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C-terminal Peptides of p53 Molecules Enhance Radiation-induced Apoptosis in Human Mutant *p53* Cancer Cells

Ken Ohnishi[1], Hiroaki Inaba[1+], Jun-ichi Yasumoto[2], Kazue Yuki[3], Akihisa Takahashi[1] and Takeo Ohnishi[1*]

Departments of [1]Biology, [2]Oral and Maxillofacial Surgery, [3]Otorhinolaryngology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan

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[+]Present address: Department of Oral Frontier Biology, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan

*Address correspondence to:

Takeo Ohnishi, Ph.D.,

Department of Biology,

Nara Medical University,

Kashihara, Nara 634-8521, Japan.

TEL: +81-744-22-3051 ext. 2264

FAX: +81-744-25-3345

e-mail: tohnishi@nmu-gw.naramed-u.ac.jp

We propose here a novel p53-targeting radio-cancer therapy using p53 C-terminal peptides for patients having mutated *p53*. Hoechst 33342 staining showed that X-ray irradiation alone efficiently induced apoptotic bodies in wild-type p53 (wt*p53*) human head and neck cancer cells transfected with a *neo* control vector (SAS/*neo* cells), but hardly induced apoptotic bodies in mutation-type p53 (m*p53*) cells transfected with a vector carrying the m*p53* gene (SAS/m*p53*). In contrast, transfection of p53 C-terminal peptides (amino acid residues 361-382 or 353-374) via liposomes caused a remarkable increase of apoptotic bodies in X-ray-irradiated SAS/m*p53* cells, but did not enhance apoptotic bodies in X-ray-irradiated SAS/*neo* cells. In immunohistochemical analysis, positively stained cells for active type caspase-3 were observed at high frequency after X-ray irradiation in the SAS/m*p53* cells pre-treated with p53 C-terminal peptides. In SAS/*neo* cells, positively stained cells for active type caspase-3 were observed with X-ray irradiation alone. Furthermore, protein extracts from X-ray-irradiated SAS/m*p53* cells showed higher DNA-binding activity of p53 to p53 consensus sequence when supplemented *in vitro* with p53 C-terminal peptides than extracts from non-irradiated SAS/m*p53* cells. These results suggest that radiation treatment in the presence of p53 C-terminal peptides is more effective for inducing *p53*-mediated apoptosis than radiation treatment alone or p53 C-terminal peptide treatment alone, especially in m*p53* cancer cells. This novel tool for enhancement of apoptosis induction in m*p53* cells might be useful for p53-targeted radio-cancer therapy.

Introduction

Activation of p53 is well known to induce apoptosis[1] and cell growth arrest[2, 3] through the expression of downstream genes and to play an important role in the suppression of tumor growth. We have recently reported that patients bearing wt*p53* show a high survival rate after radiotherapy due to positive Bax and negative Bcl-2 regulation[4, 5]. It seems that *p53* is an efficient predictive indicator of the outcome of radiotherapy for cancer. Mutated p53 (mp53) proteins are defective in DNA-binding to specific DNA sequences (p53 consensus sequence; p53CON) located upstream of *p53*-regulated genes[6, 7] due to misfolding of the p53 proteins and can not exert a suppressive effect on tumor growth. Mutations of *p53* genes are observed in more than 50% of human cancer cells[8, 9]. Attempts to develop advanced cancer therapies to

overcome this problem are underway. *p53* gene therapy has been developed as a useful cancer therapy[10-12] and applied to patients carrying m*p53* as well as wild-type *p53* (wt*p53*)[13-15]. However, overexpression of wt*p53* may carry a risk of inducing growth arrest in normal cells. Growth arrest of normal cells may adversely affect the physical strength of patients. From this point of view, there are still difficulties in the clinical application of *p53* gene therapy.

