive systemic inflammatory response that led to fever, disseminated intravascular coagulation, multi-organ failure and the eventual death of an 18-year-old patient [33,34]. Besides the potential of a lethal outcome, immunogenic toxicities attenuate the therapeutic efficacy by affecting the kinetics of delivered genes and gene products.

3. Kinetics of Ad vector-delivered gene and gene product

It is known that different viruses are cleared from the blood stream by Kupffer cells (KC) [35]. However, blood clearance varies among different viruses [35]. Important determinants of virus clearance from the blood stream include interaction between viral components and cellular receptors, and virion size. The net charge of the viral particle also affects the clearance kinetics [36]. Interventions that slow the

clearance of Ad vectors from the blood stream might favor tissue- or tumor-specific targeting approaches based on systemic delivery [37,38]. Understanding the kinetics of blood clearance and biodistribution of Ad vectors would be beneficial to the advance of their application as therapeutic agents.

Several groups have verified that KC played a central role in clearing the Ad particles from the blood stream by blocking the KC of mouse liver [26,39,40]. The Curiel group's as well as our own, indicated rapid clearance of Ad vectors from the blood of mice, with a half-life of less than 3 min and negligible levels of the Ad vectors remaining in the blood 30 min after injection (Fig. 3A) [38,41,42]. To examine the role of the liver in the blood clearance of Ad vectors, we determined the amounts of Ad vector DNA in the mouse. In accordance with the other groups' data [40,43], we found that 98% of the intravenous dose had accumulated in the mouse liver 1 h after injection. Forty-eight hours after injection, 43% of the input Ad5 DNA persisted in the liver (Fig. 3B) [41]. These data confirm that, following intravenous administration, Ad vectors are predominantly delivered to the liver. Besides the strong interaction between viral components and cellular receptors (e.g. the fiber-CAR and RGD motif of the penton base-αv integrins), the inclination of Ad vectors to the mouse liver may also be attributed to the anatomical properties of the liver sinasoid [43,44]. The accumulation of Ad vectors in the liver may itself be toxic. We further investigated the cellular distribution of Ad vector genomes in mouse livers after intravenous injection, and found them to be equally distributed in the parenchymal cells (PC; hepatocytes) and nonparenchymal cells (NPC; Kupffer cells and endothelial cells), when 1.5×10^{10} particle of Ad vectors were intravenously injected into the mouse [41]. Despite the high uptake of Ad vectors by the NPC, the Ad vector-mediated transduction efficiencies in the NPC were much lower than those in the PC, indicating the uptake of Ad vectors by the NPC is a function of phagacytosis rather than a receptor-mediated infectious pathway [41]. This result of the high uptake of Ad vectors by the NPC combined with low transduction efficiencies is consistent with previous reports of nonlinear dose responses of Ad transduction in the liver [45,46]. Those results suggested that there was a viral dose threshold effect for efficient liver transduction of Ad vectors and that NPC played a central role in this threshold effect, such that low doses of Ad vectors were efficiently taken up by the NPC without appreciable transgene expression, while high doses saturated the NPC and were able to productively transduce the PC. Hence, depleting the Kupffer cells or blocking their uptake before Ad vector administration might be helpful in reducing the Ad vector dose for systemic route gene therapy [45,46].

