Development of p53 Protein Transduction Therapy Using Membrane-permeable Peptides and the Application to Oral Cancer Cells[1]

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Abstract

Recent studies suggest that several proteins can transverse biological membranes through protein transduction. The protein transduction domains of these proteins, 10–16 residues long, have been identified as critical domains for the protein transduction. Poly-arginine peptide also has the ability of protein transduction. Here, we show that the protein delivery system using 11 poly-arginine peptides (11R) is a powerful tool for the transduction of the biologically active tumor suppressor protein, p53, to suppress the proliferation of oral cancer cells. The 11R-fused p53 proteins (11R-p53) effectively penetrated across the plasma membrane of the cancer cells and translocated into the nucleus. The proteins induced the activity of the p21/WAF promoter and inhibited the proliferation of human oral cancer cells, in which the *p53* gene was mutated. The effect was equivalent to that of the adenovirus-mediated *p53* gene transduction system. Moreover, 11R-p53 enhanced the cisplatin-dependent induction of apoptosis of the cells. These data suggest that this protein transduction method may become a promising cancer therapy.

Introduction

Viral vector-mediated delivery of tumor suppressor genes such as *p53* represents a powerful strategy for cancer therapy (1). The viral vector is useful to transduce exogenous genes into various human cells *in vitro* and *in vivo* (1). However, previous studies have indicated that virus-mediated gene therapy has some safety problems, such as inflammatory response, toxicity, and random integration of viral vector DNA into the host chromosomes (1). Recent studies have shown that liposomes are able to deliver exogenous genes with minimal toxicity *in vivo* (2–4). However, the efficiency of gene transduction is worse than that of the viral-mediated gene transfer method at present (1).

The HIV type 1 Tat protein can enter cells when added to culture media (5, 6). The PTD[3] of the Tat protein, which contains a high proportion of arginine and lysine residues, has been identified as being responsible for the ability to penetrate the plasma membrane (7). It has been shown to serve as a carrier to direct the uptake of heterologous proteins into cells by generating genetic in-frame PTD fusion proteins (8–10). Interestingly, the PTD can deliver the biologically active form of β-galactosidase, which consists of $>$1000 amino acids, to all tissues including the brain *in vivo* (11). Recent studies have shown that poly-arginine, 6–12 residues, also has the same transduction activity as the PTD of the Tat protein (12–14). Moreover, we have shown that a protein transduction method using 11 poly-arginine peptides (11R) is useful for protein delivery in cells and brain slices and in the control of the localization of the delivered protein in cells (14). The protein transduction method has some advantages over viral vector-mediated gene transduction therapy in terms of safety, low toxicity, and random integration of vector DNA. In this study, we showed that p53 protein-fused 11R (11R-p53) was efficiently delivered in oral cancer cell lines and inhibited the proliferation of these cells.

Materials and Methods

Cell Lines and Cell Culture. The oral cancer cell line NOS-1 was a gift from Drs. Oku and Komori (Kobe University, Kobe, Japan). The oral cancer cell lines SAS, HSC-3, and HSC-4 were provided by the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). These cells were maintained in GIT medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 100 units/ml penicillin and 100 units/ml streptomycin. SAOS-2 cells were provided by Riken Gene Bank (Riken, Wako, Japan). The cells were maintained in DMEM with 10% fetal bovine serum.

Plasmid Construction and Purification of 11R-p53 Fusion Protein. We constructed a bacterial expression vector, p11R-HA, to produce genetic in-frame 11R fusion proteins. The vector p11R-HA was constructed by modification of pET-21a(+) vector (Novagen, Madison, WI). Briefly, the vector was tagged with a 6-histidine leader, followed by an

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[3] The abbreviations used are: PTD, protein transduction domain; HA, hemagglutinin antigen; MOI, multiplicity of infection; WST, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; GFP, green fluorescent protein.

11-arginine flanked by glycine and glutamic acid residues (for free bond rotation of the domain) in the COOH terminal. Full-length human wild-type p53 cDNA was subcloned into *Bam*HI/*Eco*RI-digested p11R-HA vector.

The constructed plasmids were transformed into BL21-DE3 *Escherichia coli* cells. The proteins were expressed in the cells by induction with 0.1 mM isopropyl-1-thio-βD-galactopyranoside. The expressed proteins were purified as described previously (14). After we collected the bacterial pellets, the pellets were sonicated and denatured in lysis buffer containing 20 mM HEPES (pH 8.0), 100 mM NaCl, 2 M urea, and 20 mM imidazole. The denatured soluble fraction was applied to a column of Ni-NTA agarose (Invitrogen, San Diego, CA) to purify His-tagged 11R-p53 proteins.

