The NH₂ Terminus of Influenza Virus Hemagglutinin-2 Subunit Peptides Enhances the Antitumor Potency of Polyarginine-mediated p53 Protein Transduction*

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Protein transduction therapy is a newly developing method that allows proteins, peptides, and biologically active compounds to penetrate across the plasma membrane by being fused with cell-penetrating peptides such as polyarginine. Polyarginine-fused p53 protein penetrates across the plasma membrane of cancer cells and inhibits the growth of the cells. However, the protein is often entrapped inside macropinosomes in the cytoplasm. Therefore, high dose concentrations of the protein are needed for it to function effectively. To overcome this problem, in the present study, polyarginine-fused p53 was linked with the NH₂-terminal domain of influenza virus hemagglutinin-2 subunit (HA2), which is a pH-dependent fusogenic peptide that induces the lysis of membranes at low pH levels. The protein was capable of efficiently translocating into the nucleus of glioma cells and induced p21WAF1 transcriptional activity more effectively than did polyarginine-fused p53 protein. Moreover, low concentrations of the protein significantly inhibited the growth of cancer cells. These results suggest that protein transduction therapy using polyarginine and HA2 may be useful as a method for cancer therapy.

The cellular delivery of various biological compounds such as bioactive protein has been improved recently by conjugating the compounds to short peptides known as cell penetrating peptides (CPPs)¹ or protein transduction domains (PTDs)¹(1, 2). The PTD of human immunodeficiency virus type-1 TAT protein, which consists of an 11-mer polyarginine (11R), efficiently delivers peptides and proteins into cells (11, 12). The 11R-fused p53 protein (p53-11R) is delivered effectively into cancer cells and has transcriptional regulatory activity there (13). Moreover, p53-11R inhibits the proliferation of the cancer cells (13). However, a high concentration (>1 μM) and repeated administration of p53-11R are needed for transcriptional activation and the growth inhibition of cancer cells (13). Entrapment of the transduced protein in macropinosomes may weaken the effect of p53-11R. For protein delivery to become a promising method for clinical cancer therapy, this problem must be overcome

Several viruses have acquired endosomal escape mechanisms that take advantage of the low pH in mature endosomes of mammalian cells (14). The NH₂-terminal 20-amino acid peptide of the influenza virus hemagglutinin-2 protein (HA2) is well characterized as a pH-sensitive fusogenic peptide that destabilizes lipid membranes at low pH levels (14, 15). A recent study (10) has shown that TAT-PTD fusion proteins are internalized rapidly by lipid raft-dependent macropinocytosis. After internalization via the macropinocytotic pathway, the proteins are carried to macropinosomes, where most of them are then degraded (10). In order for the molecules delivered by CPPs to function in the cell, they generally must reach the cytosol. Therefore, protein delivery into the cytosol of target cells via macropinosomal escape is an important route of delivery.

The CPP, consisting of an 11-mer polyarginine (11R), efficiently delivers peptides and proteins into cells (11, 12). The 11R-fused p53 protein (p53-11R) is delivered effectively into cancer cells and has transcriptional regulatory activity there (13). Moreover, p53-11R inhibits the proliferation of the cancer cells (13). However, a high concentration (>1 μM) and repeated administration of p53-11R are needed for transcriptional activation and the growth inhibition of cancer cells (13). Entrapment of the transduced protein in macropinosomes may weaken the effect of p53-11R. For protein delivery to become a promising method for clinical cancer therapy, this problem must be overcome.

