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# Ultraviolet Radiation-Induced Apoptosis Is Inversely Correlated with the Expression Level of Poly(ADP-ribose) Polymerase

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**The present study was conducted to elucidate whether the expression level of poly(ADP-ribose) polymerase (PARP) is related to the ultraviolet radiation (UV)-induced apoptosis. After treatment of the mammalian cell lines HeLa $S_3$ and Chinese hamster ovary (CHO) with 50 J/m$^2$ UV, induction of apoptosis was determined by several means during 24 h post-incubation. Incidence of apoptosis was much lower in CHO than HeLa $S_3$ cells based on the percentage of apoptotic cells in terms of morphological changes in nucleus or direct counting of viable cells and qualitative or quantitative DNA fragmentation. Interestingly, when the expression level of PARP was measured by western blotting, the amounts of PARP that was retained at each time point inversely correlated with the incidences of apoptosis in these cells. Concomitant with generation of the 85 kDa fragment, 116 kDa PARP disappeared in HeLa $S_3$ within 6 h after UV treatment, whereas a fair amounts of 116 kDa band was still retained in CHO cells at 36 h post-incubation. This inverse relationship was also observed in the adaptive response system, in which cells were treated with a high dose of UV after pretreatment with a low dose. As expected, typical adaptive responses appeared in CHO cells but not in HeLa cells, showing greater cell viability and lesser DNA fragmentation. During the adaptive response in CHO cells, PARP was expressed at much higher level compared to the single, high dose-treated cells. Interestingly, even though PARP was induced at 6 h post-incubation in both cell types, its expression was more prominent in CHO cells. Thus, our data indicate that the retained level of intact PARP against UV damage inversely correlates with incidence of apoptosis in mammalian cells, and also suggest that a machinery to protect the PARP degradation against UV damage exists in CHO but not in HeLa $S_3$ cells.**

Morphological changes during apoptosis are usually associated with double strand cleavage of nuclear DNA at the linker regions between nucleosomes, producing ladders of DNA fragments of 180-200bp (Wyllie et al., 1980; Batistatou and Greene, 1993; Dedra et al., 1993). Even though the mechanism of apoptosis is poorly understood, it is generally accepted that many different signals of apoptosis ultimately lead 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 efforts were made to elucidate the mechanism of DNA damaging agent-induced apoptosis (Martin et al., 1993). Poly(ADP-ribose)polymerase (PARP) has been known to have a biologically significant role in the rejoining of DNA strand breaks (Durkacz et al., 1980), possibly by recognizing the conformational change in DNA in the regions of single or double strand breaks produced by DNA damaging agents (Ikejima et al., 1990). PARP is an abundant and highly conserved chromatin-bound protein in eukaryotes, and catalyzes polyADP-ribosylation of DNA binding proteins (Lindahl et al., 1995; D'Amours et al., 1999). Some investigators suggested that the inactivation of PARP might play a role in the biochemical changes that accompany apoptosis (Rice et al., 1992), even though many studies proposed that the activation of PARP contributes to the induction of apoptosis (Manome et al., 1993). Whereas Negri et al. (1993) reported that the activation process of ADP-ribosylation occurs in cells showing the typical features of apoptosis, Homburg et al. (2000) suggested that the activation of PARP induced by DNA break formation is involved in DNA transcription, replication, and repair. Thus, the role of

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PARP in normal cell growth and apoptosis is ambiguous. The purpose of this study is to elucidate the relationship between the level of PARP activation and the incidence of apoptosis in mammalian cells treated with ultraviolet radiation.

## Materials and Methods

### *Cell culture*

HeLa $S_3$ and CHO 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.) supplemented with 10% newborn calf serum and gentamycin (50 μg/ml).

### *UV irradiation*

Cells were cultured for more than 48 h in tissue culture petri dishes prior to UV irradiation. The growth medium was decanted from the dishes and the cells were 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). The fresh medium was added immediately after the irradiation. Analysis of adaptive responses to pretreatment with low dose UV was performed with the procedure described by Lee et al. (2000).