Recombinant Adenovirus Preparation. The recombinant adenovirus containing wild-type human p53 cDNA, chicken β-actin promoter, and SV40 polyadenylation signal was produced using the Adenovirus Expression Vector kit (Takara, Tokyo, Japan). The recombinant adenoviruses containing p53 cDNA (pAdex-p53 or virus-p53) were purified and concentrated using the CsCl step-gradient method as described previously (15). The concentrated adenoviruses were dialyzed with PBS and 10% glycerol and stored at −80°C until use. Recombinant adenovirus of lacZ (pAdex-lacZ) was also produced following the manufacturer's protocol.

Western Blot Analysis and Immunocytochemistry. Western blot analysis for p53 was carried out at high stringency, essentially as described previously (16). Briefly, the harvested cells were lysed in a boiled buffer containing 1% SDS with a sonicator. Samples containing 100 μg of total protein were run on SDS-PAGE gels, and then transferred to a nitrocellulose membrane (Hybond ECL; Amersham). The blots were probed with primary antibodies against p53 (1:1000; Pab 1801; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Immunocytochemistry was performed as described previously (14). Briefly, the cells were fixed with 4% paraformaldehyde, and then incubated with monoclonal anti-p53 antibodies (1:100; Pab 1801; Santa Cruz Biotechnology) and polyclonal anti-α-tubulin antibodies (1:200; C-20; Santa Cruz Biotechnology). After the antibodies were washed off, the cells were incubated with FITC-conjugated secondary antibodies (1:200; Sigma Japan) and rhodamine-conjugated secondary antibodies (1:100; Sigma Japan) or rhodamine phalloidin (1:10,000; Molecular Probes, Eugene, OR) instead of the rhodamine-conjugated secondary antibodies.

Reporter Assay for p53-driven Transactivation. The reporter assay was performed as described previously (17). The luciferase reporter vector pGL2-basic (Promega) that inserted a 2.4-kbp fragment of human p21/WAF1 promoter into the *Hin*dIII site was a gift from Drs. T. Akiyama (Tokyo University, Tokyo, Japan) and K. Yoshikawa (Osaka University, Osaka, Japan). The luciferase reporter vector was transfected into ~70% confluent SAOS-2 cells in 35-mm dishes by the calcium phosphate method. The cells were then either infected with each concentration of virus-p53 adenovirus or incubated with each concentration of 11R-p53. After 36 h, the cells were harvested, and the luciferase activities were measured with a luminometer using a reagent kit (Tokyo Ink, Tokyo, Japan). The background luciferase activity was subtracted from all experiments.

Cell Proliferation Assay. Cell proliferation was assessed at each time point after infection by pAdex-p53 or the addition of 11R-p53 by measuring the conversion of the triazolium salt WST-1 to formazan according to the manufacturer's instructions (Roche).

Assay for Cisplatin-dependent Apoptosis. Each of the cancer cells (~70% confluent) cultured on coverslips in 35-mm dishes was either transfected with virus-p53 (50 MOI) or added to 11R-p53 (1 μM). After 24 h, the cells were treated with 0.2 μg/ml cisplatin (Pharmacia) for 4 h. The cells were then washed with PBS twice, and new medium was added. One μmol of 11R-p53 was added to the new medium again. The cells were fixed with 4% paraformaldehyde (pH 7.4) 36 h after adding the cisplatin, and then Hoechst dye staining was performed. The fixed cells were treated with 3.3 μM Hoechst 33342 (Sigma Japan) for 15 min at room temperature and observed with a fluorescence microscope. Apoptotic cells were identified by the presence of highly condensed or fragmented nuclei.

