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Expression of Poly (ADP-ribose) Polymerase During Apoptosis Induced by Ultraviolet Radiation in HeLa S_3 Cells

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Induction of apoptosis allows the organism to get rid of abnormal cells and also of tumor cells. Understanding the mechanism involved in Ultraviolet radiation (UV) induced apoptosis may improve its therapeutic efficacy. In this study, we present expression of poly (ADP-ribose) polymerase (PARP) during apoptosis induced by UV in HeLa S_3 cells. Four different assays were performed in this study: morphological assessment of apoptotic cells and cell viability, DNA fragmentation analysis by agarose gel electrophoresis, quantitative assay of fragmented DNA, and expression of PARP by the western blot analysis. The percentages of apoptotic HeLa S_3 cells irradiated with 75 J/m^2 UV was increased continuously from 3 hrs incubation. DNA ladder pattern was appeared at 6 hrs. The amount of nucleosomal DNA fragments in cells treated UV increased from 3 to 12 hrs incubation and gradually decreased. The cleavage of PARP in HeLa S_3 cells irradiated with UV was induced, and the cleavage of PARP was more delayed in the cells pretreated with 5 J/m^2 UV and subsequently irradiated with 75 J/m^2 UV than that in the cells only irradiated with 75 J/m^2 UV. Thus these data suggest that the cleavage of PARP relates with DNA fragmentation associated with apoptosis.

Key Words: Apoptosis, DNA fragmentation, HeLa S_3 cells, PARP

INTRODUCTION

Apoptosis does not only play an important role in the development and maintenance of tissue homeostasis but also represents an effective mechanism by which harmful cells can be eliminated. Morphological changes of apoptosis are usually accompanied by internucleosomal DNA fragmentation (Wyllie et al., 1980; Batistatou and Greene, 1993), and associated with double strand cleavage of nuclear DNA at the linker regions between nucleosomes, and produces ladders of DNA fragments that are size of 180~200 bp (Dedra et al., 1993). Cells have a mechanism converse various extracellular signals to intracellular common signals in apoptotic process. But the mechanism of apoptosis is poorly understood yet, it is generally accepted that many different signal of apoptosis ultimately leads to activation of an endogeneous endonuclease that cuts DNA between the nucleosomes in the linker regions (Arends et al., 1990).

Many DNA damaging agents including radiation and anticancer drugs are known to induce apoptosis and some efforts were made to elucidate the mechanism of DNA damaging agent induced apoptosis (Martin et al., 1993). Poly (ADP-ribose) polymerase (PARP) is a 116 kDa nuclear enzyme that catalyzes the Poly(ADP-ribosylation) of various nuclear proteins with NAD as substrate, and because it is activated by binding to DNA ends or strand breaks, PARP has been suggested to contributed to cell death by depleting the cell of NAD and ATP (Berger et al., 1983). Negri et al. (1993) suggested that activation of Poly(ADP-ribosylation) process occurs in cells that show the typical features of apoptosis. PARP undergoes proteolytic cleavage into 89-kDa and 24-kDa fragments that contain the active site and the DNA binding domain of the enzyme, respectively, during drug-induced apoptosis in a variety of cells (Kaufmann et al., 1993). Some investigators suggested that the inactivation of PARP might play a role in the biochemical changes that accompany apoptosis (Rice et al., 1992), even through many

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studies proposed that the activation of PARP contribute to the induction of apoptosis (Manome et al., 1993). The purpose of this study is to study the level of PARP expression and its cleavage during apoptosis induced by UV in HeLa S_3 cells.

MATERIALS AND METHODS

1. Cell culture

HeLa S_3 cells were used throughout this investigation. Monolayer cultures of these cell lines were grown at 37℃ in humidified 5% CO_2 incubator using Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% newborn calf serum and gentamycin (50 μg/ml).

