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Suppression of Proliferation of Poliovirus and Porcine Parvovirus by Novel Phenoxazines, 2-Amino-4,4α-dihydro-4α-7-dimethyl-3*H*-phenoxazine-3-one and 3-Amino-1,4α-dihydro-4α-8-dimethyl-2*H*-phenoxazine-2-one

Akiko IWATA,[a] Teruhide YAMAGUCHI,[a] Kouei SATO,[b] Noriko YOSHITAKE,[c] and Akio TOMODA*[,d]

[a] *Division of Cellular and Gene Therapy Products, National Institute of Health, Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–0098, Japan:* [b] *The Institute of Saitama Red Cross Cente; 8–3–41 Kamiochiai, Saitama, Saitama 338–0001, Japan:* [c] *Third Department of Internal Medicine, Tokyo Medical University; and* [d] *Department of Biochemistry and Intractable Immune System Disease Research Center, Tokyo Medical University; 6–1–1 Shinjuku, Tokyo 160–0022, Japan.*
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The present study aimed at investigating the antiviral effects of 2-amino-4,4α-dihydro-4α-7-dimethyl-3*H*-phenoxazine-3-one (Phx-1) and 3-amino-1,4α-dihydro-4α-8-dimethyl-2*H*-phenoxazine-2-one (Phx-2) on 6 representative viruses: poliovirus, porcine parvovirus, simian virus 40 (SV-40), herpes simplex virus-1 (HSV-1), Sindbis virus, and vesicular stomatitis virus (VSV). Phx-1 and Phx-2 suppressed the proliferation of poliovirus in Vero cells and that of porcine parvovirus in ESK cells at concentrations between 0.25 μg/ml and 2 μg/ml, when the cells were treated with Phx-1 and Phx-2 for 1 h and then inoculated with these viruses. The proliferation of the other viruses, SV-40, HSV-1, Sindbis virus, and VSV, in the host cells was not influenced by Phx-1 or Phx-2 at concentrations less than 20 μg/ml. The results suggest that Phx-1 and Phx-2 may be useful to prevent the proliferation of poliovirus and porcine parvovirus infection and may contribute to developing new antiviral drugs in future.

Key words phenoxazine; poliovirus; porcine parvovirus

The development of antiviral drugs has been undertaken in parallel with that of vaccines, so as to overcome viral infections. Vaccination has been adopted to prevent several viral infections due to poliovirus, poxvirus, influenza virus *etc.* However, the usefulness of the antiviral drugs discovered so far seems to be restricted by the adverse effects of the drugs and the appearance of drug-resistant viruses.[1,2]

On the other hand, Tomoda *et al.* found that relatively water-soluble phenoxazines were biosynthesized by the reaction of *o*-aminophenol and its derivatives with human and bovine hemoglobin.[3—5] Among these phenoxazines, 2-amino-4,4α-dihydro-4α-7-dimethyl-3*H*-phenoxazine-3-one (Phx-1) has been demonstrated to have anticancer activity.[6,7] It was also shown that Phx-1 exerts an immunosuppressive effect on the activated lymphocytes such as B cells and T cells[8,9] and the activated mast cells.[10] Therefore, it seems of interest to investigate the effects of water-soluble phenoxazines on the proliferation of viruses in the host cells. We briefly reported that the proliferation of poliovirus inoculated to Vero cells was inhibited by Phx-1.[11] This discovery prompted us to investigate the effects of water-soluble phenoxazines on various kinds of viruses, because there is a possibility that a new antiviral drug may be developed through such an investigation. The present manuscript deals with studies on the antiviral effects of Phx-1 and 2-amino-4,4α-dihydro-4α-7-dimethyl-3*H*-phenoxazine-3-one (Phx-2) on 6 representative viruses: poliovirus, porcine parvovirus, simian virus (SV-40), herpes simplex virus-1 (HSV-1), Sindbis virus, and vesicular stomatitis virus (VSV).

MATERIALS AND METHODS

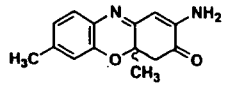
Phx-1, Phx-2, Cells and Viruses Phx-1 and Phx-2 were prepared by reaction of bovine hemoglobin with 2-amino-5-methylphenol and 2-amino-4-methylphenol, respectively, as previously described.[4,5] The chemical structures of Phx-1 and Phx-2 are shown in Fig. 1. Phx-1 or Phx-2 was dissolved in dimethyl sulfoxide (DMSO) before use to reach a concentration of 20 mM, and then was diluted with α-minimum essential medium (α-MEM).

African green monkey kidney cells (Vero cells), were generously supplied by the Japanese Cancer Research Resources Bank (Tokyo, Japan). The ESK cells (embryonic swine kidney cells line)[12] were kindly donated by Dr. J. Koga (JCR Co., Japan).

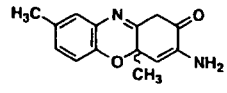
Cells were maintained in α-MEM supplemented with 10% fetal calf serum (FCS, Sigma Co., Ltd., St. Louis, MO, U.S.A.), and 30 mg/l kanamycin sulfate RPMI 1640 medium containing 10% heat-inactivated FCS, at 37 °C under moisturized air containing 5% CO_2.

Poliovirus (strain Sabin 1) was also donated by Dr. Koga. Porcine parvovirus (strain 90HS), SV-40, Sindbis virus, and HSV-1 (strain F) were the generous donation of Dr. M. Kohase (National Institute of Infectious Diseases). VSV (strain NJ) was the gift of Dr. H. Kita (Suntory Center Institute, Japan).

The supernatants of Vero cells infected with poliovirus, HSV-1, Sindbis virus, and VSV were used as the virus samples. The supernatant of ESK cells infected with porcine parvovirus was used as the porcine parvovirus sample. CV-1 cells were infected with SV-40 virus, and then 5 d after infec-



Phx-1



Phx-2

Fig. 1. Chemical Structures of Phx-1 and Phx-2

* To whom correspondence should be addressed. e-mail: tomoda@tokyo-med.ac.jp

Table 1. Effects of Phx-1 or Phx-2 on the Proliferation of Poliovirus Inoculated to Vero Cells at Different Concentrations of Phx-1 and Phx-2, Estimated by $TCID_{50}$[a]

	Phx-1 (μg/ml)					Phx-2 (μg/ml)				
	0	0.25	0.55	1	2	0	0.25	0.5	1	2
$TCID_{50}$	62	32	32	16	17	63	63	63	63	14

a) $TCID_{50}$ was defined as dilution ratio of the virus to generate 50% disruption of the cells.

tion, the supernatant was saved as the SV-40 sample. To remove the cell debris from the collected virus suspension, each suspension was centrifuged at 450×*g* for 10 min. After removing the debris, the resulting stock viruses were aliquoted and stored at −80 °C until use.

