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Original Article

2-Methoxyestradiol Enhances p53 Protein Transduction Therapy-Associated Inhibition of the Proliferation of Oral Cancer Cells through the Suppression of NFκB Activity

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Protein transduction therapy using poly-arginine peptide can deliver the biologically active proteins. A previous study showed that 11 poly-arginine fused p53 protein (11R-p53) effectively penetrated across the plasma membrane and inhibited the proliferation of oral cancer cells. However, the intracellular half-life of the delivered protein was less than 36 h. Previous studies also showed that 2-methoxyestradiol (2-ME), an endogenous non-toxic estrogenic metabolite, induces the stabilization of the wild-type p53 protein in human cancer cells posttranscriptionally. In the present study, we examined whether 2-ME induced the stabilization of 11R-p53 and had an inhibitory effect on the proliferation of oral cancer cells. The application of 2-ME significantly enhanced the inhibitory effect of 11R-p53 on the proliferation of oral cancer cells. However, 2-ME had no effect on the intracellular half-life of 11R-p53 in oral cancer cells. Of interest is the finding that 2-ME suppressed the transcriptional activity of NFκB, which has an important role in tumorigenesis, but did not affect p53 transcriptional activity. These results suggest that 2-ME synergistically enhances the 11R-p53-induced inhibition of the proliferation of oral cancer cells through the suppression of NFkB transcription.

Key words: tumor, TAT, poly arginine, gene therapy, protein therapy

The human immunodeficiency virus (HIV) TAT protein transduction system has been shown to transduce biologically active proteins into cells [1-3]. However, this method has critical disadvantages in terms of its low efficiency of transduction and its influence on neuronal plasticity [4, 5]. To overcome these problems, a novel transduction system utilizing eleven poly-arginine (11R) has been developed [4]. 11R is highly efficient in delivering proteins into cells in multiple organs and is non-toxic with respect to neuronal function [4].

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Recent advances in molecular oncology have led to the identification of proteins that are quantitatively, qualitatively or genetically altered in cancer cells. p53 protein is one of the altered proteins in cancer cells, and is mutated or deleted in more than half of human tumors [6]. Therefore, tumor suppressor protein therapy with wild-type p53 protein has been proposed as a possible therapy for malignancies lacking active p53. A previous study showed that 11R-fused p53 protein effectively penetrates across the plasma membrane of oral cancer cells (> 95% cells) and translocates into the nucleus [7]. The protein induces the activity of the p21/WAF1 promoter and inhibits the proliferation of human oral cancer cells, and its effect is equivalent to that of adenovirus-mediated p53

gene therapy [7]. However, the half-life of 11R-p53 is less than 36 h [7]. Therefore, repeated transduction of 11R-p53 is needed for the inhibition of oral cancer cell proliferation [7]. Although the immunogenicity of the transduced protein has not been determined, repeated administration of protein therapy may initiate an immune response. The development of protein therapy to transduce stable proteins or of methods to stabilize the delivered proteins is important in order to initiate clinical trials of protein therapy.

2-Methoxyestradiol (2-ME) is a metabolic byproduct of estrogen and an anti-mitotic agent that is present in human urine and blood [8]. 2-ME has been shown to inhibit the growth of various tumor cells through the induction of apoptosis [9, 10]. 2-ME can potently inhibit superoxide dismutase, resulting in enhanced formation of reactive oxygen species and thus toxicity to tumor cells [11]. Also, 2-ME interferes with NFκB transcriptional activity, which plays an important role in tumorigenesis and which has been shown to be aberrant in many types of cancer cells and primary tumors [12-14]. Moreover, previous studies have shown that 2-ME induces stabilization of the wild-type p53 protein in human cancer cells posttranscriptionally [15, 16], and that adenoviral-vector-mediated p53 gene transfer followed by 2-ME treatment causes significant growth inhibition in human lung cancer cell lines [15]. These results suggest that 2-ME might induce the stabilization of 11R-p53 in oral cancer cells and enhance the inhibitory effect of 11R-p53 on the proliferation of oral cancer cells.

In the present study, we examined whether 2-ME reduced the degradation of 11R-p53 and enhanced the anti-tumor effect of the protein.

Materials and Methods

Cell culture. The oral cell line NOS-1, which was established from a patient with oral squamous cell carcinoma and in which the *p53* gene was point-mutated at site 248, was a gift from Drs. N. Oku and T, Komori (Kobe University, Kobe, Japan). The oral cancer cell line SAS, which contains the wild-type *p53* gene, and the HSC-3 and HSC-4 cell lines were provided by the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). All cell lines were maintained in GIT medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 100 units/ml penicillin and 100 units/ml streptomycin. The cells were cultured in a 37 °C incubator with 5% CO_2.