2. Mutagen treatment

Cells were cultured for more than 48 hrs in tissue culture petridishes prior to Ultraviolet radiation (UV) irradiation, the growth medium was decanted from the petridishes and the cells washed three times with phosphate buffered saline (PBS). Cells were then exposed to various doses of 254 nm UV from mercury germicidal lamps at an incident dose rate of 1 J/m^2/sec. The dose rate was determined by UVX digital radiometer No. A030848 (San Gabrial, CA 911778 USA). The fresh medium was added immediately after irradiation.

3. Cell viability assay

Cell viability was measured by hemocytometer using the trypan blue dye exclusion. Trypsinized cells were incubated with 0.4% trypan blue solution (Sigma) for 10 mins, and more than 200 cells were scored on a hemocytometer. Viable and nonviable cells were counted by inverted microscopy.

4. Morphological assessment of apoptosis

Acridine orange/ethidium bromide (AO/EB) staining of apoptotic cell. This method was carried out as described by Inohara et al. (1997) with slight modification. The 25 μl of apoptotic cell suspension ($5\times10^5 \sim 1\times10^6$/ml) was incubated with 1 μl of AO/EB solution (1 part of 100 μg/ml AO in PBS; 1 part of 100 μg/ml EO in PBS) for 5~10 mins and mixed gently. Each sample was mixed just prior to microscopy and quantification, and then evaluated immediately. The 10 μl of cell suspension was placed onto a microscopic slide, and covered with a glass coverslip, and then examined at least 300 cells on a fluorescence microscope using a fluorescent filter and a 200× objective.

5. Quantitative and DNA fragmentation analysis

The quantification of DNA fragmentation was carried as described by McConkey et al. (1989) with slight modifications. Cells treated with UV in a 100 mm culture dish were lysed in 0.33 ml of buffer containing 5 mM Tris (pH 8.0), 20 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% Triton X-100. After incubation for 15 mins on ice, samples were centrifuged for 10 mins at 10,000 rpm to separate the intact chromatin (pellet) from the fragmented DNA (supernatant). Pellets were resuspended in 0.33 ml of a buffer containing 10 mM Tris (pH 8.0) and 1 mM EDTA. Pellets and supernatant fractions were separately assayed for DNA content using the diphenylamine reagent containing 1.5% diphenylamine, 1.5% sulfuric acid and 0.008% acetaldehyde in glacial acetic acid. DNA fragmentation was quantified by measuring the ratio of the DNA content in supernatant fraction to the total DNA content (supernatant plus pellet). The apoptotic nature of cells were examined by agarose gel electrophoresis of their nuclear DNA using the method of Waring (1990). For visualization of fragmented DNA, the supernatant fraction containing fragmented DNA was extracted two times with phenol and once with chloroform. Extracted DNA fragments were precipitated in 67% ethanol, 0.3 M sodium acetate at -70℃ for overnight, and then resuspended in a buffer containing 10 mM Tris (pH 8.0), 1 mM EDTA and 30 μg/ml RNase, prior to electrophoresis in 1.8% agarose gel as described by Jones et al. (1989).

6. Western blot analysis of Poly(ADP-ribose) polymerase proteins

Cells were washed three times in cold PBS. Samples were then diluted with an equal volume of 2 × sodium dodecyl sulfate (SDS) sample buffer and heated for 5 mins at 100℃. Samples were loaded to equivalent amount (30 μg/lane) on one-dimensional SDS-polyacrylamide gel and subjected to electrophoresis. After electrophoresis, western blot analysis was done according to the technique of Towbin et al. (1979) with slight modifications. Membrane was soaked in methanol for 10 secs and washed in distilled water for 5 mins. Blotting was performed at 0.35 A for 1 hr. And the membrane was dried by air drying. The membrane was treated with primary antibody (monoclonal anti-PARP;

CII10) for 1 hr, washed three times with PBS-TritonX-100 (PBST). That was treated with secondary antibody (polyclonal anti-mouse) was for 1 hr, followed by three times with PBST. The membrane was treated with Enhanced Chemileuminescence Liquid (ECL), and expose to X-ray film.