Determination of Viral Infectivity The infectious titer of virus suspension was determined using indicator cells. Poliovirus and porcine parvovirus were introduced into Vero cells and ESK cells, respectively.[13] The cells were seeded in a 96-well microplate (Asahi Technoglass Co., Ltd., Tokyo) at a density of 2×10^5 to 3×10^5 cells per well in culture medium. They were then cultured at 37 °C, for 2 d. Various concentrations of Phx-1 or Phx-2 solution [final concentration: 0 μg/ml (DMSO alone), 0.25, 0.5, 1 and 2 μg/ml] were then added to the cells in each well, and these were subsequently incubated for 1 h. After 1 h, the supernatant was removed from the well by an aspirator. At this time, poliovirus or porcine parvovirus which had been serially diluted with α-MEM to obtain a 50% tissue culture infectious dose ($TCID_{50}$, defined as dilution ratio of the virus to generate 50% disruption of the cells), as performed conventionally,[2] was added to Vero cells or ESK cells, respectively, in each well. Cell cultures were incubated for 1 h at 37 °C. Post infection $TCID_{50}$ cultures were then fed with α-MEM containing various concentrations of Phx-1 or Phx-2 [final concentration: 0 (DMSO alone), 0.25, 0.5, 1 and 2 μg/ml] and were incubated at 37 °C for 2 or 3 d.

After that period, the disruption of Vero cells or ESK cells was examined by the method described by Satoh *et al.*,[14] and the infectivity of poliovirus or porcine parvovirus to these cells was estimated. The estimation of the viruses SV-40, HSV-1, Sindbis virus and VSV was essentially in agreement with the method described by Satoh *et al.*[14]

Effects of Phx-1 and Phx-2 on Cell Viability We examined the effects of Phx-1 and Phx-2 on the viability of Vero cells, ESK cells, and CV-1 cells in the presence of various concentrations of these phenoxazines and without addition of viruses. There was no significant disruption of these cells at various concentrations of Phx-1 and Phx-2 up to 50 μg/ml, indicating that these phenoxazines do not affect the viability of the cells at the concentrations of used to examine the viruses.

RESULTS AND DISCUSSION

We initially studied the effects of Phx-1 and Phx-2 on the proliferation of poliovirus, a non-enveloped and single strand RNA virus, inoculated to Vero cells. Since $TCID_{50}$ is defined as the dilution ratio of the virus to generate 50% disruption of the cells, a lower value of $TCID_{50}$ means that the viral proliferation is being suppressed in the host cells. We found that

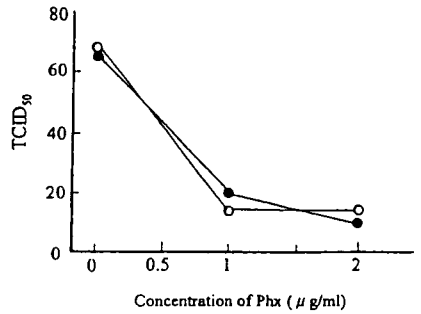


Fig. 2. Antiviral Effects of Phx-1 and Phx-2 on Porcine Parvovirus

The infectious titer of porcine parvovirus suspension was determined using ESK cells, as described in Materials and Methods. Antiviral effects of Phx-1 (●) or Phx-2 (○) were expressed by $TCID_{50}$ (defined as dilution ratio of the virus to generate 50% disruption of the cells) at different concentrations of these phenoxazines (μg/ml of Phx-1 or Phx-2).

$TCID_{50}$ of poliovirus was decreased according to the increase in the concentrations of Phx-1 or Phx-2 (Table 1). Namely, Phx-1 suppressed the proliferation of poliovirus inoculated to Vero cells at all concentrations tested between 0.25 μg/mg and 2 μg/ml, and reached maximal antiviral activity at 1 μg/ml. Phx-2 also inhibited the proliferation of poliovirus inoculated to Vero cells at 2 μg/ml (Table 1). Such inhibition was observed when the cells were treated with Phx-1 or Phx-2 for 1 h and then inoculated with poliovirus. On the contrary, the proliferation of poliovirus was not suppressed by Phx-1 or Phx-2 when Vero cells were inoculated with the virus together with various concentrations of Phx-1 or Phx-2 (data not shown). These results may be explained by the facts that these phenoxazines do not exert virucidal activity against poliovirus directly, but some mechanisms preventing the attachment or the intracellular proliferation of poliovirus in the host cells, are apparently revoked during 1 h incubation of the host cells with Phx-1 or Phx-2. The detailed biochemical changes in the host cells are not yet clear.

Figure 2 shows the inhibitory effects of Phx-1 and Phx-2 against porcine parvovirus, a non-enveloped and single strand DNA virus, as determined by the changes in $TCID_{50}$. These phenoxazines showed antiviral effects on porcine parvovirus at 1 μg/ml and 2 μg/ml. Such inhibition was observed when the cells were treated with Phx-1 or Phx-2 for 1 h and then inoculated with porcine parvovirus. However, Phx-1 or Phx-2 did not exert antiviral effects on porcine parvovirus when ESK cells were inoculated with the virus together with Phx-1 or Phx-2 (data not shown).

