Preparation of a novel adenovirus formulation with artificial envelope of multilayer polymer-coatings: Therapeutic effect on metastatic ovarian cancer

CHIEKO YOSHIHARA[1], KATSUYUKI HAMADA[2] and YOSHIYUKI KOYAMA[1]

[1]Department of Human Life Science, Otsuma Women's University Graduate School, Sanbancho, Chiyoda-ku, Tokyo; [2]Department of Obstetrics and Gynecology, School of Medicine, Ehime University, Shitsukawa, Toon, Ehime, Japan

Received September 7, 2009; Accepted October 30, 2009

DOI: 10.3892/or_00000691

Abstract. Layer-by-layer deposition of the ionic polymers onto adenovirus particles afforded the multilayer-coated virus vectors. The infectivity of the virus in the presence of anti-adenovirus antibody increased as the layer number and the viruses with five or six polymer layers allowed relatively high efficiency of reporter gene expression *in vitro*. Therapeutic effect of the intraperitoneal injection of the oncolytic adenovirus with quintal polymer multilayers on the mice bearing intraperitoneal metastatic ovarian cancer was examined. All the control mice injected with PBS died within 21 days after the tumor inoculation. On the other hand, the mice injected with the multilayer-coated oncolytic virus lived much longer and seven eighths of them lived >60 days without apparent accumulation of ascites. These approaches would open a new way to create a novel, safe and efficient viral gene therapy.

Introduction

Recombinant adenovirus has been accepted as a useful vector for cancer gene therapy because of its high infection efficiency and capacity for transgene expression in both proliferating and non-proliferating cells. More than thousand cancer patients have received the virus through systemic or local administration. Although, in some cases, clinical benefits were reported, transgene efficiency of the virus is not as high as expected from the *in vitro* experimental data.

Several studies have shown the importance of neutralizing antibodies in preventing infection and gene transfer (1). Adenovirus is not an integrating virus and repeated administration is usually required in the clinical use. It will develop an immune response that limits the subsequent vector administration. Pre-existing antibodies are also found in >90% of human adult. Neutralization of adenovirus by the antibodies can lead to inefficient gene expression.

Their high tropism to the liver would be the second important hurdle that might limit the use of adenovirus-based *in vivo* gene transfection (2). Following systemic administration in a murine model, >90% of the virus soon localizes to the liver. Not only intravenous, but intraperitoneal injection of adenovirus also results in a high accumulation of the virus in the liver, because of the lymph drainage from the peritoneal cavity (3).

The induction of strong innate immune responses by macrophages and dendritic cells is another hurdle, which induces the release of proinflammatory cytokines or chemokines such as IL-6, TNF and IFN-γ (4). These hurdles strongly limit the application of adenovirus to only intratumoral administration.

On the other hand, epithelial ovarian cancer is the fourth most frequent cause of cancer death among women. Most ovarian cancer patients have intraperitoneally disseminated disease, which is associated with a poor prognosis. Gene therapy with intraperitoneal administration of adenovirus would be one of the most rational treatments of the metastatic tumor in the peritoneal cavity. The solution to the hurdles mentioned above is required to use adenoviral vectors for the treatment of ovarian metastatic tumors by repeated administration to the peritoneal cavity (5,6).

One of the promising ways to protect virus from neutralizing antibody and reduce the innate immune responses would be carrier cell systems. In those systems, first, cultured carrier cells are infected by the therapeutic virus and then the viral particle-containing cell fragments derived from the carrier cells are administered and engulfed by target cancer cells. Oncolytic adenovirus in the system showed high infectivity even under the presence of antibody and also very high therapeutic effect after intratumor injection (7), though successful treatment by the system with systemic or intraperitoneal administration has not been established.

