[11] Lin, E.; Nemunaitis, J. Oncolytic viral therapies. *Cancer Gene Ther.* **2004,** *11*, 643-664.
[12] Russell, S. J. RNA viruses as virotherapy agents. *Cancer Gene Ther.* **2002,** *9*, 961-966.
[13] Kinoh, H.; Inoue, M.; Washizawa, K.; Yamamoto, T.; Fujikawa, S.; Tokusumi, Y.; Iida, A.; Nagai, Y.; Hasegawa, M. Generation of a recombinant Sendai virus that is selectively activated and lyses human tumor cells expressing matrix metalloproteinases. *Gene Ther.* **2004,** *11*, 1137-1145.
[14] Aghi, M.; Martuza, R. L. Oncolytic viral therapies - the clinical experience. *Oncogene* **2005,** *24*, 7802-7816.
[15] Barber, G. N. VSV-tumor selective replication and protein translation. *Oncogene* **2005,** *24*, 7710-7719.
[16] Stojdl, D. F.; Lichty, B. D.; tenOever, B. R.; Paterson, J. M.; Power, A. T.; Knowles, S.; Marius, R.; Reynard, J.; Poliquin, L.; Atkins, H.; Brown, E. G.; Durbin, R. K.; Durbin, J. E.; Hiscott, J.; Bell, J. C. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* **2003,** *4*, 263-275.
[17] Norman, K. L.; Lee, P. W. Reovirus as a novel oncolytic agent. *J. Clin. Invest.* **2000,** *105*, 1035-1038.
[18] Stoeckel, J.; Hay, J. G. Drug evaluation: Reolysin--wild-type reovirus as a cancer therapeutic. *Curr. Opin Mol. Ther.* **2006,** *8*, 249-260.
[19] Shmulevitz, M.; Marcato, P.; Lee, P. W. Unshackling the links between reovirus oncolysis, Ras signaling, translational control and cancer. *Oncogene* **2005,** *24*, 7720-7728.
[20] Kimata, H.; Takakuwa, H.; Goshima, F.; Teshigahara, O.; Nakao, A.; Kurata, T.; Sata, T.; Nishiyama, Y. Effective treatment of disseminated peritoneal colon cancer with new replication-competent herpes simplex viruses. *Hepatogastroenterology* **2003,** *50*, 961-966.
[21] Takakuwa, H.; Goshima, F.; Nozawa, N.; Yoshikawa, T.; Kimata, H.; Nakao, A.; Nawa, A.; Kurata, T.; Sata, T.; Nishiyama, Y. Oncolytic viral therapy using a spontaneously generated herpes simplex virus type 1 variant for disseminated peritoneal tumor in immunocompetent mice. *Arch. Virol.* **2003,** *148*, 813-825.
[22] Teshigahara, O.; Goshima, F.; Takao, K.; Kohno, S.; Kimata, H.; Nakao, A.; Nishiyama, Y. Oncolytic viral therapy for breast cancer with herpes simplex virus type 1 mutant HF 10. *J. Surg. Oncol.* **2004,** *85*, 42-47.
[23] Mathis, J. M.; Stoff-Khalili, M. A.; Curiel, D. T. Oncolytic adenoviruses - selective retargeting to tumor cells. *Oncogene* **2005,** *24*, 7775-7791.
[24] Post, D. E.; Khuri, F. R.; Simons, J. W.; Van Meir, E. G. Replicative oncolytic adenoviruses in multimodal cancer regimens. *Hum. Gene Ther.* **2003,** *14*, 933-946.
[25] Bischoff, J. R.; Kirn, D. H.; Williams, A.; Heise, C.; Horn, S.; Muna, M.; Ng, L.; Nye, J. A.; Sampson-Johannes, A.; Fattaey, A.; McCormick, F. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* **1996,** *274*, 373-376.
[26] Ries, S.; Korn, W. M. ONYX-015: mechanisms of action and clinical potential of a replication-selective adenovirus. *Br. J. Cancer* **2002,** *86*, 5-11.
[27] Nakamura, T.; Peng, K. W.; Harvey, M.; Greiner, S.; Lorimer, I. A.; James, C. D.; Russell, S. J. Rescue and propagation of fully retargeted oncolytic measles viruses. *Nat. Biotechnol.* **2005,** *23*, 209-214.
[28] Hermiston, T. W.; Kuhn, I. Armed therapeutic viruses: strategies and challenges to arming oncolytic viruses with therapeutic genes. *Cancer Gene Ther.* **2002,** *9*, 1022-1035.
[29] Freytag, S. O.; Khil, M.; Stricker, H.; Peabody, J.; Menon, M.; DePeralta-Venturina, M.; Nafziger, D.; Pegg, J.; Paielli, D.; Brown, S.; Barton, K.; Lu, M.; Aguilar-Cordova, E.; Kim, J. H. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res.* **2002,** *62*, 4968-4976.
[30] US Food and Drug Administration (FDA). Guidance for Industry. Guidance for Human Somatic Cell Therapy and Gene Therapy. **1998**.
[31] US Food and Drug Administration (FDA). Guidance for FDA Review Staff and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs). DRAFT GUIDANCE. **2004**.
[32] European Medicines Agency (EMEA). Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products. CPMP/BWP/3088/99. **2001**.
[33] Ministry of Health, Labour and Welfare (MHLW). Notification of Pharmaceutical Affairs Bureau, Yaku-hatsu No.1062: Guidance for Assuring the Quality and Safety of the Gene Therapy Products. (in Japanese) **1995**.
[34] ICH Harmonized Tripartite Guideline. ICH-Q5D: Derivation and Characterization of Cell Substrates Used for Production of BioTechnological/Biological Products. **1997**.
[35] Working, P. K.; Lin, A.; Borellini, F. Meeting product development challenges in manufacturing clinical grade oncolytic adenoviruses. *Oncogene* **2005,** *24*, 7792-7801.
[36] Bernt, K.; Liang, M.; Ye, X.; Ni, S.; Li, Z. Y.; Ye, S. L.; Hu, F.; Lieber, A. A new type of adenovirus vector that utilizes homologous recombination to achieve tumor-specific replication. *J. Virol.* **2002,** *76*, 10994-11002.
[37] Graham, F. L.; Smiley, J.; Russell, W. C.; Nairn, R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **1977,** *36*, 59-74.
[38] Fallaux, F. J.; Bout, A.; van der Velde, I.; van den Wollenberg, D. J.; Hehir, K. M.; Keegan, J.; Auger, C.; Cramer, S. J.; van Ormondt, H.; van der Eb, A. J.; Valerio, D.; Hoeben, R. C. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum. Gene Ther.* **1998,** *9*, 1909-1917.
[39] Lusky, M. Good manufacturing practice production of adenoviral vectors for clinical trials. *Hum. Gene Ther.* **2005,** *16*, 281-291.
[40] Farson, D.; Tao, L.; Ko, D.; Li, Q.; Brignetti, D.; Segawa, K.; Mittelstaedt, D.; Harding, T.; Yu, D. C.; Li, Y. Development of Novel E1-Complementary Cells for Adenoviral Production Free of Replication-Competent Adenovirus. *Mol. Ther.* **2006,** *14*, 305-311.
[41] ICH Harmonised Tripartite Guideline. ICH-Q5A: Viral Safety Evaluation of Biotechnology Products derived from Cell Lines of Human or Animal Origin. **1997**.
[42] Hutchins, B.; Sajjadi, N.; Seaver, S.; Shepherd, A.; Bauer, S. R.; Simek, S.; Carson, K.; Aguilar-Cordova, E. Working toward an adenoviral vector testing standard. *Mol. Ther.* **2000,** *2*, 532-534.
[43] Alemany, R.; Balague, C.; Curiel, D. T. Replicative adenoviruses for cancer therapy. *Nat. Biotechnol.* **2000,** *18*, 723-727.
[44] Raper, S. E.; Chirmule, N.; Lee, F. S.; Wivel, N. A.; Bagg, A.; Gao, G. P.; Wilson, J. M.; Batshaw, M. L. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol. Genet. Metab.* **2003,** *80*, 148-158.
[45] Lichtenstein, D. L.; Wold, W. S. Experimental infections of humans with wild-type adenoviruses and with replication-competent adenovirus vectors: replication, safety, and transmission. *Cancer Gene Ther.* **2004,** *11*, 819-829.
[46] Makower, D.; Rozenblit, A.; Kaufman, H.; Edelman, M.; Lane, M. E.; Zwiebel, J.; Haynes, H.; Wadler, S. Phase II clinical trial of intralesional administration of the oncolytic adenovirus ONYX-015 in patients with hepatobiliary tumors with correlative p53 studies. *Clin. Cancer Res.* **2003,** *9*, 693-702.
[47] Small, E. J.; Carducci, M. A.; Burke, J. M.; Rodriguez, R.; Fong, L.; van Ummersen, L.; Yu, D. C.; Aimi, J.; Ando, D.; Working, P.; Kirn, D.; Wilding, G. A Phase I Trial of Intravenous CG7870, a Replication-Selective, Prostate-Specific Antigen-Targeted Onco-lytic Adenovirus, for the Treatment of Hormone-Refractory, Metastatic Prostate Cancer. *Mol. Ther.* **2006,** *14*, 107-117.
[48] DeWeese, T. L.; van der Poel, H.; Li, S.; Mikhak, B.; Drew, R.; Goemann, M.; Hamper, U.; DeJong, R.; Detorie, N.; Rodriguez, R.; Haulk, T.; DeMarzo, A. M.; Piantadosi, S.; Yu, D. C.; Chen, Y.; Henderson, D. R.; Carducci, M. A.; Nelson, W. G.; Simons, J. W. A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res.* **2001,** *61*, 7464-7472.
[49] Todo, T.; Feigenbaum, F.; Rabkin, S. D.; Lakeman, F.; Newsome, J. T.; Johnson, P. A.; Mitchell, E.; Belliveau, D.; Ostrove, J. M.; Martuza, R. L. Viral shedding and biodistribution of G207, a multimutated, conditionally replicating herpes simplex virus type 1, after intracerebral inoculation in aotus. *Mol. Ther.* **2000,** *2*, 588-595.

Received: July 05, 2006 **Revised: September 03, 2006** **Accepted: September 10, 2006**

Available online at www.sciencedirect.com

ScienceDirect

Journal of Virological Methods 143 (2007) 95–103

Journal of Virological Methods

www.elsevier.com/locate/jviromet

Optimization of the virus concentration method using polyethyleneimine-conjugated magnetic beads and its application to the detection of human hepatitis A, B and C viruses

Eriko Uchida[a], Mieko Kogi[a,b], Tadashi Oshizawa[a], Birei Furuta[a], Koei Satoh[c], Akiko Iwata[c], Mitsuhiro Murata[d], Mikio Hikata[d], Teruhide Yamaguchi[a,e,*]

[a] *Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan*
[b] *Kanazawa Institute of Technology, Nonoichi, Ishikawa, Japan*
[c] *The Institute of the Saitama Red Cross Center, Kumagaya, Saitama, Japan*
[d] *JSR Corporation, Tsukuba Research Laboratories, Tsukuba, Ibaraki, Japan*
[e] *Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan*

Received 25 September 2006; received in revised form 22 February 2007; accepted 26 February 2007
Available online 12 April 2007

Abstract

To enhance the sensitivity of virus detection by polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR), a novel virus concentration method using polyethyleneimine (PEI)-conjugated magnetic beads was developed in our previous study. However, several viruses could not be concentrated by this method. In this paper, the conditions of virus concentration were optimized to concentrate a wide range of viruses more efficiently. The PEI beads adsorbed viruses more efficiently than other cationic polymers, and the optimum virus concentration was obtained under weak acidic conditions. Mass spectrometric analysis revealed that several serum proteins, such as complement type 3, complement type 4 and immunoglobulin M (IgM), were co-adsorbed by the PEI beads, suggesting that the beads may adsorb viruses not only by direct adsorption, but also via immune complex formation. This hypothesis was confirmed by the result that poliovirus, which PEI beads could not adsorb directly, could be concentrated by the beads via immune complex formation. On the other hand, hepatitis A (HAV) and hepatitis C (HCV) viruses were adsorbed directly by PEI beads almost completely. Like poliovirus, hepatitis B virus (HBV) was concentrated efficiently by the addition of anti-HBV IgM. In conclusion, virus concentration using PEI beads is a useful method to concentrate a wide range of viruses and can be used to enhance the sensitivity of detection of HAV, HBV and HCV.

Keywords: Polyethyleneimine; Virus concentration; Immune complex; HAV; HBV; HCV

1. Introduction

Many useful biological/biotechnological medicinal products are produced from biological materials and by cell culture techniques. Recent progress in gene therapy and cell therapy products has provided new hope for the treatment of grave genetic diseases and lethal disorders. These innovative medicinal products, however, involve some risk in terms of the spread of transmissible agents and virus-mediated infectious diseases. To ensure the viral safety of biological/biotechnological products, it is important to confirm that the starting materials, intermediate products and final products are free from virus contamination. This is especially important for cell therapy products, since it is difficult to inactivate and/or remove contaminated viruses from these products.