We studied the effects of Phx-1 and Phx-2 on various representative viruses such as SV-40 (non-enveloped, double strand DNA), HSV-1 (enveloped, double strand DNA), Sindbis virus (enveloped, single and plus strand RNA), and VSV (enveloped, single strand RNA). Table 2 summarizes the

Table 2. Antiviral Activity of Phx-1 and Phx-2 on Various Species of Viruses

Type of virus	Name of virus	Antiviral activity[a]	
		Phx-1	Phx-2
sDNA, envelope (−)	Porcine parvovirus	+	+
dDNA, envelope (−)	Simian virus 40	−	−
dDNA, envelope (+)	Herpes simplex virus-1	−	−
sRNA, envelope (−) (plus strand)	Poliovirus	+	+
sRNA, envelope (+) (plus strand)	Sindbis virus	−	−
sRNA, envelope (+) (minus strand)	Vesicular stomatitis virus	−	−

a) $TCID_{50}$ was estimated as described in Materials and Methods. Then, the antiviral activity was expressed by + or −, where + shows "effective" at the concentration of Phx-1 or Phx-2 between 0.25 and 20 μg/ml, and − shows "not effective" at these concentrations.

inhibitory effects of Phx-1 and Phx-2 on these viruses, in comparison with poliovirus and porcine parvovirus. Although Phx-1 and Phx-2 showed antiviral activity against poliovirus and porcine parvovirus, these phenoxazines did not inhibit the proliferation of SV-40, HSV-1, Sindbis virus or VSV in the host cells. Therefore, it may be conceivable that non-enveloped and single strand RNA virus (coxackie virus, ECHO virus, hepatitis virus A, encephalomyocarditis virus *etc.*) or non-enveloped and single strand DNA virus (B19 virus, adeno-associated virus 2 *etc.*) may be inhibited by Phx-1 and Phx-2 as well. Increased amounts of interferon is not possible, because the inhibition of proliferation of viruses was restricted only to poliovirus and porcine parvovirus (Table 2). These views should be assessed by further examinations.

Tang *et al.*[15] reported that hypericin, a derivative of emodin exerts antiviral activity against enveloped viruses such as HSV-1, influenza virus A and Mo-Mul V, but not against the non-enveloped viruses poliovirus and adenovirus, at concentrations of 1.56 to 25 μg/ml. On the other hand, our results showed that Phx-1 and Phx-2 exerted antiviral activity only against poliovirus and porcine parvovirus (Table 1, Fig. 2). Concerning the chemical structure of emodin (1,3,8-trihydroxy-6-methylanthraquinone) and Phx-1, emodin is analogous to Phx-1, because emodin and Phx-1 (Fig. 1) are tricyclic chromophores with the methyl group at the same position, however, the former is a semiquinone type producing active oxygens,[16] while the latter is a non-semiquinone type. Such similarity and differences in the chemical structure of these compounds may be reflected to the differences in biological actions between hypericin and Phx-1 or Phx-2.

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RNA interference of PPARγ using fiber-modified adenovirus vector efficiently suppresses preadipocyte-to-adipocyte differentiation in 3T3-L1 cells

Tetsuji Hosono[a], Hiroyuki Mizuguchi[b,*], Kazufumi Katayama[c], Naoya Koizumi[b,d], Kenji Kawabata[b], Teruhide Yamaguchi[a], Shinsaku Nakagawa[c], Yoshiteru Watanabe[d], Tadanori Mayumi[e], Takao Hayakawa[f]

[a]*Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, Tokyo 158-8501, Japan*
[b]*Project III, National Institute of Health Sciences, Osaka Branch, Fundamental Research Laboratories for Development of Medicine, Asagi 7-6-8, Saito, Ibaraki, Osaka 567-0085, Japan*
[c]*Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan*
[d]*Department of Pharmaceutics and Biopharmaceutics, Showa Pharmaceutical University, Tokyo 194-8543, Japan*
[e]*Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Hyogo 651-2180, Japan*
[f]*National Institute of Health Sciences, Tokyo 158-8501, Japan*

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Abstract

The peroxisome proliferator-activated receptor (PPAR) γ is regarded as a "master regulator" of adipocyte differentiation and is abundantly expressed in adipose. To understand the biological role of PPARγ in adipose, RNA interference (RNAi) of PPARγ should be a powerful tool. 3T3-L1 cell line serves an excellent model to investigate the mechanism of preadipocyte-to-adipocyte differentiation. However, this cell line is difficult to transfect by plasmid vectors and viral vectors. We optimized the transduction of both 3T3-L1 preadipocytes and adipocytes by means of fiber-modified adenovirus (Ad) vectors. Among the various vectors tested, polylysine modification of the C-terminal of the fiber knob most markedly improved the transduction efficiency in both 3T3-L1 preadipocytes and adipocytes. Then, we examined whether fiber-modified Ad vectors with polylysine peptides expressing the small interfering RNA (siRNA) for PPARγ inhibit the differentiation of 3T3-L1 preadipocytes into adipocytes. Oil red O staining and measurement of glycerol-3-phosphate dehydrogenase (GPDH) activity indicated that the vectors effectively suppressed the differentiation of 3T3-L1 preadipocytes to adipocytes. These results suggested that the combination of fiber-modified Ad vectors containing polylysine peptides and RNAi is an effective tool for the study of the biological and physiological mechanism of adipogenesis in adiposity and diabetes using 3T3-L1 models. Ad vector-mediated RNAi for PPARγ should also be useful to clarify the biological role of the PPARγ pathway in various tissues in addition to adipose and for therapeutic application to a variety of diseases, including adiposity and diabetes.

Keywords: Adenovirus vector; PPARγ; RNA interference; Adipocyte

1. Introduction

An understanding of the biological and physiological mechanism of adipogenesis is essential for an improved understanding of adiposity and diabetes. The expression of many transcription factors and adipocyte-specific genes, including CCAAT enhancer binding proteins (C/EBP) and

Abbreviations: CAR, coxsackievirus and adenovirus receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPDH, glycerol-3-phosphate dehydrogenase; LacZ, β-galactosidase; PPARγ, peroxisome proliferator-activated receptor γ; RT-PCR, reverse transcription–polymerase chain reaction; shRNA, short hairpin RNA; siRNA, small interfering RNA.

* Corresponding author. Tel.: +81 72 641 9815; fax: +81 72 641 9816.
E-mail address: mizuguch@nihs.go.jp (H. Mizuguchi).

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adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1 (ADD-1/SREBP-1), is programmatically regulated in the process of adipogenesis (Gregoire et al., 1998; Ntambi and Young-Cheul, 2000). Among them, the peroxisome proliferator-activated receptor (PPAR) γ is regarded as a "master regulator" of adipocyte differentiation and is abundantly expressed in adipose (Tontonoz et al., 1994a,b; Vidal-Puig et al., 1997; Wu et al., 1998; Kubota et al., 1999; Berger and Moller, 2002). PPARγ is a member of the nuclear receptor superfamily and induces transcriptional activation by heterodimerization with the retinoic acid-like receptor (RXR) (Tontonoz et al., 1994a; Berger and Moller, 2002).