**EXPERIMENTAL PROCEDURES**

*Cell Lines and Cell Culture—A human malignant glioma cell line, U251-MG, was provided from Health Science Research Resources Bank (Osaka, Japan), and a malignant glioma cell line, KR158, was a gift from Dr. T. Jacks (Massachusetts Institute of Technology, Cambridge, MA). U251-MG cells contain a homozygous missense mutation corresponding to a His/Arg transition in codon 273 in p53. KR158 is a p53 gene-deficient cell line. Both cell lines were maintained in Dulbecco’s...*
modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. The cells were cultured in a 37 °C incubator with 5% CO₂. Rat primary astrocytes were prepared from a newborn Wistar rat (Japan SLC, Inc.). The cortex of the rat on postnatal day 1 was dissected, and the meninges were removed. The cortical tissues were then dissociated mechanically. The dissociated cells were plated onto collagen-coated glass slides and 96-well collagen-coated dishes. The plated cell density was approximately 10,000 cells/ml. The cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum (Invitrogen) and 5% horse serum. Cultures were maintained at 37 °C in a 95% air, 5% CO₂ humidified incubator.

Construction of p53-11R and HA2-p53-9R—Human wild-type p53 cDNA was subcloned into p11R-HA and p9R-HA vectors to produce genetic in-frame 11 (11R) and 9 polyarginine (9R) fusion proteins in the COOH-terminal, as described previously (13). Influenza virus HA2 cDNA encoding 23 amino acids (GLFAEIQEGFWGMIDGWYG) was tagged on the NH₂ terminus of p53 cDNA in p53-9R plasmids (HA2-p53-9R).

Site-directed Mutagenesis of Human p53 and HA2 cDNAs—The mutations of p53 and HA2 cDNAs were generated by site-directed mutagenesis using a PCR strategy as described previously (16). For dominant-negative p53, Arg at the site of 273 was changed to His as described previously (17). An HA2 mutant that has no destabilization activity of the lipid membrane at low pH was produced by replacing Gly at site 13 and Met at site 17 with Pro and Leu, respectively, as described previously (18). The sequences of the mutants were confirmed with an ABI 3100 sequence analyzer.

Preparation of Fluorescein Isothiocyanate-conjugated p53-11R and HA2-p53-9R and the Localization of the Proteins in Living Malignant Glioma Cells—The p53-11R and HA2-p53-9R proteins were conjugated with a green fluorescent tag using a fluorescein-EX protein labeling kit (catalog no. F-10240, Molecular Probes). After purification of the proteins, 1 µg of p53-11R, HA2-p53-9R, or HA2 cDNA was incubated with a lyophilometer using a real-time PCR machine. The localization of the transduced proteins was observed with a confocal laser microscope (FluoView, Olympus, Japan).

Western Blotting Analysis—U251-MG cells were incubated with 0.1 µM p53-11R and HA2-p53-9R. After 2 h, the cells were washed with PBS twice and placed in fresh medium in the absence of the proteins. The cells were harvested at each of the time points indicated in the text, and Western blot analysis was performed using anti-p53 monoclonal antibody. Western blot analysis for p53 was carried out at high stringency, essentially as described previously (13). Briefly, the harvested cells were homogenized by sonication in a boiled buffer containing 1% SDS. Samples containing 100 µg of total protein were electrophoresed by SDS-PAGE and then transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences). The blots were probed with primary antibody against p53 (1:1000) (catalog no. Pab 1801, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and developed with an enhanced chemiluminescence detection system (Amersham Biosciences).

Reporter Assay for p53-driven Transactivation—The reporter assay was performed as described previously (13). Briefly, the luciferase reporter vector pGL2-basic (Promega) containing a 2.4-kbp fragment of human p21 promoter was a gift from Drs. T. Akiyama (Tokyo University) and K. Yoshikawa (Osaka University). The luciferase reporter vector was transfected into 70% confluent KR158 cells in 35-mm dishes by the calcium phosphate method. After 24 h, the cells were supplemented with various concentrations of p53-11R or HA2-p53-9R and further incubated for 24 h (day 0). After washing with PBS, the cells were placed in fresh medium in the absence of 11R-p53 and HA2-p53-9R and further incubated for 96 h (day 4). The cell viability was measured using the WST-1 assay on day 0 and day 4 according to the manufacturer’s instructions (Roche Applied Science).