Polymerase chain reaction (PCR) is a highly sensitive method for the detection of virus genomes (Saiki et al., 1988). Several nucleic acid amplification test (NAT) methods other than PCR have also been developed (Alter et al., 1995; Kamisango et al., 1999; Kern et al., 1996; Notomi et al., 2000; Sarrazin et al., 2000). These tests are reported to be able to detect only some copies of virus genomes. Therefore, in many countries, NAT methods have been employed to detect specific viruses in the virus screening of blood-derived products (Willkommen et al.,

* Corresponding author. Tel.: +81 3 3700 9064; fax: +81 3 3700 9084.
E-mail address: yamaguch@nihs.go.jp (T. Yamaguchi).

0166-0934/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2007.02.014

1999). NAT methods are also thought to be useful in testing biotechnology products derived from cell lines and cell therapy products. However, since all NAT methods used for the detection of viruses have a detection limit, it is impossible to deny virus contamination completely. In order to reduce the virus risk of both biological/biotechnological products and cell therapy products, it is essential to develop more sensitive methods of virus detection. One way to improve the sensitivity of virus detection is to concentrate the target viruses before NAT testing.

Recently, a novel viral concentration method using polyethyleneimine (PEI)-conjugated magnetic beads was developed (Satoh et al., 2003). It was shown that PEI beads efficiently adsorbed many model viruses, such as simian virus 40 (SV-40), herpes simplex 1 virus (HSV-1), Sindbis virus and vesicular stomatitis virus (VSV), and that the method improved the sensitivity of NAT for the detection of virus genomes about 10 to 100 times. It has also been reported that PEI beads efficiently adsorb amphotropic murine leukemia virus, and that the virus concentration method provided sensitive detection of replication-competent retrovirus in retrovirus vector products (Uchida et al., 2004). However, several small non-enveloped viruses such as poliovirus could not be concentrated or were only partially concentrated by PEI beads (Satoh et al., 2003). In addition, the mechanism of virus adsorption by PEI beads remains to be elucidated.

In the present study, the viral concentration method using PEI beads was optimized in order to allow the efficient concentration of several viruses. It was demonstrated that poliovirus can be concentrated by PEI beads via the formation of immune complexes. In addition, it was shown that the virus concentration method using PEI beads is applicable to human infectious viruses such as the hepatitis A(HAV), hepatitis B (HBV) and hepatitis C (HCV) viruses, which are important viruses to test for in order to ensure the viral safety of biological products and cell therapy products.

2. Materials and methods

2.1. Viruses

SV-40 virus, HSV-1 (stain F), porcine parvovirus (PPV; strain 90HS) and poliovirus (strain Sabin 1) were obtained and amplified as described previously (Satoh et al., 2003). Briefly, the supernatants of Vero cells infected with HSV-1 or poliovirus were used as virus samples. CV-1 cells were infected with SV-40 virus, and 5 days after infection, the supernatant was saved as the SV-40 sample. The supernatant of ESK cells infected with PPV was used as the PPV sample. In order to remove cell debris from the collected virus suspension, each virus suspension was centrifuged at 3000 rpm for 10 min. After removing cell debris, the resulting stock viruses (SV40: 4×10^7 copies/ml; PPV: 1×10^6 copies/ml; HSV-1: 1×10^8 copies/ml; poliovirus: 2×10^7 copies/ml) were aliquoted and stored at −80 °C until use. Human adenovirus type 5 reference material (ATCC VR-1516; 5.8×10^{11} particles/ml) was obtained from the American type culture collection (ATCC) and used without amplification. HAV was obtained from ATCC (strain HM175/18f), infected into FRhK-4 cells, and the supernatant of the cell was saved 9–11 days later as the HAV sample (1×10^8 PFU/ml). The first Japanese national standard for HBV DNA (Genotype C; potency: 4.4×10^5 IU/ml) and the first Japanese national standard for HCV RNA (Mizusawa et al., 2005); genotype HCV-1b; potency: 100,000 IU/ml) were directly used as the HBV sample and HCV sample, respectively.

2.2. Preparation of PEI beads

PEI beads were prepared by coupling PEI (MW 70,000; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) with magnetic beads (IMMUTEX-MAG™; mean diameter: 0.8 μm; JSR Inc., Tokyo, Japan) by the 1-ethylene-3-(3-dimethylaminopropyl) carbodiimide coupling method, as described previously (Satoh et al., 2003). The final concentration of the PEI beads was 50 mg/ml. Different molecular weights of PEI beads were prepared as described above but including the coupling of PEI with a molecular weight (MW) of 1800 or PEI (MW 10,000) to magnetic beads, instead of PEI (MW 70,000). Polyarylamine (PAA)-conjugated magnetic beads and poly-L-lysine (PLL)-conjugated magnetic beads were prepared in the same way as PEI beads, using PAA (MW 150,000) or PLL (MW > 300,000) instead of PEI, respectively.

2.3. Virus concentration using PEI beads

The essential adsorption procedure for each virus was as follows. Virus samples were diluted with virus dilution medium (Dulbecco's modified Eagle's medium (DMEM) or DMEM supplemented with 2% fetal calf serum (FCS)). The exact concentration of the virus suspension used for each experiment is described in the corresponding figure legends. Next, 1 ml of each virus suspension was incubated with 100 μl of PEI beads for 10 min at room temperature. The complexes of virus and PEI beads were trapped by a magnetic field (Magnetic Trapper™; Toyobo Co., Tokyo, Japan) for 5 min and separated from the supernatant fraction. The virus DNA or RNA was extracted from virus-bead complex or from untreated virus suspensions (100 μl) with an SMI-TEST EX R&D Kit (Medical & Biological Laboratories Co., Nagano, Japan) following the manufacturer's instructions. Extracted DNA or RNA was dissolved in 50 μl of TE buffer (10 mM Tris–HCl (pH 7.4)/0.1 mM EDTA), and 10 μl of the solution was used for real-time PCR or RT-PCR reaction.

2.4. Effect of pH on virus concentration by PEI beads

Good's buffers with pH 4–9 (1 M MES, pH 4.0; 1 M MES, pH 5.0; 1 M MES, pH 6.0; 1 M HEPES, pH 7.0; 1 M HEPES, pH 8.0; 1 M Tricine, pH 9.0) were prepared and added to the virus dilution medium at a final concentration of 20 mM. Virus samples were then diluted with the virus dilution media at different pH values, and concentrated with PEI beads as described. The exact concentration of the virus suspension used for each experiment is described in the corresponding figure legends.

2.5. Real-time PCR and RT-PCR

Real-time PCR and reverse transcription PCR (RT-PCR) were carried out in a 50-μl reaction mixture containing 10 μl of extracted DNA or RNA, 0.5 μM of each primer set with a fluorescence probe, 25 μl of PCR master mix and, in the case of RT-PCR, a reverse transcriptase mix prepared according to the kit manual. The following real-time PCR and RT-PCR master mix kits were used: a QuantiTect Probe PCR kit (Qiagen, Hilden, Germany) for HSV-1, SV-40, adenovirus and PPV; a QuantiTect Probe RT-PCR kit (Qiagen) for poliovirus, HAV and HCV; and a Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA, USA) for HBV. The 5′-primers, 3′-primers and fluorescence probes used for the real-time PCR and RT-PCR detection of viruses are shown in Table 1. The real-time PCR and RT-PCR were performed on an ABI PRISM 7000 Sequence-Detection System (Applied Biosystems, Foster City, CA, USA).

2.6. SDS-PAGE analysis of serum proteins adsorbed on PEI beads

The virus suspension (HSV-1) diluted with DMEM supplemented with 5% FCS was incubated with PEI beads for 10 min. The fraction of serum proteins adsorbed on the beads and the untreated virus suspension were then boiled with sodium dodecly sulfate (SDS) sample buffer and applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out on a slab gel (T = 7.5%) with a BE-120 system from Biocraft (Tokyo, Japan). Protein bands were visualized by Coomassie Brilliant Blue staining.

2.7. In-gel digestion

Protein bands of interest were excised from the SDS-PAGE gel, destained three times in 50% acetonitrile and 25 mM ammonium bicarbonate for 10 min each time, and dehydrated in acetonitrile. The gel pieces were dried in a vacuum centrifugal concentrator and incubated with 10 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate at 56 °C for 60 min. After cooling to room temperature, the DTT solution was replaced with roughly the same volume of 55 mM iodoacetamide in 25 mM ammonium bicarbonate. After incubation for 45 min at room temperature in the dark, the gel pieces were washed in 25 mM ammonium bicarbonate for 5 min and dehydrated by the addition of 50% acetonitrile and 25 mM ammonium bicarbonate for 5 min. After this procedure was repeated twice, the gel pieces were dried in a centrifugal concentrator. The gel pieces were allowed to swell in 2 μl of a digestion buffer containing 25 mM ammonium bicarbonate, 0.1% octyl glucoside, and 25 ng/μl trypsin (sequence grade; Promega, Madison, WI, USA) in ice for 5 min, and then 15 μl of a digestion buffer without trypsin was added. After 30 min, the supernatant was discarded, and the gel pieces were incu-

Table 1
Primer and probe sets used for the real-time PCR and RT-PCR

Virus	Primer and probe set
HSV-1	Forward primer: 5′-GCGTCATGGTACTGGCAAG-3′ Reverse primer: 5′-TTGACTCTACGGAGCTGGCC-3′ Probe: 5′-FAM-TGGAGCTGATGCCGTAGTCGG-TAMRA-3′
SV-40	Forward primer: 5′-GACATTCCTAGGCTCACCTCACA-3′ Reverse primer: 5′-ACCTTGCCAAACTGTCCCTTAAA-3′ Probe: 5′-FAM-CTTGAAAGAAGAACCCAAAGA-TAMRA-3′
PPV	Forward primer: AACAACTACGCAGCAACTCCAATA-3′ Reverse primer: ACGGCTCCAAGGCTAAAGC-3′ Probe: 5′-FAM-AGGAGGACCTGGATTT-MGB-3′
Adenovirus*1	Forward primer: TCCGGTCCTTCTAACACACCTC-3′ Reverse primer: ACGGCAACTGGTTTAATGGG-3′ Probe: 5′-FAM-TGAGATACACCCGGTGGTCCCGC-TAMRA-3′
Poliovirus	Forward primer: 5′-CCCGAGAAATGGGACGACTA-3′ Reverse primer: 5′-TGGAGCTGTTCCGTAGGTGTAA-3′ Probe: 5′- FAM-ACATGGCAAACCTCATCAAATCCATCAATC-MGB-3′
HAV*2	Forward primer: 5′-GGTAGGCTACGGGTGAAAC-3′ Reverse primer: 5′-AACAACTCACCAATATCCGC-3′ Probe: 5′-FAM-CTTAGGCTAATACTTCTATGAAGAGATGC-TAMRA-3′
HBV*3	Forward primer: 5′-GGACCCCTGCTCGTGTTACA-3′ Reverse primer: 5′-GAGAGAAGTCCACCMCGAGTCTAGA-3′ Probe: 5′-FAM-TGTTGACAARAATCCTCACCATACCRCAGA-TAMRA-3′
HCV*4	Forward primer: 5′-TGCGGAACCGGTGAGTACA-3′ Reverse primer: 5′-CTTAAGGTTTAGGATTCGTGCTCAT-3′ probe: 5′-FAM-CACCCTATCAGGCAGTACCACAAGGCC-TAMRA-3′

Each primer set was prepared according to the original papers described below (*1 to *4) or designed using Primer Express software (Applied Biosystems). *1 Adenovirus (Ishii-Watabe et al., 2003), *2 HAV (Jothikumar et al., 2005), *3 HBV (Pas et al., 2000), *4 HCV (Martell et al., 1999).

bated overnight at 37 °C. To extract tryptic fragments, the gel pieces were shaken in 50% acetonitrile and 5% trifluoroacetic acid (TFA) for 30 min. After this procedure was repeated twice, the extraction solutions were pooled, dried in a centrifugal evaporator, and dissolved in 20 μl of 0.1% TFA. The samples were then absorbed onto reverse-phase ZipTipC18 (Millipore, Bedford, MA, USA). The resin was washed with 0.1% TFA and the peptides were eluted with 3 μl of 75% acetonitrile/0.1% TFA. The eluate was analyzed by mass spectrometry (MS) as described below.

2.8. MS and database searching

The peptide mixture (0.5 μl volume) elution was deposited onto a matrix assisted laser desorption/ionization (MALDI) target plate, and this was closely followed by the deposition of 0.5 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% TFA. MS and tandem MS (MS/MS) analysis of the peptide mixtures was performed using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). Peptide mass fingerprinting and MS/MS ion searches were performed for protein identification by a Mascot search based on the MSDB protein database.