### *Cell viability assay*

Cell viability was measured by hemacytometer using the trypan· blue dye exclusion method. Trypsinized cells were incubated with 0.4% trypan blue solution (Sigma) for 10 min, and more than 200 cells per group were scored on a hemacytometer. Viable and non-viable cells were counted under inverted microscope.

### *Morphological assessment of apoptotic cell*

Nuclear morphology was assessed by fluorescent microscopy after Hoechst 33258 staining. Cell suspensions (500 μl at $5\times10^5$-$1\times10^6$ cells/ml) were incubated with 5 μl Hoechst 33258 (1 mg/ml in dd$H_2O$) for 10 min. The samples were transferred to microcentrifuge tubes and concentrated by centrifugation at 500 rpm for 2 min. After air-drying, coverslip was placed over the cells on slide glass to reduce light diffraction and 300-400 cells per group were counted under fluorescence microscope equipped with a DAPI filter.

### *Quantitative and qualitative DNA fragmentation analysis*

Quantification of DNA fragmentation was carried out 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 min on ice, samples were centrifuged at 10,000 rpm for 10 min to separate the intact chromatin (pellet) from the fragmented DNA (supernatant). Pellets were resuspended in 0.33 ml of buffer containing 10 mM Tris, pH 8.0 and 1 mM EDTA. The pellets and supernatant fractions were separately analyzed 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 DNA content in the supernatant to total DNA content (supernatant plus pellet). Apoptotic nature of the cells was also examined by agarose gel electrophoresis of nuclear DNA according to 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. The extracted DNA fragments were precipitated with 67% ethanol and 0.3 M sodium acetate at -70℃ overnight. The DNA pellet was resuspended in buffer containing 10 mM Tris, pH 8.0, 1 mM EDTA and 30 μg/ml RNase. Electrophoresis was performed in 1.8% agarose gel as described by Jones et al. (1989).

### *Western blot analysis of poly(ADP-ribose) polymerase*

Cells were washed three times with cold PBS. Samples were then diluted with an equal volume of 2 × sodium dodecyl sulfate (SDS) sample buffer and heated for 5 min at 100℃. Samples (30 μg/lane) were loaded on one-dimensional SDS-polyacrylamide gel and subjected to electrophoresis. After the electrophoresis, western blot analysis was performed according to the technique of Towbin et al. (1979) with slight modifications. Membrane was soaked in methanol for 10 sec and washed in distilled water for 5 min. Samples were transferred to the membrane using an electroblotting apparatus at 0.35 A for 1 h and the membrane was air dried. The membrane was treated with primary antibody (monoclonal anti-PARP; CII10) for 1 h, and then washed three times with PBS-Triton X-100 (PBST). Secondary antibody (polyclonal anti-mouse) was treated for 1 h and washed three times with PBST. The membrane treated with Enhanced Chemileuminescence Liquid was exposed to X-ray film.

## Results

### *Apoptosis induction by UV*

Apoptosis was induced in both HeLa $S_3$ and CHO cells by treatment with 50 J/m$^2$ UV and the incidence of apoptosis was scored by percentages of apoptotic cells and viability during post-incubation for 3, 6, 12 or 24 h (Fig. 1). Hoechst 33258-stained cells were examined under a fluorescence microscope, and the cells con-

