Effects of Protein Kinase Inhibitors on the Accumulation Kinetics of p53 Protein in Normal Human Embryo Cells following X-irradiation

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DNA-damaging agents induce phosphorylation of the p53 protein, resulting in its accumulation in the nucleus. To clarify the signal transduction pathway(s) involved in p53 protein accumulation in normal human embryo cells following X-irradiation, the effects of three protein kinase inhibitors were examined. Quercetin, an inhibitor of heat-shock response, dose dependently suppressed the p53 accumulation induced by X-rays at more than 100 μM. No suppression, however, was observed with calphostin-C, a specific inhibitor of protein kinase C, in the range of 0.05 to 0.25 μM. Wortmannin was the most potent inhibitor of p53 accumulation. Its suppressive effect appears within a few minutes of pretreatment with a dose of 25 μM, but posttreatment was less effective. Our findings suggest that PKC is not involved in X-ray-induced p53 accumulation in normal human embryo cells and that a wortmannin-sensitive pathway acts as a sensor of DNA damage.

INTRODUCTION

Loss of p53 suppressor function owing to point mutations and deletions occurs in the development of more than 50% of human cancers, and it contributes to an increase in the number of genetic abnormalities1). Tumor suppressor p53 protein is a nuclear phosphoprotein that is required to regulate gene expression. Its products are involved in cell-cycle regulation, DNA replication, and the apoptosis of cells exposed to DNA-damaging agents, including ionizing radiation2–4). Wild-type p53 is present at low levels in normal human cells due to its short half-life. In response to the irradiation of cells, p53 protein accumulates in the nucleus5) and binds to DNA in a sequence-specific manner6). Because p53 mRNA levels are reported to remain unchanged
after treatment with ionizing radiation, posttranslational modification appears to be the major mechanism of p53 protein regulation\(^2\).

Several reports have indicated that distinct domains of p53 are phosphorylated both \textit{in vitro} and \textit{in vivo}\(^3\)–\(^5\). Although, the physiological significance of p53 protein phosphorylation has yet to be fully clarified, recent evidence indicates that the phosphorylation of specific sites of human p53 are involved in the regulation of p53 levels\(^7\)–\(^10\). For example, serine-15, whose phosphorylation is activated by DNA-damaging agents, is located near the MDM2 binding site, and its phosphorylation therefore reduces interaction between p53 and MDM2 proteins\(^7\). Because the binding of MDM2 to p53 accelerates the latter’s degradation, possibly through the ubiquitin proteasome pathway\(^11\), phosphorylation of p53 at serine-15 results in the accumulation of p53 protein, as described elsewhere\(^7\)–\(^10\). Nuclear kinases such as ATM and DNA-PK are reported to phosphorylate p53 at serine-15\(^7\),\(^9\),\(^10\), but recent studies have shown that the accumulation of p53 is intact in an SCID mouse cell line deficient in DNA-PKcs\(^13\),\(^14\). Furthermore, although the increase is delayed or is much smaller than the control level\(^8\),\(^15\),\(^16\), p53 accumulation in AT cells is accompanied by the phosphorylation of p53 at serine-15. Phosphorylation of p53 other than at serine-15 therefore also may be involved in the accumulation and activation of p53 protein.

We examined the effect of protein kinase inhibitors on the accumulation of p53 protein in normal human embryo cells irradiated with 6 Gy of X-rays. Because ionizing radiation is reported to stimulate several signal transduction pathways, including PKC\(^17\),\(^18\), MAP kinase\(^19\), and NF-κB\(^20\), we chose the three inhibitors, quercetin (3, 3’, 4’, 5, 7-pentahydroxyflavone), calphostin-C, and wortmannin. Quercetin, a bioflavonoid, inhibits the activities of several kinases, including PKC\(^21\),\(^22\), and suppresses the heat shock response\(^23\). Calphostin-C, a secondary metabolite of the fungus \textit{Cladosporium cladosporioides}, is a specific inhibitor of PKC\(^24\). Wortmannin is a microbial metabolite that inhibits PI-3 kinase and that has an inhibitory effect on both DNA-PK and ATM\(^25\). The cells used in this study were treated with the inhibitors before or after X-irradiation, after which the accumulation kinetics of p53 was examined.