2.9. Preparation of anti-mouse IgG-rabbit IgM antibody

Anti-mouse immunogloblulin G (IgG) rabbit antiserum was obtained from rabbits immunized with highly purified mouse IgG (11 mg/ml; Jackson ImmunoResearch, West Grove, PA, USA) at 11 days after immunization, when IgM titer was increased. The antiserum (3 ml) was then diluted with an equal volume of phosphate buffered saline (PBS) (−), and applied to a mouse-IgG agarose affinity column (Invitrogen). After washing with 10 ml of PBS (−), the bound fraction was eluted with 0.1 M glycine–HCl (pH 3.0) and neutralized with 1 M Tris–HCl (pH 8.0). A PEI-sepharose-6MB column was prepared by coupling PEI to CNBr-activated sepharose-6MB (GE Healthcare Bioscience, Piscataway, NJ, USA). Anti-mouse IgG rabbit antiserum purified with a mouse IgG-agarose column was applied to a PEI-sepharose-6MB column and washed with PBS (−), and the bound fraction was eluted with 1.4 M NaCl/50 mM HEPES (pH 7.6). The eluted fraction was concentrated and used as anti-mouse IgG rabbit IgM antibody (final concentration, 4 μg/ml).

2.10. Poliovirus concentration via immune complexes

When poliovirus suspension was concentrated by PEI beads via immune complex formation, anti-poliovirus 1 mouse monoclonal antibody (IgG1: 5 μl; Chemicon International, Temecula, CA, USA) and purified anti-mouse IgG rabbit IgM antibody (20 μl), or anti-poliovirus 1 mouse monoclonal antibody and human complement C1 (5 μl; Merck Biosciences/Calbiochem, Darmstadt, Germany) and C4 (3 μl; Calbiochem) were added to the virus suspension before incubation with PEI beads.

2.11. Preparation of anti-HBV IgM antibody

Anti-hepatitis B surface antigen (HBsAg) IgM antibody was prepared as follows. Rabbits were immunized with a mixture of the adw and adr subtypes of recombinant HBsAg (Advanced ImmunoChemical, Long Beach, CA, USA). Anti-HBsAg rabbit antiserum was obtained at 10 days after immunization, when IgM titer was increased. The antiserum (3 ml) was diluted with an equal volume of PBS (−), applied to a PEI-sepharose-6MB column, washed with 20 ml PBS (−), and eluted with 1.4 M NaCl/100 mM HEPES (pH 7.0). PEI-sepharose-6MB-bound fractions were pooled, desalted with a PD-10 column equilibrated with 1.2 M NaCl/50 mM HEPES buffer (pH 7.4), and purified with an ImmunoPure IgM purification kit (Pierce Biotechnology, Rockford, IL, USA). IgM fractions were concentrated and used as anti-HBsAg IgM antibody.

3. Results

3.1. Optimization of the virus concentration method using PEI beads

In order to optimize the virus concentration method using PEI beads, the relationship between the MW of PEI coupled with magnetic beads and the efficiency of the virus concentration was examined. When PEIs with average molecular masses of 1800, 10,000 and 70,000 Da were compared, the PEI of MW 70,000 Da efficiently concentrated HSV-1, while magnetic beads with the PEI of MWs 1800 and 10,000 Da could not adsorb HSV-1 (Fig. 1). Therefore, the PEI beads with MW 70,000 Da were used in the following experiments.

Next, the virus adsorption ability of PEI was compared to that of other cationic polymers. As shown in Fig. 2, PEI beads exhibited a markedly higher virus adsorption ability than PAA- or PLL-conjugated magnetic beads for all model viruses tested.

The effect of pH on the efficiency of virus concentration was then examined. HSV-1 and SV-40 virus suspensions at different

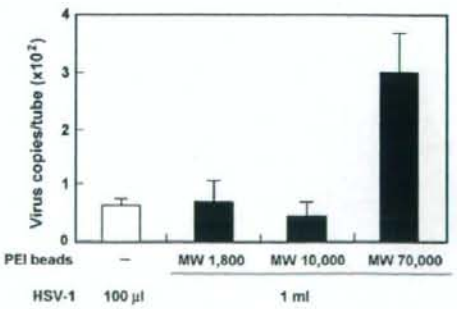


Fig. 1. Comparison of virus concentrations by magnetic beads coupled with PEIs of three different molecular weights. HSV-1 suspension (1×10^3 copies/ml, 1 ml/tube) was incubated with PEI beads whose PEI had a molecular weight of 1800, 10,000 or 70,000 Da. Viral genome DNA was extracted from the PEI bead fraction and from untreated HSV-1 suspension (100 μl). Virus copy numbers were determined by real-time PCR. Data are expressed as the mean ± S.D. ($n = 3$).

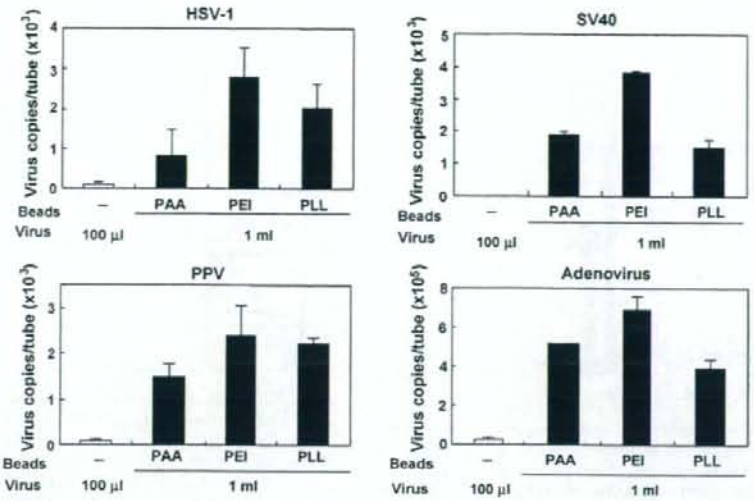


Fig. 2. Comparison of virus concentration by magnetic beads coupled with three different cationic polymers. HSV-1 (5×10^3 copies/ml), SV-40 (5×10^3 copies/ml), PPV (5×10^3 copies/ml) and adenovirus suspensions (1×10^6 copies/ml) (1 ml each) were incubated with PAA-, PEI- or PLL-conjugated magnetic beads. Viral genome DNA was extracted from each magnetic bead fraction and from untreated virus suspensions (100 µl each). Virus copy numbers were determined by real-time PCR. Data are expressed as the mean ± S.D. ($n = 3$).

pH levels (pH 5–9) were concentrated by PEI beads following the standard method. A pH levels of 6 was found to be optimal for the concentration of these viruses (Fig. 3).

3.2. Analysis of serum proteins adsorbed on PEI beads

To improve the virus concentration method using PEI beads, the serum components co-adsorbed by the beads during virus concentration were analyzed. When a virus suspension containing 5% FCS was concentrated by PEI beads and analyzed by SDS-PAGE, several proteins were specifically adsorbed by the beads (Fig. 4). Using MS and MS/MS analyses of these protein bands, complement type 3, complement type 4 and IgM heavy chain were identified as serum components concentrated by PEI beads. Since complement components and IgM were adsorbed by the beads, it is hypothesized that PEI beads may adsorb viruses not only by direct adsorption, but also via the formation of immune complexes that involve IgM antibody and/or complements.

3.3. Concentration of poliovirus by PEI beads via immune complexes

To confirm this hypothesis, concentrations of poliovirus, which PEI beads could not adsorb directly, via the formation of immune complexes were examined. Instead of anti-poliovirus IgM antibody, anti-poliovirus mouse monoclonal antibody (IgG) was used in combination with anti-mouse IgG rabbit IgM anti-

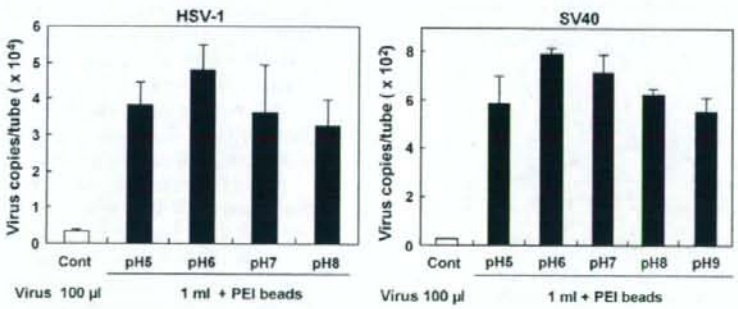


Fig. 3. Effect of pH on the efficiency of virus concentration by PEI beads. HSV-1 (5×10^4 copies/ml) and SV-40 (1×10^3 copies/ml) suspensions diluted with virus dilution medium at different pH levels (HSV-1: pH 5, 6, 7 and 8; SV-40: pH 5, 6, 7, 8 and 9) (1 ml each) were incubated with PEI beads. Viral genome DNA was then extracted from PEI bead fraction and from untreated virus suspensions (100 µl each). Virus copy numbers were determined by real-time PCR. Data are expressed as the mean ± S.D. ($n = 3$).

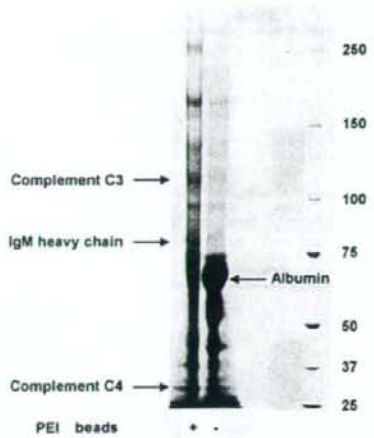


Fig. 4. Serum proteins adsorbed on PEI beads during virus concentration. HSV-1 suspension diluted with DMEM supplemented with 5% FCS was incubated with PEI beads. PEI bead fraction (+) and untreated virus suspension (−) were then boiled with SDS sample buffer and applied to SDS-PAGE. Serum protein bands concentrated by PEI beads were identified by MS/MS analysis, as shown in Fig. 5.

body to induce the formation of immune complexes. Anti-mouse IgG rabbit IgM antibody was prepared from rabbit anti-mouse IgG antiserum and purified by a mouse-IgG affinity column followed by a PEI-sepharose-6MB column. Since the PEI-sepharose-6MB column adsorbed IgM (Fig. 5) but not IgG (data not shown), the PEI-sepharose-6MB adsorbed fraction was used as the anti-mouse IgG rabbit IgM antibody. When poliovirus alone was incubated with the PEI beads, it was not adsorbed, but poliovirus was adsorbed when coincubated with anti-poliovirus IgG antibody, and a further significant improvement in the efficiency of virus concentration was achieved by the addition of anti-mouse IgG rabbit IgM along with the anti-poliovirus IgG (Fig. 6). The addition of the combination of complement C1, complement C4 and anti-poliovirus IgG to the reaction mixture of virus and PEI beads also increased the efficiency of virus concentration by PEI beads, but only when the complement system was activated by [incubation at] 37 °C (Fig. 6).

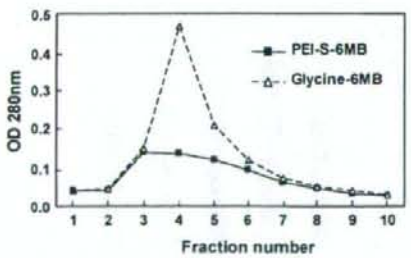
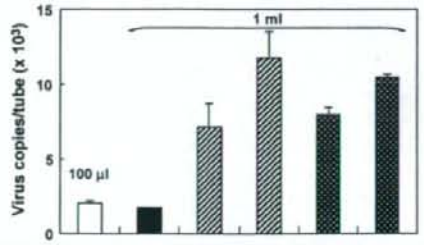


Fig. 5. Adsorption of IgM to a PEI-sepharose column. One ml of human IgM solution (1 mg/ml) was applied to a PEI-sepharose 6MB (PEI-S-6MB) column or to a control column without PEI (Glycine-6 MB) and washed with PBS (−). The eluates were fractionated into ten 1 ml fractions, and the OD280 of each fraction was determined using a spectrophotometer.



PEI beads	-	+	+	+	+	+
Anti-poliovirus mouse IgG MoAb	-	-	+	+	+	+
Anti-mouse IgG-rabbit IgM	-	-	-	+	-	-
C1 + C4	-	-	-	-	+ (r.t.)	+ (37°C)

Fig. 6. Concentration of poliovirus by PEI beads via the formation of immune complexes. Poliovirus suspension (2×10^4 copies/ml, 1 ml each) was incubated with PEI beads at room temperature or 37 °C in the absence or presence of anti-poliovirus mouse IgG monoclonal antibody, anti-mouse IgG-rabbit IgM, or a combination of complements C1 and C4. Viral genome RNA was extracted from the PEI bead fraction and from the untreated virus suspension (100 µl). Virus copy numbers were determined by real-time RT-PCR. Data are expressed as the mean ± S.D. ($n = 3$).