Results

The Expression, Localization, and Reporter Activity of 11R-p53 in SAOS-2 Cells. To demonstrate whether 11R-p53 proteins were delivered into the cells through the plasma membrane, a *p53* gene-deficient osteosarcoma cell line, SAOS-2, was incubated with 11R-p53 (1 μM), and the protein expression level was compared with that in pAdex-p53-infected cells. Western blot analysis revealed that 11R-p53 proteins were strongly expressed in SAOS-2 cells (Fig. 1*B*). The mobility shift of 11R-p53 signals was seen on SDS-PAGE gel because 11R had a positive charge (Fig. 1*B*). We also incubated SAOS-2 cells with non-11R-p53 proteins (1 μM), which were not fused with 11R, as a control. Western blotting analysis revealed that the proteins were not expressed in the cells (Fig. 1*B*). To demonstrate whether 11R-p53 was localized in the nucleus of the cells and to exclude the possibility that the protein was just attached to the surface of the cell membrane, the cells were double-stained with anti-p53 antibody (green) and anti-tubulin antibody (red; Fig. 1*Ca*). The p53 signals were localized in the nucleus of SAOS-2 cells. Moreover, the cells were double-stained with anti-p53 antibody (green) and rhodamine phalloidin (red), and serial optical sections of 2-μm steps along the Z-dimension of the immunostained cells were then collected with a confocal microscope (Fig. 1*Cb*). The p53 signals were not seen on the surface of the cell membrane. Next, we demonstrated whether the delivered 11R-p53 has a transcriptional activity in living cells. The cells were incubated with either 11R-p53 or pAdex-p53, and a reporter assay for p53 was then performed using a luciferase reporter vector driven by the p21/WAF1 promoter, which contains the p53-binding site. The transcriptional activity of 11R-p53 increased in a dose-dependent manner (Fig. 1*D*). In 1 μM 11R-p53-transduced cells, the transcriptional activity was 32-fold higher than that of control. In non-11R-p53-incubated cells, the activity was not detected (Fig. 1*D*). These results indicate that the 11R-p53 protein penetrates through the cell membrane and