RESULTS

1. Apoptosis induced by ultraviolet radiation

Fig 1. shows morphology of apoptotic cells determined by Acridine orange/ethidium bromide (AO/EB) assay in HeLa S_3 cells. Control cells stain uniformly green and can be distinguished from apoptotic cells which exhibit yellowish dots of condensed chromatin. Apoptosis cells that have lost their membrane integrity appear orange due to co-stain with EB.

Apoptosis was induced in HeLa S_3 cells by treatment with 75 J/m^2 UV and the incidence of apoptosis was scored by percentages of apoptotic cells and cell viability during post-incubation for 3, 6, 12 or 24 hrs. AO/EB-stained cells were examined under a flourecence microscope (Fig. 1), and the cells containing morphologically distorted nuclei were regarded as apoptotic cells increased continuously from 3 hrs of post-incubation in HeLa S_3 cells (Fig. 2A). And cell viability in the cells irradiated with 75 J/m^2 UV greatly decreased until 24 hrs incubation (Fig. 2B).

DNA fragmentations during apoptosis in HeLa S_3 cells irradiated with UV are shown in Fig. 3. HeLa S_3 cells were incubated for various times after treatment with 75 J/m^2 UV. In the cells severe DNA laddering showing typical apoptotic DNA fragmentation appeared at 6 hrs.

Fig. 4 represents the quantitative assay of fragmented DNA in HeLa S_3 cells irradiated with 75 J/m^2 UV and incubated for various times at 37℃. The amount of nucleosomal DNA fragments in cells treated UV increased from 3 to 12 hrs incubation and gradually decreased as the incubation time.

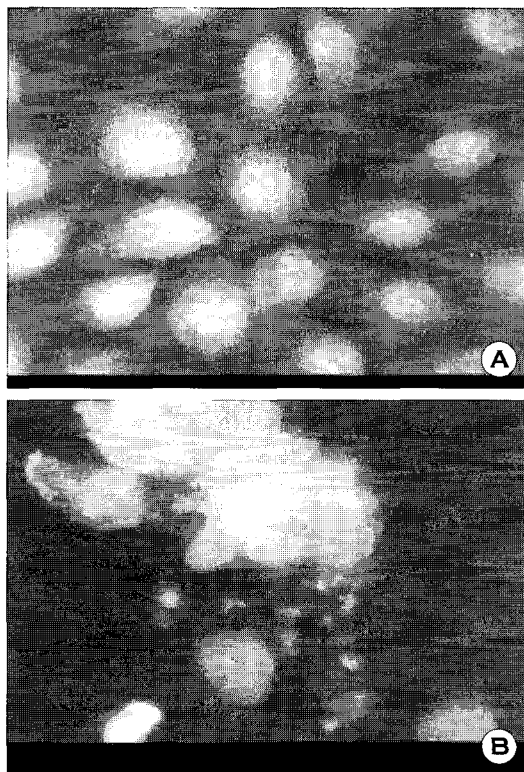

Fig. 1. Morphology of apoptotic cells stained with AO/EB in HeLa S_3 cells irradiated with UV. Control cells stain uniformly green and can be distinguished from apoptotic cells which exhibit yellowish dots of condensed chromatin **(A)**. Apoptosis cells that have lost their membrane integrity appear orange due to co-stain with EB **(B)**.