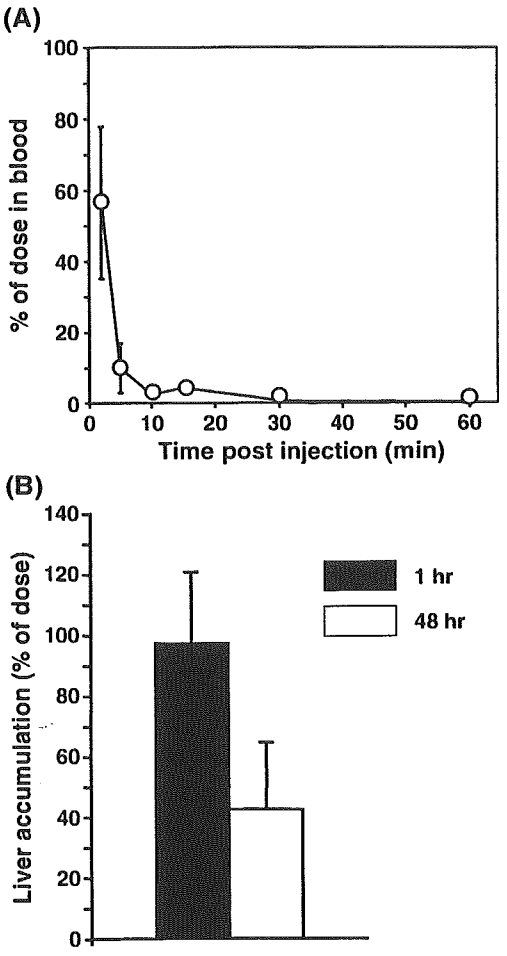

Fig. 3. Blood clearance kinetics and liver accumulation of Ad vectors after intravenous administration into mice. E1- and E3-deleted Ad vectors (1.5×10^{10} particle) were intravenously injected: (A) blood was drawn from the retro-orbital at the indicated times postinjection. (B) The livers were isolated 1 or 48 h after injection. Total DNA, including the Ad vector genome, was isolated from the blood or the livers, and slot-blot analysis was then performed. The data in this figure were published in our previous paper [41].

It is putative that transgene expression from Ad vectors is transient because of the rapid clearance of viral particles by the host immune response. However, duration of transgene expression may vary according to transgene products or animal species/strains. For example, marked variability was observed in the persistence of human alpha 1-antitrypsin (hAAT) expression delivered by an E1/E3-deleted Ad vector in different mouse strains, ranging from several weeks in the strains of C3H/HeJ and Balb/c to more than 3 months in the strains of C57Bl/6, B10.A(2R) and B10.BR [47]. This is because immunogenicity varies according to different transgene proteins, and immune responses (cellular and/or humoral) to invading virions vary according to different species or strains [48].

Overall, the kinetic features of Ad vector-delivered genes and gene products might be summarized as rapid clearance of virus from blood, liver accumulation of virus DNA and expression, and transient transgene expression. Moreover, unlike common-sense pharmaceuticals, readministration of Ad vectors is problematic due to neutralizing antibodies.

4. Approaches to improving the kinetics of Ad-delivered genes and gene products

The disadvantageous kinetics of Ad vector-delivered genes and gene products for gene therapy applications results from their immunogenic toxicities. All the potential approaches to improving the kinetics should be based on attenuating the immunologic interaction between Ad vectors and hosts, so as to extend the persistence of the virus in the blood, reduce the accumulation of the virus and transgene expression in the liver, prolong circulational or local transgene expression in the organ/tissue of interest, and make readministration possible.

4.1. Viral genome deletion

Ad vectors have been extensively engineered to reduce their immunogenicity. First-generation Ad vectors were deleted for only one or two viral early genes (E1 and E3). Cells transduced with these vectors expressed other Ad genes at low levels, inducing strong cytotoxic T-cell responses that rapidly eliminated transgene expression. Second-generation vectors that contain additional deletions in other early genes (E2 and/or E4) have shown reduced toxicity profiles compared to first-generation Ad vectors due to the decreased Ad protein synthesis in transduced cells [49–51]. However, the remaining viral gene expression still induces the T-cell response, which is difficult to overcome.

