and could radically cure any cancer subject to monitoring visually or by echographic, endoscopic, computed tomography or other types of monitoring in the outpatient clinic. This gene therapy could reduce the physical and financial burden on patients, as well as national medical expenses. Local intratumoral injection by GM-CSF/PEI/CS ternary complex thus has the potential to replace surgery for locally injectable tumors in the near future. Furthermore, it appears very likely that it will become an important method of treatment in place of anticancer drugs with the further development of systemic treatment of cancers.

Acknowledgements

This research was supported by a Grant-in-Aid from the Ministry of Education, Science, Sports, and Culture, Japan, R&D for Promoting Innovation in Regions, and the Integrated Center for Science of Ehime University. We thank K. Oka for preparing the culture medium. We thank Mr Kubo, Professor Yoshikawa, Professor Kawakami and Professor Hashida (Kyoto University) for their great help in ζ-potential measurements. We also thank Seikagaku Corporation for supplying CSs and hyaluronic acid. The authors declare that there are no conflicts of interest.

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Oncolytic plasmid: A novel strategy for tumor immuno-gene therapy

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Received April 4, 2011; Accepted October 10, 2011

DOI: 10.3892/ol.2011.467

Abstract. The oncolytic virus is expected to proliferate in and destroy tumor cells. The virus is also thought to generate antitumor immunity. Virally infected tumor cells express viral antigens on their surfaces. Such tumor cells or their fragments would be taken up by antigen-presenting cells (APCs) together with tumor-associated antigens (TAAs), and facilitated cross-priming of tumor-specific T cells. Virus-specific protein presented on the infected cells therefore played a crucial role in the enhancement of the adaptive antitumor immunity. In this study, a plasmid encoding adenovirus protein, the adenovirus death protein (ADP), was constructed, and a very fine complex of the plasmid with polyethylenimine (PEI) and chondroitin sulfate (CS) was injected into tumor-bearing mice. Transfection of the ADP gene was shown to suppress tumor growth as effectively as granulocyte-macrophage colony-stimulating factor (GM-CSF) transfection. When mice were administered plasmid coding ADP (pDNA-ADP) to generate an immune response to ADP prior to therapy, transfection of the ADP gene induced a much higher level of tumor growth suppression than that found in the non-immunized mice. An evident synergistic effect of ADP and GM-CSF genes was also observed, and at a pDNA-ADP/pDNA-GM-CSF ratio of 4:1, significant suppression of tumor growth was achieved even in the non-immunized mice.

Introduction

Since oncolytic activity attributed to replicating viruses was previously reported by De Pace (1), a number of clinical trials were conducted using viruses to treat tumors. In the 1950s, virotherapy of cancer was started with the naturally occurring oncolytic virus, a virus capable of replicating specifically in tumor cells and causing specific lysis of cancer cells but not normal cells. Advancements in virotherapy were made following the development of the genetically engineered, replication-selective oncolytic virus in 1991 (2). Such oncolytic viruses are expected to proliferate in and destroy tumor cells.

However, preclinical and clinical data suggest that in some cases virotherapy may in fact act as cancer immunotherapy. Tumor-bearing mice injected with an oncolytic virus exhibited an antitumor immune response, and local injection of the oncolytic virus often caused regression of remote as well as peripheral tumors. It should be mediated by the systemic antitumor immune response. Tumor-specific cytotoxic T lymphocytes (CTL) activity against tumor cells persisted for a long period of time, and protection against tumor rechallenge was observed (3).

Viruses are thought to generate antitumor immunity. Virally infected tumor cells would express viral antigens on their surfaces, and effectively stimulate antigen-presenting cells (APCs) to cross-prime tumor-specific T cells (4). Virus-specific proteins presented on the infected tumor cells or their fragments thus appeared to play a crucial role in the enhancement of the adaptive antitumor immunity.