Production of 11R-p53 proteins. Production of 11R-p53 was carried out as described previously [7]. Briefly, full-length human wild-type p53 cDNA was subcloned into p11R-HA vector. The plasmids were then transformed into BL21-DE3 *Escherichia coli* cells and 11R-p53 proteins were expressed in the cells by induction with 0.2 mM isopropyl-1-thio-βD-galactopyranoside. The 11R-p53 proteins were purified using a column of Ni-NTA agarose (Invitrogen, San Diego, CA, USA).

Cell proliferation assay. Cell proliferation was assessed as described previously [7]. Briefly, cells were plated in 96-well plates at a density of 4×10^3 cells/well in quintuplicate. After 24 h, the cells were incubated with 1 μM 11R-p53 with/without 10 μM 2-ME (Sigma-Aldrich Chemical, St. Louis, MO, USA). The cell medium supplemented with 10 μM 2-ME was exchanged every 24 h. Control cells received only the vehicle (0.1% DMSO). Cell growth was assessed every 24 h by measuring the conversion of the triazolium salt WST-1 (Roche Applied Science, Mannheim, Germany) to formazan according to the manufacturer's instructions.

Western blotting analysis. Western blotting analysis for p53 and actin was carried out as described previously [17]. Briefly, the harvested cells were lysed by boiling and sonication containing 1% SDS. Samples containing 50 μg of total protein were electrophoresed on 10% SDS-PAGE gels and then transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences, Piscataway, NJ, USA). The blots were probed with primary antibodies against human p53 (1:1000; Pab 1801; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and actin (1:5000; AC-40; Sigma-Aldrich Chemical, St. Louis, MO, USA). The bands were visualized using a commercial ECL detection kit (Amersham Biosciences).

Reporter assay for p53- and NFκB-driven transactivation. The reporter assays were performed as described previously [7]. For the p53 reporter assay, the luciferase reporter vector pGL2-basic (Promega, Madison, WI, USA) containing an inserted 2.4-kbp fragment of the human p21/WAF1 promoter was a gift from Drs. T. Akiyama (Tokyo University, Tokyo, Japan) and K. Yoshikawa (Osaka University, Osaka, Japan). pNFκB-TA-Luc plasmid (BD Biosciences Clontech, Palo Alto, CA, USA) was used for the NFκB reporter assay. The luciferase reporter vectors were transfected into −70% confluent NOS-1 cells in

35-mm dishes using Lipofectamine plus (Invitrogen). The cells were then incubated with 1 μM 11R-p53 and 10 μM 2-ME for 24 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 in all experiments.

Statistical analysis. Data were analyzed using either the Student's t-test to compare 2 conditions or ANOVA followed by planned comparisons of multiple conditions, and $P < 0.05$ was considered to be significant.

Results

Effect of co-treatment with 11R-p53 and 2-ME on the proliferation of oral cancer cells. A previous study showed that 1 μM 11R-p53 inhibits the proliferation of both SAS and NOS-1 cells, in which wild-type and point-mutated p53 genes are expressed, respectively [7]. In the present study, the oral cancer cells were treated with 1 μM 11R-p53 on day 0. After 4 h, the cells were washed and treated with fresh medium in the presence or absence of 10 μM 2-ME, which is an optimal concentration for the inhibition of the proliferation of cancer cells [9–11, 14]. Treatment with 11R-p53 significantly suppressed the proliferation of SAS cells (Figs. 1A and C, day 4, control, 1.56 ± 0.1; 11R-p53, 1.24 ± 0.15, n = 5 each, $^*P < 0.05$) and NOS-1 cells (Figs. 1B and C, day 4, control, 1.68 ± 0.12; 11R-p53, 1.22 ± 0.13, n = 5, $^{***}P < 0.01$) as described previously [7]. Co-application of 11R-p53 and 2-ME inhibited the proliferation of the oral cancer cells more markedly than treatment with 11R-p53 alone (Fig. 1A–C, SAS, day 4, 11R-p53 + 2-ME, 0.56 ± 0.08, n = 5, $^{**}P < 0.001$ *vs.* 11R-p53; NOS-1, day 4, 11R-p53 + 2-ME, 0.55 ± 0.13, n = 5, $^{**}P < 0.001$ *vs.* 11R-p53). Moreover, the inhibitory effect of treatment with 2-ME on the proliferation was the same as that of 11R-p53 treatment (Fig. 1 C, SAS, day 4, 2-ME, 0.91 ± 0.11, n = 5, $P > 0.05$ *vs.* 11R-p53; NOS-1, day 4, 2-ME, 1.18 ± 0.15, n = 5, $P > 0.05$ *vs.* 11R-p53). These results suggest that co-treatment with 11R-p53 and 2-ME causes enhancement of the individual effects of these agents on the proliferation of oral cancer cells.