Progress has been made in reducing T-cell responses against viral gene products expressed by transduced cells, by engineering "helper-dependent" (HD) or "gutless" or "gutted" Ad vectors, from which all viral genes are deleted except the inverted terminal repeats (ITR) sequences at the two ends and the packaging signal of the Ad genome. The HD Ad vectors are produced with a helper Ad that provides in-trans the necessary viral proteins required for replication and packaging of the HD vector (Fig. 4). This advance has improved the prospects of Ad vectors for long-term gene transfer [52]. Several application experiments have shown that the HD Ad vectors have facilitated life-long phenotypic correction in mouse models with negligible toxicity. For example, in a mouse model of hyperlipidemia, a defect correction was observed for 2.5 years with a single injection of an HD Ad vector [53]. In another mouse model of hemophilia, expression of human factor VIII was sustained for longer than 9 months [54]. However, in canine models of hemophilia A or B, only transient phenotypic correction, and in some of those cases only partial correction, was observed with no detectable toxicity using an HD Ad vector [55,56], while the same group of researchers achieved long-term phenotypic correction in a mouse hemophilia B model without toxicity by a single injection of an HD Ad vector [57]. Though the discrepancy of transgene persistence by HD Ad vectors between mouse and large animal models still needs to be elucidated, it is clear that immunogenic toxicities induced by HD Ad vectors are greatly reduced.

Compared with early generation Ad vectors, yields of HD Ad vector production need to be increased, and caution should be taken to decontaminate helper Ad. Some advances have been achieved in these aspects. Sakhuja et al. [58] developed an optimized HD Ad vector production system by generating a novel producer cell line, PERC6-Cre, which was adapted to serum-free suspension culture for bioreactor mass