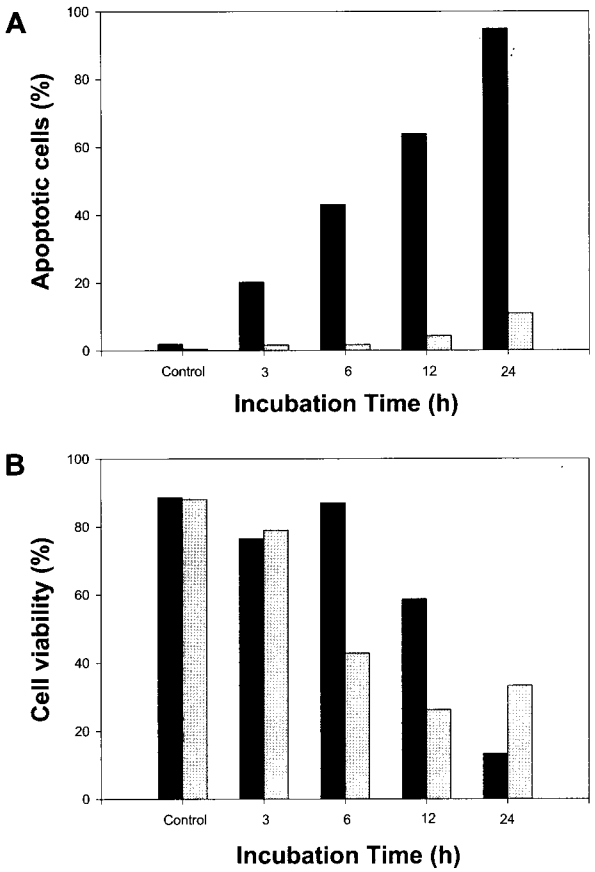


**Fig. 1.** Percentages of apoptotic cells (A) and cell viability (B) in HeLa $S_3$ (■) and CHO (▦) cells. After treatment with 50 J/m$^2$ UV, apoptotic cells showing morphological change in nuclei or viable cells excluding trypan blue dye were scored during the post incubation time points of 3, 6, 12, or 24 h.

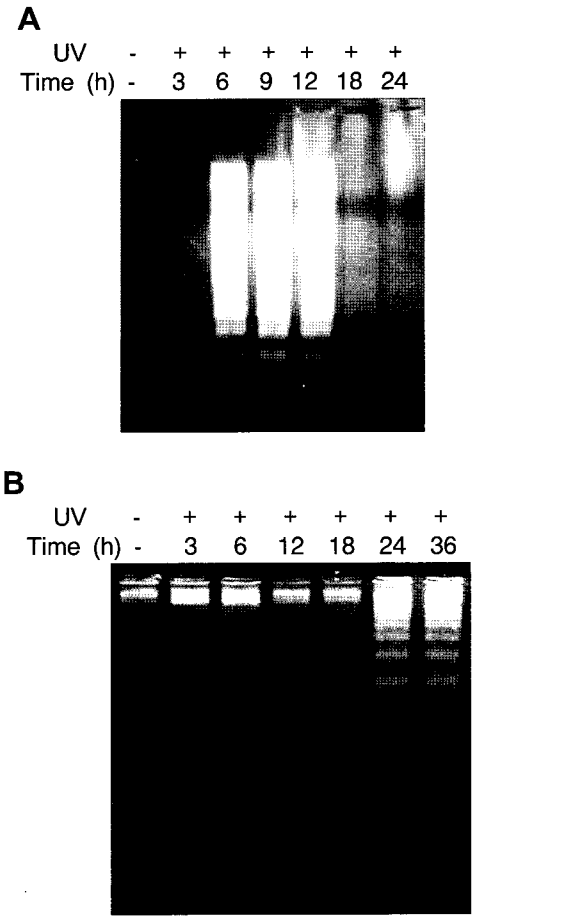


**Fig. 2.** DNA fragmenation patterns of HeLa $S_3$ (A) and CHO (B) cells. After treatment with 50 J/m$^2$ UV, the cells were incubated for various times. DNA fragments extracted from the cells at different time points of post-incubation were separated in 1.8% agarose gel.

taining morphologically distorted nuclei were regarded as apoptotic. For the viability test, cells excluding trypan blue stain were scored as viable. The number of apoptotic cells increased continuously from 3 h of post-incubation in both cell types. However, the percentages of apoptotic cells were much higher in HeLa $S_3$ than in CHO cells (Fig. 1A). Similar results were obtained in cell viability test (Fig. 1B), even though this was hard to correlate directly to the percentage of apoptotic cells as shown in Fig. 1A because the two methods assessed different aspects of apoptosis. Thus, these data suggested that HeLa $S_3$ cells were more sensitive to the UV damage than CHO cells.

