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Suppression of G1 Arrest and Enhancement of G2 Arrest by Inhibitors of Poly(ADP-ribose) Polymerase: Possible Involvement of Poly(ADP-ribosyl)ation in Cell Cycle Arrest Following γ-Irradiation

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Low-dose γ-irradiation of mouse embryonic fibroblast C3D2F1 3T3-a cells caused G1 arrest along with G2 arrest and inhibition of replicative DNA synthesis. When the cells were cultured in the presence of inhibitors of poly(ADP-ribose) polymerase [EC 2.4.2.30], such as 3-aminobenzamide, benzamide and luminol, G1 arrest of C3D2F1 3T3-a cells was suppressed and enhancement of G2 arrest was observed. In contrast, 3-aminobenzoic acid, a non-inhibitory analog of 3-aminobenzamide, did not suppress G1 arrest following γ-irradiation. These results suggest that the poly(ADP-ribosyl)ation reaction is critical for the pathway of G1 arrest and is also involved in the pathway of G2 arrest.

Key words: Poly(ADP-ribosyl)ation — G1 arrest — DNA damage — p53 — DNA repair

Exposure to DNA-damaging agents causes transient alterations in cell cycle progression.[1] G1 arrest prevents replication of the damaged DNA template and G2 arrest prevents segregation of damaged chromosomes. Both these arrests thus ensure the completion of DNA repair before the next cell cycle phase commences. It has also been suggested that, following DNA damage, abnormalities in the mechanisms of these cell cycle checkpoints are involved in cell death, apoptosis, genetic instability and carcinogenesis.[2]

The wild-type p53 apparently plays a critical role in G1 arrest, as cells in which the p53 gene is inactivated or mutated show only G2 arrest.[3] Two other factors, the ataxia-teleangiectasia (AT) gene(s) and *GADD45* have also been shown to participate in the G1 arrest pathway.[4] AT cells show no G1 arrest following γ-irradiation, and also no increase in p53 expression or induction of *GADD45* gene expression.[4] The involvement of the defective gene of Bloom syndrome patients in G1 arrest was also indicated recently.[5] Information on G2 arrest has been obtained using various yeast mutants.[6–8] For example, mutations of the *RAD9* gene abolish G2 arrest in yeast.[6] However, the mechanisms of the signal transduction pathways of G1 and G2 arrest have not yet been clarified in detail.

Since PARP[5] is a constitutive nuclear protein that recognizes DNA strand breaks and its poly(ADP-ribosyl)ation activity is activated by binding to DNA strand breaks,[9] we thought that, when it recognized DNA strand breaks following γ-irradiation, it might transduce a DNA damage signal downstream to cause G1 and G2 arrests. Here we investigated this possibility by testing whether 3-aminobenzamide and other known PARP inhibitors affect G1 and G2 arrests of mouse fibroblast cells following γ-irradiation. The results strongly suggested the involvement of PARP in the G1 and G2 arrest pathways.

MATERIALS AND METHODS

Chemicals 3-Aminobenzamide and benzamide were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was from Sigma (St. Louise, MO, USA), and nicotinamide and 3-aminobenzoic acid were from Wako Pure Chemicals (Osaka).

Cells, DNA damage The C3D2F1 3T3-a cell line, which had been established from 14-day-old mouse embryonic fibroblasts by Ogawa *et al.* (Asahikawa Medical College, Asahikawa), was maintained in Dulbecco's modified Eagle's minimum essential medium (ICN Biomedical Inc. Costa Mesa, CA, USA) supplemented with 10% fetal bovine serum. C3D2F1 3T3-a cells were inoculated at 3×10^5 per 100-mm dish and incubated for 54 h before γ-irradiation. Cells were irradiated with a ^{60}Co γ-irra-

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[5] Abbreviations used: PARP, poly(ADP-ribose) polymerase; BrdUrd, bromodeoxyuridine; FITC, fluorescein isothiocyanate.

diator at 1.07 Gy/min. Cell cycle states were assessed by pulsing cells with 10 μM BrdUrd (Sigma) for 30 min at a selected time after γ-irradiation, and then staining them with FITC-conjugated anti-BrdUrd antibody (Becton Dickinson, San Jose, CA, USA) and with propidium iodide (Sigma) to determine their DNA content. Cell survival was determined by a dye-exclusion method using trypan blue (Sigma).