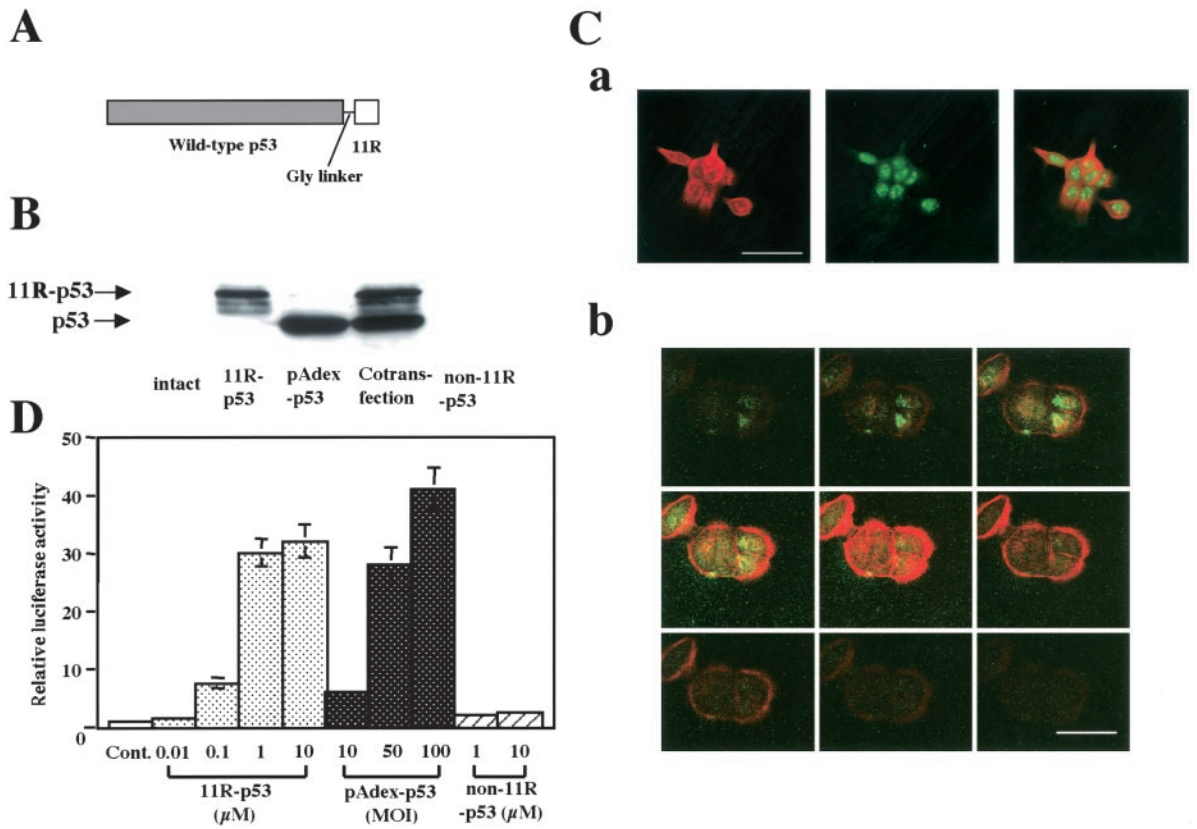

Fig. 1. The expression, localization, and reporter activity of 11R-p53 in SAOS-2 cells. *A,* construction of 11R-p53 protein. Human wild-type p53 protein was linked with 11 poly-arginine peptides at the COOH terminal using a Gly linker. *B,* the expression of 11R-p53. SAOS-2 cells were incubated with 1 μM 11R-p53 or 1 μM non-11R-p53 for 6 h and then washed with PBS once. The cells were harvested 24 h after replacement of new medium, and the lysates were used for Western blot analysis. As a control, the cells were infected with pAdex-p53 at 100 MOI. *C, a,* the localization of 11R-p53 in SAOS-2 cells. The cells were incubated with 11R-p53. After 36 h, the cells were fixed, and immunocytochemistry was performed using p53 antibody (*green*) and tubulin antibody (*red*). *Bar,* 80 μm. *b,* the images were multiple optical 2-μm step sections spanning the Z-dimension of laser scans of the cells. *Bar,* 20 μm. *D,* reporter activity of 11R-p53, non-11R-p53, and adenovirus-mediated *p53* gene transduction. SAOS-2 cells were either transduced at each concentration of 11R-p53 or were infected with each concentration of pAdex-p53 (Adeno-p53). After 36 h, the luciferase activities were measured with a luminometer. As a control (*Cont.*), the cells were incubated at each concentration of non-11R-p53. Data are represented as the means; $n = 8$ in each group; *bars,* SE.

localizes in the nucleus. Moreover, the protein has full transcriptional activity in cells.

11R-p53 Inhibits the Proliferation of NOS-1 Cells. Next, we demonstrated whether 11R-p53 inhibits the proliferation of NOS-1 cells, which has been established from a patient with oral squamous cell carcinoma and in which the *p53* gene was point mutated at site 248 (18). Each concentration of 11R-p53 or 10 μM 11R-GFP (14) was added to the culture medium at day 0. After 6 h, the medium was replaced with new medium excluding 11R-p53 or 11R-GFP. The cells were then cultured for an additional 4 days, and a WST assay was performed to demonstrate the cell proliferation every 24 h. The WST assay revealed that 0.1 μM 11R-p53 did not significantly inhibit the proliferation of NOS-1 cells at any of the time points. The growth curve of 0.1 μM 11R-p53-transduced cells was the same as that in 11R-GFP-transduced cells. In contrast, both 1 and 10 μM 11R-p53 significantly inhibited the proliferation of NOS-1 cells compared with that of 11R-GFP-transduced cells (1 μM 11R-p53, $P < 0.01$ at day 3, $P < 0.05$ at day 4 *versus* 11R-GFP on the same days; 10 μM 11R-p53, $P < 0.01$ at day 2 and day 3, $P < 0.05$ at day 4 *versus* 11R-GFP on the same days). However, the effect of 11R-p53 on the inhibition of the proliferation of the cells was weaker than that of pAdex-p53 (Fig. 2*B*). The WST assay revealed that infection of pAdex-p53 at MOIs of 20 and 100 more significantly inhibited the cell growth compared with that of 1 and 10 μM 11R-p53 at day 4 (Fig. 2, *A* and *B,* pAdex-p53 at 20 and 100 MOI, 1.05 $\pm$ 0.08 and 0.76 $\pm$ 0.025, respectively; 1 μM 11R-p53, 1.59 $\pm$ 0.04; 10 μM 11R-p53, 1.59 $\pm$ 0.09, $n = 10$ in each group; $P < 0.01$, pAdex-p53 at an MOI 20 or 100 *versus* 10 μM 11R-p53). Infection of pAdex-p53 at MOI of 300 completely inhibited the cell growth (Fig. 2*B*). However, the effect on the proliferation inhibition was attributable to virus toxicity because the infection of pAdex-lacZ at MOI of 300 also had the same effect (data not shown).

These data suggested that 11R-p53 inhibited the proliferation of NOS-1 cells, but the effect was weaker than that of the pAdex-p53 virus. One possibility is that the expression level of 11R-p53 was not sustained for a sufficient length of time in NOS-1 cells because the protein was provided only at day 0. It is well known that p53 is rapidly degraded by the