Adipocytes are thought to be derived from mesenchymal stem cells, and cell culture models using preadipocyte 3T3-L1 cell lines are extensively used to study preadipocyte-to-adipocyte differentiation (Ntambi and Young-Cheul, 2000). Treatment of 3T3-L1 adipocytes with agonists for PPARγ including thiazolidinediones (TZD), potent insulin sensitizing agents, induces preadipocyte-to-adipocyte differentiation. To understand the factors and mechanisms involved in the process of adipogenesis, studies of loss of function via knock-out/knock-down of target gene expression or gain of function via overexpression are among the most powerful methods. 3T3-L1 cells, however, are not efficiently transfectable. Viral vector-mediated transduction might improve their efficiency.

Among the viral vectors, adenovirus (Ad) vectors have been extensively used to deliver foreign genes to a variety of cell types and tissues both in vitro and in vivo. They can be easily grown to high titer and can efficiently transfer genes into both dividing and non-dividing cells. The efficiency of Ad vector-mediated transduction into 3T3-L1 cells, however, is quite low due to the scarcity of the primary receptor, the coxsackievirus and adenovirus receptor (CAR) (Orlicky et al., 2001). To overcome CAR-dependent transduction, fiber-modified Ad vectors have been developed (Krasnykh et al., 1996; Wickham et al., 1997; Dmitriev et al., 1998; Shayakhmetov et al., 2000; Havenga et al., 2001; Mizuguchi et al., 2001; Mizuguchi and Hayakawa, 2002; Koizumi et al., 2003), containing RGD peptides in the HI loop of the fiber knob, polylysine peptides in the C-terminal end of the fiber knob, or fiber proteins derived from subgroup B Ads such as Ad type 3, -11, or -35. These vectors are infected via αv integrin, heparan sulfates, or CD46 (or CD80 or CD86) on the cellular surface, respectively (Wickham et al., 1997; Dmitriev et al., 1998; Mizuguchi et al., 2001; Gaggar et al., 2003; Segerman et al., 2003; Short et al., 2004).

For studies of loss of function via knockdown of target gene expression, RNA interference (RNAi) has been shown to have great promise for both basic research and therapeutic use. 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), a 19- to 29-nt RNA, leads to the inhibition of target gene expression in a sequence-specific manner (Elbashir et al., 2001). Vector-based siRNA systems, including Ad vectors, have also been developed using RNA polymerase III promoters, such as the U6 promoter or the H1 promoter, to express siRNA (Brummelkamp et al., 2002; Miyagishi and Taira, 2002; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002; Shen et al., 2003; Zhao et al., 2003; Hosono et al., 2004).

In the present study, we established an optimal fiber-modified Ad vector for transduction of 3T3-L1 preadipocytes and adipocytes and demonstrated that the fiber-modified Ad vector expressing short hairpin RNA (shRNA) against PPARγ efficiently suppressed the differentiation of preadipocytes into adipocytes in 3T3-L1 cells.

2. Materials and methods

2.1. Cells

293 cells and 3T3-L1 cells (clonal subline of the mouse 3T3 that accumulate large amounts of triglyceride fat when the cells are in the resting state; Human Science Research Resources Bank, Japan, JCRB9014) were cultured with Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (FCS). NIH3T3 cells were cultured with minimum essential medium supplemented with 10% FCS.

2.2. Construction of stable CAR-expressing NIH3T3 cells

Mouse liver total RNA was isolated using ISOGEN reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Mouse liver cDNA was obtained from reverse transcription (RT) product for mouse liver total RNA using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Mouse CAR cDNA was amplified from mouse liver cDNA using the following primers: 5′-accatggcgcgcc-tactgtgct-3′ and 5′-ttcagtagtccttcatatttat -3′. The amplified polymerase chain reaction (PCR) product was inserted into the TA cloning vector pGEM-T Easy (Promega Corp., Madison, WI, USA) to generate pGEM-T-mCAR. CAR-expressing plasmid pCMV-mCAR was constructed by ligation of the *Eco*RI site of pcDNA3 (Invitrogen Life Technologies) with the *Eco*RI fragment of pGEM-T-mCAR, and was transfected into NIH3T3 cells by SuperFect transfection reagent (Qiagen, Inc., Valencia, CA, USA). The stable CAR-expressing cells, NIH3T3-CAR, were obtained by geneticin (G418) selection.

2.3. Plasmid and virus

pHMCA-LacZ1 contains a β-actin promoter/CMV enhancer with a β-actin intron, which was kindly provided by Dr. J. Miyazaki (Osaka University, Osaka, Japan) (Niwa et al., 1991), an *Escherichia coli* β-galactosidase (LacZ)

gene derived from pCMVβ (Clontech, Palo Alto, CA, USA), and a bovine growth hormone polyadenylation signal, flanked by *I-Ceu*I and *PI-Sce*I sites.

pHM5-H1-PPARγ was constructed by insertion of the oligonucleotides (5′-gatccccgtctgctgatctgcgagccttcaagagaggctcgcagatcagcagacttttttggaaat-3′ and 5′-ctagatttccaaaaagtctgctgatctgcgagcctctcttgaaggctcgcagatcagcagacgggg-3′) (Katayama et al., 2004) (loop sequences were underlined) into the *Bgl*II and *Xba*I sites of pHM5-H1 (Hosono et al., 2004), which is designed to express shRNA upon the insertion of an appropriate sequence into the *Bgl*II/*Xba*I site. pHM5-H1-Scramble was constructed by insertion of the oligonucleotides (5′-gatccccacgctgagtacttcgaaatttcaagagaatttcgaagtactcagcgttttttggaaat-3′ and 5′-ctagatttccaaaaaacgctgagtacttcgaaatttctcttgaaatttcgaagtactcagcgggg-3′) (loop sequences were underlined) into the *Bgl*II and *Xba*I sites of pHM5-H1. The sequence was verified on a DNA sequencer (ABI PRISM 310, Applied Biosystems, Foster City, CA, USA).