Another method to prevent the antibody neutralization would be using protective polymer-coating on the virus particles. Hydrophilic polymer layer grafted on the virus surface would shield the viral proteins reducing the

Correspondence to: Dr Yoshiyuki Koyama, Department of Human Life Science, Otsuma Women's University Graduate School, Sanbancho, Chiyoda-ku, Tokyo 102-8357, Japan
E-mail: koyama@otsuma.ac.jp

Key words: gene therapy, oncolytic adenovirus, ovarian cancer, layer-by-layer, artificial envelope

interaction with immune systems. The covalent chemical modifications of the virus with polymers to overcome the barriers have been attempted (8). However, chemical modification sometimes caused the decrease of infectivity (9,10) and reproducible production might be difficult in those chemical reactions on the heterogeneous solid particle surfaces. Although PEGylation could effectively reduce the interaction with innate immunity against virus proteins, sites of polymer attachment would be recognized as new antigenic epitopes. Repeated dosing of the PEGylated virus would, thus, produce antibodies against the new epitopes after the first dosing, diminishing the effect of the second dosing with the vectors (11).

Taking advantage of the anionic surface charge of the virus, coating by polycations has also been performed. Ionic complexes of the virus showed higher infection efficiency on cultured cells (12). Virus/polycation also showed higher *in vivo* infectivity in some cases (13). However, intraperitoneal administration of those viral complexes demonstrated no significantly improved effect in mice bearing intraperitoneal tumors (14).

Cationic coating on the virus particle could enhance the infectivity on the anionic mammalian cells. But addition of antiserum immediately neutralizes it to slightly positive charge, and strongly inhibited the infection. In order to prevent the blocking by the antibodies and establish the infection, heavier coating would be required to shield the virus surface proteins. In this study, we coated the adenovirus with layer-by-layer deposition of ionic polymers to produce the multilayer-coated virus particles. Their infectivity on the cultured cells in the presence of antibodies and therapeutic effect by the intraperitoneal injection of the multilayer-coated oncolytic adenovirus on the mice bearing metastatic peritoneal ovarian cancer were examined.

Materials and methods

Cell lines and adenoviruses. Non-small cell lung carcinoma A549 cells and murine ovarian carcinoma OVHM cells were cultured, as described previously (7). Construction, purification and plaque assay of the replication-competent adenovirus AdE3-*IAI.3B*, having a promoter IAI.3B were performed, as described previously (15).

Materials. A linear polyethyleneimine hydrochloride (PEI Max; MW 40,000) was obtained from Polyscience, Inc., Warrington, USA. HA sodium salt (from Microorganism) and protamine sulfate (PRT) were purchased from Nacalai Tesque, Inc., Kyoto, Japan).

ζ-Potential and size measurement. ζ-Potential and the size of the virus complexes were measured with a particle analyzer, Zetasizer Nano ZS (Malvern Instruments Ltd). Adenovirus/polymer complexes were prepared as follows: the adenovirus stock solution ($5x10^{11}$ pfu/ml) was diluted with 5% glucose to $1.5x10^8$ pfu/ml ($3.0x10^9$ vp/ml). It was divided into 800 μl aliquots and kept at 0˚C. Aqueous solutions of PEI and HA (2-4 μl) were added to the aliquot one after the other at 30 min intervals. ζ-Potential was measured soon after the final addition of the polymer.

In vitro transfection procedure. A549 cells were seeded onto 24-well plates at $5x10^4$ cells per well and cultured overnight in RPMI supplemented with 10 vol% fetal bovine serum (FBS), penicillin G sodium (100 U/ml) and streptomycin sulfate (0.1 mg/ml). Recombinant adenovirus with green fluorescence protein gene, Ad-*GFP*, was diluted with 5% glucose into $3x10^8$ pfu/ml. Aliquots of 666 μl were then complexed with PEI (or PRT) and HA alternately to prepare the multilayer-coated adenovirus complexes in a similar way as the ζ-potential experiments. Compositions are listed in Tables I and II. After addition of anti-adenovirus antibody solution (titer: x6000; 200 μl), the virus complex suspension was added to the wells and allowed to be incubated for 3 h at 37˚C. Fresh medium (500 ml) was then added to the wells and expression of the fluorescence protein was observed by a fluorescence microscopy after several days' incubation.