Cell cycle analysis DNA synthesis was assessed in terms of incorporation of BrdUrd, and flow cytometric analysis was carried out as follows. After incubation with 10 μM BrdUrd for 30 min, the cells were fixed in 70% ethanol, washed with phosphate-buffered saline, and resuspended in 4 N hydrochloric acid for 20 min at room temperature. Then they were treated with 0.1 M sodium tetraborate, pH 9.4, and with phosphate-buffered saline, pH 7.4, and finally with 20 μl of FITC-labeled anti-BrdUrd in phosphate-buffered saline containing 0.2% bovine serum albumin, and 0.1% Tween 20 (Sigma). They were then washed twice, passed through a 59 μm nylon filter, incubated with 5 μg/ml propidium iodide for 20 min at room temperature, and analyzed by flow cytometry (FACScan, Beckton Dickinson). Cell cycle analysis was carried out repeatedly (at least twice) to confirm the results.

RESULTS

Cell cycle states were analyzed 12 h after γ-irradiation. Typically, the cell-cycle phase distribution of mock-irra-

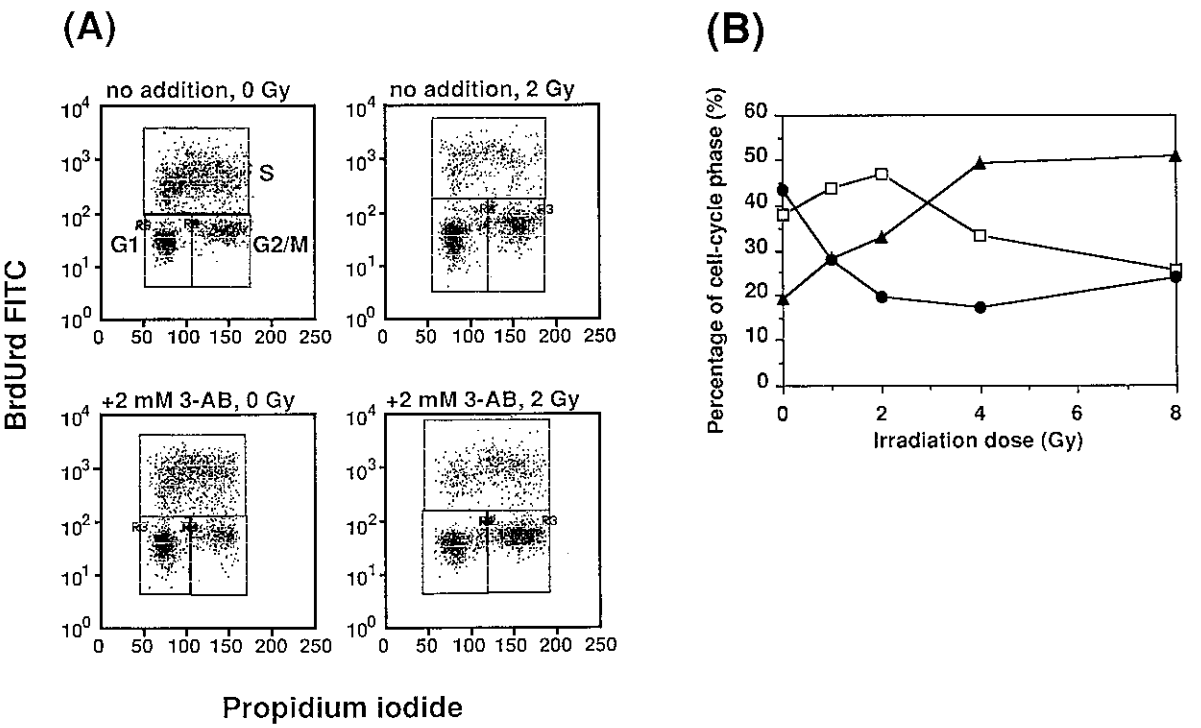

Fig. 1. Cell cycle changes of C3D2F1 3T3-a cells after exposure to γ-irradiation. (A) Cell-cycle phase distributions 12 h after 0 Gy (left panels) or 2 Gy exposure (right panels). 3-Aminobenzamide (3-AB) was present at 2 mM from 2 h before γ-irradiation (lower panels). Flow cytometric dot plots display simultaneous analysis of DNA synthesis (determined after a 30 min pulse with BrdUrd) on the ordinate and DNA content (determined by staining with propidium iodide) on the abscissa. Cell cycle populations are characterized as G1 phase (2N DNA content with no BrdUrd incorporation), S phase (variable DNA content with BrdUrd incorporation), and G2/M phase (4N DNA content with no BrdUrd incorporation during the pulse period). This experiment was repeated at least three times; results were essentially reproducible. The mean percentages of cell cycle phase$\pm$SD in typical three experiments were; with no addition at 0 Gy: 35.3$\pm$2.5% (G1 phase), 45.0$\pm$2.0% (S phase), 19.7$\pm$0.6% (G2/M phase); with no addition at 2 Gy: 45.3$\pm$3.8% (G1 phase), 23.7$\pm$5.5% (S phase), 31.0$\pm$2.0% (G2/M); with 4 mM 3-aminobenzamide at 0 Gy: 38.3$\pm$3.1% (G1 phase), 43.0$\pm$1.7% (S phase), 18.7$\pm$2.1% (G2/M phase); with 4 mM 3-aminobenzamide at 2 Gy: 34.0$\pm$2.6% (G1 phase), 26.3$\pm$3.1% (S phase), 40.0$\pm$4.0% (G2/M phase). (B) Changes in the percentage of cell-cycle phase distribution 12 h after exposure to various doses of γ-irradiation: G1 phase (□), S phase (●), G2/M phase (▲).