One of important features of p53 as a tumor suppressor is its binding activity to p53CON. Most mutations of *p53* are localized in the binding domain to p53CON[18-20]. The mutations of p53 molecules can be categorized into 2 classes[20]. “Contact mutants” such as 248W and 273H affect residues that are involved in direct contact with DNA, whereas “conformation mutants” such as 175H, 249S and 281G affect residues that are involved in maintaining the structural stability of the core domain. Understanding the DNA-binding mechanism of p53 is important for radio-therapy based on p53-mediated signal transduction. The sequence-specific DNA-binding of p53 is allosterically regulated by its C-terminal domain[19, 21, 22] and can be activated *in vitro* by C-terminal truncation or binding of the anti-p53 monoclonal antibody PAb421, which recognizes a C-terminal epitope[22]. In addition, small peptides corresponding to the C-terminal residues 369-383 of p53 can activate latent p53 for specific DNA-binding *in vitro*[23]. The sequence-specific DNA binding activity of some mp53 proteins can be rescued *in vitro* by PAb421, C-terminal truncation or a C-terminal peptide[19,24-26], and intranuclear microinjection of PAb421 or C-terminal peptide results in activation of wild-type p53 and mp53 proteins in cultured cells[23, 26, 27]. Restoring the sequence-specific DNA-binding and transactivation functions to mp53 seems to be an appealing strategy for cancer therapy. Indeed, transfection of m*p53* cancer cells with C-terminal peptides effectively caused the inhibition of cell proliferation[28] and the induction of apoptosis[29]. *In vitro* experiments showed that both “contact” and “conformation” mutants of p53 were rescued by the C-terminal peptides[30]. However, it has been described that complete activation of p53 is not accomplished effectively by the C-terminal peptides alone, suggesting that negative regulatory domain interactions are not easily disrupted[23]. To produce more effective activation of mp53, we applied radiation treatment to m*p53* cancer cells that were pre-treated with p53 C-terminal peptide. The aim of this study was to more effectively restore the ability of mp53 to induce apoptosis and to obtain fundamental data useful for radio-cancer therapy.

Results and Discussion

We first measured the incidence of apoptosis after X-ray irradiation with or without various p53-derived peptides using Hoechst staining. The rate of apoptosis significantly increased ($p<0.05$, Student's *t*-test) 48 h after treatment with C-terminal peptide (amino acid residues 361-382) in SAS/*neo* cells (3.54±0.81%) but increased less markedly in SAS/m*p53* cells (1.93±0.42%). When the cells were irradiated with X-rays at 6 Gy (D_{10} dose, 10% survival dose), the rate of apoptosis increased remarkably in SAS/*neo* cells (7.15±1.32%) but increased slightly in SAS/m*p53* cells (3.82±0.86%). Irradiation at the iso-survival dose (8 Gy) also did not significantly enhance the rate of apoptosis in SAS//m*p53* cells irradiated with 6 Gy. There was no significant difference in the rate of apoptosis between SAS/*neo* cells irradiated with X-rays alone and SAS/*neo* cells treated with X-rays and C-terminal peptide (361-382). However, a higher rate of apoptosis (6.78±1.54%) was observed in SAS/m*p53* cells treated with a combination of X-rays and C-terminal peptide (361-382) than in SAS/m*p53* cells irradiated with X-rays alone ($p<0.05$). Similarly, the rate of apoptosis in SAS/*neo* cells was significantly increased 48 h after treatment with C-terminal peptide (amino acid residues 353-374) (3.49±1.17%, $p<0.05$, compared with control level), X-rays at 6 Gy (D_{10} dose) (7.15±1.32%, $p<0.01$) or a combination of C-terminal peptide (353-374) and X-rays (8.55±2.3%, $p<0.01$). SAS/m*p53* cells also showed increased rate of apoptosis 48 h after treatment with C-terminal peptide (353-374) (2.88±0.35%, $p<0.01$), X-rays at 6 Gy (D_{10} dose) (3.82±0.86%, $p<0.01$), X-rays at 8 Gy (iso-survival dose for SAS/*neo* cells) (4.78±0.69%) or a combination of C-terminal peptide (353-374) and X-rays (6 Gy, 9.49±0.63%, $p<0.01$; 8 Gy, 9.09±1.84%, $p<0.01$). There was no significant difference in the rate of apoptosis between SAS/*neo* cells treated with X-rays alone and SAS/*neo* cells treated with a combination of X-rays and C-terminal peptide (353-374), but a significant difference in the rate of apoptosis was observed between SAS/m*p53* cells treated with X-rays (6 Gy or 8 Gy) and SAS/m*p53* cells treated with a combination of X-rays (6 Gy or 8 Gy) and the C-terminal peptide ($p<0.01$ or $p<0.05$, respectively). In contrast to the C-terminal peptide, the N-terminal peptide (amino acid residues 14-27) did not significantly increase the rate of apoptosis in SAS/*neo* and SAS/m*p53* cells. Furthermore, the rate of apoptosis was also not increased by combination treatment with X-rays and the N-terminal peptide. These results show that the induction of apoptosis by the combination of C-terminal peptide

and X-rays is p53-dependent.