In vivo transfection procedure. The oncolytic virus with five (quintet) polymer layers was prepared as follows; to a suspension of oncolytic virus, AdE3-*IAI.3B*, in 5% glucose ($2.5x10^{10}$ pfu in 5 ml) were added PEI (83.3 μg), HA (833 μg), PEI (417 μg), HA (1390 μg) and PEI (278 μg) in turn. In each step, polymers were added as 5 mg/ml solution in water at 30 min intervals. Female (C57BL/6xC3H/He) F1 mice (CLEA Japan) were immunized with subcutaneous injection of Ad-*βGal* ($1x10^{10}$ pfu/mouse) 3 weeks before use. The mice were then intraperitoneally inoculated with $1x10^6$ OVHM cells (Day 0). On days 4 and 8, the multilayer-coated adenovirus complexes were intraperitoneally administered to the mice, of which the survival days were observed every 2 days. The animal experiments were performed in accordance with the institutional ethics guidelines.

Results

Adenovirus particles showed negative surface potential in aqueous 5% glucose solution. On addition of PEI solution to the virus suspension, ζ-potential of the adenovirus particles increased as the amount of PEI and positively charged complex was obtained with PEI >0.02 μg for $2.4x10^9$ vp (PEI/virus >$8x10^{-12}$ μg/vp). Addition of more PEI further increased the potential up to 30 mV. The adenovirus/PEI complex prepared with $2.4x10^9$ vp of the virus and 0.2 μg of PEI (ζ-potential was ~15 mV) was then mixed with HA. The virus/PEI complex was effectively recharged to negative by HA. When >30 μg of HA was added, the ζ-potential reached <-50 mV, which is lower than that of the original virus particle. Since 'naked' virus particle without PEI may not interact with the like-charge HA, the virus/PEI/HA ternary complex should be formed.

PEI was again added to the complex prepared by adenovirus ($2.4x10^9$ vp), PEI (0.2 μg) and HA (2 μg) (ζ-potential = -13 mV), the surface charge increased again to a positive value. Similar charge-reversing phenomena could be repeated by the alternative addition of PEI and HA (Fig. 1), indicating the construction of layer-by-layer polymer-coatings on the virus particles like an artificial envelope (Fig. 2). Repeated reversal recharging was also observed with PRT instead of PEI. Addition of >0.03 μg of PRT recharged the surface potential of the adenovirus ($2.4x10^9$ vp) into positive

Table I. Amount of polymers added to prepare the PEI/HA multilayer-coated adenovirus.

No.	Ad (pfu)	PEI (μg)	HA (μg)	PEI (μg)	HA (μg)	PEI (μg)	HA (μg)
E-1	$2x10^8$						
E-2	$2x10^8$	0.333					
E-3	$2x10^8$	0.333	3.33				
E-4	$2x10^8$	0.333	3.33	3.33			
E-5	$2x10^8$	0.333	3.33	3.33	16.7		
E-6	$2x10^8$	0.333	3.33	3.33	16.7	3.33	
E-7	$2x10^8$	0.333	3.33	3.33	16.7	3.33	16.7
E-6(2)	$2x10^8$	0.667	6.67	3.33	11.1	2.22	
E-7(2)	$2x10^8$	0.667	6.67	3.33	11.1	2.22	11.1

Table II. Amount of polymers added to prepare the PRT/HA multilayer-coated adenovirus.

No.	Ad (pfu)	PRT (μg)	HA (μg)	PRT (μg)	HA (μg)	PRT (μg)	HA (μg)
R-5	$2x10^8$	1	3.33	10	16.7		
R-6	$2x10^8$	1	3.33	10	16.7	10	
R-7	$2x10^8$	1	3.33	10	16.7	10	16.7
R-6(2)	$2x10^8$	0.5	3.33	5	16.7	5	
R-7(2)	$2x10^8$	0.5	3.33	5	16.7	5	16.7