Effect of 2-ME on the stabilization and level of transcription of 11R-p53 in oral cancer cells. To investigate the effect of 2-ME on the stabilization of 11R-p53 in NOS-1 cells, the effect of 2-ME on the time-dependent changes in the level of 11R-p53 were observed. A high level of 11R-p53 was detected 6 h after exogenous addition of the protein to the cells in the absence of 2-ME (Fig. 2A). The level was decreased after 12 h and the low level was maintained until 72 h after 11R-p53 addition. Co-application of 10 μM

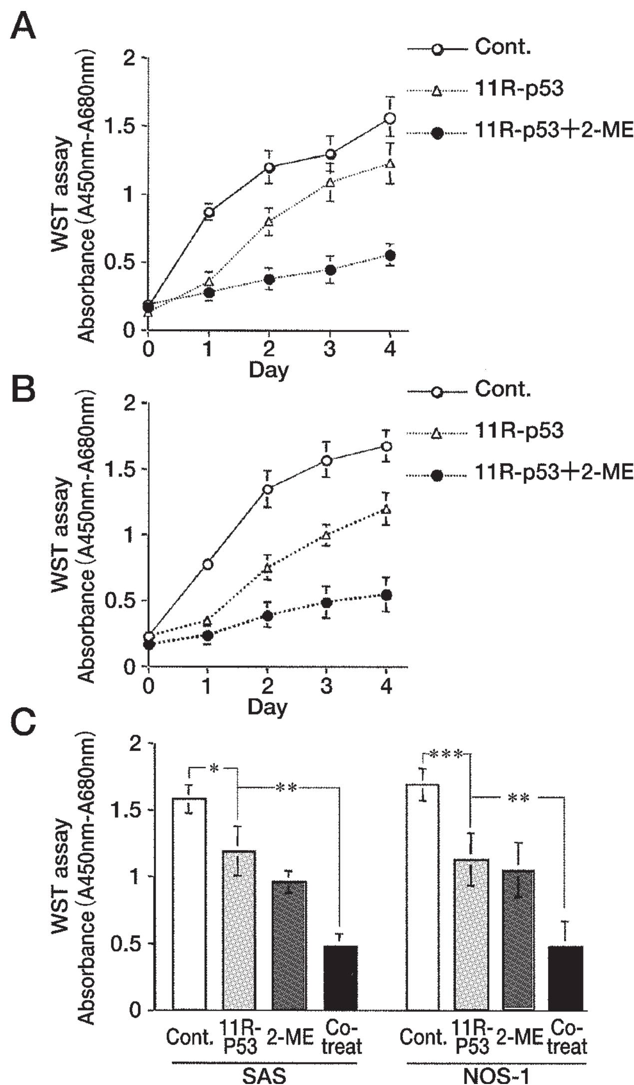

Fig. 1 2-ME induced the inhibitory effect of 11R-p53 on the proliferation of the oral cancer cell lines, SAS (**A**) and NOS-1 (**B**). 1 μM 11R-p53 with 10 μM 2-ME (11R-p53 + 2-ME) or without 2-ME (11R-p53) was added to the cells at day 0. The medium was changed to fresh medium every 24 h, and for 11R-p53 + 2-ME, 10 μM 2ME was added in the medium. Cell growth was assessed every 24 h by WST assay as described in "Materials and Methods". **C**, Comparison of the inhibitory effect of each treatment on cell growth at day 4. Data are representative of 5 independent experiments. Bars, S.D. $^*P < 0.05$, $^{**}P < 0.001$ and $^{***}P < 0.01$.

2-ME with 11R-p53 had no effect on the level of 11R-p53 (Fig. 2A). The level of 11R-p53 was high 6 h after it was added together with 2-ME, but the high level was not maintained 12 h after the addition. The level of 11R-p53 72 h after the addition was not different than the level observed in the absence of 2-ME. The effect of high-dose 2-ME (50 μM) was also examined, but 50 μM 2-ME did not affect the stabilization of 11R-p53 (data not shown).