Detection of Apoptotic Cells—Apoptotic cells were confirmed by Hoechst and TUNEL staining as described previously (19). Briefly, after fixation with 4% paraformaldehyde, KR158 cells were incubated with 0.1 µg/ml of Hoechst 33244 (Sigma) for 1 min. The morphology of the nucleus was observed with a fluorescence microscope. Apoptotic cells were identified by the presence of highly condensed or fragmented nuclei. For TUNEL staining, KR158 cells seeded in 35-mm diameter culture dishes were incubated with p53-11R or HA2-p53-9R for 2 h. After washing twice with PBS, the cells were further incubated in fresh medium for 24 h. The cells were then fixed with 4% paraformaldehyde, and TUNEL staining was performed with an in situ cell death detection kit (Roche Applied Science). After TUNEL staining, the cells were incubated with rhodamine phallolidin (1:1000) (Molecular Probes) for 1 h to detect F-actin. The staining was observed using a confocal laser microscope (model no. LSM510, Zeiss). Representative graphs are shown for experiments in which at least four randomly chosen fields containing 100 cells each were scored.

FIG. 1. Comparison of the expression (A) and the p53-driven transcriptional activity (B and C) between HA2-p53-9R and p53-11R in glioma cells. A, time-dependent changes of expression in U251-MG cells are shown. Cont., no addition of p53 protein (control); upper band, polyarginine-fused p53 proteins; lower band, endogenous p53 protein. B, p21kip1 luciferase reporter activities in HA2-p53-9R- or p53-11R-transduced cells. n = 6 each; *, p < 0.01; C, comparison of p21kip1 luciferase reporter activities in cells transduced with each type of p53 protein. KR158 cells were transduced with each protein at 0.1 µM. Cells were harvested 24 h after the protein transduction, and the luciferase reporter activity was measured. n = 6 each; *, p < 0.01; **, p < 0.001.
Statistical Analysis—Data are shown as the mean \( \pm \) S.E. Data were analyzed using either Student’s \( t \) test to compare two conditions or analysis of variance followed by planned comparisons of multiple conditions, and \( p < 0.05 \) was considered significant.

RESULTS

Time-dependent Expressional Changes of HA2-p53-9R and p53-11R in Glioma Cells—We first produced HA2-fused p53-11R protein. However, the protein was insoluble after dialysis against PBS (data not shown). Therefore, p53 protein was fused with HA2 and 9-arginine (HA2-p53-9R), which also has protein transduction activity (11). Both HA2-p53-9R and p53-11R were delivered effectively into U251-MG cells within 4 h (Fig. 1A). After 4 h, the expression of both proteins decreased in a time-dependent manner (Fig. 1A). HA2-p53-9R expression declined more rapidly than p53-11R expression in the cells.

Comparison between p53 Transcriptional Activity of HA2-p53-9R and p53-11R—to compare the transcriptional activity between HA2-p53-9R and p53-11R in glioma cells, each protein (0.1 \( \mu \)M) was transduced into KR158 cells, which lack endogenous p53, and the time-dependent p53 transcriptional activity was examined. The transcriptional activity in HA2-p53-9R-transduced cells was significantly higher than that in p53-11R-transduced cells at all of the times examined (Fig. 1B). The transcriptional activity of HA2-p53-9R was maximal 12 h after the protein transduction, and it then decreased gradually in a time-dependent manner.

To investigate whether the enhancement of the transcriptional activity of HA2-p53-9R was the result of the function of HA2, an HA2 mutant (mtHA2) that has no endosome-releasing activity was fused with p53-9R, and the transcriptional activity of the fusion protein (mtHA2-p53-9R) was examined. The transcriptional activity of mtHA2-p53-9R was significantly lower than that of HA2-p53-9R (Fig. 1C) and was the same level as that of p53-11R. Moreover, the transcriptional activity of dominantly negative p53 protein fused with HA2 and 9R (HA2-DNp53) was much lower than that of HA2-p53-9R. These results suggested that the linkage with HA2 results in increased transcriptional activity of p53 protein transduced using polyarginine (p53-11R).