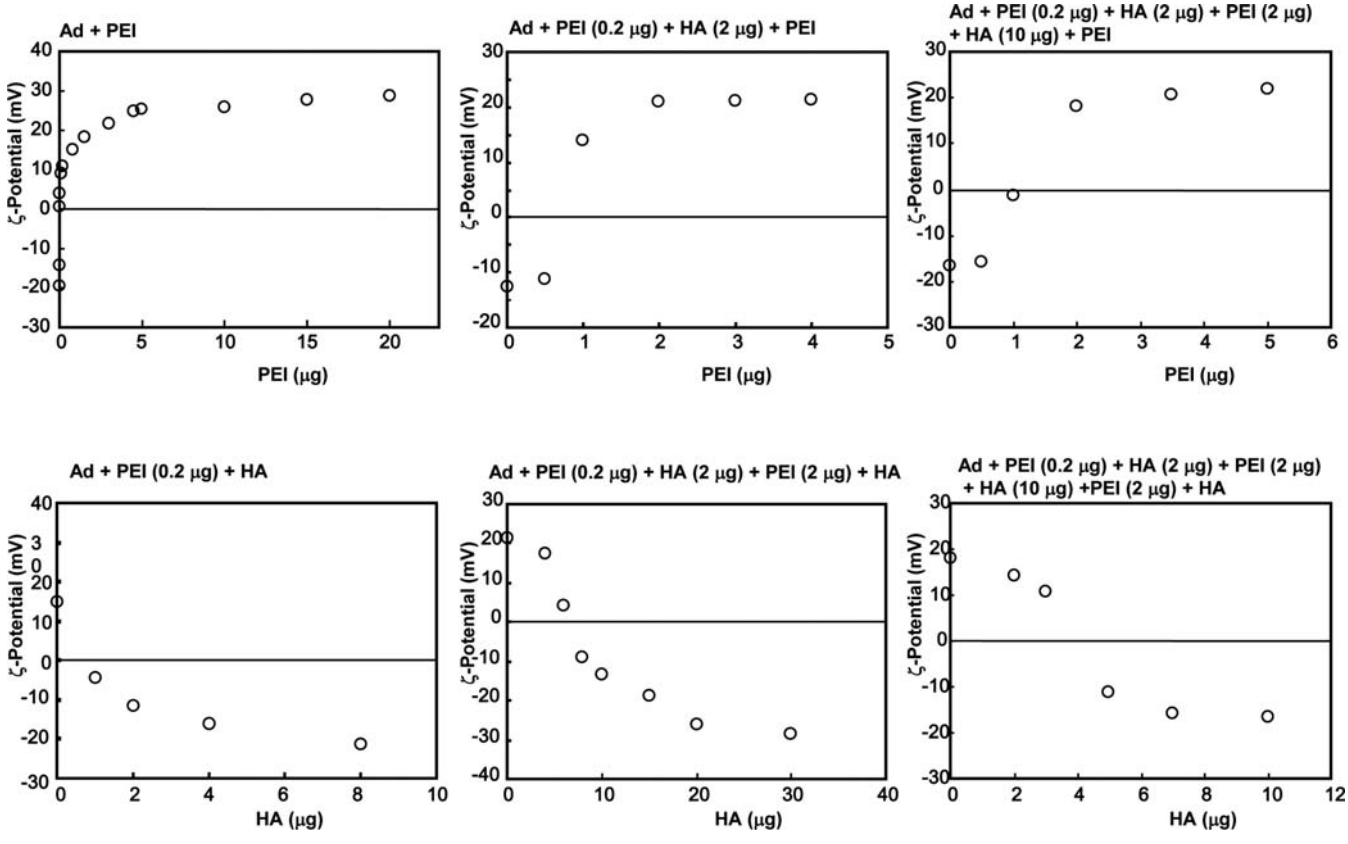

Figure 1. ζ-potential change on the adenovirus particles ($2.4x10^9$ vp) by alternative addition of PEI and HA.