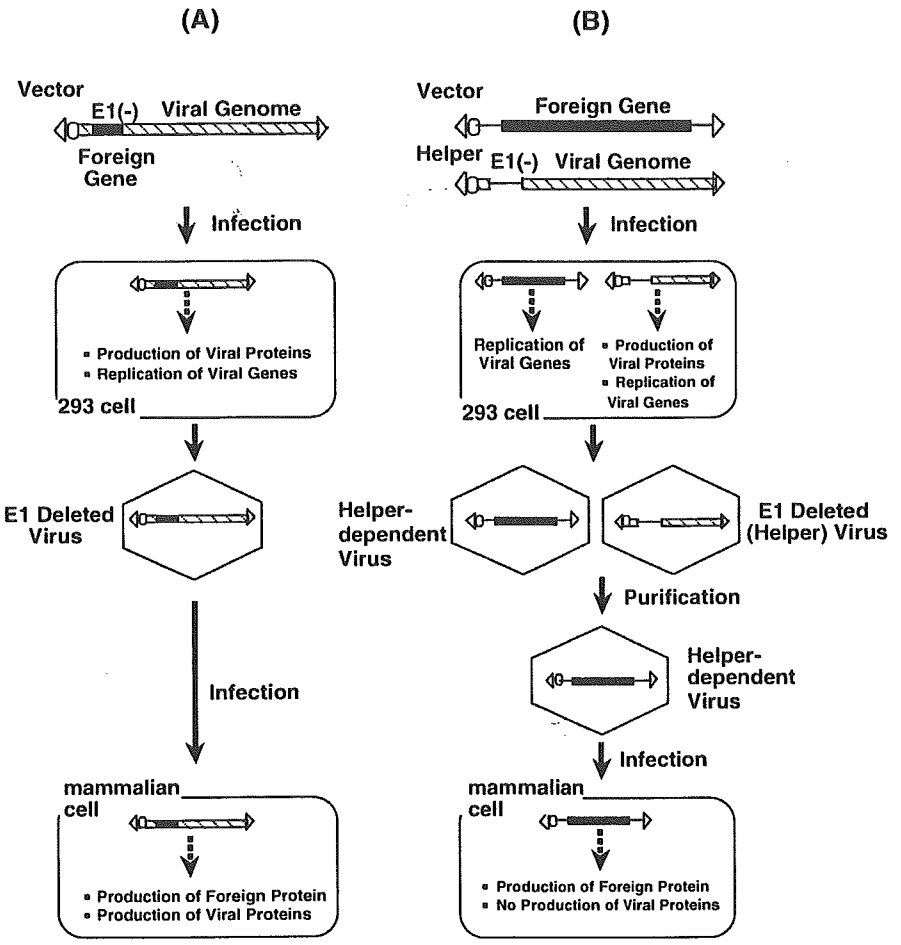

Fig. 4. Propagation diagrams of the first-generation Ad and helper-dependent Ad vectors. (A) The first-generation Ad vectors are produced in E1-transcomplementing cell lines, such as 293, 911, or PER.C6 cells. (B) Helper-dependent (HD) Ad viruses are propagated with the viral proteins provided by helper Ad viruses. To reduce the production of the helper virus, a Cre/loxP recombination system is generally utilized to excise the packaging signal from the helper virus genome [59].

production of HD Ad vectors. However, they also indicated that using the existing Cre/loxP technology to excise the packaging signal from the helper virus genome, which was originally developed by Graham and colleagues [59], could not completely eliminate the helper virus from HD Ad preparations.

With the advantages of reduced toxicity, larger packaging capacity for foreign genes of up to 36 kb, and possible persistent transgene expression, HD Ad vectors remain a powerful tool for gene therapy, though the humoral response against incoming capsid proteins shortly after administration remains a major challenge.

4.2. Modulation of the viral tropism

The broad tropism of Ad, on one hand, leads to unwanted vector uptake by many different cell types in multiple organs when the vectors are delivered systemically. Even the local delivery of Ad vectors can lead to leakage and dissemination to other tissue, resulting in toxic effects on distal sites, most notably the liver [60–62]. On the other hand, important types of target tissues are refractory to Ad infection due to CAR scarcity; these include primary tumor cells [63–66], mature skeletal muscle [67], endothelial [68,69], smooth muscle [68,69], differentiated airway epithe-

lial [70–72], lymphocytes [69,73,74], fibroblasts [68,69,75], hematopoietic cells [76] and monocyte-derived dendritic cells [77,78] and require an escalating dose of vector in order to achieve efficient gene transfer. This in turn increases vector-associated immunogenic toxicities. Hence, lack of Ad vector specificity is directly linked to the induction of massive systemic immune responses. Furthermore, localizing gene transfer by Ad vectors to specific cell types is likely to reduce immunogenic toxicities by allowing lower does to be administered. Therefore, there is a strong rationale for the development of tropism-modulated Ad vectors of enhanced specificity and gene transfer efficiency. In recent years, there have been significant efforts to improve Ad transduction efficiency to targets that are resistant to Ad infection due to CAR deficiency [79,80]. Ad vectors with the native tropism completely ablated have also been successfully developed [42,81].

4.2.1. Increasing transduction of target cells by bispecific conjugates

Douglas et al. [82] first reported the bispecific conjugate-based approach. They 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 was shown to redirect the Ad infection of target cells via the folate receptor at a high efficiency. Furthermore, when complexed with an Ad vector carrying the gene for herpes simplex virus thymidine kinase, the Fab–folate conjugate mediated the specific killing of cells that overexpress the folate receptor [82]. After that, the Fab fragment of the anti-fiber monoclonal antibody has been utilized to conjugate with several other ligands. For example, the Fab has been conjugated with (1) basic fibroblast growth factor (FGF2) to target various cells [83], including Kaposi's sarcoma cell lines [84], and ovarian cancer cells [85–87]; (2) mAb against the epidermal growth factor receptor (EGFR) to target glioma cells [64] and squamous cell carcinoma [88]; (3) an anti-CD40 mAb fragment to target dendritic cells [77]; (4) anti-angiotensin converting enzyme (ACE) mAb to target pulmonary endothelial cells [89]; (5) and an Hc fragment of tetanus toxin to target neuronal cells [90]. Theoretically, in this approach, any conjugates with one component directed against the Ad capsid and the second component directed against the cell surface protein can be applied to increase transduction of the target cells. The component directed against the Ad capsid can be the neutralizing Fab fragment of an anti-fiber monoclonal antibody as described above, a neutralizing anti-Ad knob single-chain antibody (scFV) [91–95] or the extracellular domain of CAR [96,97]. The targeting cell-binding moiety can either be natural molecules or man-made peptides identified by phage display technique [92,98,99].