We thought that adaptive antitumor immunity might be induced not only by virus infection, but also by transfection of the gene encoding viral protein into the tumor cells. Extra viral genes would produce a viral antigen in the tumor cells, and cause the stimulation of APCs as well as viral-infected cells.

The question arises as to which of the virus proteins would be most effective in inducing antitumor immunity. Oncolytic adenoviruses have usually been engineered by deletion of E1 genes that are necessary for a virus to replicate in normal cells. E3 genes were often deleted to provide more space for therapeutic gene insertion. However, E3-positive (E3+) viruses were reported to kill tumor cells 1.6-20 times more effectively in different cell lines (5). Among the E3 proteins, the adenovirus death protein (ADP) has unique properties. ADP is an adenovirus nuclear membrane glycoprotein (6), and, unlike other E3 genes, ADP is expressed at late stages of infection. Whereas almost all other E3 genes have immunomodulatory functions, ADP is responsible for the efficient lysis and release of the progeny virus from the infected cell (7-9). Adenovirus, which overexpresses ADP, was also prepared by deleting the

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Key words: gene therapy, adenovirus death protein, immunotherapy, plasmid, tumor

E3 region and reinserting the ADP gene, and was found to spread more rapidly and effectively through tumors, showing highly improved tumor growth regression (10).

In this study, plasmid DNA encoding ADP gene was constructed, and an extremely fine complex of the plasmid with polyethylenimine (PEI) and chondroitin sulfate (CS) was prepared by a previously reported method (11). The complex was injected into tumor-bearing mice, and the tumor growth inhibition effect was examined.

Materials and methods

Materials and animals. CS from shark cartilage was supplied by Seikagaku Corporation (Tokyo, Japan). Linear PEI 'MAX' (MW 40,000) was purchased from Polyscience, Inc. (Warrington, PA, USA).

Plasmid coding adenovirus ADP (pDNA-ADP) was constructed with the product of PCR conducted with ADP-S ATGACCAACACAACCAACGC and ADP-AS-1 ACTCGAGGAATCATGTCTCA as primers, the genome of adenovirus type 5 (Microbix Biosystems, Ontario, Canada) as a template, and Takara Ex Taq (Takara, Japan) as the enzyme. pDNA-ADP was first subcloned into pTAC-1, and its sequence was analyzed by ABI Sequencing Analysis. Finally, pDNA-ADP was inserted into pcDNA3.1(-) (Invitrogen, USA) with *Eco*RI and *Bam*HI, and the sequence was confirmed again by ABI Sequencing Analysis. Plasmid coding mouse granulocyte macrophage-colony stimulating factor (pDNA-GM-CSF) was similarly produced with pcDNA3.1 vector as previously reported (11). The plasmids were amplified, and purified by Mitsuwa Frontech Corp., Japan.

Cytotoxic activity of the ADP plasmid complex

Preparation of DNA complex. CS solution (267 μg in 600 μl) and PEI solution (132 μg in 300 μl), respectively, were added in this order to a solution of plasmid DNA coding ADP or luciferase (45 μg in 300 μl). The solutions were previously prepared in 7 mM phosphate buffer (PB). After 30 min, the mixture was diluted by condensed PBS to afford an isotonic solution containing a given amount of the plasmid complex.

Evaluation of the cytotoxicity. B16 cells, a mouse melanoma cell line, were seeded onto 96-well plates at $7.5x10^4$ cells per well, and cultured for 2 days in Gibco minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin G sodium (100 U/ml), and streptomycin sulfate (0.1 mg/ml). The primary growth medium was then replaced with 100 μl of fresh MEM with FBS and antibiotics. Fresh or lyophilized-and-rehydrated plasmid DNA complex suspensions prepared above were added to the cells (100 μl/well), and incubated for 4 h at 37°C. Fresh medium was added to the wells (100 μl/well), and after an additional incubation at 37°C (20 or 68 h), cell viability was measured by WST-1 assay.