Ad vectors expressing LacZ or siRNA were constructed by an improved in vitro ligation method (Mizuguchi and Kay, 1998; Mizuguchi and Kay, 1999). Briefly, pHMCA-LacZ1 was digested with *I-Ceu*I and *PI-Sce*I, and then ligated with *I-Ceu*I/*PI-Sce*I-digested pAdHM4 (Mizuguchi and Kay, 1998), pAdHM15-RGD (Mizuguchi et al., 2001), pAdHM41-K7(C) (Koizumi et al., 2003), or pAdHM34 (Mizuguchi and Hayakawa, 2002), resulting in pAdHM4-CALacZ1, pAdHM15-RGD-CALacZ1, pAdHM41-K7-CALacZ1, or pAdHM34-CALacZ1, respectively. pAdHM41-K7-H1, pAdHM41-K7-H1-PPARγ, and pAdHM41-K7-Scramble were constructed by the ligation of *I-Ceu*I/*PI-Sce*I-digested pHM5-H1, pHM5-H1-PPARγ, or pHM5-H1-Scramble, respectively, with *I-Ceu*I/*PI-Sce*I-digested pAdHM41-K7(C).

To generate the virus (Ad-CALacZ, AdRGD-CALacZ, AdF35-CALacZ, AdK7-CALacZ, AdK7-H1, AdK7-H1-PPARγ, AdK7-H1-Scramble), *Pac*I-digested Ad vector plasmids (pAdHM4-CALacZ1, pAdHM15-RGD-CALacZ1, pAdHM34-CALacZ1, pAdHM41-K7-CALacZ1, pAdHM41-K7-H1, pAdHM41-K7-H1-PPARγ, and pAdHM41-K7-Scramble, respectively) were transfected into 293 cells plated in a 60-mm dish with SuperFect (Qiagen, Inc.) according to the manufacturer's instructions. Viruses were prepared as described previously (Mizuguchi and Kay, 1998). AdK7-Null contains no transgene in the E1 deletion region. The virus was purified by $CsCl_2$ gradient centrifugation, dialyzed with the solution containing 10 mM Tris (pH 7.5), 1 mM $MgCl_2$ and 10% glycerol, and stored in aliquots at −70 °C. The determination of virus particle titer was accomplished spectrophotometrically by the method of Maizel et al. (1968).

2.4. X-gal staining

In the case of 3T3-L1 preadipocytes, 3T3-L1 cells (1×10^5 cells) were seeded into a 12-well plates. On the following day, they were transduced with Ad-CALacZ, AdRGD-CALacZ, AdF35-CALacZ, or AdK7-CALacZ (3000 or 10,000 vector particles (VP)/cell) for 1.5 h. Forty-eight hours later, LacZ production in the cells was determined by X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining.

In the case of 3T3-L1 adipocytes, 3T3-L1 cells were differentiated as described in the next section. After 6 days in culture with differentiation medium, the cells were transduced with each Ad vector (3000 or 10,000 vector particles (VP)/cell) for 1.5 h. Forty-eight hours later, LacZ production in the cells was determined by X-gal staining.

2.5. Adipocyte differentiation

Induction of adipocyte differentiation was performed as previously described (Tontonoz et al., 1994a). Two days after confluence (day 0), the medium was replaced with differentiation medium containing pioglitazone (CALBIOCHEM, San Diego, CA, USA) (3 μM), insulin (Sigma, Saint Louis, MO, USA) (150 nM), dexamethasone (Sigma) (1 μM) and 3-isobutyl-1-methylxanthine (Sigma) (100 μM), which was changed every 3 days thereafter until analysis.

Differentiation of 3T3-L1 preadipocytes to adipocytes was monitored by measurement of intracellular lipid accumulation using Oil red O staining and glycerol-3-phosphate dehydrogenase (GPDH) activity on day 9. The cells were fixed for 2 h with 10% formaldehyde in isotonic phosphate buffer and then washed with distilled water. The cells were then stained with complete immersion in a

Table 1
Adenovirus vectors used in this study

Name	Fiber type	Gene of interest
Ad-CALacZ	type 5 fiber	CA promoter+LacZ
AdRGD-CALacZ	RGD peptide in the HI-loop of the fiber knob	CA promoter+LacZ
AdK7-CALacZ	polylysine peptide in the C-terminal of the fiber knob	CA promoter+LacZ
AdF35-CALacZ	chimeric type 5 fiber tail and type 35 fiber knob and shaft	CA promoter+LacZ
AdK7-H1	polylysine peptide in the C-terminal of the fiber knob	H1 promoter
AdK7-H1-PPARγ	polylysine peptide in the C-terminal of the fiber knob	H1 promoter+shRNA for PPARγ
AdK7-H1-Scramble	polylysine peptide in the C-terminal of the fiber knob	H1 promoter+shRNA for Scramble
AdK7-Null	polylysine peptide in the C-terminal of the fiber knob	none

CA promoter: β-actin promoter/CMV enhancer with β-actin intron.

working solution (0.3%) of Oil red O for 4 h. Excess dye was removed by exhaustive washing with water. The GPDH activity was measured using a GPDH assay kit (Hokudo, Hokkaido, Japan).

2.6. Reverse transcription–polymerase chain reaction for CAR mRNAs

Total RNA was isolated using ISOGEN reagent according to the manufacturer's instructions. RT was carried out using a SuperScript First-Strand Synthesis System for reverse transcription–polymerase chain reaction (RT-PCR) according to the manufacturer's instructions. PCR amplification of the mouse CAR and GAPDH was performed in 50 μl of a solution containing 1 μl of RT products, 1 U TaKaRa Ex Taq HS and attached reagents (TaKaRa, Shiga, Japan). The sequences of the primer for PCR are as follows: CAR: forward, 5′-aattcctgctgaccgttctt-3′; reverse, 5′-tttctgccagccatggcgta-3′; GAPDH: forward, 5′-accacagtccatgccatcac-3′; reverse, 5′-tccaccaccctgttgctgta-3′. The following parameters were used: CAR: 20 s at 94 °C, 10 s at 60 °C, and 60 s at 72 °C for 35 cycles; GAPDH: 20 s at 94 °C, 10 s at 60 °C, and 60 s at 72 °C for 25 cycles. The PCR products were electrophoresed in 2.0% agarose gel. The sequence of the PCR products was confirmed by direct sequencing.