The induction of apoptosis mediated by caspase-3 activation was examined in SAS/*neo* and SAS/m*p53* cells (Fig. 2). In the case of no treatment with X-rays or C-terminal peptide, cells positively stained for active caspase-3 were hardly observed in SAS/*neo* (0%) and SAS/m*p53* cells (0%). When the cells were irradiated with 6 Gy or 8 Gy X-rays alone, the rate of the positive cells was significantly increased 48 h after the irradiation in SAS/*neo* cells (6 Gy, 8.10±1.67%), but not in SAS/m*p53* cells (6 Gy, 0.33±0.47%; 8 Gy, 0.45±0.64%). However, when SAS/m*p53* cells were treated with a combination of X-rays and C-terminal peptides, positively stained cells were frequently observed 48 h after the treatment (about 5% for both C-terminal peptides). Cells positive for 85 kDa PARP were detected in a similar pattern to that of cells positive for active caspase-3. When SAS/m*p53* cells were treated with a combination of X-rays and N-terminal peptides, the rate of the positive cells was not increased 48 h after the treatment. Immunohistochemical data on active caspase-3 and proteolysed PARP were well correlated with the data on apoptosis detected with Hoechst staining. When caspase-3 is activated during the process of apoptosis, caspase-3 is self-digested from 32-kD pro-caspase-3 to 17- and 11-kD subunits. This degradation is regulated together with Bax induction[31]. Activated caspase-3 is involved in the proteolysis of several important molecules, such as PARP and Bcl-2[32, 33]. During apoptosis, PARP is cleaved from a 116-kD intact form into 85- and 25-kD fragments, and this cleavage is considered to be a marker of apoptosis[34]. The present results suggest that the observed apoptosis of SAS/*neo* cells might be induced through a p53-dependent caspase-3 pathway through Bax activation, because the incidence of X-ray induced apoptosis in SAS/m*p53* cells was depressed as compared with that in SAS*/neo* cells. In contrast, we found effective induction of apoptosis after X-ray irradiation when we treated the cells with C-terminal peptides, but not N-terminal peptides, of p53 molecules before the irradiation (Fig. 1). It is presumed that the apoptosis observed in SAS/m*p53* cells was also induced through the p53-Bax-caspase-3 pathway after the restoration of the p53CON binding ability of mp53 by the C-terminal peptides.

The induction of apoptosis mediated by caspase-3 activation was examined in SAS/*neo* and SAS/m*p53* cells. Typical photographs of the cells positive for the active form of caspase-3 are shown in Fig. 2A. When SAS/*neo* and SAS/m*p53* cell were treated with either X-rays or C-terminal peptide (361-382), cells positively stained for

active caspase-3 were hardly observed. When the cells were irradiated with 6 Gy X-rays alone, the rate of positive cells significantly increased 48 h after the irradiation in SAS/*neo* cells (8.10±1.67%), but not in SAS/m*p53* cells (0.33±0.47%). However, when SAS/m*p53* cells were treated with the combination of X-rays (6 Gy) and C-terminal peptide (361-382), positively stained cells were frequently observed 48 h after the treatment (4.71±3.27%). Cells positive for 85-kDa PARP were detected with a pattern similar to that cells positive for active caspase-3 (Fig. 2B). When SAS/m*p53* cells were treated with the combination of X-rays and N-terminal peptide (14-27), the rate of positive cells was not increased 48 h after the treatment.