To confirm that the different sensitivity of these cell types to the UV damage is due to differences in apoptotis, quality and quantity of DNA fragmentation were analyzed. After treatment with 50 J/m$^2$ UV, HeLa $S_3$ and CHO cells were incubated for various periods. DNA fragments extracted from cells during the post-incubation were separated in 1.8% agarose gels (Fig. 2). In HeLa $S_3$ cells, severe laddering showing typical apoptotic DNA fragmentation appeared from 6 h to 12 h but disappeared after 18 h (Fig. 2A). This disappearance may represent massive cell death (see Fig. 1). On the other hand, the DNA laddering was not severe and appeared more slowly in CHO cells (Fig. 2B). When the amounts of subgenomic DNA fragments from these cells were quantified during various post-incubation time points, more fragmented DNA was observed in HeLa $S_3$ cells than in CHO cells (Fig. 3). Thus, these data indicated that, compared to CHO cells, apoptosis occurs more easily in HeLa $S_3$ cells upon UV-irradiation, and suggested that some differences in protection machinery to the UV damage exist in these cells.

*Expression of poly(ADP-ribose) polymerase*

In an effort to elucidate the mechanism underlying the different incidence of apoptosis in HeLa $S_3$ and CHO cells to the UV damage, expression level of PARP was measured by Western blotting at various post-incubation time points after the UV treatment. Western blot analysis of PARP in HeLa $S_3$ and CHO cells are shown in Fig. 4. Fig. 4A shows the expression of PARP in HeLa $S_3$ cells treated with 50 J/m$^2$ UV and

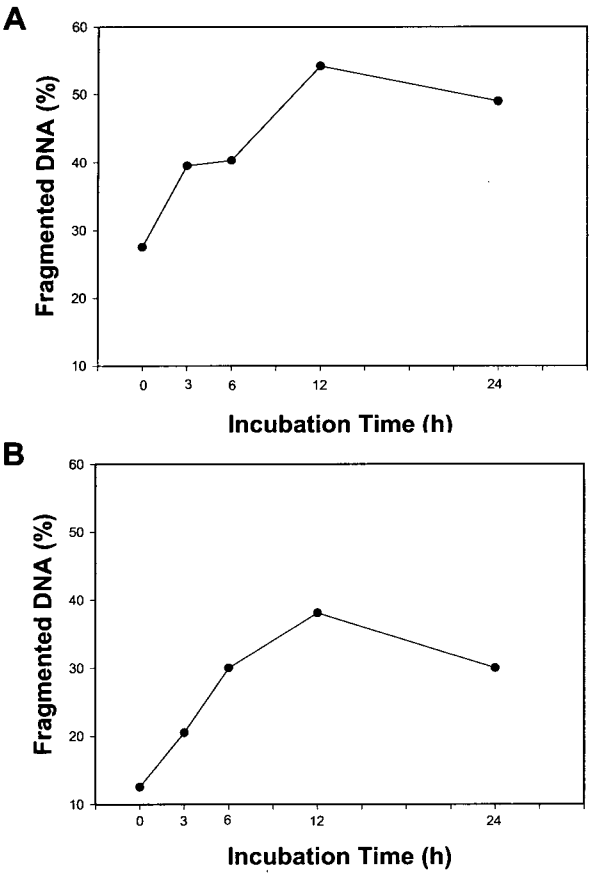


**Fig. 3.** Quantitative assay of fragmented DNA during apoptosis induced by UV in HeLa $S_3$ (A) and CHO (B) cells. After treatment with 50 J/m$^2$ UV, cells were incubated for various periods, and the extents of DNA fragmentation were determined by diphenylamine reaction.

incubated for various time afterwards. The cleavage of PARP was apparent starting from 3 h of post-incubation. The intact 116 kDa PARP disappeared from 6 h of post-incubation, concomitant with the generation of 85 kDa fragment of PARP. On the contrary, the level of PARP in CHO cells gradually decreased during the post-incubation period, so that fair amounts of intact 116 kDa PARP were still retained after 36 h (Fig. 4B). Thus, the data indicated that the amounts of intact PARP retained in the cells inversely correlated with the incidence of apoptosis after UV-irradiation.