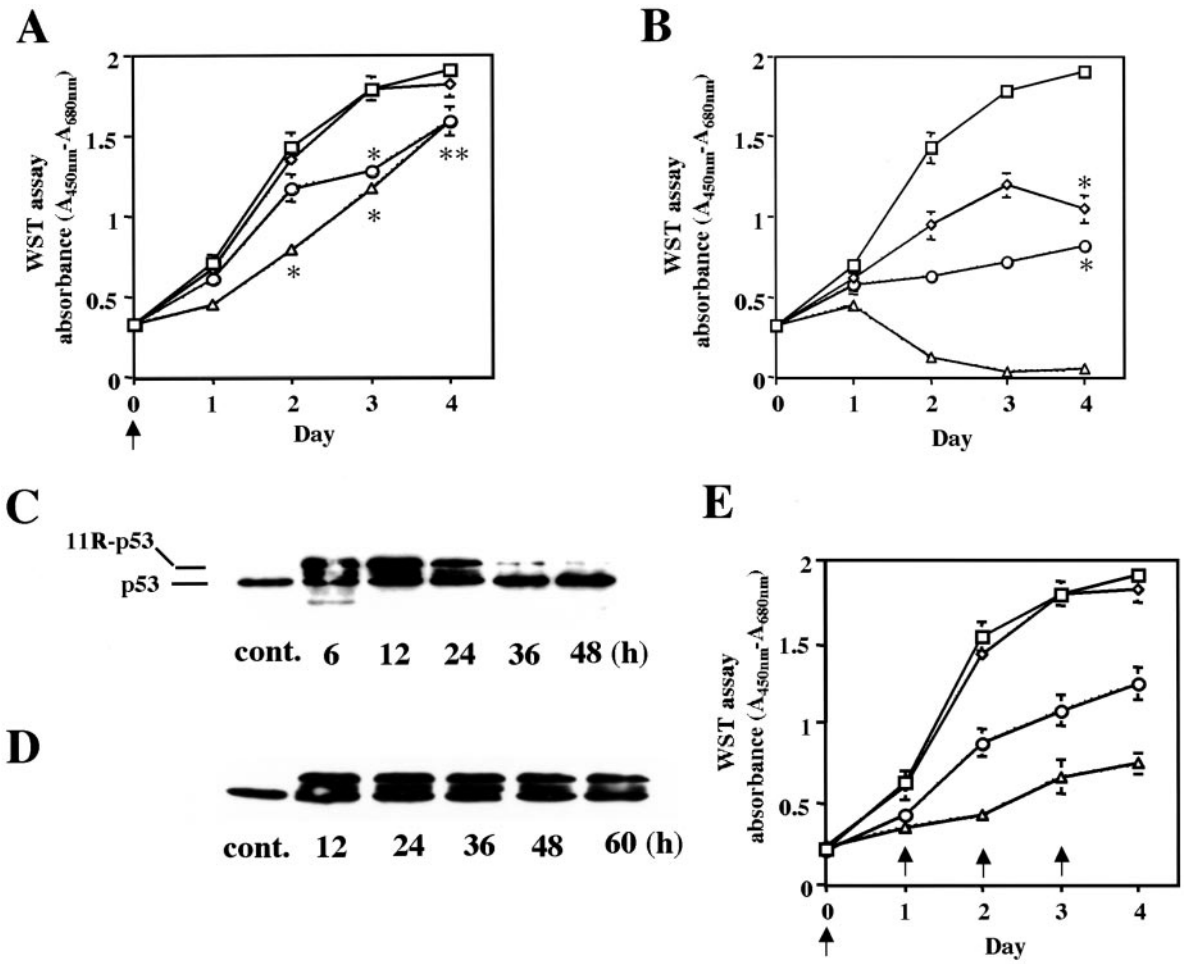

Fig. 2. Effect of 11R-p53 and pAdex-p53 on the inhibition of the proliferation of NOS-1 cells. *A,* the effect of a single transduction of 11R-p53. Each concentration of the proteins was added at day 0 (*arrow*). A WST assay was performed every 24 h. □, 10 μM11R-GFP; ◇, 0.1 μM 11R-p53; ○, 1 μM 11R-p53; △, 10 μM 11R-p53. Data are presented as means; $n = 10$ in each group; *bars,* SE. The significance of differences was calculated by the Scheffe's test following two-way ANOVA. ∗, $P < 0.01$ and ∗∗, $P < 0.05$ compared with the 11R-GFP. *B,* the effect of pAdex-p53. The viruses at an MOI of 20 (◇), 100 (○), and 300 (△) were infected at day 0. As a control, pAdex-lacZ at an MOI of 100 (□) was infected at the same time. ∗, $P < 0.01$ compared with the effect of 10 μM 11R-p53. *Bars,* SE. *C,* time course of the degradation of the 11R-p53 proteins. NOS-1 cells were harvested at each time point after the addition of the proteins (10 μM), and Western blot analysis was performed using anti-p53 antibodies. *cont,* no addition of 11R-p53. *D,* the effect of a repeat application of 11R-p53 on the protein expression. The proteins (10 μM) were applied at 0, 24, and 48 h. *cont,* no addition of 11R-p53. *E,* the effect of a repeat application of 11R-p53 on the inhibition of the proliferation of the cells. Each concentration of the proteins (□, 10 μM11R-GFP; ◇, 0.1 μM 11R-p53; ○, 1 μM 11R-p53; △, 10 μM 11R-p53) was added every 24 h for a total of four times (*arrows*). *Bars,* SE.