To examine whether p53 C-terminal peptides restore the DNA-binding activity of mp53 to p53CON, a gel mobility-shift assay was performed using whole-cell proteins extracted from m*p53*- or *neo* control vector-transfected cells (Fig. 2). Activated wtp53 extracted from X-ray-irradiated wt*p53* cells is known to bind to p53CON, which is homologous to a specific DNA sequence located upstream of the *bax* gene and which positively controls apoptosis[35]. The DNA-binding activity of whole-cell proteins was increased in SAS//*neo* cells when the cells were treated with X-rays (6 Gy) (Fig. 2Aa). Whole-cell proteins from SAS//*neo* cells showed increased DNA-binding activity when the proteins were mixed with p53 C-terminal peptide, 361-382 (20 μM). Similar DNA-binding activity was observed in proteins from X-ray-irradiated SAS/*neo* cells when the proteins were mixed with the p53 C-terminal peptide. In contrast, the whole-cell proteins from X-ray-irradiated SAS/m*p53* cells did not show any DNA-binding activity (Fig. 2Aa). The defective DNA-binding ability of p53 from SAS/m*p53* cells may be due to the dominant-negative nature of mp53 protein[36, 37]. However, when the whole-cell proteins from intact SAS/m*p53* cells were mixed with the p53 C-terminal peptide, the proteins showed a clear increase in DNA-binding activity, probably due to binding of p53 to p53CON (Fig. 2Aa). This increased DNA-binding was enhanced in the whole-cell proteins from X-ray-irradiated SAS/m*p53* cells. When unlabeled p53CON probe (100 excess compared to labeled probe) was added to the reaction mixture containing ^{32}P-labeled p53CON and proteins from X-ray-irradiated SAS/*neo* or SAS/m*p53* cells treated with the peptide, the binding of p53 to p53CON disappeared. The whole-cell proteins from *p53*-null H1299/*neo* cells did not show any DNA-binding activity with any of the treatments (Fig. 2Ab). However, the whole-cell proteins from m*p53*-transfected H1299/m*p53* cells showed

DNA-binding activity when the proteins were mixed with p53 C-terminal peptide, 361-382 (20 μM). The DNA-binding activity in whole-cell proteins from X-ray-irradiated H1299/m*p53* cells was enhanced. These results indicate that the enhancement of DNA-binding activity by the p53 C-peptide in SAS/m*p53* cells resulted from the restoration of the DNA-binding activity of not endogenous wtp53, but rather mp53. In contrast to the p53 C-terminal peptide, the p53 N-terminal peptide did not show any ability to restore the DNA-binding activity of wtp53 or mp53 (Fig. 2B).

It is reported that phosphorylation of the p53 C-terminal domain by either protein kinase C or casein kinase II[22, 38, 39], or deletion of this regulatory domain[21], activates the ability of wtp53 to bind to p53CON. The specific DNA-binding function of wtp53 appears to be regulated, in part, by the C-terminal peptide[19, 21, 22, 24, 40]. The C-terminal domain dissociates from the DNA-binding domain to permit the specific DNA- binding activity of wtp53. C-terminal peptide is thought to disrupt association of the C-terminus with the DNA-binding domain of p53[23]. In contrast to wtp53, mp53 proteins are not activated by C-terminal phosphorylation[24]. This phenomenon may indicate that the DNA-binding domain of mp53 has an increased affinity for the C-terminus, preventing efficient activation of mp53 through phosphorylation of C-terminal residues. Addition of the C-terminal peptide may weaken the strong binding affinity between the mutant DNA-binding domain and the C-terminus through efficient competition with the C-terminal peptides. Binding of the C-terminal peptides to the DNA-binding domain may stabilize the interaction between the DNA-binding domain and DNA[28]. However, in the present study, treatment with the C-terminal peptide alone did not significantly increase the incidence of apoptosis in SAS/m*p53* cells. Complete activation of mp53 may not be caused by the C-terminal peptide alone, as suggested previously[23]. This notion is supported by the present findings that the DNA-binding activity of mp53 is strongly enhanced by X-ray irradiation and p53 C-terminal peptide treatment.

SAS/mp53 cells were transfected with m*p53* and thus harbored a “contact mutant” of p53. The mechanism of complete activation of the “contact mutant” of p53 induced by combined treatment with X-rays and the C-terminal peptides is still not clear. However, it is possible that some modification(s) of p53 molecules such as radiation-induced phosphorylation, acetylation, sumoylation or poly(ADP-ribosyl)ation may participate in the complete activation of mp53. Wild-type p53 is stabilized and

activated through phosphorylation of p53 at serine 15/serine 20 by ATM and Chk2 and regulates downstream genes[41]. A recent report[42] has shown that phosphorylation of p53 at serine 46 contributes to radiation-induced apoptosis, with *p53*-dependent damage-inducible nuclear protein 1 (p53DINP1) acting as a cofactor for the putative p53-serine 46 kinase. The mp53 whose activity is restored by the C-terminal peptides may gain the ability to induce serine 46-mediated apoptosis through p53-regulated apoptosis-inducing protein 1 (p53AIP1) after irradiation[43]. It has also been reported that the DNA-binding activity of p53 is enhanced by acetylation of the C-terminus of p53 and sumoylation of lysine 386. These modifications via the radiation-induced signaling pathway may play important roles in the complete activation of "contact mutants" of p53.