3.4. Application of the virus concentration method using PEI beads to human hepatitis viruses

The virus concentration method using PEI beads was applied to human HAV, HBV and HCV. Fig. 7 shows the effect of pH on the virus concentration efficiency. HAV was efficiently adsorbed by the PEI beads (Fig. 7A). The number of viral copies obtained in the PEI bead fraction when using 1 ml of virus suspension was about 10-fold the number extracted from untreated virus suspension (100 µl), suggesting that the concentration of HAV almost reached the predicted level. Neither the presence or absence of serum nor the pH condition affected the efficiency of the HAV concentration. HCV was also efficiently adsorbed by PEI beads, even in the presence of 2% FCS, and the optimum pH was found to be 5 (Fig. 7C). On the other hand, the efficiency of HBV concentration by PEI beads was lower than the efficiencies of HAV and HCV concentrations. The number of viral copies obtained in the PEI bead fraction under the optimum condition of pH 5 without serum was about six-fold the number extracted from untreated virus suspension (Fig. 7B). The presence of FCS significantly reduced the adsorption of HBV by PEI beads.

In order to improve the concentration of HBV obtained by PEI beads, anti-HBV IgM antibody was prepared and the concentration of HBV via immune complex formation was examined. As shown in Fig. 8, the concentration of HBV by PEI beads was improved by the addition of anti-HBV IgM antibody. Under the optimum condition, the number of viral copies obtained in the PEI bead fraction was more than seven-fold the number extracted from the untreated virus suspension even in the

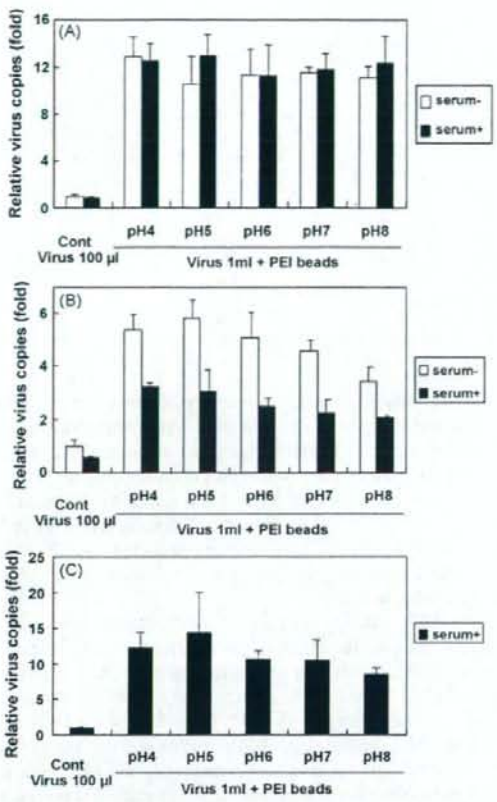


Fig. 7. Effect of pH on the concentration of HAV, HBV and HCV by PEI beads. HAV (A), HBV (B), and HCV (C) were diluted with virus dilution media of different pH levels supplemented with or without 2% FCS. Virus suspensions (HAV: 5×10^4 PFU/ml; HBV: 8.8×10^3 IU/ml; HCV: 1×10^3 IU/ml; 1 ml/tube) with different pH levels were incubated with PEI beads. Viral genome DNA and RNA were then extracted from PEI bead fraction and analyzed by real-time PCR and RT-PCR. Data are expressed as the mean ± S.D. ($n = 3$).

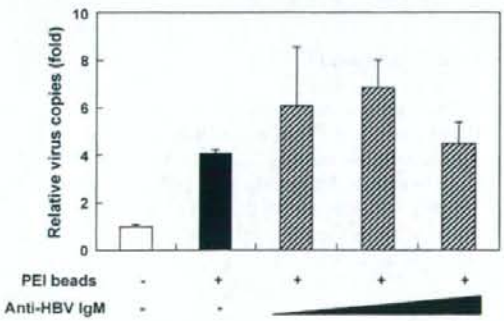


Fig. 8. Effect of anti-HBV IgM antibody for HBV concentration by PEI-beads. HBV suspensions (8.8×10^3 IU/ml; 1 ml/tube) were incubated with PEI beads in the absence or presence of 5, 15 or 50 µl of anti-HBV IgM antibody. Viral genome DNA was then extracted from the PEI bead fraction and analyzed by real-time PCR. Data are expressed as the mean ± S.D. ($n = 3$).

presence of serum. Therefore, the virus concentration achieved by PEI beads was shown to be enhanced by the formation of immune complexes.

Table 2 shows a summary of virus concentrations by PEI beads for all of the viruses examined. A wide range of viruses, including small non-enveloped viruses and human hepatitis viruses (HAV, HBV and HCV), were efficiently concentrated by PEI beads under the optimum condition, either directly or by the formation of immune complexes.

4. Discussion

In the present study, the virus concentration method using PEI beads (Satoh et al., 2003) was optimized, and was applied to human hepatitis A, B and C viruses.

First, the effects of various cationic polymers, PEI molecular weights, and pH values were examined in order to determine the optimal conditions for virus concentration. Among PEI beads with three different molecular weights (1800, 10,000 and 70,000 Da), only the PEI whose MW was 70,000 Da was able to adsorb viruses (Fig. 1). With respect to the cationic polymers, PEI magnetic beads showed a higher virus adsorption ability than PAA- or PLL-conjugated magnetic beads (Fig. 2). The optimum pH for the concentration of model viruses by PEI beads was subacidic (Fig. 3). The virus adsorption mechanism of PEI beads remains unclear. However, it is hypothesized that the positively charged field of the PEI molecule may interact tightly with the negative charge of surface lipids or the negatively charged surface proteins on viruses (Satoh et al., 2003). PEI is a polycationic polyamine with the highest cationic charge density among existing polymers (Futami et al., 2005). PEI has a branched backbone containing primary, secondary and tertiary amine groups. In contrast, PAA is a linear polycation having only primary amine groups, and PLL is a linear polycation with primary and secondary amine groups. Therefore, it is suggested that the high-density cationic charge of PEI and its branched structure on the surface of the magnetic beads may be important for efficient virus adsorption. According to the analysis of Owada et al. (1999), the interaction between PEI-coated membranes and human immunodeficiency virus type 1 (HIV-1) or plasma protein may be dependent on the surface area of each particle, and this fundamental principle was consistent with their observation that PEIs with higher MWs bound more intensely to HIV-1. This is also consistent with the data that PEI with a MW of 70,000 Da was able to adsorb viruses more efficiently than PEIs of 1800 Da or 10,000 Da.

In order to improve the efficiency of virus concentration by PEI beads, the serum components co-adsorbed by the beads were analyzed. MS analysis revealed that several proteins, including complement type 3, complement type 4 and IgM, were specifically co-adsorbed by PEI beads during virus concentration (Fig. 4), suggesting that the beads were able to adsorb immune complexes that involved IgM antibody and/or complements. Therefore, it is hypothesized that in addition to direct adsorption, PEI beads may adsorb viruses via the formation of immune complexes. This hypothesis was confirmed by the fact that PEI beads were able to adsorb poliovirus under con-

Table 2
Summary of concentration of viruses by PEI beads

Viruses	Natural host	Virus genome	Envelope	Size (nm)	PEI beads concentration
Model viruses cytomegalovirus (CMV)	Simian	DNA	+	180–200	+
Herpes symplex virus type 1 (HSV-1)	Human	DNA	+	150–200	+
Vesicular stomatitis virus (VSV)	Bovine	RNA	+	70–150	+
Amphotropic murine leukemia virus	Murine	RNA	+	80–110	+
Sindbis virus	Human	RNA	+	60–70	+
Adenovirus type 5	Human	DNA	−	70–90	+
Simian virus 40 (SV40)	Simian	DNA	−	40–50	+
Porcine parvovirus (PPV)	Porcine	DNA	−	18–24	+
Poliovirus sabin 1	Human	RNA	−	25–30	+[a]
Human hepatitis viruses hepatitis B virus (HBV)	Human	DNA	+	40–45	+[a]
Hepatitis C virus (HCV)	Human	RNA	+	40–50	+
Hepatitis A virus (HAV)	Human	RNA	−	25–30	+

[a] Concentrated by the addition of antibodies.

ditions which fostered immune complex formation, such as the addition of anti-poliovirus mouse IgG antibody with anti-mouse IgG rabbit IgM, or the addition of anti-poliovirus IgG antibody with activated complements (Fig. 6). Poliovirus is a very small (25–30 nm) non-enveloped virus, and could not be concentrated by PEI beads in our previous study (Satoh et al., 2003). Another possible explanation is that the increase in the surface area of virus particles due to the formation of immune complexes enhances the interaction between the poliovirus and the PEI beads, as hypothesized by Owada et al. (1999).

The results obtained from model viruses suggest that the virus concentration method using PEI beads may be applicable to a wide range of viruses. Therefore, this method was applied to human hepatitis viruses. A recent study reported that in some HAV patients, the duration of the viremic phase persisted for more than 1 year with low viral load levels (10^3–10^4 HAV genome equivalents/ml) (Normann et al., 2004). In the case of HBV, the presence of occult HBV infection (HBV DNA positivity in the setting of negative serum hepatitis B surface antigen) has been documented, and the majority of these infections were associated with low viral loads (<10^5 copies/ml) (Minuk et al., 2004). Several studies have demonstrated high rates of transmission of HCV through transfusions with extremely low viral loads (Operskalski et al., 2003). HCV is particularly infectious during the early window period, with levels as low as 1 viral copy in 20 ml plasma able to transmit infection by transfusion (Busch et al., 2003), though intermittent low-level HCV viremia can occur as long as 2 months before the periods of exponential increase in viral load (Glynn et al., 2005). Therefore, it is extremely important to develop a highly sensitive detection method for these viruses. In the present study, it was possible to concentrate HAV and HCV by PEI-beads to almost the predicted levels (Fig. 7). In contrast, HBV was not fully concentrated even under optimum conditions around pH 5. Therefore, the concentration of HBV via the formation of immune complexes was tested. As expected, the concentration of HBV was improved by the addition of anti-HBV IgM antibody (Fig. 8), indicating that the virus concentration method using PEI beads is applicable for the concentration and sensitive detection of HAV, HBV and HCV by PCR and RT-PCR reaction.

To enhance/establish the utility of this virus concentration method using PEI meads for viral safety of biological products and cell therapy products, examination using actual patient sera and different genotypes/subtypes of each virus may be required. In a preliminary experiment, it is confirmed that this PEI beads method can be used for hepatitis virus samples spiked in human plasma. Applicability to different genotypes will be examined using a Japanese genotype panel of HBV and HCV, which will be available soon.

PEI beads may be applicable not only for virus concentration but also for the efficient infection of viruses. Scherer et al. (2002) report that superparamagnetic nanoparticles coated with PEI enhanced the infection of adenovirus and retrovirus vectors under a magnetic field. This infection method (magnetofection) also enhanced the infection of measles virus (Kadota et al., 2005). In a preliminary experiment, the PEI beads used in the present study also enhanced the infectivity of several viruses under a magnetic field. Therefore, it is suggested that PEI beads may be useful for the sensitive detection of both virus genomes and virus infectivity.

In conclusion, the present study demonstrates that the virus concentration method using PEI beads is effective for the concentration and sensitive detection of a wide range of viruses, including HAV, HBV and HCV.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Health and Labor Science Research (H17-SAISEI-021) from the Japanese Ministry of Health, Labor and Welfare, as well as by a Grant-in-Aid for Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation. The authors are grateful to Ms. Momoko Komesu for her technical assistance.

References

Alter, H.J., Sanchez-Pescador, R., Urdea, M.S., Wilber, J.C., Lagier, R.J., Di Bisceglie, A.M., Shih, J.W., Neuwald, P.D., 1995. Evaluation of branched DNA signal amplification for the detection of hepatitis C virus RNA. J. Viral Hepat. 2, 121–132.

Busch, M.P., Hirschkorn, D.F., Herring, B.L., Delwart, E.L., McAuley, J., Murthy, K.K., Alter, H.J., 2003. Defining the minimal infectious dose and infectious wind[o]w period for HCV using plasma donor panels and the chimpanzee transfusion model. Transfusion 43, S107–S140.

Futami, J., Kitazoe, M., Maeda, T., Nukui, E., Sakaguchi, M., Kosaka, J., Miyazaki, M., Kosaka, M., Tada, H., Seno, M., Sasaki, J., Huh, N.H., Namba, M., Yamada, H., 2005. Intracellular delivery of proteins into mammalian living cells by polyethylen[e]imine-cationization. J. Biosci. Bioeng. 99, 95–103.

Glynn, S.A., Wright, D.J., Kleinman, S.H., Hirschkorn, D., Tu, Y., Heldebrant, C., Smith, R., Giachetti, C., Gallarda, J., Busch, M.P., 2005. Dynamics of viremia in early hepatitis C virus infection. Transfusion 45, 994–1002.