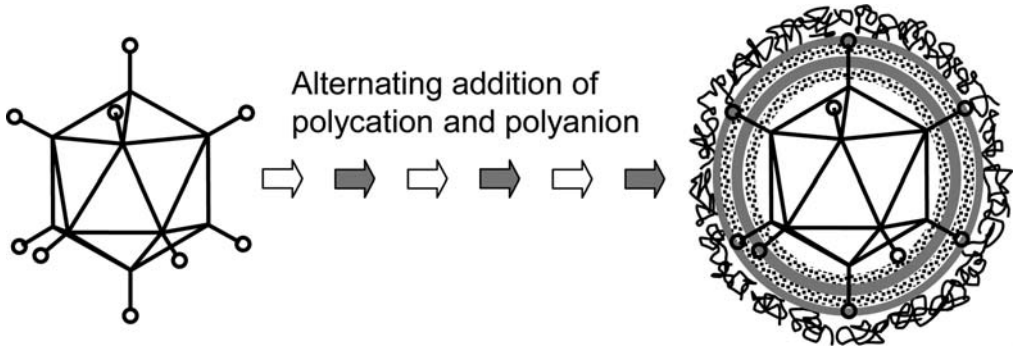

Figure 2. Preparation of adenovirus complex with artificial envelope of multilayer polymer-coatings.

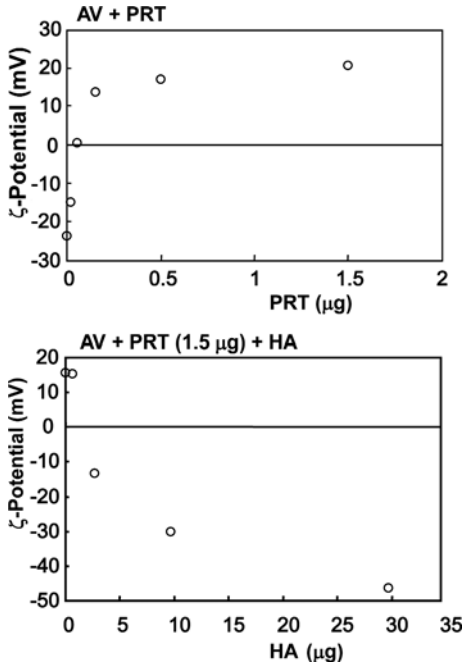

Figure 3. ζ-potential change on the adenovirus particles ($2.4x10^9$ vp) by alternative addition of PRT and HA.

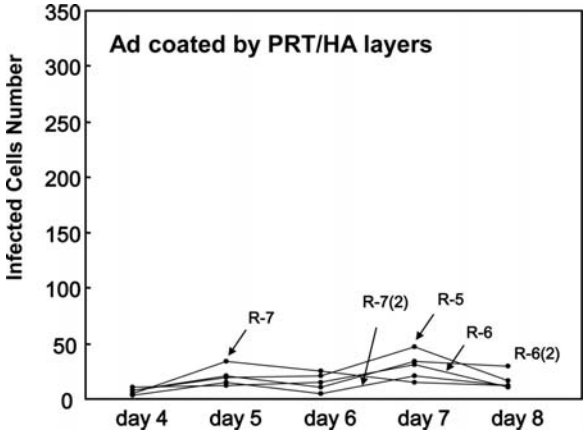

Figure 4. Number of the GFP expression-positive cells in the well after infection by PRT/HA-coated adenoviruses.

(Fig. 3). It was again reduced by HA and highly negative virus particles (<-40 mV) were obtained by the polyanion. Further alternative addition of PRT and HA also resulted in the repeated charge reversal.

The expression of GFP by the virus complexes prepared by alternate addition of PEI (or PRT) and HA was examined on the cultured A549 cells with or without antibody. In the absence of the antibody, infection was established by all the virus complexes in one day on almost 100% of the cells, as well as the naked virus. Under the existence of the antibody, the virus complexes comprising PRT and HA induced scarce production of fluorescence protein on the cells (Fig. 4). In the case with the virus coated by PEI and HA, infection was not established until day 4, but 5 days after the treatment, the cells slowly began to show the reporter gene expression. As shown in Fig. 5, the number of the reporter gene expression-positive cells increased as the layer number and the viruses with five and six polymer layers showed the highest extragene expression level.