Therapeutic effect of the ADP plasmid complex

Preparation of freeze-dried DNA complex. CS solution (594 μg in 118.7 μl) and PEI solution (294 μg in 58.8 μl), respectively, were added in this order to a plasmid DNA coding ADP or mouse GM-CSF solution (100 μg in 4.82 ml). All the solutions were previously prepared in 7 mM PB. After standing for 20 min, the complex of plasmid-ADP and that made of plasmid-GM-CSF was mixed at the given ratio. Dextran solution in H_2O (10%, 50 μl) was added, and the mixture was freeze-dried, and stored at 4°C. It was rehydrated with 250 μl of H_2O just before use.

Immunization. Immunization was performed by intramuscular injection of pDNA-ADP (10 μg in 50 μl PBS) into the left posterior leg of male ddY mice 3 weeks prior to inoculation of B16 cells.

Therapeutic effect on the tumor-bearing mice. Male C57BL/6 mice (5 weeks) were inoculated subcutaneously with $2.0\text{-}2.7x10^6$ B16 cells. When the size of the tumor reached 3-4 mm in diameter, the animals were intratumorally injected with the rehydrated plasmid complex (containing 100 μg of plasmid) five times every other day. The tumor diameter was measured every day for 25 days, and the tumor size was calculated as $(4/3)\pi ab^2$, where a is the long axis and b the short axis of the solid tumor. The care and use of laboratory animals followed the guidelines for animal experiments of the institutes involved.

Results

Preparation of the small DNA complex particle suspension. Extremely small DNA complex particles were obtained by mixing the plasmid/CS premixed solution with PEI at a very low concentration, followed by the lyophilyzed-and-rehydrated condensation procedure, as previously reported (11). Particle size analysis using a laser-diffraction particle size analyzer showed that 50% of the complexes were no more than 150 nm in diameter.

Cytotoxic activity of the pDNA-ADP complex. The cultured B16 melanoma cells were treated with the pDNA-ADP complex, and the survival percentage compared to the non-treated control was estimated by WST-1 assay. The survival rate in the cells treated with pDNA-luciferase complex was also simultaneously examined. As shown in Fig. 1, after 24 h incubation with 10 μg/ml of pDNA-ADP complex, cell survival was almost 60%, as was the case with the pDNA-luciferase complex. After 72 h treatment with the pDNA-ADP complex, a reduction of approximately 50% was observed, while the cells transfected with the luciferase gene still showed a relatively high rate (75%).

Therapeutic effect of the pDNA-ADP complex on tumor-bearing mice. Small DNA/PEI/CS complex was prepared with pDNA-ADP, and intratumorally injected five times every other day into immunized or non-immunized male C57BL/6 mice bearing subcutaneous B16 tumors. The results of the pDNA-ADP complex and the pDNA-GM-CS complex are shown in Fig. 2. Rapid growth of the tumor was observed in all the control non-treated mice. In the non-immunized mice, the pDNA-ADP complex showed as effective a suppression of tumor growth as the pDNA-GM-CSF complex, which exhibited a high therapeutic effect, as shown in our previous study (11). When mice were administered pDNA-ADP prior to therapy to generate an immune response to ADP, transfection of the ADP gene induced a much higher level of tumor growth suppression than in the non-immunized mice, whereas

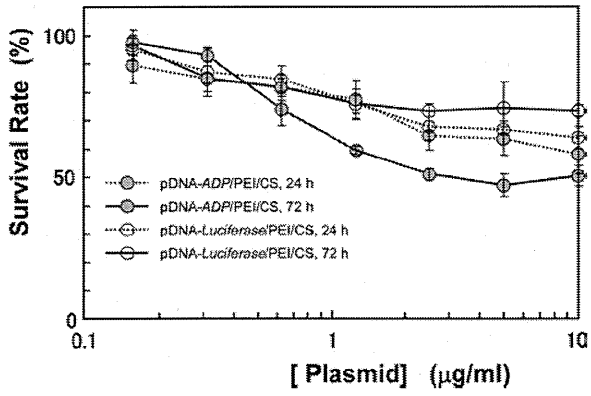

Figure 1. Cytotoxicity of plasmid complexes. Each complex was incubated with B16 melanoma cells for 24 or 72 h at the given concentrations.