Ishii-Watabe, A., Uchida, E., Iwata, A., Nagata, R., Satoh, K., Fan, K., Murata, M., Mizuguchi, H., Kawasaki, N., Kawanishi, T., Yamaguchi, T., Hayakawa, T., 2003. Detection of replication-competent adenoviruses spiked into recombinant adenovirus vector products by infectivity PCR. Mol. Ther. 8, 1009–1016.

Jothikumar, N., Cromeans, T.L., Sobsey, M.D., Robertson, B.H., 2005. Development and evaluation of a broadly reactive TaqMan assay for rapid detection of hepatitis A virus. Appl. Environ. Microbiol. 71, 3359–3363.

Kadota, S., Kanayama, T., Miyajima, N., Takeuchi, K., Nagata, K., 2005. Enhancing of measles virus infection by magnetofection. J. Virol. Meth. 128, 61–66.

Kamisango, K., Kamogawa, C., Sumi, M., Goto, S., Hirao, A., Gonzales, F., Yasuda, K., Iino, S., 1999. Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. J. Clin. Microbiol. 37, 310–314.

Kern, D., Collins, M., Fultz, T., Detmer, J., Hamren, S., Peterkin, J.J., Sheridan, P., Urdea, M., White, R., Yeghiazarian, T., Todd, J., 1996. An enhanced-sensitivity branched-DNA assay for quantification of human immunodeficiency virus type 1 RNA in plasma. J. Clin. Microbiol. 34, 3196–3202.

Martell, M., Gomez, J., Esteban, J.I., Sauleda, S., Quer, J., Cabot, B., Esteban, R., Guardia, J., 1999. High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. J. Clin. Microbiol. 37, 327–332.

Minuk, G.Y., Sun, D.F., Greenberg, R., Zhang, M., Hawkins, K., Uhanova, J., Gutkin, A., Bernstein, K., Giulivi, A., Osiowy, C., 2004. Occult hepatitis B virus infection in a North American adult hemodialysis patient population. Hepatology 40, 1072–1077.

Mizusawa, S., Okada, Y., Horiuchi, Y., Tanaka, T., Sato, K., Kaneko, K., Sasaki, Y., Tanaka, T., Tomono, T., Tomomizu, T., Hayami, S., Hijikata, M., Hirako, I., Mayumi, M., Mikami, K., Mishiro, S., Miyamoto, S., Muta, K., Weimer, T., Gierman, T., Komuro, K., Yamaguchi, T., 2005. Establishment of the first national standard for nucleic acid amplification technology assay for HCV RNA. Jpn. J. Transfus. Med. 51, 515–519.

Normann, A., Jung, C., Vallbracht, A., Flehmig, B., 2004. Time course of hepatitis A viremia and viral load in the blood of human hepatitis A patients. J. Med. Virol. 72, 10–16.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucl. Acids Res. 28, E63.

Operskalski, E.A., Mosley, J.W., Tobler, L.H., Fiebig, E.W., Nowicki, M.J., Mimms, L.T., Gallarda, J., Phelps, B.H., Busch, M.P., 2003. HCV viral load in anti-HCV-reactive donors and infectivity for their recipients. Transfusion 43, 1433–1441.

Owada, T., Miyashita, Y., Motomura, T., Onishi, M., Yamashita, S., Yamamoto, N., 1999. Anti-HIV-1 activity of an ionically modified porous polypropylene membrane determined by filtration of a viral suspension. Microbiol. Immunol. 43, 141–151.

Pas, S.D., Fries, E., De Man, R.A., Osterhaus, A.D., Niesters, H.G., 2000. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. J. Clin. Microbiol. 38, 2897–2901.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A., 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239, 487–491.

Sarrazin, C., Teuber, G., Kokka, R., Rabenau, H., Zeuzem, S., 2000. Detection of residual hepatitis C virus RNA by transcription-mediated amplification in patients with complete virologic response according to polymerase chain reaction-based assays. Hepatology 32, 818–823.

Satoh, K., Iwata, A., Murata, M., Hikata, M., Hayakawa, T., Yamaguchi, T., 2003. Virus concentration using polyethyleneimine-conjugated magnetic beads for improving the sensitivity of nucleic acid amplification tests. J. Virol. Meth. 114, 11–19.

Scherer, F., Anton, M., Schillinger, U., Henke, J., Bergemann, C., Kruger, A., Gansbacher, B., Plank, C., 2002. Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo. Gene Ther. 9, 102–109.

Uchida, E., Sato, K., Iwata, A., Ishii-Watabe, A., Mizuguchi, H., Hikata, M., Murata, M., Yamaguchi, T., Hayakawa, T., 2004. An improved method for detection of replication-competent retrovirus in retrovirus vector products. Biologicals 32, 139–146.

Willkommen, H., Schmidt, I., Lower, J., 1999. Safety issues for plasma derivatives and benefit from NAT testing. Biologicals 27, 325–331.

HUMAN GENE THERAPY 18:74–80 (January 2007)

DOI: 10.1089/hum.2006.129

Technical Report

Rapid Construction of Small Interfering RNA-Expressing Adenoviral Vectors on the Basis of Direct Cloning of Short Hairpin RNA-Coding DNAs

HIROYUKI MIZUGUCHI,[1,2] NAOKO FUNAKOSHI,[1] TETSUJI HOSONO,[3] FUMINORI SAKURAI,[1] KENJI KAWABATA,[1] TERUHIDE YAMAGUCHI,[3] and TAKAO HAYAKAWA[4]

ABSTRACT

In the conventional method for constructing an adenoviral (Ad) vector expressing small interfering RNA (siRNA), short hairpin RNA (shRNA)-coding oligonucleotides are introduced downstream of a polymerase III (or polymerase II)-based promoter cloned into a shuttle plasmid. An siRNA expression cassette, which is cloned into the shuttle plasmid, is then introduced into the E1 deletion region of the Ad vector plasmid by *in vitro* ligation or homologous recombination in *Escherichia coli*, and the linearized plasmid is transfected into 293 cells, generating an Ad vector expressing siRNA. Therefore, two-step plasmid manipulation is required. In this study, we developed a method by which shRNA-coding oligonucleotides can be introduced directly into the Ad vector plasmid. To do this, we constructed a new vector plasmid into which the human U6 promoter sequence was cloned in advance. Unique restriction enzyme sites were introduced at the transcription start site of the U6 promoter sequence in the vector plasmid. Luciferase and p53 genes were efficiently knocked down by Ad vectors generated by the new method and expressing siRNA against the target gene. This method should be useful for RNA interference-based experiments, and should make it easy to construct an siRNA-expressing Ad vector library for functional screening.

INTRODUCTION

RNA INTERFERENCE (RNAi), which mediates the sequence-specific suppression of gene expression in a wide variety of eukaryotes by double-stranded RNA homologies to the target gene (Scherer and Rossi, 2003), is a powerful tool for the knockdown of gene expression. Transduction of synthetic small interfering RNA (siRNA; 19 to 29 nucleotides of RNA) or the promoter-based expression of siRNA in the cells results in sequence-dependent degradation of target mRNA and subsequent reduction of target gene expression. Most promoter-based RNAi systems express short hairpin RNA (shRNA), which is then trimmed by Dicer, generating functional siRNA. Polymerase III-based promoters, such as the small nuclear RNA U6 promoter or the human RNase P RNA H1 promoter, are widely used for the expression of shRNA (siRNA) (Scherer and Rossi, 2003), although polymerase II-based promoters are also used (Xia *et al.*, 2002; Shinagawa and Ishii, 2003). The promoter-based method has an advantage in that viral vectors as well as nonviral vectors can be used for delivery of the siRNA expression unit, whereas only nonviral vectors are used for delivery of synthetic siRNA.

Recombinant adenoviral (Ad) vectors have been used extensively to deliver foreign genes to a variety of cell types and tissues both *in vitro* and *in vivo* (McConnell and Imperiale, 2004; Volpers and Kochanek, 2004). They can be easily grown to high titers and can efficiently transfer genes into both dividing and nondividing cells. Therefore, Ad vector-mediated

[1]National Institute of Biomedical Innovation, Osaka 567-0085, Japan.
[2]Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 567-0871, Japan.
[3]National Institute of Health Sciences, Tokyo 158-8501, Japan.
[4]Pharmaceuticals and Medical Devices Agency, Tokyo 100-0013, Japan.

delivery of an siRNA expression unit, in which a promoter-based shRNA expression cassette is delivered into the cell by the Ad vector, provides a valuable tool for both gene function studies and therapeutic applications.

Construction of Ad vectors used to be a time-consuming and labor-intensive procedure, but several improved methods to facilitate the construction of Ad vectors have been developed (reviewed in Mizuguchi *et al.*, 2001). The homologous recombination method in E1-complementing cell lines (i.e., 293 cells) has been the most widely used method for generating recombinant Ad vectors, and it has greatly contributed to the widespread use of Ad vectors (Bett *et al.*, 1994). The major limitations of this approach are the low frequency of the recombination event and the tedious and time-consuming plaque purification procedure required to select the recombinant virus of interest, because a relatively high percentage of the virus produced is wild type (in most cases, 20–70%), due to recombination with the Ad sequence integrated into the chromosomes of 293 cells. The improved *in vitro* ligation method (Mizuguchi and Kay, 1998, 1999) and the homologous recombination method in *Escherichia coli* (He *et al.*, 1998), which are commercially available from Clontech (Palo Alto, CA) and Invitrogen (Carlsbad, CA), respectively, have now become widely used, because these systems overcome the limitations of the homologous recombination method in 293 cells. To construct an Ad vector expressing siRNA by these two methods, shRNA-coding oligonucleotides are introduced downstream of the polymerase III (or polymerase II)-based promoter cloned in a shuttle plasmid. An shRNA (siRNA) expression cassette, which is cloned in the shuttle plasmid, is then introduced into the E1 deletion region of the Ad vector plasmid, which clones a full Ad genome, by simple *in vitro* ligation or homologous recombination in *E. coli*. The resulting plasmid is then linearized and transfected into 293 cells, generating an Ad vector expressing siRNA. Therefore, two-step *E. coli* transformation and plasmid manipulation is required for the improved *in vitro* ligation method, whereas three-step *E. coli* transformation and plasmid manipulation is required in the homologous recombination method in *E. coli* (because a special *E. coli* strain is used in the latter method, retransformation into a normal stain of *E. coli* is required) (reviewed in Mizuguchi *et al.*, 2001).

In the present study, we developed a simple method for generating an Ad vector expressing siRNA, in which shRNA-coding oligonucleotides could be directly introduced into an Ad vector plasmid containing the human U6 (hU6) promoter sequence. Unique restriction enzyme sites were introduced at the transcription start site of the hU6 promoter sequence cloned into the Ad vector plasmid. Two types of modified hU6 promoter sequence were constructed to develop this method. Using this method, only one-step *E. coli* transformation is required to generate an Ad vector plasmid containing an siRNA expression cassette.

MATERIALS AND METHODS

Cells

A549 and 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). A549-Luc cells, which are stable transformants with luciferase expression, were cultured in DMEM supplemented with 10% FCS. For construction of A549-Luc cells, A549 cells were transfected with luciferase-expressing plasmid pGL3-Control-RSVneo, which contains the simian virus 40 (SV40) promoter/enhancer-luciferase cDNA-SV40 p(A) sequence and the neomycin expression cassette, using SuperFect transfection reagent (Qiagen, Valencia, CA). pGL3-Control-RSVneo was constructed by insertion of the Rous sarcoma virus (RSV) promoter-driven neomycin expression cassette into pGL3-Control (Promega, Madison, WI). Monoclonal A549 cells stably expressing luciferase (A549-Luc) were obtained by geneticin (G418) selection.

Plasmid and virus

The hU6 promoter sequence was amplified from human genomic DNA (Clontech), using the following primers: hU6-S1, hU6-AS1, and hU6-AS2 (Table 1). The hU6a and hU6b promoter sequences were amplified with hU6-S1/hU6-AS1 and hU6-S1/hU6-AS2 primer sets, respectively (see Fig. 2). These promoter sequences were introduced into pHM5 (Mizuguchi and Kay, 1999), and were then transferred into the E1 deletion region of the vector plasmid pAdHM4.1, a derivative of pAdHM4 (Mizuguchi and Kay, 1998) (the *Xba*I site outside the Ad genome of pAdHM4 was deleted), by an *in vitro* ligation method using the I-*Ceu*I and PI-*Sce*I sites (Mizuguchi and Kay, 1998, 1999), resulting in pAdHM4-hU6a and pAdHM4-hU6b, respectively (Fig. 1A). To construct a vector plasmid containing an shRNA-coding sequence against luciferase, oligonucleotides 1/2 and 3/4 were synthesized (Table 1), annealed, and cloned into the *Cla*I and *Xba*I sites of pAdHM4-hU6a or the *Swa*I and *Xba*I sites of pAdHM4-hU6b, generating pAdHM4-hU6a-Lu and pAdHM4-hU6b-Lu, respectively. The target sequence for siRNA is bp 158 to 176 of luciferase cDNA. For the construction of vector plasmid containing shRNA-coding sequence against p53 (Brummelkamp *et al.*, 2002), oligonucleotides 5/6 and 7/8 were used for cloning into the *Cla*I and *Xba*I sites of pAdHM4-hU6a or the *Swa*I and *Xba*I sites of pAdHM4-hU6b, generating pAdHM4-hU6a-p53 and pAdHM4-hU6b-p53, respectively. The target sequence for siRNA is bp 775 to 793 of human p53 cDNA.