Electron microscopic image of the virus complex with quintal polymer multilayers revealed that the virus complex prepared by repeated addition of PEI and HA had thick coating on the surface (Fig. 6). There are also aggregates consisting of several virus particles (Fig. 6; right), in which each particle stick to each other with some space between the hexagonal capsid, most probably by the PEI/HA polymer complex layers.

Therapeutic effect of the multilayer-coated virus on the mice bearing ovarian cancer cells inoculated into peritoneal cavity was then investigated. Oncolytic virus complex with quintal PEI/HA multilayers, which corresponds to E-6(2) in the *in vitro* experiment, was prepared and intraperitoneally injected into the tumor-bearing mice. All the control mice injected with PBS died within 21 days after the tumor inoculation. On the other hand, the mice injected with the quintet multilayer-coated oncolytic viruses lived significantly longer ($P<0.05$) and seven eighths of them lived >60 days without apparent accumulation of ascites (Fig. 7).

Discussion

Since the polyion complexes are known to stick easily to each other in the high ionic strength solutions (16), the adenovirus particles were complexed with polyions in aqueous 5% glucose solution to avoid aggregation. Addition of PEI to $1.2x10^8$ pfu ($2.4x10^9$ vp) of the virus, the ζ-potential of the

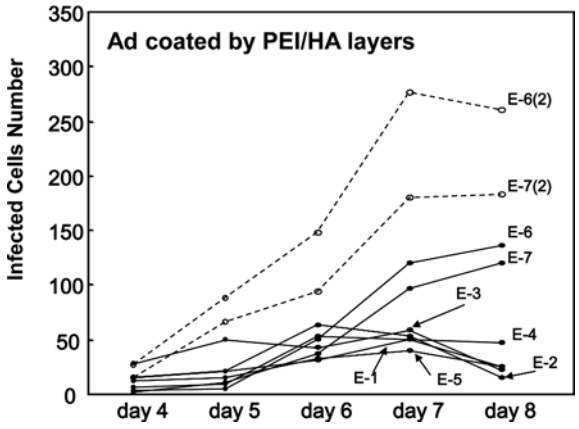

Figure 5. Number of the GFP expression-positive cells in the well after infection by PEI/HA-coated adenoviruses.

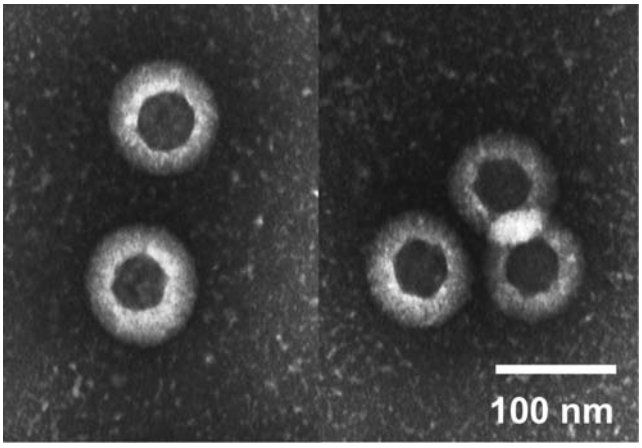

Figure 6. Electron microscopic image of the adenovirus complex with quintal polymer multilayers comprising PEI and HA.

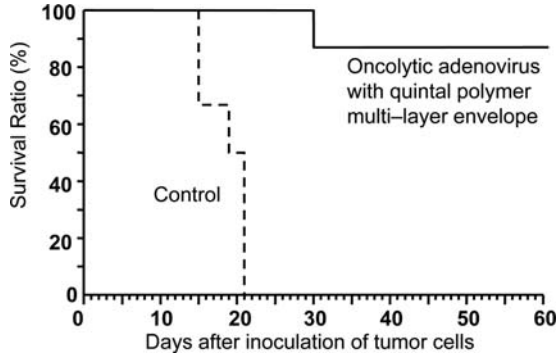

Figure 7. Survival days of the mice after intraperitoneal inoculation of ovarian cancer with or without intraperitoneal administration of oncolytic adenovirus complex with quintal polymer multilayers.

virus increased to positive at PEI >0.02 μg and reached almost plateau at PEI >5 μg (PEI/virus >$2x10^{-9}$ μg/vp). Inflection point appeared at around PEI = 0.03 μg, where 270 molecules of PEI should be attached to one virus particle, assuming all the PEI molecules were deposited onto the virus at this point.