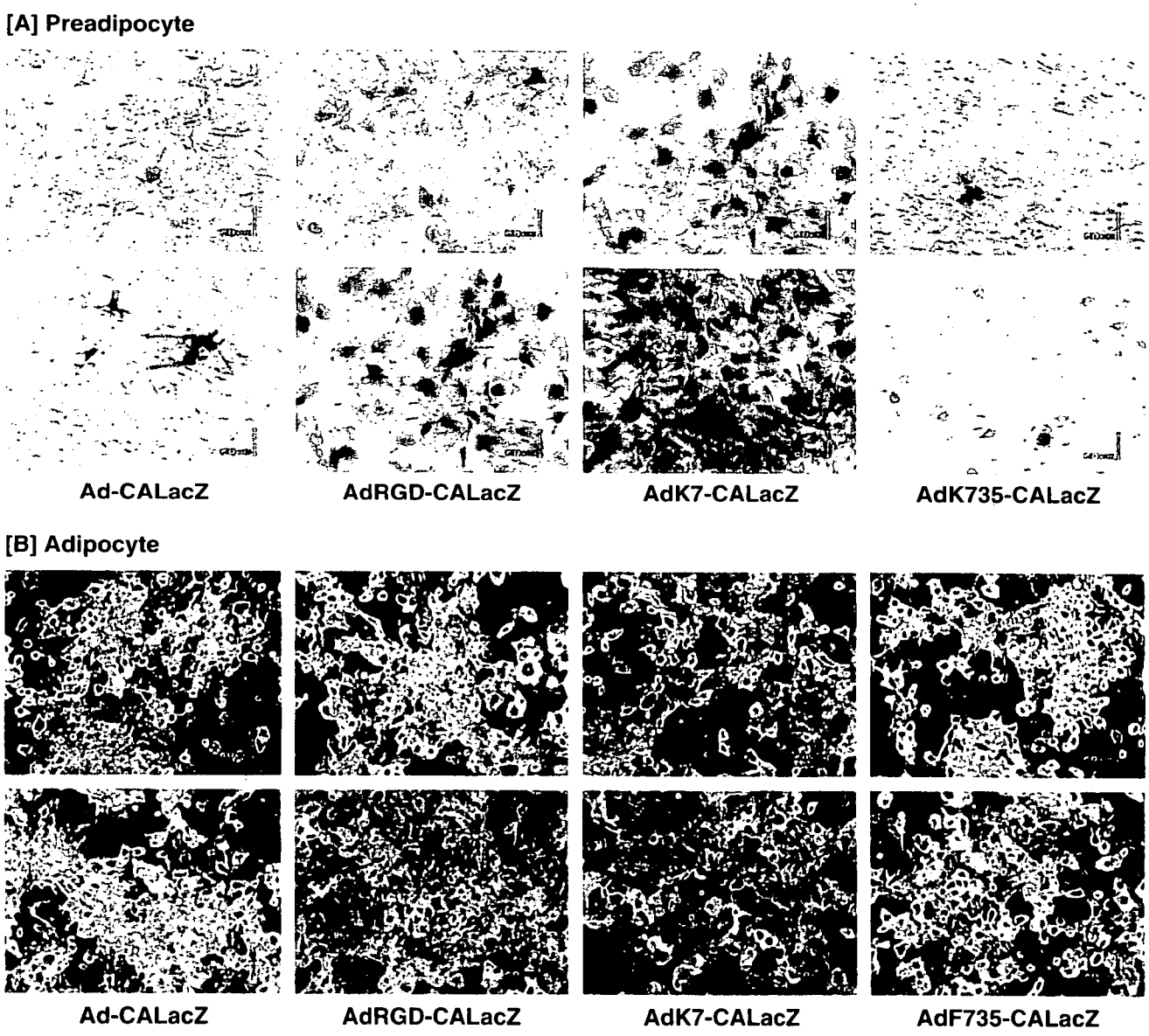

Fig. 1. Comparison of the transduction efficiency of various types of fiber-modified Ad vector into 3T3-L1 preadipocytes and adipocytes. 3T3-L1 preadipocytes (A) and adipocytes (B), which were cultured in differentiation medium containing pioglitazone, insulin, dexamethasone and 3-isobutyl-1-methylxanthine for 6 days, were transduced with Ad-CALacZ, AdRGD-CALacZ, AdK7-CALacZ or AdF35-CALacZ (3000 or 10,000 VP/cells) for 1.5 h. After 48 h in culture, LacZ expression was determined by X-gal staining.

2.7. Western blotting for PPARγ proteins

The cell extracts were prepared in lysis buffer (25 mM Tris [pH 7.5], 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 150 mM NaCl) containing a cocktail of protease inhibitors (Sigma). The protein content was measured with a Bio-Rad assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. The protein samples (10 μg) were electrophoresed on 12.5% SDS–polyacrylamide gels under reducing conditions, followed by electrotransfer to Immobilon-P membranes (Millipore, Bedford, MA, USA). After blocking in Block Ace (Dainippon Pharmaceuticals, Osaka, Japan), the filters were incubated with antibodies against PPARγ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and GAPDH (Trevigen, Gaithersburg, MD, USA), followed by incubation in the presence of peroxidase-labeled horse anti-mouse IgG antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) or peroxidase-labeled goat anti-rabbit IgG antibody (Cell Signaling Technology, Inc.). The filters were developed using chemiluminescence (ECL Western blotting detection system; Amersham Biosciences, Piscataway, NJ, USA). The signals were read using a LAS-3000 (FUJIFILM, Tokyo, Japan), and quantified by Image Gauge Software (FUJIFILM).

3. Results

3.1. Optimization of fiber-modified Ad vectors for the transduction of 3T3-L1 adipocytes and preadipocytes

The Ad vector is known to transduce 3T3-L1 preadipocytes with very low efficiency (Orlicky et al., 2001; Orlicky and Schaack, 2001; Ross et al., 2003). Therefore, we first optimized the transduction of 3T3-L1 adipocytes as well as preadipocytes by means of fiber-modified Ad vectors, which exhibit different tropism with the conventional Ad vector.