*Apoptosis induced by pre-treatment with UV*

We next tested whether there is a relationship between the incidence of apoptosis and the retention level of intact PARP by analyzing adaptive responses of the cells after pre-treatment with low dose UV. To induce adaptive responses, cells were pre-treated with 2 J/m$^2$ UV and subsequently with 50 J/m$^2$ UV. At various post-incubation time points, the levels of DNA fragmentation and PARP were measured. The control groups were treated only with a high dose UV and then incubated for various time periods. The results are

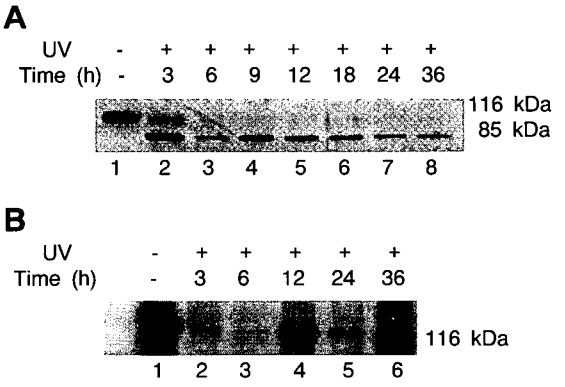


**Fig. 4.** Western blot analysis of PARP expression in HeLa $S_3$ (A) or CHO (B) cells. Cells were treated with 50 J/m$^2$ UV, and the level of PARP expression was detected by western blotting using CII 10 antibody, which recognizes both 116 kDa intact PARP and its 85 kDa cleavage product.

shown in Fig. 5. In contrast to the control groups, the cells pre-treated with a low dose UV retained a fair amounts of intact PARP at 6 h post-incubation (compare lanes 3 and 6 in Fig. 5A). However, the 85 kDa fragment was generated at 12 h post-incubation (lanes 7 and 8 in Fig. 5A). On the other hand, the DNA fragmentation patterns were similar in both groups (Fig. 5B). This suggested that PARP cleavage was inhibited during the early stage of adaptive

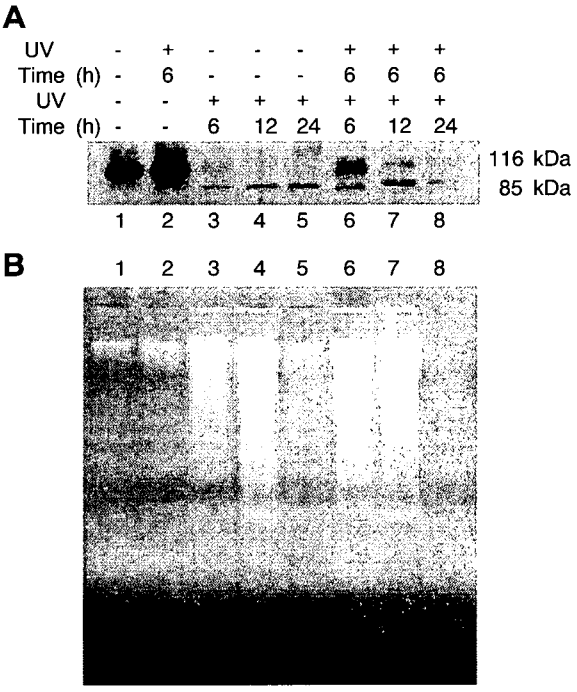


**Fig. 5.** PARP expression (A) and DNA fragmentation (B) in HeLa $S_3$ cells during adaptive response to the pre-treatment of low dose (2 J/m$^2$) of UV. Lane 1: control. Lane 2: cells incubated for 6 h after treatment with low dose UV. Lanes 3, 4, and 5: cells incubated for 6, 12, and 24 h after treatment with 50 J/m$^2$ UV. Lanes 6, 7, and 8: cells incubated for 6, 12 or 24 h after treatment with low dose UV and subsequently with 50 J/m$^2$ UV.