The original intact hU6 promoter sequence, derived from an *Eco*RI/*Sal*I fragment of piGene hU6 (iGENE Therapeutics, Tsukuba, Japan), was also introduced into the *Sph*I and *Sal*I sites of pHM5 (Mizuguchi and Kay, 1999), resulting in pHM5-ihU6. pHM5-ihU6 was then digested with *Sal*I and *Xba*I, and ligated with oligonucleotides 9 and 10, resulting in pHM5-hU6. In this case, oligonucleotides 11/12 and 13/14 (for the shRNA-coding sequence against luciferase and p53, respectively) were introduced into the *Bsp*MI site of pHM5-hU6 according to the report of Miyagishi *et al.* (2004) and the manufacturer's instructions (iGENE Therapeutics); and then an siRNA expression cassette was inserted into the E1-deletion region of pAdHM4 (Mizuguchi and Kay, 1998), using the I-*Ceu*I and PI-*Sce*I sites, resulting in pAdHM4-hU6-Lu and pAdHM4-hU6-p53, respectively. The sequence was verified with a DNA sequencer (ABI PRISM 310; Applied Biosystems, Foster City, CA).

Viruses (Ad-hU6-Lu, Ad-hU6a-Lu, Ad-hU6b-Lu, Ad-hU6-p53, Ad-hU6a-p53, and Ad-hU6b-p53) were prepared by the

TABLE 1. OLIGONUCLEOTIDES USED IN THE PRESENT STUDY

Oligonucleotide	*Sequence of oligonucleotide (5'–3')*
hU6-S1 primer	aaggtcgggcaggaagagggcctа
hU6-AS1 primer	ggtctagaagt*atcga*tttcgtctttccacaagatatat (*Xba*I and *Cla*I recognition sequences are underlined and italicized, respectively)
hU6-AS2 primer	ggtctagaagt*atttaaat*cgtcctttccacaagatatataa (*Xba*I and *Swa*I recognition sequences are underlined and italicized, respectively)
Oligonucleotide 1	c*gacgctgagtacttcgaaat*tcaagagaa*atttcgaagtactcagcg*ttttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 2	ctagatttccaaaaaa*cgctgagtacttcgaaat*ctcttgaa*atttcgaagtactcagcgt* (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 3	cc*acgctgagtacttcgaaat*tcaagagaa*atttcgaagtactcagcg*ttttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 4	ctagatttccaaaaaa*cgctgagtacttcgaaat*ctcttgaa*atttcgaagtactcagcgtgg* (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 5	c*ggactccagtggtaatctac*ttcaagaga*gtagattaccactggagtc*ttttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 6	ctagatttccaaaaa*gactccagtggtaatctac*tctcttgaa*gtagattaccactggagtc* (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 7	cc*gactccagtggtaatctac*ttcaagaga*gtagattaccactggagtc*ttttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 8	ctagatttccaaaaa*gactccagtggtaatctac*tctcttgaa*gtagattaccactggagtc*gg (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 9	tcgacctgcaggcatgcaagcttc (BspMI recognition sequences are underlined)
Oligonucleotide 10	ctaggaagcttgcatgcctgcagg (BspMI recognition sequences are underlined)
Oligonucleotide 11	cacc*acgctgagtacttcgaaat*ttcaagaga*atttcgaagtactcagcg*ttttt (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 12	gcataaaaaa*cgctgagtacttcgaaat*ctcttgaa*atttcgaagtactcagcgt* (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 13	cacc*gactccagtggtaatctac*ttcaagaga*gtagattaccactggagtc*ttttttt (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 14	gcataaaaaa*gactccagtggtaatctac*tctcttgaa*gtagattaccactggagtc* (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)

transfection of a *Pac*I-linearized vector plasmid (pAdHM4-hU6-Lu, pAdHM4-hU6a-Lu, pAdHM4-hU6b-Lu, pAdHM4-hU6-p53, pAdHM4-hU6a-p53, and pAdHM4-hU6b-p53, respectively) into 293 cells as described previously (Mizuguchi and Kay, 1998). Ad vectors containing only the original intact hU6 promoter sequence (without a target sequence; Ad-hU6) were similarly constructed with pHM5-hU6 and pAdHM4. The virus was purified by $CsCl_2$ gradient centrifugation; dialyzed with a solution containing 10 m*M* Tris (pH 7.5), 1 m*M* $MgCl_2$, and 10% glycerol; and stored in aliquots at −70°C. Determination of virus particle (VP) titers and infectious titers was accomplished spectrophotometrically by the method of Maizel *et al.* (1968) and with an Adeno-X rapid titer kit (Clontech), respectively. The infectious titer-to-particle ratio was 1:36 for Ad-hU6, 1:31 for Ad-hU6-Lu, 1:28 for Ad-hU6a-Lu, 1:24 for Ad-hU6b-Lu, 1:22 for Ad-hU6-p53, 1:12 for Ad-hU6a-p53, and 1:15 for Ad-hU6b-p53.

Adenovirus-mediated gene transduction and luciferase assay

A549 cells (2×10^5 cells) were seeded into a 12-well dish. The next day, they were transduced with the Ad vectors for 1.5 hr. Determination of luciferase production in the cells and extraction of cellular protein for Western blotting were performed after a 72-hr culture period. Luciferase production in the cells was measured with a luciferase assay system (PicaGene LT 2.0; produced by Toyo Ink [Tokyo, Japan] for Wako [Kyoto, Japan])

Western blotting for p53

Cell extracts were prepared in lysis buffer (25 m*M* Tris [pH 7.5], 1% Triton X-100, 0.5% sodium deoxycholate, 5 m*M* EDTA, 150 m*M* NaCl) containing a cocktail of protease inhibitors (Sigma, St. Louis, MO). The protein content was measured

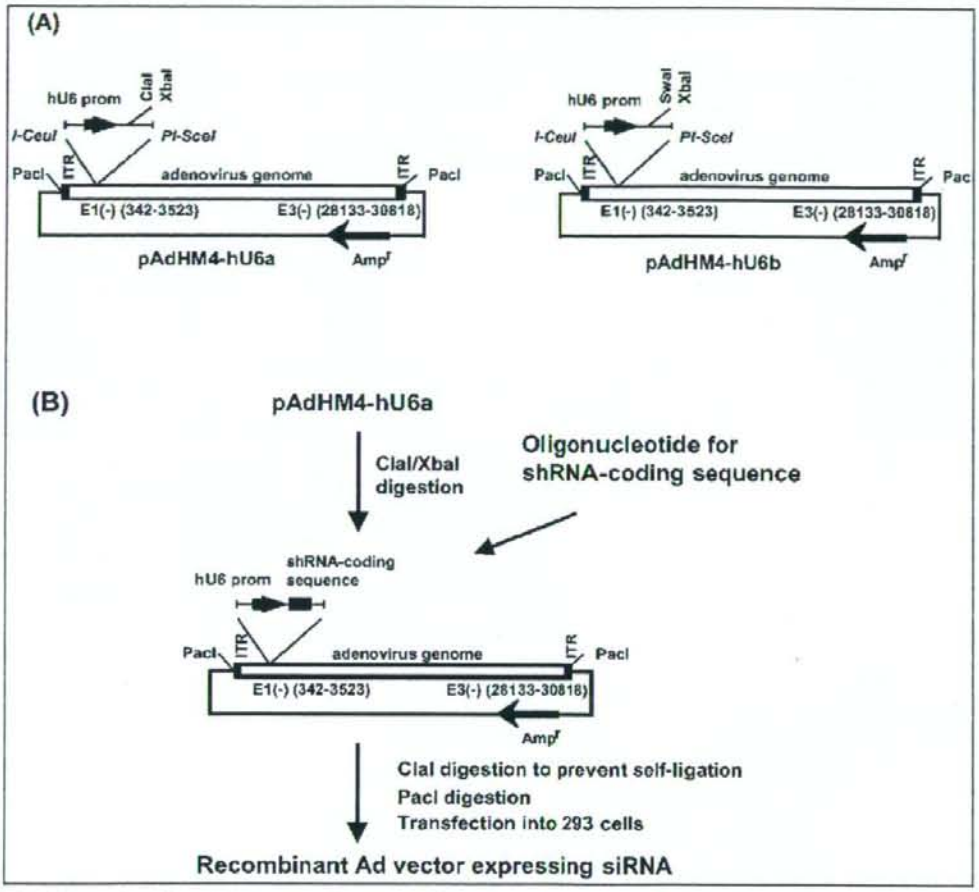


FIG. 1. Vector plasmids and the construction strategy for Ad vectors expressing siRNA. (**A**) Vector plasmids pAdHM4-hU6a and pAdHM4-hU6b. pAdHM4-hU6a contains a unique *ClaI* site at the transcription start site of the hU6 promoter sequence and an *XbaI* site downstream from the promoter sequence. pAdHM4-hU6b contains a unique *SwaI* site at the transcription start site of the hU6 promoter sequence and an *XbaI* site downstream from the promoter sequence. (**B**) Construction strategy for the Ad vector expressing siRNA. pAdHM4-hU6a was digested with *ClaI*/*XbaI* and ligated with oligonucleotides for the shRNA-coding sequence. Ligation products were then digested with *ClaI* to prevent the generation of nonrecombinant parental plasmid. The resulting plasmid was linearized by digestion with *PacI* and transfected into 293 cells, generating recombinant Ad vectors expressing siRNA. pAdHM4-hU6b was similarly used.

with a Bio-Rad assay kit (Bio-Rad, Hercules, CA), using bovine serum albumin as the standard. Protein samples (10 μg) were electrophoresed on sodium dodecyl sulfate (SDS)– 12.5% polyacrylamide gels under reducing conditions, followed by electrotransfer to Immobilon-P membranes (Millipore, Bedford, MA). After blocking in nonfat dry milk, the filters were incubated with antibodies against p53 (Santa Cruz Biotechnology, Santa Cruz, CA) and actin (Oncogene Research Products/EMD Biosciences, San Diego, CA), followed by incubation in the presence of peroxidase-labeled goat anti-mouse IgG antibody (American Qualex Antibodies, San Clemente, CA) or peroxidase-labeled goat anti-mouse IgM antibody (Oncogene Research Products/EMD Biosciences). The filters were developed by chemiluminescence (ECL Western blotting detection system; GE Healthcare, Piscataway, NJ). The signals were read with an LAS-3000 (Fujifilm, Tokyo, Japan), and quantified with Image Gauge software (Fujifilm).

RESULTS AND DISCUSSION

Rapid and efficient construction of Ad vectors expressing siRNA offers the promise of using RNAi in the context of both gene function analysis and therapeutic applications. In the present study, we developed a simple method for constructing Ad vectors expressing siRNA, based on only one-step *in vitro* ligation. To do this, we first constructed an Ad vector plasmid containing the E1- and E3-deleted Ad genome and the hU6 pro-

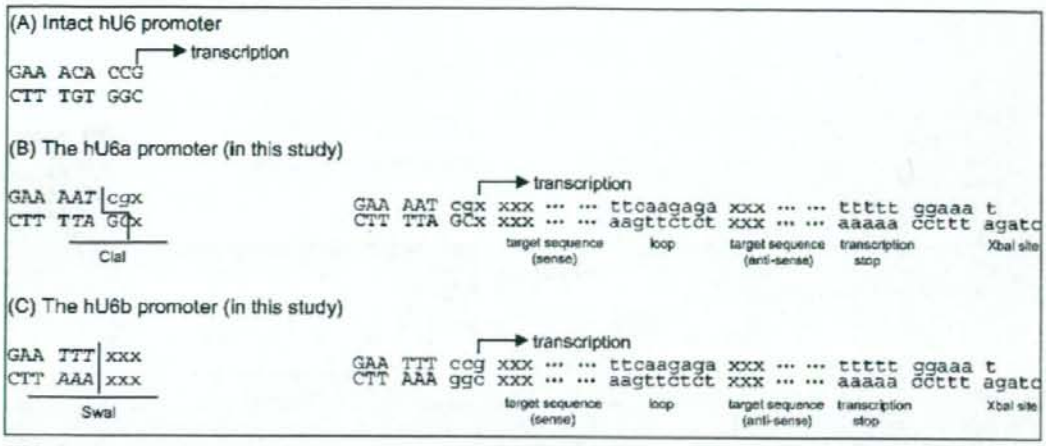


FIG. 2. Sequences at the transcription start site of the new hU6 promoter. (**A**) Intact hU6 promoter sequence. (**B**) hU6a promoter sequence. In this promoter, the *ClaI* site is placed at the transcription start site. (**C**) hU6b promoter sequence. In this promoter, a *SwaI* site is placed at the transcription start site. shRNA-coding oligonucleotides to be synthesized for each promoter are shown as lower-case letters on the right-hand side.