diated C3D2F1 3T3-a cells was 35% in G1 phase, 45% in S phase, and 20% in G2/M phase (upper left panel of Fig. 1A). After γ-irradiation at 2 Gy, the cell-cycle phase distribution changed to 48% in G1 phase, 21% in S phase, and 31% in G2/M phase (upper right panel of Fig. 1A). The cell number in G1 phase increased from 35% to 48%, while the cell number in G2 phase increased from 20% to 31%. Thus, substantial G1 arrest was observed along with G2 arrest. However, at γ-ray doses higher than 2 Gy, G2 arrest became marked and G1 arrest was not observed (Fig. 1B). So the effect of the PARP inhibitor 3-aminobenzamide on cell cycle phase distribution was examined at the γ-irradiation dose of 2 Gy. Since millimolar concentrations of 3-aminobenzamide have been reported to suppress PARP activity in intact cells within 1 h after addition,[10] 2 m*M* 3-aminobenzamide was added 1 or 2 h before γ-irradiation in the following experiments. The cell-cycle phase distribution of the 3-aminobenzamide-treated unirradiated cells was 35% in G1 phase, 44% in S phase, and 21% in G2/M phase (lower left panel of Fig. 1A). When cells were incubated with 3-aminobenzamide from 2 h before γ-irradiation at 2 Gy, the cell-cycle phase distribution changed as shown in the lower right panel in Fig. 1A (36% in G1 phase, 25% in S phase, and 39% in G2/M phase). The cell number in G1 phase did not significantly increase while the cell number in G2 phase increased about 2-fold. That is, G1 arrest was almost completely suppressed and G2 arrest was enhanced by 2 m*M* 3-aminobenzamide treatment. This suppression of G1 arrest and enhancement of G2 arrest were similarly observed with 4 m*M* 3-aminobenzamide treatment (Fig. 2). Survival of the cells irradiated at 2 Gy either in the absence or in the presence of 4 m*M* 3-aminobenzamide was unchanged at cell harvest, 12 h after γ-irradiation (data not shown).