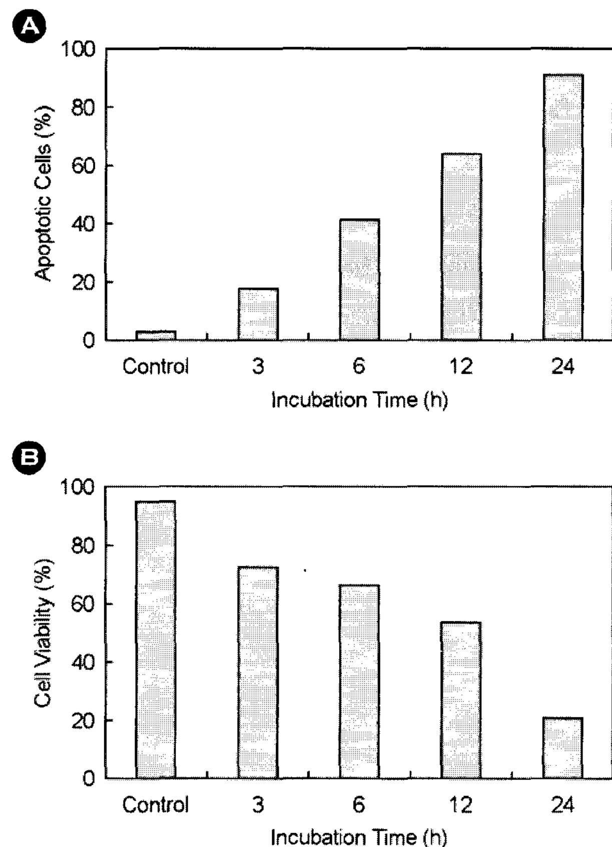

Fig. 2. Percentages of apoptotic cells **(A)** and cell viability **(B)** during apoptosis induced by UV in HeLa S_3 cells. Cells were irradiatted with 75 J/m^2 UV and then incubated for 3, 6, 12 or 24 hrs at 37℃. C represents the control.

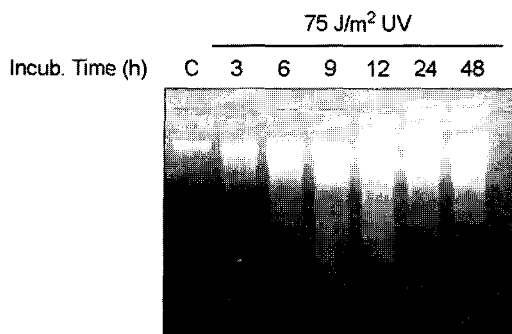

Fig. 3. Photograph of agarose gel electrophoresis of extracted DNA in HeLa S_3 cells irradiated with UV. Cells were irradiated with 75 J/m^2 UV and then incubated for 3, 6, 9, 12, 24 or 48 hrs at 37℃. DNA fragments extracted from the cells at different time pionts of post-incubation were separated in 1.8% agarose gel. Lane C represents the control.

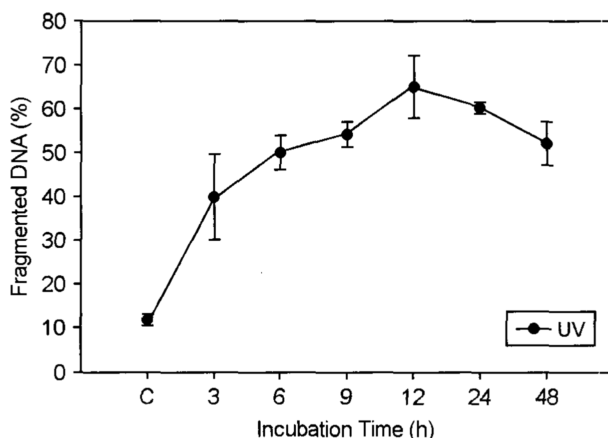

Fig. 4. Quantitative assay of fragmented DNA during apoptosis induced by UV in HeLa S_3 cells. Cells were irradiatted with 75 J/m^2 UV and then incubated for 3, 6, 9, 12, 24 or 48 hrs at 37℃. C represents the control. Quantitative assay of fragmented DNA was determined by diphenylamine reaction.