ubiquitin-proteasome-mediated pathway (19). We demonstrated time-dependent changes of the expression level of 11R-p53 (Fig. 2*C*). The protein levels of 11R-p53 were higher between 6 and 12 h after the addition of 11R-p53. However, the level rapidly decreased 36 h after the addition (Fig. 2*C*). In contrast, the expression of endogenous p53 protein was unchanged at each time point. To maintain the protein level of 11R-p53 in NOS-1 cells, we added 11R-p53 every 24 h. Western blotting analysis revealed that the repeated application of 11R-p53 maintained the expression level of the proteins (Fig. 2*D*) The WST assay showed that the repeated application of the proteins strongly inhibited the proliferation of NOS-1 cells. The effect of 10 μM 11R-p53 was equivalent to that of pAdex-p53 at an MOI of 100 [10 μM 11R-p53, 0.63 ± 0.06 ($n = 10$); pAdex-p53 at 100 MOI, 0.75 ± 0.05 ($n = 10$)]. These data show that p53 protein transduction using 11R inhibits the proliferation of oral cancer cells expressing the mutated *p53* gene.

11R-p53 Inhibits the Proliferation of Various Types of Oral Cancer Cells. Next, we demonstrated whether 11R-p53 inhibits the proliferation of other types of oral cancer cells. SAS cells contain the wild-type *p53* gene (20). HSC-3 cells harbor an insertional mutation between codon 305 and 306 (21), and in HSC-4 cells, a heterozygous missense mutation corresponding to a CAG → CGG transition in codon 248 was seen in the *p53* gene (20). Western blotting analysis revealed that the 11R-p53 proteins were effectively delivered into all types of these cells (Fig. 3*A*). In Fig. 3*B,* we compare the effect of the proteins on the inhibition of the proliferation of these four cell lines. The 11R-p53 (1 μM) was applied every 24 h, for a total of four times, and the WST assay was performed 96 h after the first application of the proteins. As