Because G1 arrest is evident only at a low irradiation dose, an irradiation dose-response study was performed at 0, 0.25, 0.5, 1, 2, and 4 Gy. The suppression of G1 arrest by 3-aminobenzamide was evident at 0.25 to 2 Gy and the enhancement of G2 arrest was also observed at 0.5 to 4 Gy (data not shown). A time course study showed that G1 arrest became evident from 12 h after γ-irradiation at 2 Gy (top panel of Fig. 2). This G1 arrest was almost completely suppressed by 4 m*M* 3-aminobenzamide treatment, which also caused about 2-fold increase in the number of cells arrested in G2. This treatment resulted in not only enhancement of G2 arrest, but also delay of the peak of G2 arrest for several hours (Fig. 2).

Next, PARP inhibitors other than 3-aminobenzamide were used. Since benzamide and luminol have been used at concentrations of 1–5 m*M* and 0.25–1 m*M*, respectively, for cultured cells,[11] we chose 4 m*M* benzamide and 1 m*M* luminol for the present study. These PARP

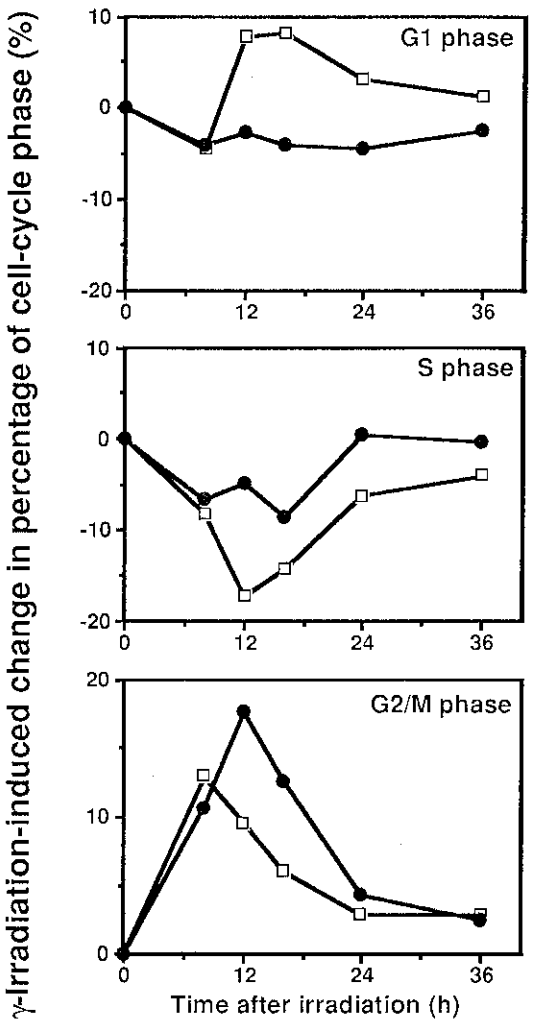

Fig. 2. Time course of the effects of 3-aminobenzamide on cell-cycle phase distribution following γ-irradiation. C3D2F1 3T3-a cells were inoculated at 1×10^5 cells per 100-mm dish and cultured for 54 h before γ-irradiation at 2 Gy. Cells were cultured in the absence (□) or presence (●) of 4 m*M* 3-aminobenzamide from 2 h before γ-irradiation. γ-Irradiation-induced change in the percentage of cell-cycle phase were calculated by subtracting (the cell-cycle phase percentage after mock-irradiation) from (the cell-cycle phase percentage after γ-irradiation). The time course experiment was repeated once; results were essentially reproducible.

inhibitors, benzamide and luminol, suppressed G1 arrest and enhanced G2 arrest, as in the case of 3-aminobenzamide. In contrast, 3-aminobenzoic acid (4 m*M*), a non-inhibitory analog, did not significantly alter the cell-cycle phase distribution. Twelve hours after γ-irradiation at 2 Gy, the mean γ-irradiation-induced changes of G1 phase percentages in several experiments were 11.3% for con-

trol cells, −2.0% for benzamide-treated cells, −4.8% for luminol-treated cells and 9.4% for 3-aminobenzoic acid-treated cells. The mean γ-irradiation-induced changes of G2 phase percentages in the above experiments were 10.0% for control cells, 16.0% for benzamide-treated cells, 13.5% for luminol-treated cells, and 9.5% for 3-aminobenzoic acid-treated cells.