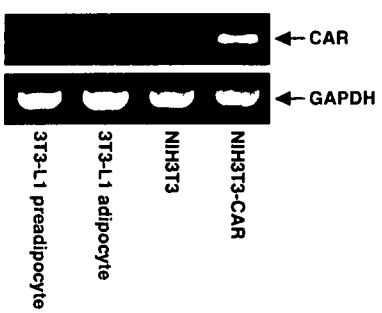

Fig. 2. RT-PCR analysis of CAR mRNA expression in 3T3-L1 preadipocytes and adipocytes. Total RNA was isolated from 3T3-L1 preadipocytes and adipocytes differentiated for 6 days, and RT-PCR analysis was performed as described in Materials and methods. NIH3T3 and NIH3T3-CAR cells were also analyzed as a negative and positive control of CAR mRNA expression, respectively.

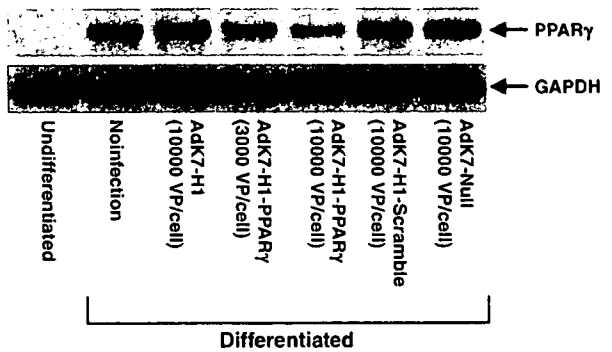

Fig. 3. Suppression of PPARγ expression in 3T3-L1 cells transduced with AdK7-H1-PPARγ. 3T3-L1 preadipocytes were transduced with each Ad vector for 1.5 h. On the following day, the cells reached confluence. From 3 days after Ad treatment, the cells were cultured with differentiation medium containing pioglitazone, insulin, dexamethasone and 3-isobutyl-1-methylxanthine for 4 days. Proteins were then extracted from the cells, and the levels of PPARγ expression were examined by Western blotting. The GAPDH bands served as an internal control for equal total protein loading.

The 3T3-L1 preadipocytes were infected with LacZ-expressing Ad vectors containing modified fiber proteins (Ad-CALacZ, AdRGD-CALacZ, AdF35-CALacZ and AdK7-CALacZ) (Table 1 and Fig. 1A). Ad-CALacZ contains the wild-type fiber, AdRGD-CALacZ contains an RGD peptide motif in the HI-loop of the fiber knob, AdK7-CALacZ contains a polylysine peptide in the C-terminal of the fiber knob, and AdF35-CALacZ contains a fiber protein derived from Ad type 5 fiber tail and Ad type 35 fiber knob and shaft. As shown previously (Orlicky et al., 2001; Orlicky and Schaack, 2001; Ross et al., 2003), Ad-CALacZ was inefficient for transduction of 3T3-L1 preadipocytes. AdK7-CALacZ was the most effective in transducing the LacZ genes. Nearly 100% of 3T3-L1 preadipocytes were transduced by AdK7-CALacZ at 10,000 vector particles (VP)/cell. AdRGD-CALacZ mediated higher levels of LacZ expression than Ad-CALacZ but lower levels than AdK7-CALacZ, while AdF35-CALacZ was ineffective.

We then examined the transduction efficiency of 3T3-L1 adipocytes using various types of Ad vectors. 3T3-L1 preadipocytes differentiate into mature, lipid droplet-containing adipocytes when stimulated with an appropriate hormonal cocktail containing insulin, dexamethasone and 3-isobutyl-1-methylxanthine. Pioglitazone, the ligand of PPARγ, enhances adipocyte differentiation of 3T3-L1 cells. 3T3-L1 preadipocytes were cultured with a differentiation medium containing pioglitazone for 6 days and then transduced with Ad vectors (Fig. 1B). Under the differentiated conditions, AdK7-CALacZ showed high transduction efficiency (67% LacZ-positive cells), although its efficiency was slightly lower than that in 3T3-L1 preadipocytes. AdRGD-CALacZ also showed high transduction efficiency (59% LacZ-positive cells). Ad-CALacZ and AdF35-CALacZ were ineffective. No cytotoxicity or other negative effects on cell function were observed in either 3T3-L1 preadipocytes or adipocytes.