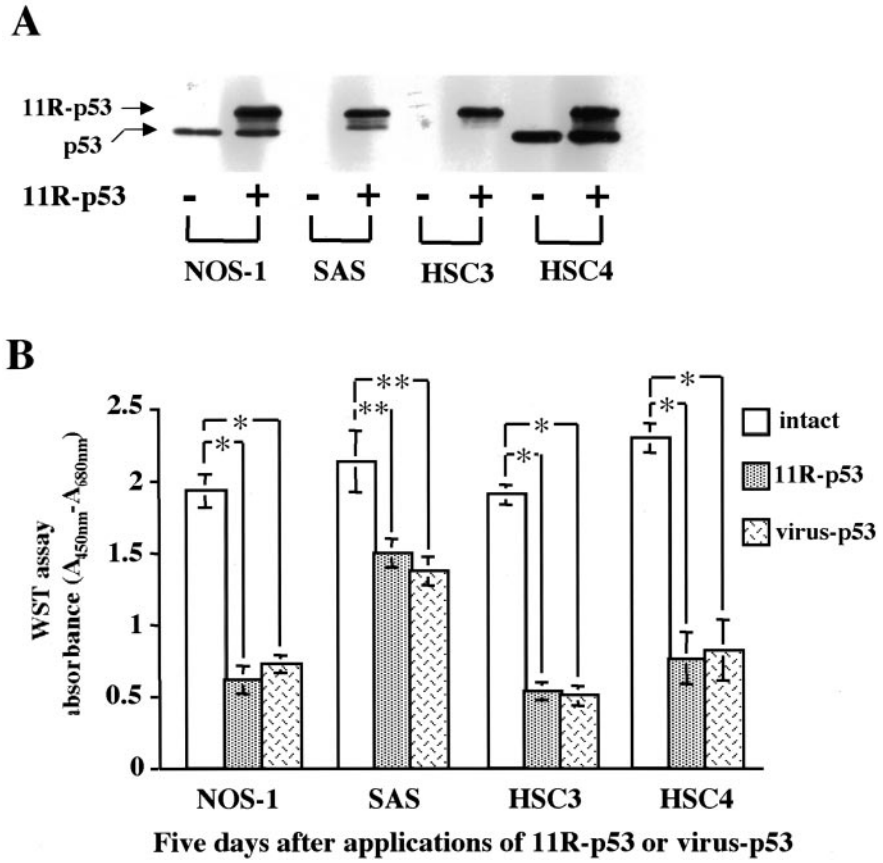

Fig. 3. Comparison of the effect of 11R-p53 on the inhibition of the proliferation of oral cancer cell lines, NOS-1, SAS, HSC-3 (*HSC3*), and HSC-4 (*HSC4*). Each of the cell lines was repeatedly applied with 1 μM 11R-p53 every 24 h for 4 days or infected with virus-p53 at 100 MOI at day 0. Western blotting analysis and the WST assay were then performed 5 days after the first application of 11R-p53 and infection of pAdex-p53. *A,* expressions of 11R-p53 and the endogenous proteins. *B,* the effect of 11R-p53 and virus-p53 on the inhibition of the proliferation of each of the cell lines. Data are represented as the means; $n = 6$ in each group; *bars,* SE. The significance of differences was calculated by the Scheffe's test following two-way ANOVA. $*$, $P < 0.005$ and $**$, $P < 0.01$ compared with the intact cells of each cell line.

a control, each of the cell lines was infected with pAdex-p53 at an MOI of 100. The 11R-p53 proteins significantly inhibited the proliferation of all of the cell lines. In all of the cell lines, moreover, the effect of the inhibition was the same as that of pAdex-p53 (Fig. 3*B*).

11R-p53 Enhances the Effect of Cisplatin on the Induction of Apoptosis of NOS-1 Cells. Previous studies have shown that transduction of the wild-type *p53* gene into cancer cells enhances the effect of anticancer drugs on the induction of apoptosis of the cancer cells (22, 23). We demonstrated whether the transduction of 11R-p53 increased the effect of cisplatin on the induction of apoptosis of NOS-1 cells. The transduction of 11R-p53 *per se* did not induce apoptosis (Fig. 4, *E* and *G*). However, the transduction of the protein enhanced the cisplatin-dependent induction of apoptosis of the cells (Fig. 4, *F* and *G*). The treatment with cisplatin after 11R-p53 transduction markedly induced apoptosis of the cells compared with the treatment with cisplatin only (Fig. 4*G,* CDDP + 11R-p53, 57 $\pm$ 6.7%; CDDP only, 35.2 $\pm$ 7.3%; $n = 100$ in each group). Moreover, the combination of overexpression of p53 and cisplatin might inhibit the cell viability of the cancer cells because the cell number in Fig. 4, *D* and *F,* was fewer than that of other treatments. Therefore, we compared the cell viability among each condition. The transduction of 11R-p53 significantly enhanced the cisplatin-dependent inhibition of the cell viability (Fig. 4*F*). These effects of 11R-p53 on the induction of chemosensitivity were the same as that of virus-p53.

Discussion

A large number of studies have attempted to evaluate the potential of tumor suppressor gene delivery in cancer therapy (1). Adenovirus-mediated *p53* gene delivery is being tested in human clinical trials to treat non-small cell carcinoma, head and neck cancers (24). However, a number of reports have indicated that virus-mediated gene therapy has some safety problems, such as inflammatory response, toxicity, and random integration of viral vector DNA into the host chromosomes (1). Direct delivery of tumor suppressor proteins such as p53 may reduce these problems. In the present study, we showed that 11R-p53 markedly inhibited the proliferation of oral cancer cells and the effect was at the same level as that of adenovirus-mediated *p53* gene transfection. Our data suggest that protein transduction therapy may become a promising cancer therapy. A recent study has shown that a protein fused with the HIV-1 Tat protein-transduction domain (RKKRRQRRR) or arginine-rich peptides (RRRRRRRRR) efficiently penetrated the epidermis to the same extent as the dermis of the s.c. layer *in vivo*, when the protein was sprayed on animal skin (25). This characteristic is useful for the treatment of oral cancer because we are able to directly transduce 11R-p53 into the tumor with ointment.

We also considered the disadvantage of the protein transduction method in the present study. The expression level of 11R-p53 was not maintained for >36 h. We needed repeated