On addition of PEI, the ζ-potential increased rapidly and then kept rising slowly for 20 min. Further mixing with HA also showed rapid decrease, followed by a slow decrease for 15-20 min. Polyions were thus added to the virus complex at 30 min intervals. In each step, effective charge reversing was observed (Fig. 2), indicating the formation of layer-by-layer multi-polymer-coating on the virus particles as an artificial envelope. Polymer multilayer envelope could also be formed by the alternative addition of the appropriate amount of PRT and HA (Fig. 3).

Those viruses coated by the artificial polymer envelope were expected to avoid neutralization by the antibody. The expression of GFP by the virus complexes having PEI (or PRT)/HA alternative multilayers was thus examined on the cultured A549 cells with or without antibody. Virus complexes with the polymer envelope were prepared by the addition of adequate polymer in each step to alter the surface charge, but the amount was set to minimum to avoid the precipitation of the complex of excess polymers.

In the absence of antibody, all the virus complexes established the infection of almost 100% of the cells in one day, indicating they still maintained high infectivity in spite of the thick envelopes. Under the existence of the antibody, the viruses coated by the polymers showed only slight reporter gene expression within the first 4 days. But, on day 5, the cells treated with the virus complex comprising more than quintet PEI/HA layers started to become fluorescently luminous. To improve the infectivity under the presence of antibody, repeated disposition of the polyions was required. Thickness of the piled polymer layers should be increased to some extent to efficiently prevent the antibody-neutralizing. Late expression of the reporter gene by the multilayer-coated viruses is due to the period required to peel off the polymer-coatings, or to the different cell-uptake mechanism for the ligand-shielded viruses. Plasmid/PEI complexes are known to be far more efficient in mediating the cell transfection than plasmid/PRT complexes, though the reason is still not clear. Lower infectivity of the virus complexes comprising PRT than those with PEI might be for a similar reason as the plasmid/PRT complex, such as intracellular destination.

The population of the positive cells is still very small (<1%), but, concerning that the cell should be infected by several virus particles to express the adequate fluorescence protein being detectable by the fluorescence microscopy, infection must have been established in considerable proportion of the cells. The relatively higher infectivity of the adenoviruses with the polymer envelope was then expected to induce high therapeutic effect on the tumor-bearing mice. The complex formation behavior of the virus at the higher concentration such as used for the *in vivo* therapeutic experiments [(virus) = $2.5x10^9$ pfu/ml] was examined. In each step, the polymer addition effectively reversed the surface potential and the multilayer complexes were obtained similarly to the diluted virus suspension. Virus complex with quintal polymer multilayers, which is corresponding to E-6(2), and showed the highest infectivity in the *in vitro* experiment, was prepared with oncolytic adenovirus and intraperitoneally injected into the mice having intraperitoneal metastatic ovarian cancer. The virus with quintet multilayer envelope showed significantly high therapeutic effect, and most mice injected with the virus complex lived >60 days without accumulation of ascites (Fig. 7).

The remarkable therapeutic effect of the multilayer-coated virus complexes would be attributed to the protection against antibody-neutralizing, diminishing the innate immune response and reduced drainage from the peritoneal cavity which allows the long regional duration of the virus complex particles.

In conclusion, layer-by-layer polymer-coated on the adenovirus showed relatively higher infectivity on the cultured cells even in the presence of anti-adenovirus antibodies. The polymer-coated virus also exhibited the highly efficient therapeutic effect in the treatment of intraperitoneally metastatic ovarian cancer. These approaches to construct the artificial polymer multilayer envelope on the exterior of the adenovirus particles would open a new way to overcome the hurdles of virus-mediated gene delivery and create a novel strategy of gene therapy using recombinant adenovirus as a safe and efficient tool.

Acknowledgements

This study was supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture, Japan.