4.2.2. Increasing transduction of target cells by genetic modification of the fiber

Since the fiber stretches out from the capsid and plays a central role for Ad binding to the native receptors, many attempts have focused on genetic modifications of the fiber. This approach can be divided into two main sub-approaches.

One is to incorporate ligands into the fiber knob. In order not to destroy the fiber trimerization, and to facilitate the ligands to access their cognate receptor, the HI loop and C-terminal of the Ad fiber knob have been found to be most appropriate to accommodate the foreign ligands [100,101]. We and other groups showed that Ad vectors containing the RGD motif in the HI loop greatly increased by as much as 3 orders the efficiency of gene delivery to a variety of CAR-deficient cells including primary and established ovarian cancer cells [102,103], squamous cell carcinoma [104], leukemia [105,106], rhabdomyosarcoma [107], dendritic cells [108,109], glioma [94,105], pancreatic cancer cells and primary human endothelia cells [103]. These results indicate that the integrin family could be very efficient mediators for expanding the native tropism to various CAR-deficient cells by RGD-modified Ad vectors. Currently, the RGD-modified Ad vector is being tested in a phase I clinical trial of ovarian cancer and recurrent cancer of the oral cavity and oropharynx [80]. Besides the RGD motif, there are reports of inserting the peptide SIGYPLP (Ser-Ile-Gly-Tyr-Pro-Leu-Pro), which was discovered by phage display to show high affinity to vascular endothelial cells [92], in the HI loop to increase transduction of vascular endothelial cells [110] and cancer cells [111]. In terms of the incorporation of foreign peptide into the C-terminal of fiber knob, we and another group found that a peptide containing seven lysine residues could be

inserted to increase transduction efficiency to a variety of CAR-deficient cells [69,112].

Another sub-approach is fiber-pseudotyping. Since Ads that belong to subgroup B, such as Ad11, Ad14, Ad16, Ad21, Ad35, and Ad50, recognize CD46 as the primary cellular receptors [113,114], fiber (knob, or knob and shaft) substitution could alter the tropism of Ad5 vectors. This strategy was first reported by Gall et al. [115]. They constructed a chimeric Ad5 vector by replacing the Ad5 fiber gene with the fiber gene from Ad7 (although Ad7 belongs to subgroup B, its receptor has not been identified), and found altered tropism to Ad5 vectors. Shayakhmetov et al. [76] constructed an Ad5 vector with a chimeric fiber (Ad5 tail/Ad35 shaft/Ad35 knob) and showed increased transduction of CD34+ cells relative to the unmodified Ad5 vector. Also, the same group incorporated the Ad11 fiber to Ad5 and found enhanced infection of human hematopoietic progenitor cells [116]. An Ad5-based vector containing the Ad16 fiber shaft and knob domains yielded an 8- and 64-fold increase in gene transfer to endothelial and smooth muscle cells as compared to Ad5 [117] and an 150-fold increase in gene transfer to cultured synoviocytes as compared to Ad5 [118]. Replacement of only the knob domain of the fiber can also alter viral tropism. Stevenson et al. [119] demonstrated that replacement of the Ad5 fiber knob with the Ad3 fiber knob (which, though it also belongs to subgroup B, has been shown not to use CD46 as a high-affinity attachment receptor [113,114,120]) improved gene delivery to human fibroblasts and head and neck cancer cells when compared to unmodified Ad5. Takayama et al. [121] generated a dual-knob mosaic Ad virus by incorporating both Ad5 and Ad3 knobs in the same particle, which displayed infectivity enhancement and tropism expansion by utilizing either receptor, CAR or the Ad3 receptor, for virus attachment to cells.