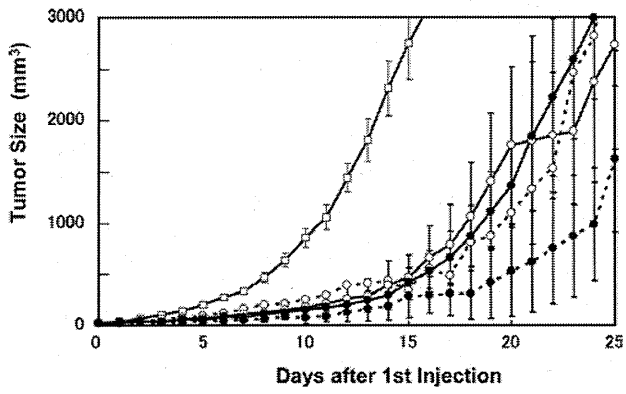

Figure 2. *In vivo* effects of tumor treatment with pDNA-ADP complex (closed circle), or pDNA-GM-CSF complex (open circle), on the non-immunized mice (solid line) or the mice pre-immunized by intramuscular injection of pDNA-ADP (dotted line). The open square expresses the results with control non-treated mice. Each complex containing 100 μg of the plasmid was intratumorally injected five times every other day into male C57BL/6 mice bearing subcutaneous B16 melanoma solid tumors. Tumor volume was expressed as the mean ± SE (n=5).

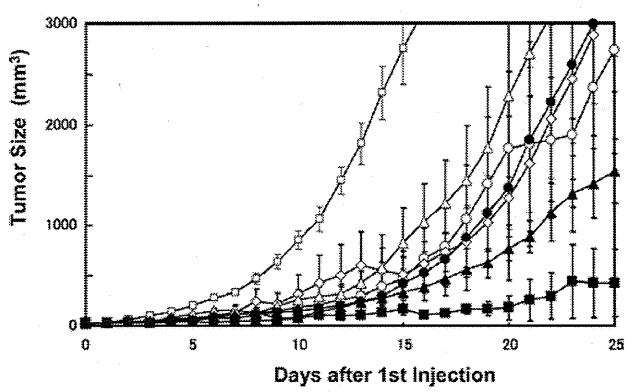

Figure 3. *In vivo* effects of tumor treatment with the complexes comprising pDNA-ADP+pDNA-GM-CSF at ratios of 0:5 (open circle), 1:4 (open diamond), 2:3 (open triangle), 3:2 (closed triangle), 4:1 (closed square), and 5:0 (closed circle) on the non-immunized mice. The open square expresses the results with control non-treated mice. Each complex containing 100 μg of the plasmid was intratumorally injected five times every other day into male C57BL/6 mice bearing subcutaneous B16 melanoma solid tumors. Tumor volume was expressed as the mean ± SE (n=5).

the therapeutic effect by the GM-CSF gene was no different between the immunized and non-immunized groups.

The effect of the simultaneous co-transfection of pDNA-ADP and pDNA-GM-CSF (total 100 μg of the plasmid per mouse) was examined at various pDNA-ADP/pDNA-GM-CSF ratios on the non-immunized mice. As shown in Fig. 3, an evident synergistic effect of the genes was observed, and significant suppression of tumor growth was observed even in the non-immunized mice. The highest therapeutic effect was obtained at plasmid-ADP/plasmid-GM-CSF at a ratio of 4:1, and the tumor volume was maintained <5 times as large as at the beginning of treatment for 20 days, while that in the control mice became 10 times larger in 10 days.