moter sequence in the E1 deletion region, pAdHM4-hU6a and pAdHM4-hU6b (Fig. 1A). By introducing the hU6 promoter sequence into the vector plasmid in advance, the cloning step of the gene of interest from the shuttle plasmid to the vector plasmid, which is an essential step in the conventional method for constructing Ad vectors (namely, the improved *in vitro* ligation method [Mizuguchi and Kay, 1998, 1999] and homologous recombination method in *E. coli* [He *et al.*, 1998]), can be skipped. To make it possible to directly clone the shRNA-coding oligonucleotides downstream of the hU6 promoter sequence, hU6 promoters containing unique restriction enzyme sites at the transcription start site have been developed. The new hU6 promoter sequences contain a *ClaI* or *SwaI* site around the transcription start site and an *XbaI* site downstream from the promoter (Figs. 1 and 2). These enzyme sites were selected because they do not cut the E1- and E3-deleted Ad genome. Because the transcription of shRNA might be influenced by the mutated sequences around the transcription start site, two types of hU6 promoters, differing by only a few nucleotides, were constructed. The hU6a promoter sequence contains a *ClaI* site, whereas the hU6b promoter sequence contains a *SwaI* site. *ClaI*, *SwaI*, and *XbaI* sites are unique in the vector plasmids pAdHM4-hU6a and pAdHM4-hU6b. To generate a recombinant vector plasmid for Ad vectors expressing siRNA, oligonucleotides for shRNA against the target gene were synthesized, annealed, and ligated with *ClaI*/*XbaI*-digested pAdHM4-hU6a or *SwaI*/*XbaI*-digested pAdHM4-hU6b. Oligonucleotides were designed so that recombinant vector plasmid containing the shRNA-coding sequence is redigested with *XbaI*, but not with *ClaI* or *SwaI*. By designing oligonucleotides like the one described above, the generation of self-ligated plasmid can be avoided by digestion of the ligation products with *ClaI* or *SwaI*. On the right side of Fig. 2, DNA sequences, including the shRNA-coding sequence around the transcription start site of the hU6 promoter, are shown. Oligonucleotides that must be synthesized for the shRNA-coding sequence are shown as lower-case letters. By using the method developed in the present study, we could easily generate Ad vectors expressing siRNAs against luciferase and human p53. More than 90% of the recombinant Ad vector plasmids contained the correct insert. Because the *ClaI*- (or *SwaI*-) and *XbaI*-digested pAdHM4-hU6a and pAdHM4-hU6b can be stored at −20°C, only the ligation-based introduction of oligonucleotides into these sites of the vector plasmid would be required for the construction of an appropriate vector.

To examine the function of Ad vectors expressing siRNA against luciferase (Ad-hU6a-Lu and Ad-hU6b-Lu), the efficiency of knockdown of luciferase expression in A549-Luc cells, which stably express luciferase, was examined by treatment with Ad-hU6a-Lu or Ad-hU6b-Lu (Fig. 3). Ad-hU6-Lu, in which the hU6 promoter contains the original intact sequence even after introduction of an shRNA-coding sequence, was used as a positive control. To generate Ad-hU6-Lu, the shRNA-coding sequence was first introduced downstream from the hU6 promoter sequence cloned into the shuttle plasmid, according to the report of Miyagishi *et al.* (2004) and the manufacturer's instructions (iGENE Therapeutics): the shRNA expression cassette was then introduced into the E1 deletion region of the Ad vector plasmid pAdHM4 (Mizuguchi and Kay, 1998). Transfection of a *PacI*-digested vector plasmid into 293 cells generated Ad-hU6-Lu. Ad-hU6, which contains the intact hU6 promoter without the shRNA-coding sequence, and Ad-hU6-p53, which contains the intact hU6 promoter with the shRNA-coding sequence against human p53, were similarly constructed and used as negative controls. Data showed that Ad-hU6a-Lu and Ad-hU6b-Lu suppressed luciferase expression in A549-Luc cells as efficiently as Ad-hU6-Lu, in a dose-dependent manner (Fig. 3). Ad-hU6 and Ad-hU6-p53 showed no effects on luciferase expression. Ad-hU6a-p53 and Ad-hU6b-p53 (these Ad vectors are used in Fig. 4) also had no influence on luciferase expression (data not shown). The RNAi effect of luciferase expression was relatively weak compared with that of p53 (de-

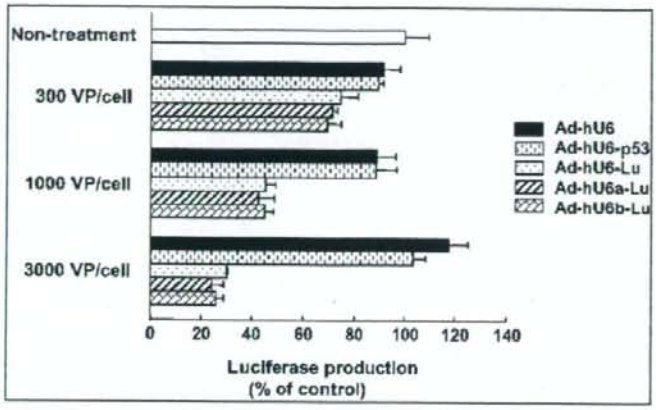


FIG. 3. Suppression of luciferase expression by Ad vector expressing siRNA. A549-Luc cells, which stably express luciferase, were transduced for 1.5 hr with Ad-hU6, Ad-hU6-p53, Ad-hU6-Lu, Ad-hU6a-Lu, or Ad-hU6b-Lu at 300, 1000, or 3000 VP/cell. After culturing for 72 hr, luciferase production in the cells was measured by luminescence assay. Data are expressed as means and SD (n = 4).

scribed below). This difference probably occurred because the A549-Luc cells were expressing luciferase from a strong viral promoter (SV40 promoter and enhancer) and because the levels of luciferase expression were higher than those of endogenous p53 expression.

We next examined the RNAi effect of the siRNA-expressing Ad vector generated in the present study on the endogenous gene. As a model, we silenced p53 expression in A549 cells (Fig. 4). Ad-hU6a-p53 and Ad-hU6b-p53 were generated, and Ad-hU6, Ad-hU6-Lu, and Ad-hU6-p53 were also used. Ad-hU6-p53 contains the intact hU6 promoter sequence, including the transcription start site, even after introduction of the shRNA-coding sequence. A549 cells were transduced with a 300- or 1000-VP/cell of each Ad vector, and cultured for 3 days. Levels of p53 expression were examined by Western blotting. Expression of actin was also measured as an internal control. Expression of p53 in A549 cells was efficiently decreased by treatment with Ad-hU6a-p53 and Ad-hU6b-p53 as well as with Ad-hU6-p53. Levels of p53 expression in cells treated with Ad-hU6-p53, Ad-hU6a-p53, or Ad-hU6b-p53 at 1000 VP/cell were decreased to 7, 2, and 5%, respectively, relative to cells treated with Ad-hU6, according to Image Gauge software (Fujifilm) (in the case of 300 VP/cell, they were decreased to 53, 24, and 30%, respectively). The efficiency of p53 silencing by treatment with Ad-hU6-p53 was slightly lower than that with Ad-hU6a-p53 or Ad-hU6b-p53. This reduced efficiency is likely due to the approximately 1.5 to 2 times lower infectious titer-to-particle titer ratio of Ad-hU6-p53 in comparison with those of Ad-hU6a-p53 and Ad-hU6b-p53. Ad-hU6 and Ad-hU6-Lu did not decrease the level of p53 expression (Fig. 4). These results indicate that new hU6 promoters containing *Cla*I or *Swa*I sites at the transcription start site should transcribe as efficiently as the original hU6 promoter, and that Ad vectors containing the new hU6 promoters efficiently silence target gene expression. Different vector systems (pAdHM4-hU6a and pAdHM4-hU6b) should be used according to the specific purpose.

To facilitate the construction of an siRNA expression plasmid, the U6 and H1 promoters, which contain *Apa*I, *Bbs*I, *Bgl*II, *Eco*RV, *Sal*I, and *Xba*I sites, etc., at the transcription start site, have been developed (Brummelkamp *et al.*, 2002; Lee *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002; Boden *et al.*, 2003). All types of promoters worked efficiently, and could be widely used for efficient RNAi, although the efficiency (activity) of the mutated promoters described above has not been compared with that of the intact promoter. The present study clearly showed that the mutated hU6 promoter, at least one having a *Cla*I or *Swa*I site at the transcription start site and an *Xba*I site downstream of the promoter sequence, is similar in activity to the intact hU6 promoter and would not influence the function of the promoter.

The method using polymerase chain reaction (PCR)-based amplification of shRNA together with the U6 promoter followed by subsequent cloning of the complete expression cassette directly into the Ad vector genome is another strategy for one-step construction of recombinant Ad plasmids containing an siRNA expression cassette. In this method, however, the procedures described below are required for preparation of insert DNA: (1) ordering of the PCR primer, (2) PCR, (3) purification of the PCR product, (4) restriction enzyme digestion and purification of the PCR product, and (5) ligation. In our present system, only the following procedures are required: (1) ordering of the oligonucleotides, (2) hybridization of the oligonucleotides, and (3) ligation. Thus, the present method would be much easier and would allow any laboratory to easily construct

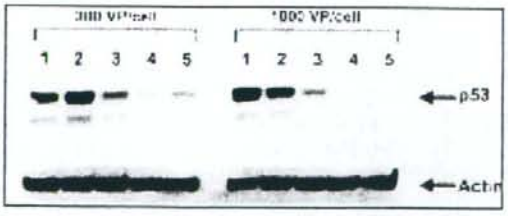


FIG. 4. Suppression of human p53 expression by Ad vector expressing siRNA. A549 cells were transduced for 1.5 hr with Ad-hU6 (lane 1), Ad-hU6-Lu (lane 2), Ad-hU6-p53 (lane 3), Ad-hU6a-p53 (lane 4), or Ad-hU6b-p53 (lane 5) at 300 or 1000 VP/cell, and then cultured for 3 days. Proteins were then extracted from the cells, and the levels of p53 expression were examined by Western blotting. The actin bands served as an internal control for equal total protein loading. The extra (lower) bands of p53 are nonspecific.

Ad vectors expressing siRNA for gene transfer studies and therapeutic applications.

Various types of promoters that are based on polymerase II as well as polymerase III have been developed to transcribe shRNA (siRNA) (Xia *et al.*, 2002; Shinagawa and Ishii, 2003). Although the present study applied the most commonly used U6 promoter for simple and efficient construction of siRNA-expressing Ad vectors, this method could easily be applied to vectors using other promoters including polymerase II-based promoters. This method can also easily be combined with various types of improved Ad vectors, such as Ad vectors containing capsid modification (Koizumi *et al.*, 2003, 2006; Mizuguchi and Hayakawa, 2004; Kurachi *et al.*, 2006) or Ad vectors belonging to different subgroups to modify tropism (Sakurai *et al.*, 2003), and Ad vectors containing a tetracycline-inducible RNAi system (Hosono *et al.*, 2004). The method developed in the present study should be a powerful tool for the application of RNAi, and might facilitate the development of an siRNA-expressing Ad vector library for functional screening.

ACKNOWLEDGMENTS

The authors thank Risako Nagahashi-Nakano and Tomomi Sasaki for technical assistance. This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan.

REFERENCES

BETT, A.J., HADDARA, W., PREVEC, L., and GRAHAM, F.L. (1994). An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc. Natl. Acad. Sci. U.S.A. **91,** 8802–8806.

BODEN, D., PUSCH, O., LEE, F., TUCKER, L., SHANK, P.R., and RAMRATNAM, B. (2003). Promoter choice affects the potency of HIV-1 specific RNA interference. Nucleic Acids Res. **31,** 5033–5038.

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

HE, T.C., ZHOU, S., DA COSTA, L.T., YU, J., KINZLER, K.W., and VOGELSTEIN, B. (1998). A simplified system for generating recombinant adenoviruses. Proc. Natl. Acad. Sci. U.S.A. **95,** 2509–2514.

HOSONO, T., MIZUGUCHI, H., KATAYAMA, K., XU, Z.L., SAKURAI, F., ISHII-WATABE, A., KAWABATA, K., YAMAGUCHI, T., NAKAGAWA, S., MAYUMI, T., and HAYAKAWA, T. (2004). Adenovirus vector-mediated doxycycline-inducible RNA interference. Hum. Gene Ther. **15,** 813–819.

KOIZUMI, N., MIZUGUCHI, H., UTOGUCHI, N., WATANABE, Y., and 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.