**MATERIALS AND METHODS**

**Cell cultures**

Normal human embryo cell (HE49) cultures were maintained in Eagle’s Minimum Essential Medium (MEM; Nissui, Tokyo) supplemented with 0.2 mM serine, 0.2 mM asperate, 1.0 mM pyruvate, 10 mM HEPES, and 10% fetal bovine serum (FBS; Trace Bioscience, Australia), as described elsewhere\(^26\). Totally, 3 \(\times\) 10\(^5\) cells were inoculated in a 25 cm\(^2\) culture flask then incubated at 37°C in a humidified atmosphere with 5% CO\(_2\) and 95% air until 70–80% confluency was reached.

**X-irradiation**

X-irradiation was done the dose rate of 0.425 Gy/min with an X-ray generator (M-150 WE; Softex, Osaka) operated at 150 kVp and 5mA and equipped with an external filter of 0.1 mm Cu and 1 mm Al\(^27\). Approximately 14.1 minutes was needed for the 6 Gy irradiation of the cells
placed on an irradiation stage 30-cm the focus.

Treatment with protein kinase inhibitor

Quercetin (Sigma Chemical Co. USA) was dissolved in dimethyl sulphoxide (DMSO; Sigma) giving a 100 mM stock solution that was stored at 4°C. Stock solutions of Calphostin-C (Wako Pure Chemicals, Tokyo, Japan) and wortmannin (Wako Pure Chemicals, Tokyo, Japan) were dissolved in DMSO yielding a 50 mM stock solution that was stored at –20°C. Cells were treated with an inhibitor before or immediately after irradiation, or with the inhibitor alone, then incubated to extract proteins.

Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS) containing 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride. After freezing them at –30°C, the cell lysates were centrifuged at 15, 000 rpm for 10 min at 4°C. The supernatant obtained was stored at 4°C before use. The total protein concentration was measured by the BCA protein assay (Pierce, Rockford, USA) using an absorption maximum of 562 nm. An 8 μg protein obtained by SDS-PAGE was transferred electrophoretically to a polyvinyl difluoride (PVDF) membrane with the aid of transfer buffer (100 mM Tris, 192 mM Glycine). Loading homogeneity and transfer efficiency were checked by staining the gel with Coomassie brilliant blue R-250 (Sigma Chemical Co., USA). The membrane was blocked by incubating it overnight with blocking solution (10% skim milk), after which it was incubated with anti-p53 monoclonal antibody (Clone BP53–12, Lab vision, Fremont, USA). The membranes were incubated with biotinylated anti-mouse Ig monoclonal antibody (Amersham Japan Co., Ltd, Tokyo, code RPN. 1001) and streptavidin/biotinylated alkaline phosphatase conjugate (Amersham Japan Co., Ltd, Tokyo, code RPN. 1234) to make visible the bound primary antibodies. Then the membranes were incubated with 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) and nitroblue tetra-zolium chloride (NBT) (GIBCO BRL) for color development. The band obtained by Western blotting was quantitated densitometrically, and the relative amount calculated.

RESULTS

Accumulation of p53 protein after irradiation

Cells were irradiated with various doses of X-rays then incubated for 2 h before the extraction of total protein. As shown in Fig. 1, p53 protein was present constitutively in the unirradiated normal HE 49 cells, and its content increased after X-irradiation. Dose-dependent increases in p53 protein were detected after irradiation with 1 to 4 Gy of X-rays. At more than 4 Gy, however, p53 accumulation reached a plateau. At doses of irradiation between 4 and 8 Gy, the p53 protein content was approximately two fold that of the unirradiated cells. In subsequent experiments cells were irradiated with X-rays at the dose of 6 Gy.
Effects of quercetin on p53 accumulation

The effects of the protein kinase inhibitors on this accumulation were examined to determine the signal transduction pathways involved. Cells treated with various concentrations of quercetin (between 100 to 200 μM) were irradiated with 6 Gy of X-rays and after irradiation cultured for 2 h in medium containing quercetin before the extraction of protein. Treatment of HE49 cells with quercetin alone produced an increase in the p53 protein content, which depended on the concentration of quercetin used (Fig. 2). The accumulation of p53 protein in HE49 cells irradiated with X-rays was suppressed as the quercetin dose increased; 100 μM of quercetin only slightly suppressed accumulation, whereas 200 μM completely inhibited it (Figs. 2B and 2D).