We have already shown that *p53* status can be used as a predictive marker for response to radiation. Transfection of m*p53* into SAS cells renders the cells resistant to X-rays and suppresses X-ray-induced apoptosis due to dominant-negative nature of mp53[44]. These results suggest that radiosensitivity of cancer cells is p53-dependent. Thus, an effective therapeutic response is expected for wt*p53* cancer cells but not for m*p53* ones. Unfortunately, the p53 function as a tumor suppressor is frequently deficient in human cancer cells[8, 9]. To overcome the radio-resistance of m*p53* cancer cells, many investigators have vigorously searched for molecules which have the ability to induce normal function of p53 in mp53 or inactive p53. Besides p53 C-terminal peptides, the fluorescein-labeled peptide Fl-CDB3[45], an alkaloid ellipticine[46], the small compound CP-31398[47] and PRIMA-1[48] have been reported as chaperone molecules for p53. In addition, we have reported that glycerol restores the ability of mp53 to induce WAF1 expression[49] and X-ray-induced apoptosis[44], resulting from a conformational change of mp53 to the wild-type conformation[44]. Such experimental strategies based on manipulation of mp53 or inactive p53 by these molecules may provide new radio-cancer therapies effective for patients carrying m*p53* tumors.

Materials and Methods

Cells. A cell line of human head and neck squamous cell carcinoma, SAS cells (provided by JCRB, Tokyo, Japan), and *p53*-null human lung cancer cells, H1299 (a gift from Dr. M. Oren, Weizmann Institute of Science, Rehovot, Israel), were cultured at 37°C in Dulbecco's Modified Eagle's medium containing 10% (v/v) fetal bovine serum, penicillin (50 U/ml), streptomycin (50 μg/ml) and kanamycin (50 μg/ml) (DMEM-10).

SAS and H1299 cells were transfected with plasmid pC53-248, which contains an m*p53* gene (codon 248, from Arg to Trp) that encodes a dominant negative mp53, or with the control vector pCMV-Neo. These plasmids were provided by Dr. B. Vogelstein, Johns Hopkins Oncology Center, MD, USA. The stable transfectants SAS/m*p53* and SAS/*neo* were selected with G418 (200-400 μg/ml, Sigma Chemical Co., St. Louis, MO), and used for the present experiments. The detailed procedure for transfection is described elsewhere[50].

Peptide treatment. Cells in DMEM without serum or antibiotics were treated with synthesized p53 C-terminal peptide (amino acid residues 361-382, Gly-Ser-Arg-Ala-His-Ser-Ser-His-Leu-Lys-Ser-Lys-Lys-Gly-Gln-Ser-Thr-Ser-Arg-His-Lys-Lys; Shimazu, Tokyo, Japan; at a final concentration of 20 μM) or p53 N-terminal peptide (amino acid residues 14-27, Leu-Ser-Gln-Glu-Thr-Phe-Ser-Asp-Leu-Trp-Lys-Leu-Leu-Pro; Shimazu; at a final concentration of 20 μM) mixed with 5 μl of liposomes (1 μg/1 μl) (Biotechnologies Inc., Montreal, Canada) and were incubated at 37°C for 12 h. Then, the medium was replaced with DMEM with serum and antibiotics and the cells were incubated at 37°C for 24 h until X-ray irradiation. After X-ray irradiation, the cells were incubated at 37°C for 48 h until sampling.

X-ray irradiation. For the X-ray irradiation treatment, subconfluent cells in DMEM-10 in 96-well dishes were exposed to X-rays (Gy) using a 150-kVp X-ray generator (Model MBR-1520R, Hitachi, Tokyo, Japan) with total filtration using a 0.5-mm aluminum plus 0.1-mm copper filter, and then incubated at 37°C in a conventional humidified 5% CO_2 incubator.