The effect of 2-ME on the expression of endogenous p53 was next examined in various oral cancer cell lines. HSC-3 cells harbor an insertion mutation between codons 305 and 306. The p53 protein is prematurely terminated and the predicted molecular weight is 42 kDa. In HSC-4 cells, a heterozygous missense mutation corresponding to a CAG (Gln) → CGG (Arg) transition in codon 248 was seen in the p53 gene. Each cell line was incubated with

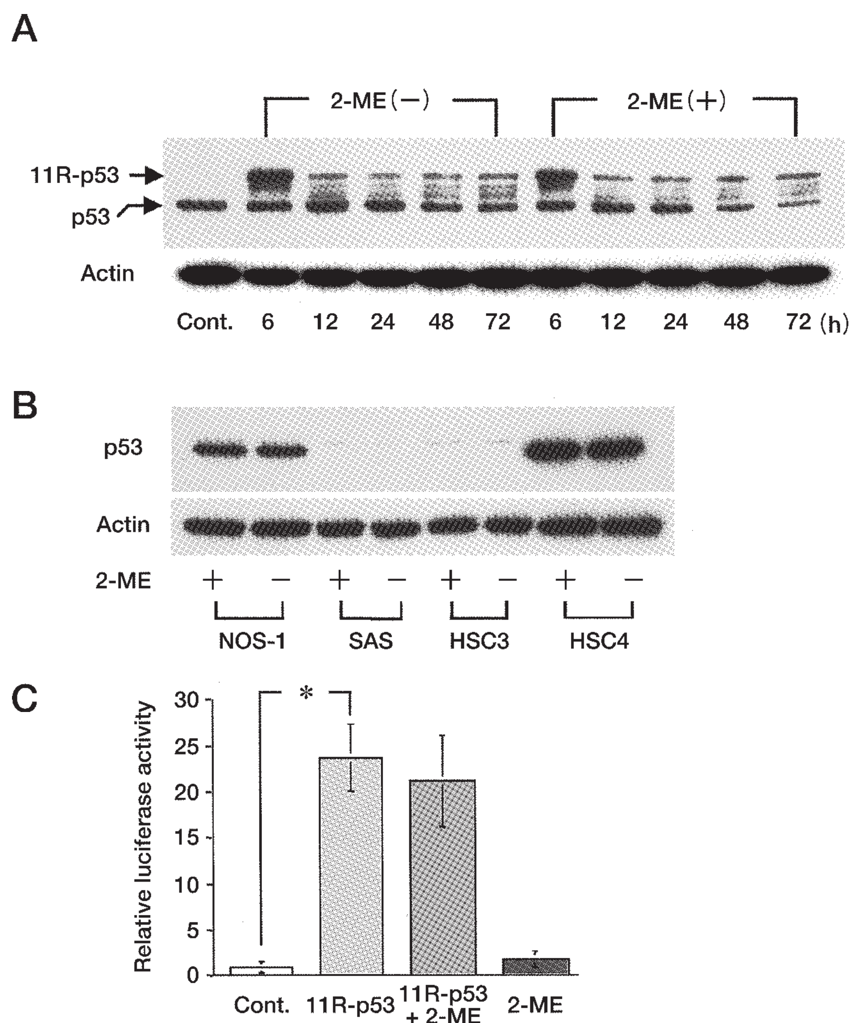

Fig. 2 A, Time course of the degradation of the 11R-p53 proteins and the effect of 2-ME on the degradation in NOS-1 cells. After incubation with 11R-p53 for 4 h, the cells were washed with PBS twice and were then incubated with fresh medium in the presence or absence of 2-ME (10 μM). The medium was replaced with fresh medium with/without 2-ME (10 μM) every 24 h. The cells were collected at the indicated time-points, and Western blotting analysis was performed using anti-p53 and anti-actin antibodies. B, Effect of 2-ME on endogenous p53 expression in oral cancer cell lines. Each cell line was incubated with 10 μM 2-ME for 48 h. The cells were then collected and Western blotting analysis was performed using anti-p53 and anti-Actin antibodies. The predicted molecular weight of the p53 protein in HSC-3 cells is 42 kDa. C, Effect of 2-ME (10 μM) on p53 reporter activity. Data are representative of 5 independent experiments. Data represent means ± S.D. $^*P < 0.0001$.