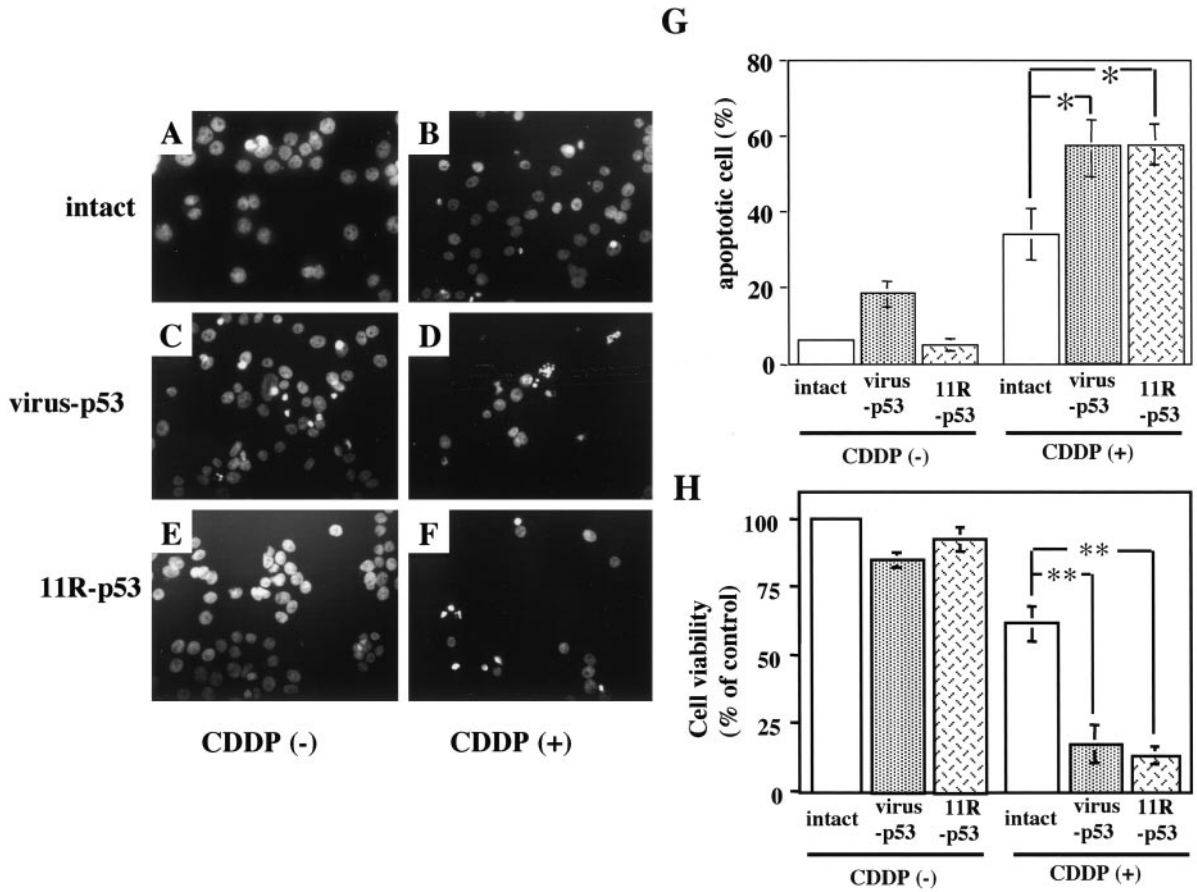

Fig. 4. Effects of 1 μM 11R-p53 (*E* and *F*) and virus-p53 (*C* and *D*) on cisplatin (*CDDP*)-induced apoptosis of NOS-1 cells. Apoptotic morphology of the cells in the absence of cisplatin (*A, C,* and *E*) or in the presence of cisplatin (*B, D,* and *F*) was observed by Hoechst staining. *G,* analysis of apoptotic cells among each treatment. The numbers of apoptotic nuclei were counted from representative photomicrographs and are represented as a percentage of the total number of nuclei counted ($n = 100$). Data are represented as the means; *bars,* SE. The significance of difference was calculated by the Scheffe's test following two-way ANOVA. $*$, $P < 0.01$. *H,* cell viability among each treatment. Cell viability was assessed using WST-1 assay. WST-1 assay was performed 36 h after adding the cisplatin. Data of cell viability are represented as percentages relative to control (intact) values (100%); *bars,* SE. The significance of difference was calculated by the Scheffe's test following two-way ANOVA. $**$, $P < 0.001$.

transduction of 11R-p53 for the inhibition of oral cancer cell proliferation. This problem may be solved by the following methods. A previous study showed that 2-methoxyestradiol induces the stabilization of the wild-type p53 protein in human cancer cell lines posttranscriptionally (26). Transduction of 11R-p53 with 2-methoxyestradiol may induce stabilization of 11R-p53. Another possible method is the transduction of the mutated p53 protein, which is resistant to ubiquitin-proteosome-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-proteosome-mediated degradation (19). Studies to develop a transduction method for stable 11R-p53 proteins are currently in progress in our laboratory.

In conclusion, we have provided three important findings: (*a*) the p53 protein-fused 11 arginine (11R-p53) was delivered and functioned in cancer cells; (*b*) transduction of 11R-p53 inhibited the proliferation of oral cancer cells; and (*c*) 11R-p53 enhanced the CDDP-induced apoptosis of oral cancer cells. These effects of 11R-p53 were the same as those of adenovirus-mediated delivery of the *p53* gene. Our p53 protein transduction therapy is a novel method of cancer therapy and should be a promising approach.

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