Hoechst staining. Induction of apoptosis was analyzed by detection of apoptotic bodies. The cells collected from 35-mm dishes were fixed with 1% glutaraldehyde (Nakalai Tesque, Kyoto, Japan) in PBS at 4°C, washed with PBS, stained with 0.2 mM Hoechst 33342 (Sigma Chemical Co.) and then 3 different microscopic fields of the cells were observed by a person who was blind to the source of the specimen. One hundred cells were evaluated per field and a total of 300 cells was evaluated for each analyzed point and used for statistical analysis (Student's *t*-test).

Immunocytochemistry. The cells plated on 96-well plates were rinsed with PBS (-) 48 hours after X-ray irradiation and fixed with methanol. The active caspase-3 and 85kDa PARP proteins of methanol-fixed cells were stained by using the avidin-biotin peroxidase complex method with a HISTOFINE SAB-PO(R) kit (Nichirei Co., Tokyo,

Japan). Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol at room temperature for 30 min. The sections were exposed for 10 min to an excess of blocking solution (10 % goat serum), and were subsequently treated with a 1:1000 dilution of polyclonal active caspase-3 antibody (Pharmingen, San Diego, California) or a 1:100 dilution of polyclonal 85 kDa PARP antibody (Promega Co., Madison, Wisconsin) overnight at room temperature. The cells were then incubated sequentially with secondary antibody and the avidin-biotin peroxidase complex was allowed to form and was stained with diaminobenzidine. For quantitative analysis, 100 cells were evaluated in 3 independent random fields by a person who was blind to the source of the specimen, and thus a total of 300 cells was evaluated for each analyzed point and used for statistical analysis (Student's t-test).

Gel mobility-shift assay. Whole cell extracts were prepared from intact cells (about 2 x 10^7 cells) and X-ray-irradiated cells 6 h after X-ray irradiation according to the method described elsewhere[50]. The binding activity of p53 to p53 consensus sequence (p53CON) was measured by a gel mobility-shift assay[50] using as a probe synthetic double-stranded DNA fragment encoding the p53CON (5'-GGACATGCCCGGGCATGTCC-3', Japan Bioservice, Niiza, Saitama, Japan) that is located upstream of the *bax* gene. For the *in vitro* assay of the effectiveness of the peptides, whole cell extracts were mixed with p53 C-terminal peptides or N-terminal peptide (20 μM) for 30 min at room temperature.

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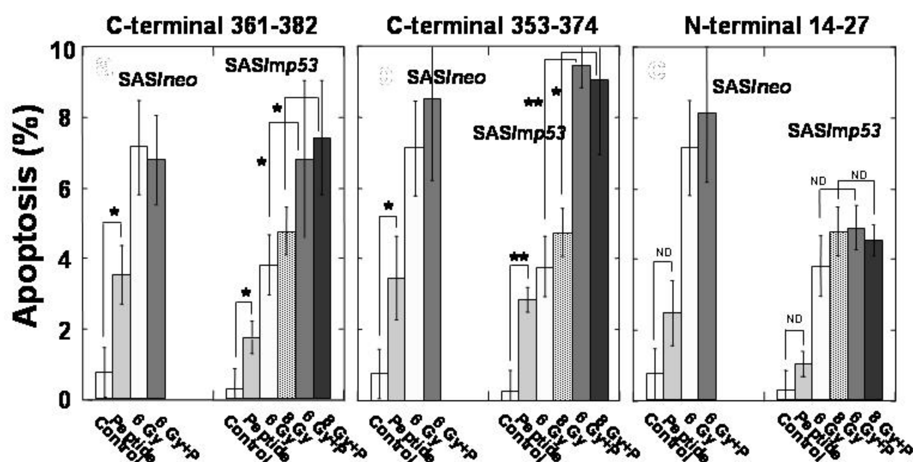

Figure 1. Incidence of apoptosis analyzed with Hoechst33342 staining. The cells treated with (**a**) p53 C-terminal peptide (361-382), (**b**) p53 C-terminal peptide (353-374) or (**c**) p53 N-terminal peptide (14-27) were incubated at 37˚C for 48 h after X-ray irradiation (6 Gy, D_{10} dose for SAS/*neo* cells; 8 Gy, iso-survival dose for SAS/m*p53* cells), fixed with glutaraldehyde, stained with Hoechst 33342, and then observed under a fluorescence microscope. *, p<0.05; **, p<0.01; ND, not significantly different (Student's *t*-test)