In addition to the approaches at the level of transduction, increasing transduction of the target cells can also be achieved to some extent at the level of transcription by using cell-specific promoters. Furthermore, combining transductional and transcriptional targeting seems to be an attractive strategy to enhance the targeting effect of Ad vectors. Reynolds et al. [122] reported that in an Ad vector, the combination of transductional targeting by linking the Fab fragment of an anti-Ad5 knob antibody to the anti-ACE (pulmonary endothelial marker) monoclonal antibody mAb, and an endothelial-specific promoter (flt-1) resulted in a synergistic, 300,000-fold improvement in the selectivity of transgene expression for the lung versus the usual site of vector sequestration, the liver. Barnett et al. [123] obtained great synergistic targeting effect in cancer cells using a similar dual targeting strategy with the target molecule being epidermal growth factor receptor (EGFR), which is overexpressed on many tumor cells; the specific promoter was the osteocalcin gene 2 promoter, which has specificity for osteoblasts and osteoblastic metastatic lesions. Nicklin et al. [111] also observed the synergistic targeting effect in certain cancer cells by combining transductional targeting (incorporating the SIGYPLP peptide into the fiber knob) with transcriptional targeting (via the FLT-1 promoter).

4.2.3. Ablation of the native tropism

Though the above tropism-modified Ad vectors could greatly improve transduction efficiency to many CAR-deficiency cells, when systemically administered, sometimes vector dissemination, resulting in liver accumulation, is still unavoidable. To create a strictly targeted Ad vector, two basic requirements are thought to be necessary: interaction of Ad with its native receptors must be completely removed and novel tissue-specific ligands must be added to the virus capsid (Fig. 5).

The capsid proteins determine the tropism of Ad. The fact that Ad5 uses multiple receptors such as CAR, αv integrin and heparan sulfate to transduce various cells implies that the Ad5 capsid must be multi-engineered to abolish its native tropism. Several groups including us have shown that vectors with the ablation of only CAR-binding, i.e., vectors in which the AB, DE, or FG loop of the fiber knob was mutated, do not change the systemic gene-transfer properties [124–127]. Vectors with the ablation of only αv-integrin-binding also show similar or slightly decreased liver transduction compared with wild-type Ad vectors [126]. Furthermore, the length [128–130] and the KKTK motif of the fiber shaft [81] have been reported to influence Ad5-mediated in vivo gene transfer. We supposed that Ad5 tropism would be determined by at least three factors: the fiber knob, the fiber shaft and the RGD motif at the penton base. Thereby, we developed a triple-mutant Ad5 vector by