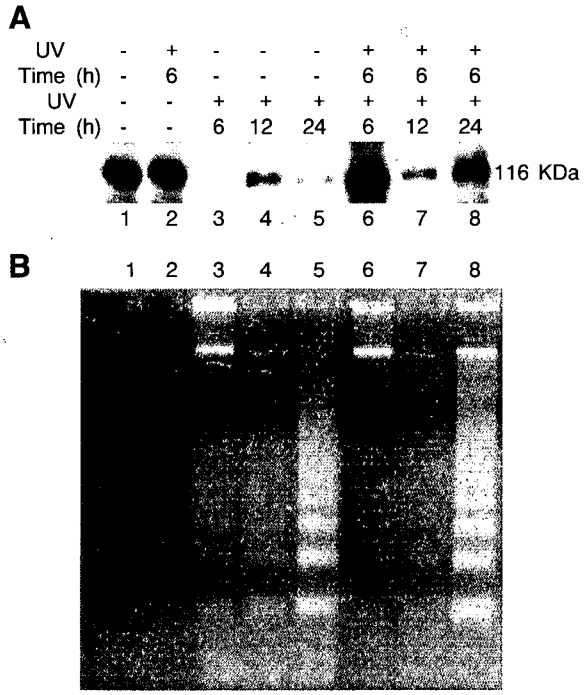


**Fig. 6.** PARP expression (A) and DNA Fragmentation (B) in CHO cells during adaptive response to the pre-treatment of low dose (2 J/m$^2$) of UV. Lane 1: control. Lane 2: cells incubated for 6 h after treatment with low dose UV. Lanes 3, 4, and 5: cells incubated for 6, 12, and 24 h after treatment with 50 J/m$^2$ UV. Lanes 6, 7, and 8: cells incubated for 6, 12, or 24 h after treatment with low dose UV and subsequently with 50 J/m$^2$ UV.

responses, but that this was not enough to prevent the DNA fragmentation. When the same experiment was performed with CHO cells, the expression level of PARP was greatly enhanced in the pre-treated groups, even higher than that of controls that were not treated with UV at all, at 6 h post-incubation, and fairly high levels of intact PARP were retained in subsequent time points (Fig. 6A). Accordingly, the levels of DNA fragmentation were also greatly reduced during post-incubation (Fig. 6B). Thus, these data indicated that the level of intact PARP and incidence of apoptosis highly correlated with each other during the adaptive response of cells against UV. The results also suggested that the level of intact PARP was important in the apoptotic pathway in response to UV-irradiation. Furthermore, the fact that the amounts of PARP in the 6 h post-incubation during adaptive response were even higher than the basal level suggested that de novo synthesis of PARP might be induced during adaptive response of CHO cells against UV. It was also possible that PARP was protected from degradation during the adaptive response. On the other hand, PARP degraded rapidly in HeLa $S_3$ cells in the same condition.