11R-p53 for 48 h and was then collected (Fig. 2B). 2-ME did not induce the expression of endogenous p53 in any of the cell lines. These results suggest that 2-ME had no effect on the expression or stabilization of 11R-p53 and p53 in oral cancer cells.

We also examined the effect of 2-ME on the level of transcriptional activity of 11R-p53 in NOS-1 cells (Fig. 2 C). Transduction of 11R-p53 induced the p53 transcriptional activity (23.5 ± 1.7 fold compared with the control, n = 6 each, $^*P < 0.0001$). The p53 transcriptional activity in the cells incubated with both 11R-p53 and 2-ME was the same as that in 11R-p53-transduced cells (11R-p53 + 2-ME, 21 ± 3.7 fold, n = 6, $P > 0.05$ *vs.* 11R-p53), indicating that 2-ME had no effect on the 11R-p53 transcriptional activity. Moreover, the application of 2-ME did not induce endogenous p53 transcriptional activity without 11R-p53 (2-ME, 1.7 ± 0.9, n = 6, $P > 0.05$ *vs.* control).

Inhibitory effect of 2-ME on the NFκB transcriptional activity. Previous studies have shown that 2-ME interferes with NFκB transcriptional activity, resulting in the inhibition of the proliferation of cancer cells and the induction of apoptosis of the cells [12–14]. The effects of 11R-p53 and 2-ME on the level of NFκB transcriptional activity in NOS-1 cells were examined (Fig. 3). A high level of NFκB activity was observed in the intact cells, and 11R-p53 protein transduction had no effect on the level of transcriptional activity (1.1 ± 0.1, n = 5, $P > 0.05$ *vs.* Cont.). In contrast, co-application of 11R-p53 and 2-ME significantly reduced the level of NFκB transcriptional activity (0.23 ± 0.12, n = 5, $^*P < 0.001$ *vs.* Cont.). Application of 2-ME alone also inhibited the level of NFκB activity to the same extent as co-application of 11R-p53 and 2-ME (Fig. 3, 0.21 ± 0.05, n = 5, $^*P < 0.001$ *vs.* Cont.). These results suggest that 2-ME inhibits the NFκB transcriptional activity in the oral cancer cells.

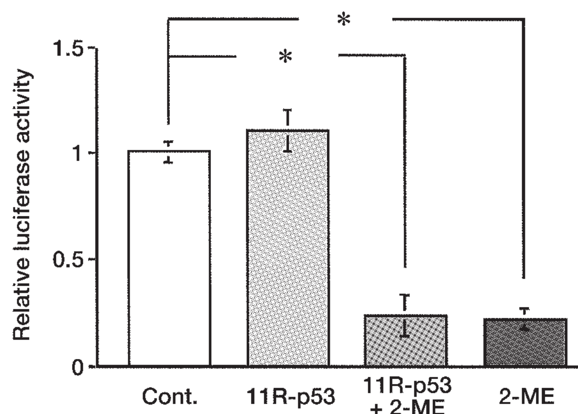

Fig. 3 Effect of 11R-p53 and 2-ME on NFκB transcriptional activity in NOS-1 cells. The cells were transfected with pNF B-TA-Luc plasmid. After 24 h, the cells were incubated with 1 μM 11R-p53 and/or 10 μM 2-ME for 24 h and were then harvested, and the luciferase activities were measured. Data represent means ± S.D. $^*P < 0.001$

Discussion

The results of the present study provided the three following important findings: 1) Co-application of 11R-p53 and 2-ME significantly inhibited the proliferation of oral cancer cells compared with the application of 2-ME alone; 2) 2-ME had no effect on the stabilization or expression of either 11R-p53 or endogenous p53 protein in oral cancer cells; 3) 2-ME reduced the NFκB transcriptional activity but had no effect on the p53 transcriptional activity.