To examine the effect of the length of treatment on the accumulation of p53 protein, cells were treated with 140 μM of quercetin. These quercetin-treated cells were irradiated with 6 Gy of X-rays and incubated for different periods in medium containing 140 μM of quercetin. p53 protein accumulations were detected after treatments with quercetin and X-rays (Fig. 3A). The accumulation patterns differed completely. The accumulation of p53 protein increased with the length of quercetin treatment, the maximum being at 10 h and the level decreasing thereafter. In the case of X-irradiation, the peak p53 accumulation appeared between 1–3 h after irradiation. If quercetin treatment and X-irradiation induce p53 accumulation by different pathways, the induc-
The kinetics of p53 protein accumulation after combined treatment with quercetin and X-rays, however, was very similar to that after treatment with quercetin alone (Fig. 3B). Interestingly, p53 protein accumulation was lower 1 and 3 h after combined treatment with quercetin and X-rays, as compared with the kinetics of p53 accumulation after treatment with X-rays alone (Figs. 3A and 3B).

**Effects of Calphostin-C on p53 protein accumulation**

The specific protein kinase C inhibitor calphostin-C was used to investigate the function of protein kinase C as a mediator of p53 protein regulation after ionizing radiation. HE49 cells were treated for 12 h with calphostin-C in a range of doses from 0.05 to 0.25 μM then irradiated with 6 Gy of X-rays. The irradiated cells were incubated for 2 h more before the extraction of protein. Calphostin-C had no inhibitory effect on the accumulation of p53 protein, even at high doses, when compared to treatment with X-rays alone (Figs. 4A and 4B); whereas, calphostin-C alone
Fig. 3. Effects of quercetin on the kinetics of p53 accumulation. HE49 cells were treated with 140 μM of quercetin then immediately irradiated with 6 Gy of X-rays. After irradiation, the cells were cultured in medium containing quercetin before the extraction of total cell protein. The culture period includes the time required for X-irradiation (about 14.1 min). Total cell extracts were prepared at each indicated time. The amount of p53 protein was determined by Western-blot analysis using anti-p53 antibody. A: Accumulation kinetics of p53 after treatment with quercetin and X-rays (○) or quercetin alone (♦). B: Accumulation kinetics of p53 protein after combined treatment with X-rays and quercetin (▲). C: Theoretical accumulation kinetics of p53 protein after combined treatment with X-rays and quercetin (■). The theoretical value was calculated using the formula [(Relative amount of p53 protein induced by X-rays + the relative amount of p53 protein induced by quercetin) - the relative amount of p53 protein in the control cells (1.0)]. The dotted line represents the experiment values in Figure 3B. QCT; quercetin.

slightly decreased the p53 protein level at high doses (Figs. 4a and 4C).

Cells were treated with 0.05 μM calphostin-C to investigate its time-dependent effects on p53 accumulation. Calphostin-C-treated cells were irradiated with 6 Gy of X-rays then incubated
Effects of calphostin-C on p53 protein accumulation. HE49 cells were treated with various concentrations of calphostin-C for 12 h before irradiation. After irradiation with 6 Gy of X-rays, the cells were incubated for 2 h more in medium containing calphostin-C before the extraction of total cellular protein. The incubation time includes the time required for X-irradiation (about 14.1 min). The amount of p53 protein was determined by Western-blot analysis using anti-p53 antibody. A: p53 protein levels after calphostin-C treatment alone. B: Band intensities from panel A. C: p53 protein levels after combined treatment with calphostin-C and X-rays. D: Band intensities from panel C. +X: 6 Gy-irradiated.

Effects of wortmannin on p53 protein accumulation
To further clarify the mechanism of p53 protein stabilization, we tested the protein kinase inhibitor wortmannin. HE49 cells treated with various concentrations of wortmannin were irradiated with 6 Gy of X-rays. After irradiation, the cells were incubated for 2 h before the extraction in medium containing 0.05 μM calphostin-C for different periods before the extraction of protein. Treatment with calphostin-C decreased the p53 protein level over time (Fig. 5A); whereas, approximately a two fold accumulation of p53 protein was detected 2 h after combined treatment with calphostin-C and X-rays (Fig. 5B). The p53 accumulation pattern for the combined with X-ray and calphostin-C treatment is the same as that for X-irradiation alone(Fig. 5C). This means that calphostin-C did not inhibit p53 protein accumulation after X-irradiation.
Fig. 5. Effects of calphostin-C on p53 accumulation. HE49 cells were irradiated with 6 Gy of X-rays then treated with calphostin-C (0.05 \( \mu \)M). After irradiation, the cells were incubated in medium containing 0.05 \( \mu \)M of calphostin-C for the times indicated, after which total cell protein was extracted. The incubation period includes the time required for X-irradiation (about 14.1 min). The amount of p53 protein was determined by Western-blot analysis using anti-p53 antibody. A: Kinetics of p53 protein accumulation after treatment with X-rays (●) and calphostin-C (○). B: Kinetics of p53 protein accumulation after combined treatment with calphostin-C and X-rays (△). C: Theoretical accumulation kinetics of p53 protein after combined treatment with X-rays and quercetin (□). Theoretical value was calculated using the formula: [Relative amount of p53 protein induced by X-rays + the relative amount of p53 protein induced by quercetin - the relative amount of p53 protein in the control cells (1.0)]. The dotted line represents the experimental values in Figure 5B. Cal-C: calphostin-C.
Fig. 6. Effect of wortmannin on p53 accumulation in X-irradiated HE 49 cells. Cells were irradiated with 6 Gy of X-rays then treated with wortmannin at doses between 5 and 40 μM. After irradiation, the cells were incubated in medium containing wortmannin for 2 h, after which total cellular protein was extracted. The incubation period includes the time required for X-irradiation (about 14.1 min). The amount of p53 protein was determined by Western-blot analysis using anti-p53 antibody. A: p53 protein levels after wortmannin treatment alone. B: Band intensities from panel A. C: p53 protein levels after combined treatment with wortmannin and X-rays. D: Band intensities from panel C. +X: 6 Gy-irradiated.