Discussion

Cytotoxic activity of the pDNA-ADP complex. ADP has been reported to be associated with a cell lytic activity of adenovirus. Transfection of the ADP gene to cultured cells was therefore expected to show cytotoxic activity. Induction of cytotoxicity by ADP-transfection was higher than that by pDNA-luciferase, but not as high as expected from the cell lytic activity reported. The duration of the plasmid-gene expression, or the protein production may not be sufficient to express strong cell lytic phenomena. Otherwise, ADP may not be capable of cell killing activity by itself. Another adenovirus death factor, E4orf4, was also found to induce cell death and suggested to collaborate with ADP in the induction of cell lysis and progeny release (12).

Therapeutic effect of the pDNA-ADP complex on tumor-bearing mice. In the non-immunized mice, the pDNA-ADP complex was found to be as effective in the suppression of tumor growth as GM-CSF. The effect of the ADP transfection was strongly enhanced by pre-immunization of mice with ADP-coding plasmid. Pre-existing anti-ADP antibody or ADP-specific CTL in the immunized mice would be attributed to the rapid response in the early therapeutic stage.

In *in vivo* therapy for tumors, GM-CSF is often used as an immunomodulatory agent. Oncolytic viruses engineered to secrete GM-CSF have been reported to show higher antitumor activity (13). Co-injection of GM-CSF-expressing virus also enhanced the anticancer effect of oncolytic viruses (14). The simultaneous co-transfection of the ADP and GM-CSF genes was subsequently attempted to examine the synergistic effect of the proteins on tumor growth suppression. As expected, a much higher therapeutic effect was obtained even in the non-immunized mice at a plasmid-ADP/plasmid-GM-CSF ratio of 3:2 and 4:1. GM-CSF is known to recruit and stimulate DCs in viral tumor therapy (15). The activity of DCs against ADP-expressing tumor cells would be enhanced by the cytokines.

In our previous study, an allogeneic model, B16 melanoma cells in ddY mice, was used allowing pDNA-GM-CSF to exhibit complete regression of the tumor (11). In the present study, a syngeneic model was employed in which tumor growth is more rapid than in the allogeneic model, and the effect of the transfection of the GM-CSF gene was not so strong as to completely inhibit the tumor growth. In the syngeneic model, tumor cells may escape from immune surveillance since the

cells are altered cells derived from genetically identical mice. GM-CSF expression may not be enough to prevent tumor escape in a syngeneic model.

Tumor escape from the immune system is most often caused by weak immunogenicity of tumor-associated antigens (TAAs), and has been a major problem in immunotherapy. Various DNA vaccine encoding TAAs have been developed and applied in antitumor immunotherapy. However, the activation of immunity against poorly immunogenic tumor antigens is difficult. Weber *et al* reported that vaccination of mice with DNA encoding cancer differentiation antigens is ineffective when self-DNA is used (16). These authors showed a high potential of the orthologous DNA from another species. To conquer the disadvantage of weak immunogenicity, Bergman *et al* developed the xenogeneic DNA vaccination (17). Injection of the plasmid encoding human tyrosinase into dogs bearing melanoma was capable of breaking tolerance against a self-tumor differentiation antigen, leading to high clinical responses.

In this study, a viral protein, ADP, was transfected on the tumor cells. ADP is an integral membrane glycoprotein that localizes to the inner and outer nuclear membrane and the Goldi apparatus. Some ADP molecules may also exist on the cell surface membrane, or at least appear on the apoptotic tumor cell fragments. These fragments would be captured by and stimulate APCs to mature and cross-prime T cells against both ADP and TAAs.

Since Tang *et al* demonstrated that plasmid DNA induces the formation of antibodies against an encoded protein (18), there has been rapid progress in the DNA vaccine, and it has now been accepted as a promising therapy for tumors. Prior identification of each tumor-related antigen is required for peptide-based immunotherapy. The transfection of ADP is apparently effective in various types of tumor. The elicitation of immune response towards tumor antigens by the transfection of such viral protein genes into tumor cells is likely to be a breakthrough in genetic immunotherapy.

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