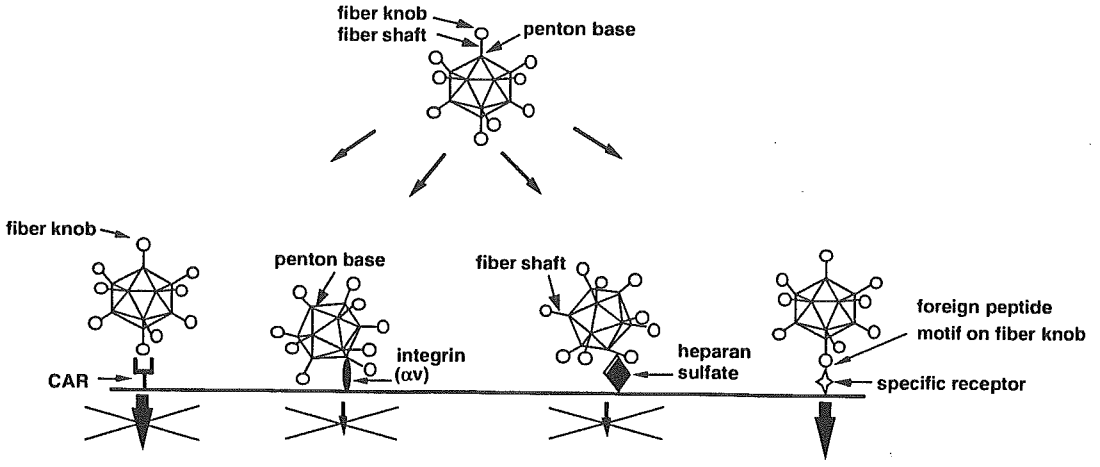

Fig. 5. Schematic diagram of targeted Ad vectors with the original tropism ablated. With the triple mutations in the fiber knob, fiber shaft and penton base, the CAR-, αv intergrin- and heparan sulfate-binding activities of the Ad viral capsid are completely ablated. Targeting effects can be achieved by either the bispecific conjugate or the foreign ligands incorporated into the fiber knob.

(1) mutating the fiber knob to ablate CAR-binding interaction, (2) replacing the Ad5 shaft with a shorter shaft from Ad35, which contains no KKTK motif to ablate binding with heparan sulfate, and (3) depleting the RGD motif at the penton base to ablate αv-integrin-binding interaction. As expected, this triple-capsid-mutant Ad5 vector exhibited little tropism to any organs (Fig. 6). Compared with the wild-type Ad5 vector, it showed 30,000-fold lower mouse liver transduction [42]. This indicates that to ablate the original tropism of Ad vectors, all three parts of the capsid (fiber knob, shaft and penton base) associated with the original tropism should be simultaneously blocked. Smith et al. [81] utilized a similar strategy and reached the same conclusion as ours.

Due to the multiple mutation of the capsid, the triple-mutant Ad vectors could not be produced by the original protocol using 293 cells. To support the propagation of our triple-mutant Ad5 vector, we generated a mutant 293 cell line stably expressing wild-type Ad5 fiber protein (Fiber-293 cells). It is possible to produce this mutant Ad vector to high titer using Fiber-293 cells. Furthermore, for the convenient display of foreign ligands, both the HI loop and C-terminal region of the fiber knob and the region of the RGD motif of the penton base were designed to have unique restriction sites. Therefore, by using our simple in vitro ligation method, the targeting ligands can easily be displayed in the capsid of our triple-mutation Ad vector [42]. We suggest that our triple-mutation Ad vector provide for a platform for future targeted Ad vector development. Future efforts should be directed into exploring novel ligands for specific tissue targeting.

Our triple-mutant Ad vector described here should be easily combined with other approaches such as transductional targeting, transcriptional targeting and even deletion of viral genomes to create less immunogenic vectors. Such combination will no

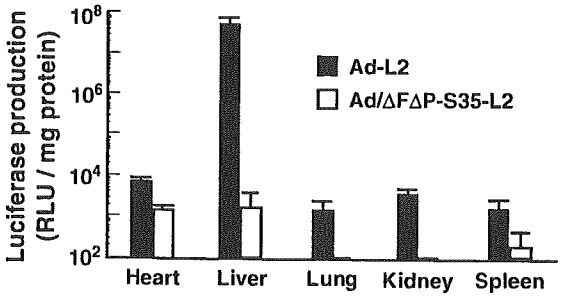

Fig. 6. Biodistribution of a transgene product (luciferase) in mice after the systemic administration of Ad-L2 (conventional Ad vectors) or Ad/ΔFΔP-S35-L2 (with triple mutations in the regions of fiber knob, fiber shaft and penton base). Ad-L2 or Ad/ΔFΔP-S35-L2 (3.0×10^{10} VP) were intravenously injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were isolated, and luciferase production was measured by luminescent assay. All data represent the mean±S.E. of five mice. The data in this figure were published in our previous paper [42].