KOIZUMI, N., KAWABATA, K., SAKURAI, F., WATANABE, Y., HAYAKAWA, T., and MIZUGUCHI, H. (2006). Modified adenoviral vectors ablated for coxsackievirus–adenovirus receptor, α_v integrin, and heparan sulfate binding reduce *in vivo* tissue transduction and toxicity. Hum. Gene Ther. **17,** 264–279.

KURACHI, S., KOIZUMI, N., SAKURAI, F., KAWABATA, K., SAKURAI, H., NAKAGAWA, S., HAYAKAWA, T. and MIZUGUCHI, H. (2006). Characterization of capsid-modified adenovirus vectors containing heterologous peptides in the fiber knob, protein IX, or hexon. Gene Ther. (in press).

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

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

McCONNELL, M.J., and IMPERIALE, M.J. (2004). Biology of adenovirus and its use as a vector for gene therapy. Hum. Gene Ther. **15,** 1022–1033.

MIYAGISHI, M., SUMIMOTO, H., MIYOSHI, H., KAWAKAMI, Y., and TAIRA, K. (2004). Optimization of an siRNA-expression system with an improved hairpin and its significant suppressive effects in mammalian cells. J. Gene Med. **6,** 715–723.

MIZUGUCHI, H., and HAYAKAWA, T. (2004). Targeted adenovirus vectors. Hum. Gene Ther. **15,** 1034–1044.

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

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

MIZUGUCHI, H., KAY, M.A., and HAYAKAWA, T. (2001). Approaches for generating recombinant adenovirus vectors. Adv. Drug Deliv. Rev. **52,** 165–176.

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

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

SAKURAI, F., MIZUGUCHI, H., and HAYAKAWA, T. (2003). Efficient gene transfer into human $CD34^+$ cells by an adenovirus type 35 vector. Gene Ther. **10,** 1041–1048.

SCHERER, L.J., and ROSSI, J.J. (2003). Approaches for the sequence-specific knockdown of mRNA. Nat. Biotechnol. **21,** 1457–1465.

SHINAGAWA, T., and ISHII, S. (2003). Generation of Ski-knockdown mice by expressing a long double-strand RNA from an RNA polymerase II promoter. Genes Dev. **17,** 1340–1345.

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

VOLPERS, C., and KOCHANEK, S. (2004). Adenoviral vectors for gene transfer and therapy. J. Gene Med. **6,** S164–S171.

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

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

Address reprint requests to:
Dr. Hiroyuki Mizuguchi
National Institute of Biomedical Innovation
Asagi 7-6-8, Saito
Ibaraki, Osaka 567-0085, Japan

E-mail: mizuguch@nibio.go.jp

Received for publication September 3, 2006; accepted after revision November 12, 2006.

Published online: December 22, 2006.

10 ヒト細胞治療薬の品質と安全性確保について

■山口（やまぐち）　照英（てるひで）
国立医薬品食品衛生研究所　生物薬品部

山口　照英
1976年神戸大学大学院理学研究科修工課程終了。同年東京都臨床医学総合研究所炎症研究室研究員，87年国立衛生試験所生物化学部主任研究，91年同第3室長，2002年国立医薬品食品衛生研究所遺伝子細胞医薬部長，06年同生物薬品部長。
研究テーマはバイオ医薬品及び細胞治療薬の品質・安全性確保技術の開発。趣味は読書。

Key words :細胞治療薬，薬事法，細胞・組織加工医薬品，品質と安全性

Abstract
再生医療・細胞治療の開発が急速に進んでいるが，臨床研究や高度先進医療としての開発に比較し薬事法の規制を受ける細胞治療薬の開発が遅れているといわれている。より再生医療・細胞治療を広く国民に提供して行くには，臨床研究等の成果を生かし薬事法の規制のもとに細胞組織加工医薬品等（細胞治療薬；医薬品や医療機器を含む）として開発することが望まれる。本稿では，細胞治療薬を適切に開発して行くために求められるウイルス等の感染因子に対する安全確保，製法の確立や恒常性の維持，品質管理のありかた等について概説した。

はじめに

発生学や幹細胞研究の飛躍的な進展に加え，種々の細胞への分化誘導や増幅法などの培養技術やバイオテクノロジー応用技術の進歩により，ヒトまたは動物の細胞や組織を培養，加工し，さまざまな疾患の治療に用いる細胞治療薬やそれを用いた医療技術の開発が進んでいる。さらに，これらの開発では治験や臨床研究といった異なるアプローチがとられており，医薬品･医療用具といった薬事法上の規制のかかる製品開発を目指す場合ばかりでなく，高度先進医療としての実用化を目指している場合もある。本稿では，特に薬事法の規制を受ける細胞治療薬（細胞・組織加工医薬品等）の指針で求められている開発に当たっての要点を概説する。さらには各国の規制状況との比較を行い，実用化において特に注意を払うべき点について考察したい。

細胞治療薬は，極めて複雑な構造を持ち，かつ生きているというダイナミックな特性を併せ持つことから，従来の医薬品に適用されていた品質管理や，非臨床試験や臨床試験の必要事項は必ずしも適用出来るわけではない。さらに，生きた細胞を投与するために，これまでのバイオ医薬品等のように高度な精製やウイルス不活化・除去工程を適用することが困難であり，安全性に関して特別な配慮が必要とされる。厚生労働省からは，**表1**にあげたような細胞治療薬に関するいくつかの指針や基準が出されている。特に，平成12年に出された医薬発第1314号通知の別添1[1]，及び別添2[2]は，ヒト細胞治療薬の規制の根幹をなす指針である。別添1は，細胞治療薬の製造に当たって，その採取行為から加工，製造における取り扱いや使用に当たっての基本的要件を示している。別添2は，ヒト由来細胞治療薬に焦点をあて，その品質･安全性・有効性確保のための要件をまとめたもので，承認申請のみならず治験前の確認申請で求められる資料についても明らかにされている。この確認申請の制度は，細胞治療薬については未知・未経験の要素が多いことから，その治験

Quality control and Safety of human cell therapy products : Teruhide Yamaguchi : National Institute of Health Sciences. Division of Biological Chemistry and Biologicals.

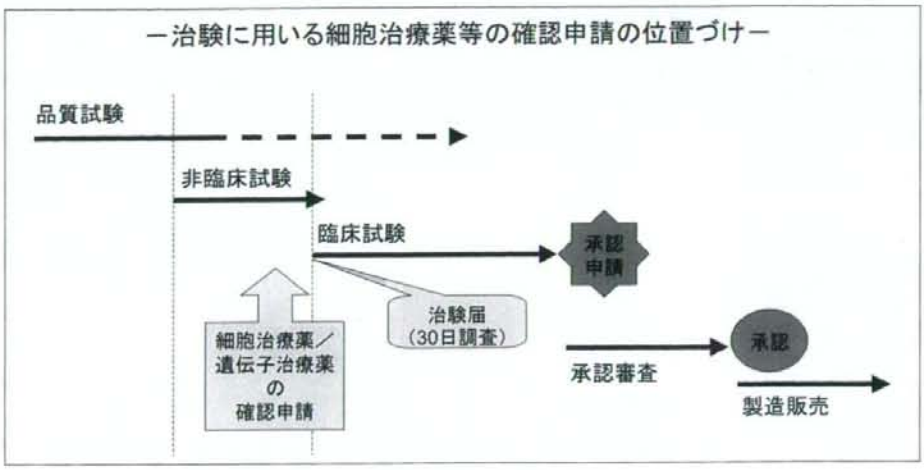


図1　薬事法に基づく先端医薬品の品質・安全性の確保

表1　我が国における細胞治療薬（再生医療）に関連する指針や通知

細胞・組織利用医薬品等の取扱い及び使用に関する基本的考え方（医薬発第1314号 別添1）
ヒト由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針（医薬発第1314号 別添2）
生物由来製品及び特定生物由来製品の指定並びに生物由来原料基準の制定等について（医薬発第052001号）
生物由来製品に関する感染症定期報告制度について（医薬発第051508号）

を開始する前に一定の品質・安全性を担保することを目的として，厚生労働大臣にその確認を求めるとされているものである（図1）。確認申請は，治験の申請前に行われ，その段階で必要とされるデータはあくまでも安全に治験を実施するに足る品質・安全性が確保されているかを確認するためのものである。このような確認申請は，他には遺伝子治療薬にのみ適用されている制度である。

第1314号については現在見直し作業が進行中であり，指針をヒト自己由来細胞製品とヒト同種由来細胞製品に分ける予定である。それぞれの指針については，今後の議論を通じて変更される可能性があり，本稿ではこれまでの指針に沿った概説を行うと共に，必要に応じて現時点で示されている改正案についても触れていくことにする。

1．ヒト由来細胞治療薬関連指針の概要

1）ヒト由来細胞を用いた細胞・組織加工医薬品等の定義

細胞治療に用いる細胞・組織加工医薬品等とは，ヒトあるいは動物由来の細胞・組織を加工した医薬品又は医療用具と定義される。本稿では前述したように，細胞・組織加工医薬品等を細胞治療薬と略す。前記した通知や指針は，細胞治療に用いる医薬品や医療用具を企業が開発しようとする場合を対象としており，細胞治療の臨床研究については対象外とされている。

2）「指針」の対象とする範囲

「指針」等の対象とする範囲として，輸血用血液製剤，移植医療としての骨髄移植，臍帯血移植，ヒト皮膚や骨等を直接利用する医療行為は含まれていない。また細胞・組織の加工としては，*in vitro*での増殖，薬剤処理による細胞の活性化あるいは生物学的特性の改変，遺伝子工

学的改変を指し，単なる遠心操作等の細胞・組織の分離や抗生物質処理及びガンマ線等の滅菌，冷凍，解凍は含まれない。欧米では，我が国で移植医療として分類される製品についても細胞治療薬として規制がかけられており，この点が大きな違いである。日米欧の細胞治療薬（細胞治療製品）の規制の違いについては，表2を参照されたい。

3）細胞治療薬等の品質や安全性面での問題点

「指針」の安全性面で最も重視されている点はウイルス等の感染症伝播をいかに防止するかである。細胞治療に用いる細胞は滅菌や高度な精製といった処理ができないため，原材料や製造に用いられる試薬や血清等へのウイルス等の混入を如何に防止するかが最重要課題となる。また，製品に感染因子が混入した場合，患者ばかりでなく患者の家族や医療従事者等へも感染が広がる危険性があり，公衆衛生の観点も含め十分な対策が求められる。この点は，欧米のガイドラインとも共通している点であり，細胞治療薬の基本的要件である。

しかし，ウイルス試験にも検出限界があり，また未知のウイルスの存在も考えられるため，ウイルスの潜在を前提とした対策が求められる。原材料となる細胞・組織に関する記録や最終製品の製造記録や試験及び検査記録の保存，可能であれば採取した細胞・組織の一部を保管することが求められている。これは，将来患者に当該製品が原因と推定されるような感染症が発症した場合の原因解明を可能とするための措置である。また，製品が生物由来原料基準に基づき，特定生物由来製品や生物由来製品の指定を受けた場合には，それぞれの指定に応じた上乗せ的な安全対策が必要となる。

2．指針等で求められる細胞治療薬の要件

1）基原または発見の経緯及び外国等における使用状況

細胞治療薬の開発の経緯やその特徴などについて明らかにすることが求められる。また，外国等での使用状況についても明らかにする必要がある。一方，ヒト由来細胞を用いた細胞治療薬の開発では，先行して実施された国内での臨床研究の技術移転をうけているケースも多くあり，技術移転を受けた臨床研究の実施状況についての情報も提供されなければならない。

2）原材料となる細胞・組織の由来と選択基準

第1314号指針では，原材料として用いられる細胞・組織が自己由来であるか非自己であるかを明確にすることが求められるが，改正予定の指針では自己と同種とに分けられる予定である。細胞・組織の入手方法及びその生物学的特徴について説明し，細胞・組織を選択した理由を明らかにする必要がある。原材料となる細胞・組織の特性と適格性について，形態学的特徴，増殖特性，生化学的指標，免疫学的特徴，特徴となる細胞由来産生物質等，遺伝型や表現型から適切な指標を選択して解析し，明らかにすることが求められる。

特にHBV，HCV，HIV，HTLVや，必要に応じてパルボウイルスB19，サイトメガロウイルスやEBウイルスについて血清学的試験や核酸増幅法等の検査を行う必要がある。さらに，細菌や真菌等の試験が必要となる。また，問診や病歴等も考慮した上で，ドナーとしての適格性を評価する必要がある。この場合，ヒト同種由来細胞製品のみならず，ヒト自己由来細胞製品についても，製造工程での作業従事者の安全性，他の製品に対する交差汚染防止の観点から，ウイルス等の必要な試験の実施を考慮することが求められる。ウイルス等の検査においては，ＰＣＲ等を用いても検出出来ないウインドウ期の存在があることから，適切な時期に再検査を行うことが推奨されている。

3）採取行為及び利用の妥当性

細胞・組織採取時のドナーに対する説明及び同意の内容を明らかにし，細胞の採取部位，採取方法が科学的及び倫理的に適切であることを