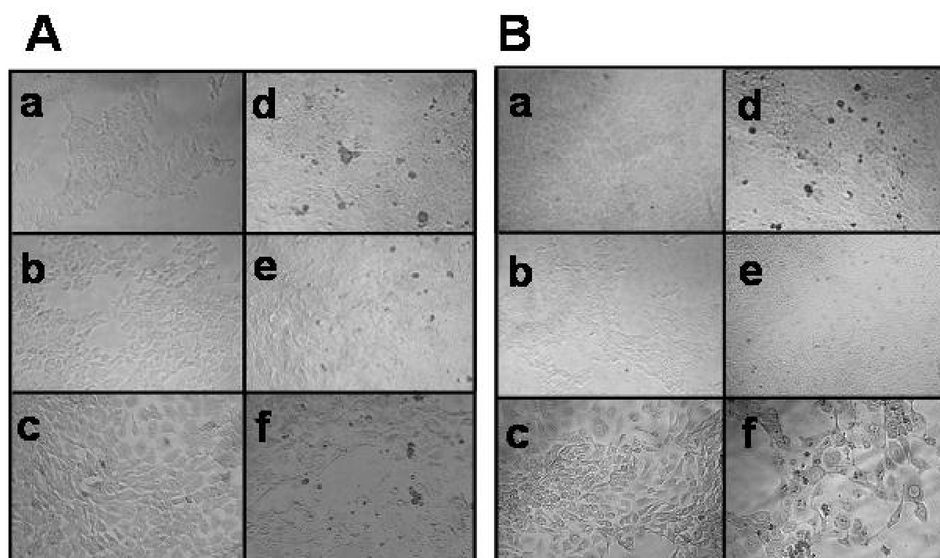

Figure 2. Typical photographs of positive cells stained immunohistochemically for active caspase-3 (A) and 85 kDa PARP (B). ***a***, control SAS/*neo*; ***b***, control SAS/m*p53*; ***c***, C-terminal peptide (361-382)-treated SAS/m*p53*; ***d***, X-rays (6 Gy)-irradiated SAS/*neo*; ***e***, X-rays (6 Gy)-irradiated SASm*p53*; ***f***, C-terminal peptide (361-382)- and X-rays (6 Gy)-treated SAS/m*p53*. Dark-colored cells are positive cells. Cells were fixed and stained 48 h after each treatment.

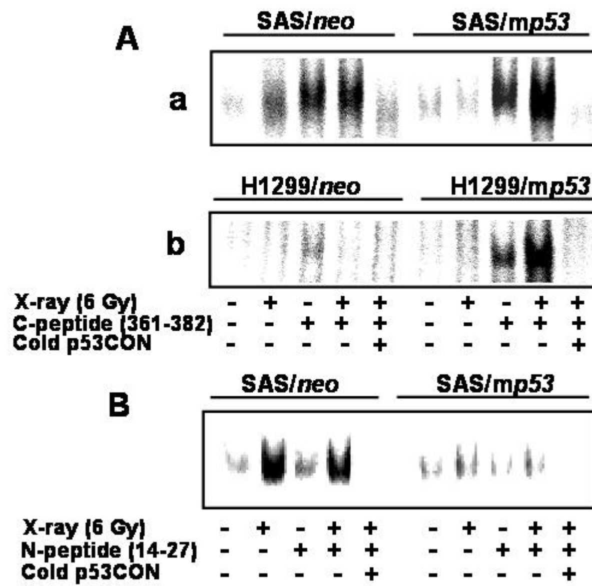

Figure 3. Effects of p53 C-terminal peptides on DNA-binding activity of the whole cell proteins to p53CON. **A**, p53 C-terminal peptide (361-382) was mixed with whole cell proteins extracted from SAS/*neo* and SAS/m*p53* cells (**a**) and H1299/*neo* and H1299/m*p53* cells (**b**) and then the DNA-binding activity of p53 was measured with the gel mobility-shift assay; **B**, p53 N-terminal peptide (14-27) was mixed with whole cell proteins extracted from SAS/*neo* and SAS/m*p53* cells and the DNA-binding activity was measured.