2. Expression of Poly(ADP-ribose)polymerase

Cells were irradiated with 75 J/m^2 UV, and the level of PARP expression was detected by western blotting using CII10 antibody, which recognizes both 116 kDa intact PARP and its 85 kDa fragments. Fig. 5 shows the western blot analysis of the expression of PARP in HeLa S_3 cells treated 75 J/m^2 UV and then incubated for 3, 6, 9, 12, 18, 24 or 48 hrs at 37℃. When cells were incubated for 3 hrs after treatment with 75 J/m^2 UV, the cleavage of PARP was induced. The intact 116 kDa PARP disappeared from 6 hrs of post-incubation, concomitant with the generation of 85 kDa fragment of PARP.

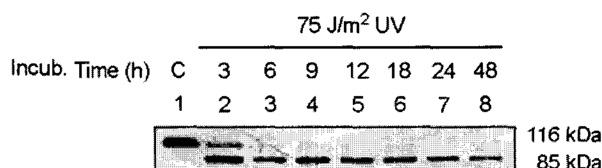

Fig. 5 Western blot analysis of PARP expression in HeLa S_3 cells treated with 75 J/m^2 UV. Cells were irradiated with UV and then incubated for 3, 6, 9, 12, 18, 24 or 48 hrs at 37℃ (lane 2~8). Lane 1 represents the control. PARP was detected by western blotting using CII10 antibody, which recognizes both 116 kDa intact PARP and its 85 kDa cleavage product.

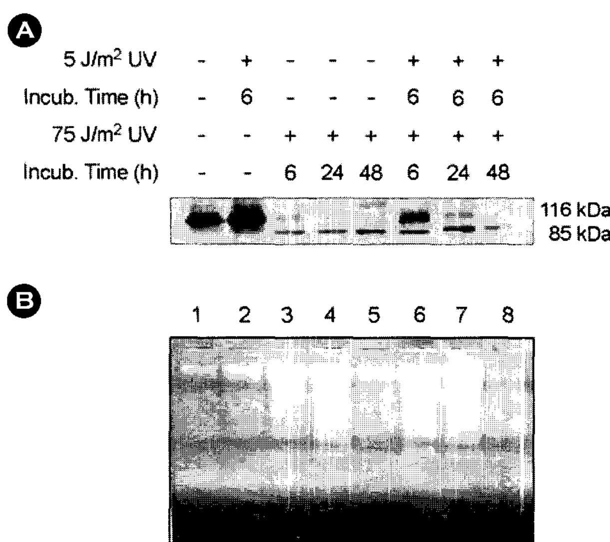

Fig. 6 Western blot analysis of PARP expression and DNA fragmntation in HeLa S_3 cells. Cells were treated with 5 J/m^2 UV and subsequently treated with 75 J/m^2 UV followed by incubation for various times. Lane 1: control. Lane 2: cells incubated for 6 hrs after treatment with 5 J/m^2 UV. Lane 3, 4 and 5: cells incubated for 6, 24 and 48 hrs after treatment with 75 J/m^2 UV. Lane 6, 7 and 8: cells irradiated with 5 J/m^2 UV and subsequently treated with 75 J/m^2 UV followed by incubation for 6, 24 or 48 hrs.

3. Apoptosis induced by pretreatment with low dose of UV

Expression of PARP and DNA fragmentation in HeLa S_3 cells pretreated with 5 J/m^2 UV and subsequently treated with 75 J/m^2 UV following incubation for 6, 24 or 48 hrs are shown in Fig. 6. The cleavage of PARP was not shown in cells treated with 5 J/m^2 UV. When cells were incubated for 6 hrs after treatment with 75 J/m^2 UV, the cleavage of PARP was induced. The intact 116 kDa PARP disappeared from 6 hrs of post-incubation, concomitant with the generation of 85 kDa fragment of PARP (Fig. 6A). When cells were pretreated with 5 J/m^2 UV and subsequently treated with 75 J/m^2 UV following incubation for 6 or 12 hrs, 116

kDa PARP was appeared (Fig. 6A). And DNA fragmentation in cells pretreated with 5 J/m^2 UV and subsequently treated with 75 J/m^2 UV was similar to that in cells treated with 75 J/m^2 UV alone (Fig. 6B). Thus, these results indicated that the amounts of intact PARP retained in the cells inversely correlated with the incidence of apoptosis after treatment with UV.