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

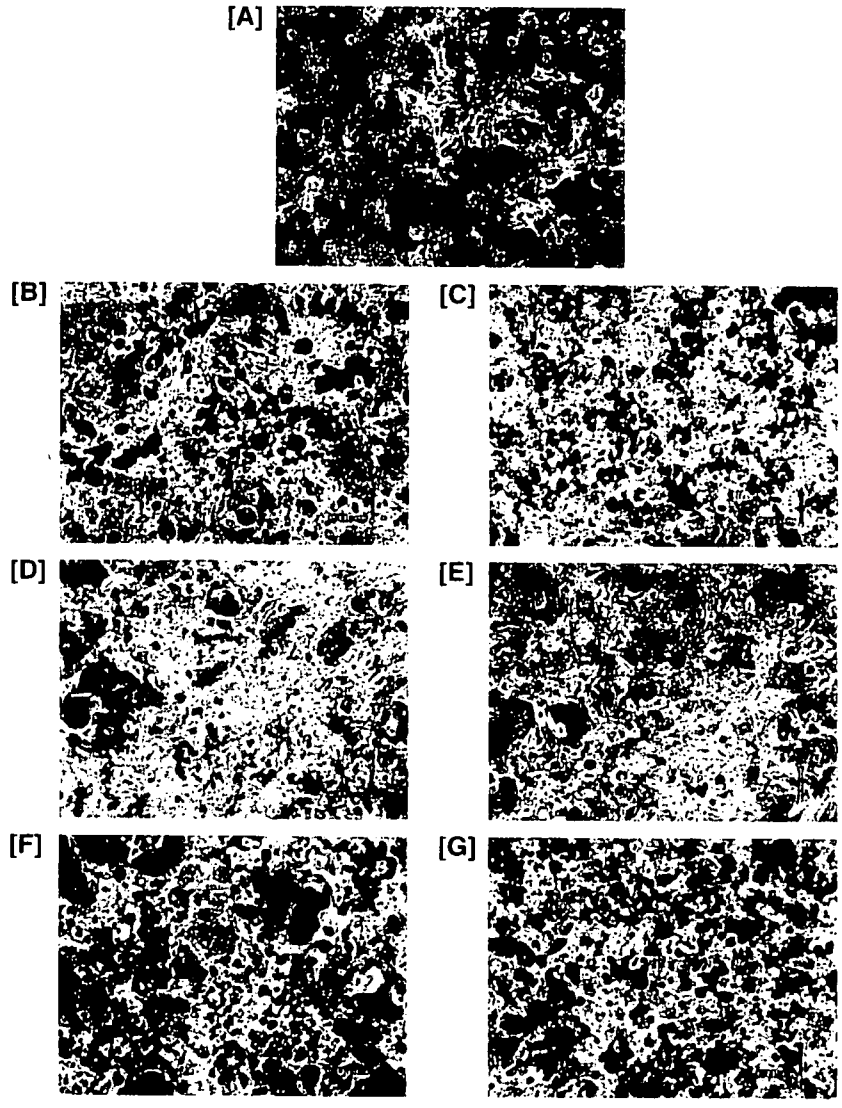

Fig. 4. Suppression of preadipocyte-to-adipocyte differentiation by transduction with AdK7-H1-PPARγ. 3T3-L1 preadipocytes were transduced with each Ad vector for 1.5 h. On the following day, the cells reached confluence. From 3 days after Ad treatment, the cells were cultured with differentiation medium 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.

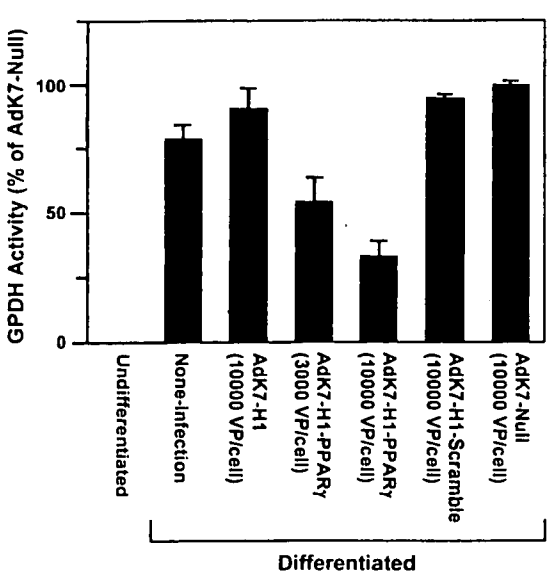

Fig. 5. Suppression of the fatty synthesis on 3T3-L1 cells transduced with AdK7-H1-PPARγ. The cells and virus were treated as described in the legends of Fig. 4. The fatty synthesis was determined by the measurement of GPDH activity in 3T3-L1 cells. Data were expressed as percentage of the GPDH activity 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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〔The 2nd Annual Meeting of JHUPO〕

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

Yukio Yamamoto, Yoshiko Akita, Shigeyuki Tai, Susumu Fukasaku, Teruhide Yamaguchi, Tadashi Oshizawa, Kazuko Yamaoka, Mariko Shimamura and Tadahiko Hazato

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

Yukio Yamamoto[1], Yoshiko Akita[1], Shigeyuki Tai[2], Susumu Fukasaku[3], Teruhide Yamaguchi[4], Tadashi Oshizawa[4], Kazuko Yamaoka[1], Mariko Shimamura[1] and Tadahiko Hazato[1]

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

[1] Tokyo Metropolitan Institute of Medical Science, Honkomagome 3-18-22, Bunkyo-ku, Tokyo 113-8613, Japan.
[2] Tokyo Metropolitan Bokutoh Hospital, Koutoubashi 4-23-15, Sumida-ku, Tokyo 130-8515, Japan.
[3] School of Medicine, Juntendo University, Hongo 2-1-1, Bunkyo-ku, Tokyo 113-8421, Japan.
[4] National Institute of Health Science, Kamiyoga 1-18-1, Setagaya,-ku, Tokyo 158-8501, Japan.
Corresponding address: Yukio Yamamoto, Ph.D., Department of Medical Biology, The Tokyo Metropolitan Institute of Medical Science, Honkomagome 3-18-22, Bunkyo-ku, Tokyo 113-8613, Japan.
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.

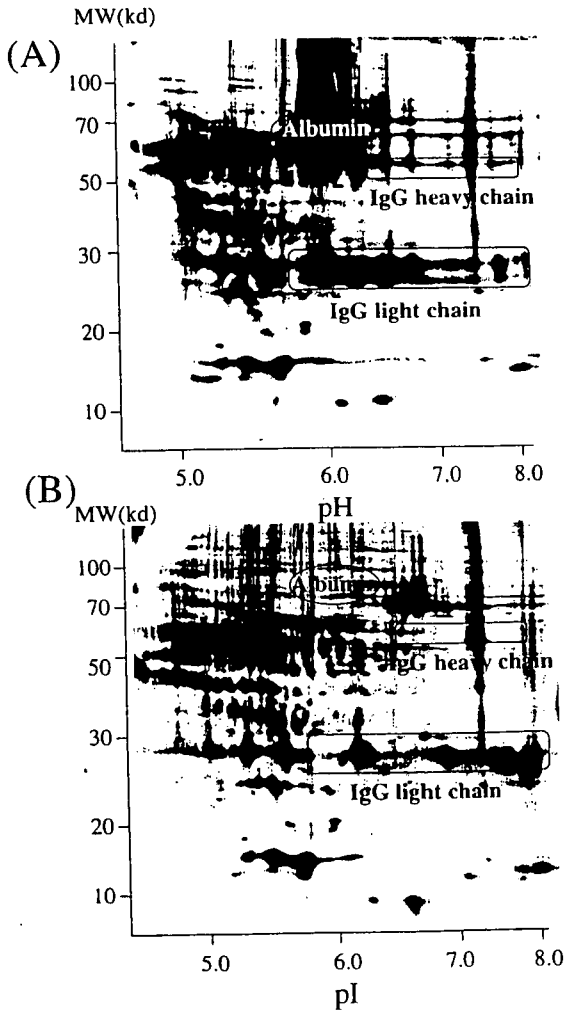

Fig. 1. Effect of pretreatment for albumin and IgG removal from cerebrospinal fluids.

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.