DISCUSSION

The generation of DNA strand breaks following γ-irradiation has been shown to stimulate the synthesis of poly(ADP-ribose).[12] The precise function of poly(ADP-ribose) polymer in DNA repair and cell cycle regulation is not clear, but it has been suggested to be involved in altering chromatin structure or modulating the enzyme activities essential for DNA repair through poly(ADP-ribosyl)ation of nuclear proteins. Here, using PARP inhibitors, we examined whether PARP participates in cell cycle checkpoint regulation following γ-irradiation. We used C3D2F1 3T3-a cells for this purpose because after γ-irradiation they showed both G1 and G2 arrests and also an increase in the p53 protein level. p53 gene status in C3D2F1 3T3-a cells was examined and no mutation was detected in exons 5 to 9.[13] In the present work, we demonstrated that 3-aminobenzamide and other known PARP inhibitors suppressed G1 arrest and enhanced G2 arrest of C3D2F1 3T3-a cells following γ-irradiation. There was a possibility that 3-aminobenzamide caused apparent G1 arrest suppression at 2 Gy by sensitizing cells to γ-irradiation, leading to marked G2 arrest concealing G1 arrest. But this possibility was ruled out because G1 arrest was also suppressed by 3-aminobenzamide at a lower dose than 2 Gy, where G2 arrest was not profound enough to conceal G1 arrest.

Kastan *et al.* recently proposed a pathway for G1 arrest transduction following γ-irradiation in which the cell recognizes DNA damage and rapidly increases the level of p53 protein.[3] Then p53 functions as a transcription factor and up-regulates the expression of several effector genes including *GADD45* and *WAF1/CIP1*.[14] In the G1 arrest pathway, genes defective in ataxia-teleangiectasia and in Bloom syndrome are also suggested to be involved.[4,5] However, the molecular mechanisms of G1 arrest pathway have not yet been clarified precisely. In the present study we found that 3-aminobenzamide and the other PARP inhibitors suppressed G1 arrest following γ-irradiation. These PARP inhibitors block the poly(ADP-ribosyl)ation reaction in the cells, indicating that G1 arrest is dependent on poly(ADP-ribosyl)ation. Therefore it is suggested that PARP is a G1 arrest determinant. However, we have not established at which signal-transduction step of G1 arrest PARP is required, or whether PARP is involved in the p53-dependent G1 arrest pathway.

On the other hand, PARP does not seem to play a critical role in induction of G2 arrest. But the enhanced and prolonged G2 arrest induced by PARP inhibitors indicates that PARP may function in release from G2 arrest. 3-Aminobenzamide was reported to delay the repair of both single- and double-strand breaks caused by ionizing radiation.[15,16] Thus, delay of DNA repair could be a direct cause of the enhancement and prolongation of G2 arrest induced by PARP inhibitors.

In this study, we also examined the effects of other types of PARP inhibitors than 3-aminobenzamide, i.e., benzamide and luminol, on cell cycle arrest following γ-irradiation. These two PARP inhibitors clearly suppressed G1 arrest and enhanced G2 arrest in C3D2F1 3T3-a cells after γ-irradiation, as did 3-aminobenzamide. These results suggest that inhibition of PARP activity caused suppression of G1 arrest and enhancement of G2 arrest, and it seems unlikely that these chemicals caused cell cycle modulation by inhibiting some enzyme activity other than PARP. The disturbance of G1 arrest and enhancement of G2 arrest by PARP inhibitors might have contributed to the enhancement of γ-irradiation cytotoxicity observed by Nduka *et al.*[17] Elucidation of the precise mechanisms of modulation of G1 and G2 arrests by PARP will require extensive study, including PARP gene-disruption experiments.

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