Protein transduction therapy using agents such as poly-arginine and TAT peptides is useful for the delivery of biologically active proteins. However, protein transduction therapy has a disadvantage in terms of the short-term expression of the delivered proteins. The present and previous studies show that the intracellular half-life of the delivered 11R-p53 is less than 24 h [7]. Previous studies have shown that 2-ME induces posttranscriptional stabilization of the wild-type p53 protein in human cancer cells [15, 16], and that adenoviral-vector-mediated p53 gene transfer followed by 2-ME treatment causes significant growth inhibition in human lung cancer cell lines [15]. In the present study, we investigated whether 2-ME enhanced the stabilization and expression of 11R-p53 in oral cancer cells. However, 2-ME had no effect on the stabilization or expression of either 11R-p53 or endogenous p53 protein in oral cancer cells. It is hard to explain the discrepancy between the previous and present studies because the mechanism of 2-ME-dependent induction of the stabilization of p53 protein is unclear. One possibility is that the origin of the cancer cell lines was different. Previous studies have shown the effect of 2-ME on the induction of p53 protein in human lung cancer cell lines and colorectal carcinoma cell lines [15, 16]. A method to stabilize the transduced 11R-p53 in oral cancer cells needs to be developed. A previous study showed that a p53 mutant in which lysine residues

370, 372, 373, 381, 382 and 386 were replaced by arginine residues was resistant to ubiquitin-proteosome-mediated degradation, and that the transcriptional activity was higher than that of wild-type p53 [18]. This result suggests that stable expression of the fusion protein of the p53 mutant with 11R is maintained in oral cancer cells. Another possible method of stabilization is the transduction of a phosphorylated form of 11R-p53. Phosphorylation of p53 at Ser15 and/or Ser20 induces stabilization of p53 by inhibiting of the binding with MDM2, which is critical for ubiquitin-proteosome-mediated degradation [19]. Direct addition of phospho-11R-p53, which was previously phosphorylated *in vitro*, may solve the problem of the stabilization of 11R-p53.

In the present study, the growth of SAS cells with wild-type p53 gene was suppressed by wild-type p53 protein transduction. The expression of p53 is regulated by the action of Mdm2, an oncogenic E3 ligase, to induce p53 ubiquitination and degradation [20, 21]. The human Mdm2 is overexpressed in a wide range of human cancers in which wild-type p53 is expressed, and the overexpression of Mdm2 serves to inactivate p53 function in these cells [22, 23]. These results may suggest that Mdm2 overexpresses in SAS cells and induces the rapid degradation of p53. Therefore, 11R-p53 transduction may effectively inhibit the proliferation of the cells.

Interestingly, the present results showed that 2-ME inhibited the NFϰB transcriptional activity. Numerous studies have examined the tumor-inhibiting potential of 2-ME [8-11, 14, 24]. Moreover, co-application of adenoviral-vector-mediated p53 gene transfer and 2-ME causes significant growth inhibition in human lung cancer cell lines [15]. However, the exact mechanism by which 2-ME inhibits cancer cell proliferation is unknown. A very recent report showed that 2-ME preferentially inhibits the growth of brain tumor cells by blocking cell cycle progression in the G_2/M phase and induces apoptosis of the tumor cells [14]. In addition, 2-ME reduces the transcriptional activity and DNA binding activity of NFϰB but does not influence the expression of p53 [14]. The present results agree with those results, and show that 2-ME did not alter p53 expression or a p53 transcriptional activity. NFϰB has been suggested to have a wide-ranging role in tumor progression, and its activity is associated with angiogenesis and metastasis [12, 13]. Also it is known to be required for the transforming properties of many oncogenes, such as Bcr-Abl, Ras and the human T cell lymphotrophic virus 1 (HTLV 1) protein Tax [25-28]. These results suggest that NFϰB activation may be crucial for cancer cell growth and that 2-ME may inhibit cell growth through the inhibition of NFϰB activation, not through a p53-mediated pathway in oral cancer cells.

In contrast, we previously showed that 11R-p53 inhibited the proliferation of oral cancer cells without any significant induction of apoptosis [7]. Moreover, 11R-p53 induces the activity of the promoter of p21/WAF, which mediates G1 arrest via its inhibitory effects on the cyclin-dependent kinases (CDKs) required for S-phase entry [29, 30]. These results may suggest that 11R-p53 inhibits the growth of oral cancer cells by means of the induction of cell-cycle arrest, and that the mechanism of the growth inhibition induced by 11R-p53 is different from that induced by 2-ME. Therefore, co-application of 11R-p53 and 2-ME might cause a significant additive inhibitory effect on the proliferation of oral cancer cells compared with the independent application of each agent alone in the present study. 2-ME is a natural metabolic by-product of estrogen and causes no adverse reactions such as hair loss, intestinal damage or infection [31]. These results suggest that co-application of 11R-p53 and 2-ME has great potential as a chemotherapeutic treatment for oral cancer.

In conclusion, 2-ME had no effect on the stabilization or expression of 11R-p53, but significantly inhibited oral cancer proliferation. Our current findings may be useful for the development of a novel oral cancer therapy.

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