accumulation of p53 protein in the X-irradiated cells (approximately 49%), approximately 80% suppression being produced by 25 μM or higher doses.

Cells were pretreated with 25 μM of wortmannin for various periods (between 8 and 120 min) then irradiated with 6 Gy of X-rays to determine whether transient treatment is effective. After irradiation, the cells were incubated for 2 h in wortmannin-free medium before the extraction of protein (Fig. 7). A minimum of 8 min pretreatment with wortmannin completely eliminated the X-ray-induced p53 protein accumulation (Figs. 7A and 7B).

Next, we examined the inhibitory effect of continuous treatment with wortmannin on p53 protein accumulation. HE49 cells were treated with 25 μM wortmannin for 8 min, after which the medium was changed to wortmannin-free medium. The cells then were incubated for various periods, ranging from 0 to 120 min, after which they were irradiated with 6 Gy of X-rays and incubated for 2 h more before the extraction of protein. p53 accumulation was suppressed by pretreatment with wortmannin for 8 min, but the inhibitory effect decreased once the wortmannin
Fig. 7. Effect of pretreatment with wortmannin on p53 accumulation in X-irradiated HE 49 cells. Cells were treated with 25 μM wortmannin for various periods of 8 to 120 min. After treatment, the cells were immediately irradiated with 6 Gy of X-rays then incubated in wortmannin-free medium for 2 h after which total cellular protein was extracted. The incubation period includes the time required for X-irradiation (about 14.1 min). The amount of p53 protein was determined by Western-blot analysis using anti-p53 antibody. A: p53 protein levels. B: Band intensities from panel A. +X: 6 Gy-irradiated.

was washed away (Fig. 8). No significant suppression was observed 1 h after removal of the wortmannin.

Effects of posttreatment with wortmannin on p53 accumulation

After irradiation with 6 Gy of X-rays, the medium was immediately changed to medium containing 25 μM wortmannin, and the HE 49 cells were incubated for various periods ranging from 5 to 120 min. Protein was extracted 2 h after irradiation. Posttreatment with wortmannin was less effective than pretreatment (Fig. 9), similar inhibitory effects being found only when the cells were treated continuously with wortmannin for more than 60 min.
We investigated the effects of the protein kinase inhibitors, quercetin, calphostin-C, and wortmannin on the X-ray-induced accumulation of p53 protein in normal human embryo cells. Both quercetin and wortmannin treatment suppressed the accumulation of p53 protein dose dependently, but there was no inhibition with calphostin-C. Although quercetin inhibits protein kinase C, its effect is non-specific\(^{21,22}\). It also is reported to suppress PI-3 and MAP kinase\(^{22,29}\). Our findings suggest that activation of PKC is not always required for the activation of p53 by ionizing radiation. Evidence suggests that Ca\(^{2+}\) and phospholipid-dependent protein kinase C (PKC) are stimulated after cells are exposed to ionizing radiation\(^ {17}\). Moreover, serine-378 of the p53 protein is phosphorylated by PKC, indicative that PKC is involved in the regulation of p53.