Time-dependent Changes of Subcellular Localization of p53-11R and HA2-p53-9R—To investigate whether HA2-p53-9R translocated effectively into the nucleus, p53-11R and HA2-p53-9R were conjugated with a green fluorescent tag, and U251-MG cells were transduced into each protein. The subcellular localization of each transduced protein was compared 30 min and 2 h after protein transduction in living cells. Moreover, to exclude the possibility that the proteins were just attached to the surface of the cell membrane, serial optical sections of 3-\( \mu \)m
step sections along the z dimension of the cells were then collected with a confocal microscope. Both p53-11R and HA2-p53-9R were observed in the cell membrane and perinuclear region after 30 min, and there was no expression of the protein observed in the nucleus (data not shown). After 2 h, HA2-p53-9R was observed in the nucleus and cell body, whereas p53-11R remained localized in the cell membrane and perinuclear region. (Fig. 2). These results suggested that HA2-p53-9R translocates more effectively into the nucleus than p53-11R.

Inhibitory Effect of HA-p53-9R on the Growth of Glioma Cells—
A previous study (13) has shown that a single administration of 1 μM p53-11R fails to inhibit the growth of oral cancer cells and that repeated administration every 24 h is needed for the inhibition of their growth. The present study also showed that a single application of 1 μM p53-11R did not inhibit the growth of glioma cells 3 or 4 days after the protein transduction (Fig. 3A). In contrast, HA2-p53-9R inhibited the growth of the cells in a dose-dependent manner (Fig. 3). Lower concentrations (0.01 and 0.1 μM) of HA2-p53-9R had no effect on the growth of glioma cells 1 or 2 days after the protein transduction. However, HA2-p53-9R at the higher concentrations inhibited the cell growth 4 days after protein transduction (Fig. 3). The maximal inhibitory effect on the growth of cells was achieved with 1 μM HA2-p53-9R (Fig. 3). To confirm that the inhibitory effect of 1 μM HA2-p53-9R was not caused by a toxic effect of HA2, HA2 peptides fused with 11R (HA2–9R) were transduced into the cells. HA2–9R (1 μM) did not affect the growth of glioma cells (Fig. 3B). Moreover, protein transduction of 1 μM HA2-DNp53-9R or 1 μM mtHA-p53-9R had no inhibitory effect on the cell growth (Fig. 3B).

Induction of the Apoptosis of Glioma Cells by HA2-p53-9R—
The effect of HA-p53-9R on the induction of the apoptosis of KR158 cells was investigated next. Treatment with 1 μM p53-11R did not induce apoptosis of the glioma cells, whereas 1 μM HA2-p53-9R markedly induced apoptosis of the cancer cells (Fig. 4). In contrast, the transduction of HA2-p53-9R did not induce the apoptosis of primary astrocytes (Fig. 5A and B) or inhibit the growth of the cells (Fig. 5C). Moreover, p53-11R also did not affect the growth or the apoptosis of primary astrocytes, whereas pAdex-p53 at an m.o.i. of 500 significantly induced apoptosis and inhibited the cell growth of primary astrocytes (Fig. 5).

DISCUSSION
Protein transduction therapy of p53 is useful for the inhibition of the proliferation of cancer cells (13, 20). However, the useful-
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treatment is limited because of the necessity of high doses for the inhibition and because only short-term expression occurs in the cells (12, 13). A previous study (13) showed that repeated protein transduction of p53-11R was needed for the inhibition of cancer cell proliferation. Although the immunogenicity of transduced proteins has not been studied in detail, repeated administration of protein therapy may initiate an immune response. Before initiating clinical trials of protein transduction therapy, the problems of protein therapy noted above must be overcome. In the present study, we showed that the linkage of HA2 peptides with p53-11R significantly enhanced the transcriptional activity and the antitumor effect of p53-11R. A single application of 0.01 μM HA2-p53-9R inhibited the growth of glioma cells, whereas a single application of 1 μM p53-11R failed to inhibit cell growth. Moreover, HA2-p53-11R did not affect the cell growth of normal glial cells. These results suggest that transduction therapy using p53 protein fused with HA2 may overcome the disadvantages of p53 protein therapy for cancer, allowing it to become a promising modality of cancer therapy.