DISCUSSION

During apoptosis, a complex death program becomes initiated that ultimately leads to the fragmentation of the cell. A number of morphological and biochemical features are typical of apoptosis (Zakeri et al., 1995), but some of them are not obligatory. Wyllie (1980) showed that the morphological changes of apoptosis are associated with double strand cleavage of nuclear DNA at the linker regions between nucleosome. Agarose gel electrophoresis displays the internucleosomal DNA from apoptotic cells in a typical ladder pattern, whereas the DNA cleavage in necrotic cells is random and is seen as a smear (Arends et al., 1990). But the nucleosomal DNA ladders are not always associated with apoptosis (Rusnak et al., 1996). DNA ladder was shown in human leukemia HL 60 cells at 3 h after treated with 250 J/m^2 UV (Yoon et al., 1996), and also shown in human Jurkat T cells treated with 60 J/m^2 UV (Chen et al., 1996). In the present study, DNA ladder patterns in HeLa S_3 cells irradiated with 75 J/m^2 UV were shown at 6 hrs. These results are generally consistent with other's reports. Thus, the inidence of apoptosis is variable depending on the cell type.

In HL 60 cells treated with topoisomerase inhibitors, the percentages of apoptotic cells were increased up 60% after 5 hrs (Alexandre et al., 2000). And the percentages of apoptotic cells Syrian hamster embryo cells treated with camptothecin or etoposied were below 8% (Alexandre et al., 2000). In the present study, the percentages of apoptotic HeLa S_3 cells irradiated with 75 J/m^2 UV was increased continuously from 3 hrs incubation. Thus, the percentages of apoptotic cells is variable depending on the cell type or apoptotic agents.

On the other hands, some evidences indicate that a protease is a component of the mammalian cell death pathway (Gagliardini et al., 1994; Tewari and Dixit, 1995). Caspase-3 is a protease that cleaves PARP to the signature 85 kDa apoptotic fragment (Tewari et al., 1995), this Caspase-3 plays a central role in the execution of the apoptotic program (Alnemri et al., 1996). Kaufmann et al. (1993) found that the 116 kDa nuclear protein poly(ADP-ribose) polymerase was specifically cleaved to produce an 85 kDa fragment in many forms of apoptosis. The 116 kDa PARP effectively cleaved into its 85 kDa fragment in human U937pMEP cells treated with TNF-α for 12 hrs (Monney et al., 1998). In HL 60 cells treated with 40 μg/ml etoposide, the cleavage of PARP was shown, whereas serum-deprived Syrian hamster embryo SHE cells was not shown (Alexandre et al., 2000). In an effort to elucidate mechanisms underlying the different incidences of apoptosis in HeLa S_3 cells, expression levels of PARP were measured. We found that the amount of intact PARP retained in the cells were inversly correlated with the incidences of apoptosis at different time points of the post-incubation in HeLa S_3 cells irradiated with a single high dose of UV. The inverse relationship between the level of intact PARP and the incidence of apoptosis was also observed in the DNA repair system, in which the cells were irrated with high dose UV after pre-treatment with a low dose. In this study, cleavage of PARP in HeLa S_3 cells irradiated with 75 J/m^2 UV was detected at 3 hrs incubation, and 116 kDa PARP was disappeared after incubation for 6 hrs. Thus typical DNA fragmentation coincided with the PARP cleavage in HeLa S_3 cells irradiated with 75 J/m^2 UV. These results suggest that the retained level of intact PARP in the damaged cells is important in commitment to the apoptotic pathway, and the ability to protect the degradation of PARP after the UV induced damage is different in cell. And these data suggest that the cleavage of PARP relates with DNA fragmentation associated with apoptosis. To elucidate the detailed molecular mechanisms of DNA fragmentation associated with apoptosis, further studies are necessary.

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