**DISCUSSION**

Fig. 8. Effects of prolonged treatment of HE49 cells with wortmannin on the accumulation of p53 protein. Cells were treated for 8 min with 25 \(\mu\)M of wortmannin then incubated in wortmannin-free medium for the indicated periods. After the wortmannin treatment, the cells were immediately irradiated with 6 Gy of X-rays then incubated for 2 h more in wortmannin-free medium after which total cell protein was extracted. The incubation period includes the time required for X-irradiation (about 14.1 min). The amount of p53 protein was determined by Western-blot analysis using anti-p53 antibody. A; p53 protein levels. B: Band intensities from panel A.
In fact, in vitro studies have shown that PKC enhances the sequence-specific DNA binding capacity of p53. Furthermore, calphostin-C inhibits p53 activation in vivo. There also have been contradictory findings that PKC may not be involved in p53 activation. For example, Milne et al. reported that the C-terminal domain of p53 is phosphorylated by PKC in vitro studies but that in vivo studies the phosphorylation of p53 at these sites does not seem to be carried out directly by PKC. Our present findings support this and suggest that a protein kinase(s) other than PKC is responsible for p53 accumulation in normal human embryo cells in response to ionizing radiation.

Our findings also show that a few minutes of pretreatment with wortmannin is sufficient to suppress p53 accumulation, indicative that a wortmannin-sensitive pathway may provide the major signal transducer for the p53 protein. Wortmannin inhibits PI-3 kinase and has recently

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**Fig. 9.** Posttreatment effects of wortmannin on p53 accumulation in X-irradiated HE49 cells. Cells were irradiated with 6 Gy of X-rays then treated immediately with 25 μM of wortmannin for the periods indicated. The cells then were cultured in wortmannin-free medium. Total cellular proteins was extracted 2 h after irradiation. The amount of p53 protein was determined by Western-blot analysis using anti-p53 antibody. A: p53 protein levels. B: Band intensities from panel A. +X: 6 Gy-irradiated.
been shown to suppress the activities of both DNA-PK and ATM kinase\(^{25}\). Recent studies have shown that ATM phosphorylates p53 on a single residue, serine-15\(^{9,10}\), but this same phosphorylation has been detected in cells that lack the ATM function, in response to ionizing radiation\(^{8}\). Furthermore, previous studies indicated that accumulation takes place even in AT cells with defective ATM function\(^{15,16}\), the speed of accumulation being slower and/or the amount accumulated less as compared to normal cells. These results suggest that the ATM function is not the only function required in the phosphorylation of p53 protein.

As reported elsewhere, DNA-PK is a candidate for this other type of kinase. DNA-PK is a serine/threonine kinase that depends on the presence of DNA strand breaks for its activity\(^{32}\). It phosphorylates human p53 on serines 15 and 37 \textit{in vitro}\(^{33}\). In addition, the phosphorylation of p53 at serines 15 and 37 by DNA-PK is reported to inhibit interaction with MDM2 \textit{in vitro} studies\(^{7}\), as does phosphorylation of the amino terminus of MDM2 by DNA-PK\(^{34}\). Woo et al.\(^{35}\) however, recently showed that p53 can increase in cells lacking DNA-PK. It has also been reported that p53 accumulation also is reported to be normal in SCID (severe combined immunodeficient) cells defective in DNA-PK activity owing to DNA damage\(^{13,14}\). Although ATM may compensate for a DNA-PK deficiency, wortmannin is reported to inhibit the activities of both DNA-PK and ATM. An unidentified factor, initiated from the sensor DNA damage in response to ionizing radiation, also may be involved in p53 accumulation and activation.

We found that a few minutes pretreatment with wortmannin efficiently suppressed p53 accumulation (Fig. 7). As shown in Figure 8, approximately 2h incubation was required to restore the p53 response to X-rays. This suggests that wortmannin-sensitive factor(s) levels are limited and may not be regulated after X-irradiation. Posttreatment was less effective than pretreatment. Inhibitory effects similar to those for the 8 minute pretreatment were obtained with more than 60 minutes of posttreatment. A wortmannin-sensitive pathway probably responds immediately after irradiation and, once activated, inhibits this pathway. We speculate that a wortmannin-sensitive pathway functions as a sensor of DNA damage.

In summary, we showed that quercetin suppresses the X-ray-induced accumulation of p53 protein, whereas a specific protein kinase C inhibitor has no effect. Wortmannin is a most effective inhibitor for the suppression of p53 protein accumulation, because only a few minutes of pretreatment is effective, whereas posttreatment is not. These findings suggest that PKC does not function in X-ray-induced p53 accumulation in normal human embryo cells and that a wortmannin-sensitive pathway(s) may act as a sensor of DNA damage.

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