The mechanism of protein transduction of p53-11R into cells remains unknown. A recent study (10) showed that TAT fusion proteins were internalized rapidly by lipid raft-dependent macropinocytosis, and most of the internalized proteins were trapped in macropinosomes. The present results showed that the transduction of HA2-fused p53-11R resulted in elevated transcriptional activity of p53-11R within 6 h, whereas the transduction of the fusion product with an HA mutant with no ability to disrupt macropinosomes had no effect on the transcriptional activity. Moreover, HA2-p53-9R translocated more effectively into the nucleus compared with p53-11R. These results suggest that the majority of polyarginine fusion proteins such as p53-11R may be entrapped in macropinosomes and that the fusion of HA2 may effectively release these proteins from macropinosomes in cancer cells, although there was no measurement of macropinosome trapping/escape performed in the present study.

The present results showed that normal astrocytes had low susceptibility to p53 protein transduction-mediated apoptosis and inhibition of cell growth. In contrast, adenosivirus-mediated p53 gene therapy at an m.o.i. of 500 induced apoptosis and inhibited the cell growth of the cells. Although we did not show the molecular mechanism responsible for divergent activities of HA-p53-9R between glioma cells and normal astrocytes in the present study, previous studies (21–23) have led to a hypothesis regarding the mechanism. Previous studies have shown that the overexpression of exogenous wild-type p53 protein has suppressive activity in tumor cells, although it does not apparently induce detrimental effects in normal cells. E2F, which is a transcription factor, forms a complex with p53, and this complex stimulates the apoptotic function of p53 (24). In normal cells, the activity of E2F is regulated tightly by the tumor suppressor Rb. Rb inhibits the activity of p53 through interaction with E2F (24). In tumor cells, in contrast, the Rb pathway is often inactivated, resulting in the deregulation of E2F activity (25). The deregulated E2F could then complex with and promote p53 to induce apoptosis. This may partly explain the sensitivity of glioma cells to p53 protein transduction therapy. The present results may provide a rationale for the development of p53 protein therapy approaches to tumor treatment without the need to target tumor cells, which is one of the most critical steps in tumor protein therapy with suppressive agents.

The data presented here show that HA2-p53-9R was more rapidly degraded in glioma cells compared with p53-11R (Fig. 1). It is well known that endogenous wild-type p53 protein is rapidly degraded by the ubiquitin-proteasome pathway in the nucleus (26). These results suggest that HA2-p53-9R released from macropinosomes may translocate into the nucleus, where it may be broken down rapidly by the ubiquitin-proteasome system. In contrast, p53-11R may be kept longer in macropinosomes than HA2-p53-9R protein and may not undergo rapid degradation by the ubiquitin-proteasome system. Moreover, these results suggest that p53 protein transduction therapy to transduce stable p53 protein could be further improved. One possibility is the transduction of mutated p53 protein that is resistant to ubiquitin-proteasome-mediated degradation. Simultaneous mutation of lysine residues 370, 372, 373, 381, 382, and 386 of the p53 protein to arginine residues generates a p53 molecule with potent transcriptional activity that is resistant to ubiquitin-proteasome-mediated degradation (27). Studies to develop a transduction method for stable 11R-p53 proteins fused with HA2 are currently in progress in our laboratory.

Acknowledgments—We thank A. Kemori and T. Ogawa for technical assistance, T. Akiyama and K. Yoshikawa for luciferase reporter vector and p21WAF1 promoter, and H. Matsushita and T. Jacks for KR158 cells.

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J. Biol. Chem. 2005, 280:8285-8289.
doi: 10.1074/jbc.M412430200 originally published online December 16, 2004

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