References

1. Kreppel F and Kochanek S: Modification of adenovirus gene transfer vectors with synthetic polymers: a scientific review and technical guide. Mol Ther 16: 16-29, 2007.
2. Alemany R and Curiel DT: CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. Gene Ther 8: 1347-1353, 2001.
3. Johnson M, Huyn S, Burton J, Sato M and Wu L: Differential biodistribution of adenoviral vector *in vivo* as monitored by bioluminescence imaging and quantitative polymerase chain reaction. Hum Gene Ther 17: 1262-1269, 2006.
4. Muruve DA: The innate immune response to adenovirus vectors. Hum Gene Ther 15: 1157-1166, 2004.
5. Vasey PA, Shulman LN, Campos S, Davis J, Gore M, Johnston S, Kirn DH, O'Neill V, Siddiqui N, Seiden MV and Kaye SB: Phase I trial of intraperitoneal injection of the E1B-55-kd-gene-deleted adenovirus ONYX-015 (dl1520) given on days 1 through 5 every 3 weeks in patients with recurrent/ refractory epithelial ovarian cancer. J Clin Oncol 20: 1562-1569, 2002.
6. Muller CY, Coleman RL, Rogers P, Merritt JA, Parker K and Miller DS: Phase I intraperitoneal adenoviral p53 gene transfer in ovarian cancer. Proc Am Soc Clin Oncol 20: Abs 1025, 2001.
7. Hamada K, Desaki J, Nakagawa K, Zhang T, Shirakawa T, Gotoh A and Tagawa M: Carrier cell-mediated delivery of a replication-competent adenovirus for cancer gene therapy. Mol Ther 15: 1121-1128, 2007.
8. O'Riordan CR, Lachapelle A, Delgado C, Parkes V, Wadsworth SC, Smith AE and Francis GE: PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody *in vitro* and *in vivo*. Hum Gene Ther 10: 1349-1358, 1999.
9. Alemany R, Suzuki K and Curiel DT: Blood clearance rates of adenovirus type 5 in mice. J Gen Virol 81: 2605-2609, 2000.
10. Xiong Z, Cheng Z, Zhang X, Patel M, Wu JC, Gambhir SS and Chen X: Imaging chemically modified adenovirus for targeting tumors expressing integrin $\alpha_v\beta_3$ in living mice with mutant herpes simplex virus type 1 thymidine kinase PET reporter gene. J Nucl Med 47: 130-139, 2006.
11. Croyle M, Chirmule N, Zhang Y and Wilson J: 'Stealth' adenoviruses blunt cell-mediated and humoral immune responses against the virus and allow for significant gene expression upon readministration in the lung. J Virol 75: 4792-4801, 2001.
12. Dodds E, Piper T, Murphy S and Dickson G: Cationic lipids and polymers are able to enhance adenoviral infection of cultured mouse myotubes. J Neurochem 72: 2105-2112, 1999.
13. McKay T, MacVinish L, Carpenter B, Themis M, Jezzard S, Goldin R, Pavirani A, Hickman ME, Cuthbert AW and Coutelle C: Selective *in vivo* transfection of murine biliary epithelia using polycation-enhanced adenovirus. Gene Ther 7: 644-652, 2000.
14. Lanuti M, Kouri C, Force S, Chang M, Amin K, Xu K, Blair I, Kaiser LR and Albelda S: Use of protamine to augment adenovirus-mediated cancer gene therapy. Gene Ther 6: 1600-1610, 1999.
15. Hamada K, Kohno S, Iwamoto M, Yokota H, Okada M, Tagawa M, Hirose S, Yamasaki K, Shirakata Y, Hashimoto K and Ito M: Identification of the human IAI.3B promoter element and its use in the construction of a replication-selective adenovirus for ovarian cancer therapy. Cancer Res 63: 2506-2512, 2003.
16. Trubetskoy VS, Loomis A, Slattum PM, Hagstrom JE, Budker VG and Wolff JA: Caged DNA does not aggregate in high ionic strength solutions. Bioconjugate Chem 10: 624-628, 1999.