## Discussion

A number of morphological and biochemical features are typical of apoptosis (Zakeri et al., 1995). Even though the nucleosomal DNA ladders are not always associated with apoptosis (Rusnak et al., 1996), Wyllie (1980) showed that the morphological changes during apoptosis are associated with double strand cleavage of nuclear DNA at the linker regions between nucleosome. Agarose gel electrophoresis displays the inter-nucleosomal 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). DNA ladder was shown in human leukemia HL60 cells 3 h after they were treated with 250 J/m$^2$ UV (Yoon et al., 1996), and also in human Jurkat T cells treated with 60 J/m$^2$ UV (Chen et al., 1996). However, the incidence of apoptosis is different depending on cell types. In HL60 cells treated with topoisomerase inhibitors, the percentages of apoptotic cells were increased up to 60% after 5 h, whereas the percentages of those in Syrian hamster embryo cells treated with camptothecin or etoposide were below 8% (Alexandre et al., 2000). Similarly, as demonstrated in this study, when DNA ladder patterns were measured in the 50 J/m$^2$ UV-treated HeLa $S_3$ and CHO cells, the percentages of apoptotic cells were increased up to 95% in HeLa $S_3$ cells, whereas of 11% in CHO cells of 24 h post-incubation (Fig. 1). Thus, the incidence of apoptosis is variable depending on the cell types, even after the same treatment. This may imply that the machineries involved in DNA repair and/or other sensing machineries to external apoptotic stimuli are variable in different cell types.

In an effort to elucidate mechanisms underlying the different incidences of apoptosis in HeLa $S_3$ and CHO cells, expression levels of PARP were measured. We found that the amounts of intact PARP retained in the cells were inversely correlated with the incidences of apoptosis at different time points of the post-incubation in CHO and HeLa $S_3$ cells treated with a single high dose of UV (Figs. 1-4). The inverse relationship between the level of intact PARP and the incidence of apoptosis was also observed in the adaptive response system, in which the cells were treated with a high dose UV after pre-treatment with a low dose (Figs. 5 and 6). Further studies will be necessary to confirm our finding using other cell types and also with other apoptosis-inducing agents.

Evidence indicates that a protease is a component of the mammalian cell death pathway (Gagliardini et al., 1994; Tewari and Dixit, 1995). Caspase-3 is known as a protease that cleaves PARP to the 85 kDa fragment (Tewari and Dixit, 1995). Caspase-3 also 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 the 85 kDa fragment in many forms of apoptosis. The 116 kDa PARP was effectively cleaved into the 85 kDa fragment in human U937pMEP cells treated with TNF-α for 12 h (Monney et al., 1998). The cleavage was

shown in HL60 cells treated with 40 μg/ml etoposide, but not in serum-deprived hamster embryo SHE cells (Alexandre et al., 2000). In this study, we found that the 116 kDa disappeared in HeLa $S_3$ within 6 h after UV treatment, whereas a fair amount was still retained in CHO cells at 36 h post-incubation (Fig. 4). Thus, our data suggest that a machinery to protect the PARP degradation against UV damage exists in CHO but not in HeLa cells.

Pre-treatment of HL60 cells with calpain inhibitor did not prevent DNA fragmentation or plasma membrane damage, whereas pre-treatment with pan-caspase inhibitor Z-VAD-fmk resulted in inhibition of DNA fragmentation (Wood and Newcomb, 1999). In a previous study, we tested the effect of low doses of mutagenes on adaptive response, and found that the change in glycoconjugates in plasma membrane correlates with the induction of apoptosis even though the effect of the change is different depending on cell types (Lee et al., 1998; Lee et al., 2000). In this study, we found that PARP was induced at 6 h post-incubation in CHO cells during the adaptive response to pre-treatment with a low dose UV. Furthermore, the retained level of intact PARP in CHO cells pre-treated with 2 J/$m^2$ UV and subsequently treated with 50 J/$m^2$ UV was higher than the basal level (Fig. 6A). In accordance, DNA fragmentation was greatly reduced in these cells (Fig. 6B). 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 tissue types. It should be mentioned, however, that the amounts of PARP at 6 h post-incubation during the adaptive response are even higher than the basal level in CHO cells whereas PARP degrades rapidly in HeLa $S_3$ cells. These results suggest that *de novo* synthesis of PARP is induced during the adaptive response of CHO cells against UV-irradiation and the underlying mechanism of PARP induction during the adaptive response must be clarified in further studies.

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