DNA-binding activity of the N-terminal cleavage product of poly(ADP-ribose) polymerase is required for UV mediated apoptosis

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SUMMARY

The role of the N-terminal cleavage product of poly(ADP-ribose) polymerase (PARP) on UV mediated apoptosis was investigated in cultured HeLa cells. Ultrastructural analysis of cells expressing caspase-resistant PARP (PARPD214A) revealed the typical features of necrosis following UV treatment. However, cells co-expressing PARPD214A with the N-terminal fragment of PARP containing the DNA-binding domain underwent apoptosis instead of necrosis. In this study, we have demonstrated that the DNA-binding activity of the N-terminal fragment of PARP is important for the execution of apoptosis. Point mutations were introduced in the DNA-binding sites of the N-terminal fragment. Cells co-expressing PARPD214A with the mutated N-terminal fragments neither stimulated apoptosis nor prevented necrosis in response to UV irradiation. The present study proposes that the DNA-binding activity of the N-terminal fragment of PARP in UV treated cells prevents cellular ATP depletion, a mechanism by which necrotic cell death is triggered.

Key words: Cell death, Apoptosis, Necrosis, Poly(ADP-ribose) polymerase, ATP depletion

INTRODUCTION

Two fundamental types of cell death with distinguishing morphological and biochemical features have been previously described. Necrosis has been considered as a passive cell death that occurs under non-physiological conditions, while apoptosis has been regarded as an active cellular suicide that is tightly regulated by genetic programs (Ellis et al., 1991). Nevertheless, these two different types of cell death seem to share some common signal paths. For example, hypoxia was reported to induce both types of cell death, and anti-apoptotic proteins, Bcl-2 and Bcl-XL, suppressed hypoxia-induced necrosis as well as apoptosis (Shimizu et al., 1996). Recent studies have shown that the activation of caspase is required for the execution of apoptosis, while without caspase activation cells die from necrosis (Hirsch et al., 1997; Lemaire et al., 1998). These studies have suggested that caspase activation is a key control element that determines the choice between the two type of cell death.

Poly(ADP-ribose) polymerase (PARP), an abundant nuclear enzyme which is activated by DNA interruptions, binds rapidly to DNA breaks through its N-terminal DNA-binding domain and utilizes NAD+ to synthesize poly(ADP-ribose) on a variety of nuclear proteins such as proteins involved in DNA metabolism including DNA polymerases (Yoshihara et al., 1985; Ohashi et al., 1986), topoisomerases (Ferro and Olivera, 1984; Darby et al., 1985), DNA dependent protein kinase (Ruscetti et al., 1998), p53 (Wesierska-Gadek et al., 1996) and PARP itself (de Murcia and de Murcia, 1994); or nuclear structural proteins including histones (Huletsky et al., 1985; Yoon et al., 1996) and lamins (Pedraza-Reyes and Alvarez-Gonzalez, 1990). PARP is known to be cleaved by a caspase during apoptosis. The cleavage of PARP yields a 25 kDa N-terminal fragment containing two zinc fingers and an 89 kDa C-terminal fragment containing the automodification domain and the NAD+ binding domain (Kaufmann et al., 1993). Several studies have attempted to interpret the biological meaning of PARP cleavage during apoptosis. Oliver et al. (1998) reported delayed apoptosis in cells expressing an uncleavable PARP mutant following CD95 stimulation. More direct evidence for the involvement of PARP cleavage in apoptosis was provided by Smulson et al. (1998) who demonstrated the irreversible binding of the N-terminal fragment of PARP with DNA from apoptotic mouse lymphoma cells.

It was proposed that the intracellular ATP level influences the mode of cell death and that an adequate level of ATP is required for the progression of apoptosis (Eguchi et al., 1997; Leist et al., 1997). The intracellular level of ATP is directly affected by the catalytic activity of PARP; thus, the apoptotic process, which is an energy requiring process, may well be influenced by PARP activity. The positive roles of PARP cleavage in the maintenance of cellular ATP and the execution of apoptosis were shown by Herceg and Wang (1999) using cells expressing a caspase-resistant PARP mutant in a PARP-null background. Expression of the N-terminal fragment of PARP containing the DNA-binding domain was reported to be a trans-dominant inhibitor of the resident PARP in mammalian
cells (Kupper et al., 1990; Molinete et al., 1993; Schreiber et al., 1995). In the present study, we examined the hypothesis that the N-terminal fragment of PARP stimulates apoptosis but prevents necrosis by contributing to the maintenance of cellular energy during the course of cell death.

**MATERIALS AND METHODS**

**Reagents and antibodies**
Radioactive chemicals including [3H]NAD+ (0.5 mCi/m mole) and [35S]methionine (1,000 Ci/m mole) were obtained from AmershamPharmacia Biotech (UK). Anti-green fluorescent protein (GFP) monoclonal antibody was from Clontech (USA).

**Vectors, mutagenesis and transfection**
The DNA fragments encoding full length PARP and PARP mutants were cloned in-frame to pEGFP-C1 vector (Clontech, USA) to produce GFP-fusion proteins. Site-directed mutagenesis was performed using the procedures of Quick Change Site-directed Mutagenesis (Stratagene, USA). For the active transport of GFP-fused PARP and its mutants into the nuclei, the nuclear localization signal of SV40 large T-antigen (Kalderon et al., 1984) was inserted between GFP and the N terminus of PARP using oligomers of 5’GGAAATTTC- AAGCTTTCGCAAGAAGAGCAGAAATCGACGCC3’ and 5’GGCCGTGCAATTTGCTTTCCTTTCGGAAGCTTGAA TT-CC3’. The vector constructs encoding GFP-fused PARP and its mutants were transfected into HeLa cells by employing FuGENE6 transfection reagent (Boehringer Mannheim, Germany). Expression of GFP was evaluated by fluorescence microscopic observation (Ex./Em.: 490 nm/510 nm).

**In vitro transcription/translation and PARP cleavage**
Recombinant PARP proteins were produced using the T7 Quick Coupled Transcription/Translation System (Promega, USA). The plasmids used were pGEM-7zf(+)-PARP and pGEM-7zf(+)-PARPD214A. The cleavage of in vitro translated [35S]PARP or [35S]PARPD214A by caspase-3 was examined as described (Tewari et al., 1995). [35S]PARP or [35S]PARPD214A was incubated with Escherichia coli BL21(DE3) cell lysate containing human caspase-3 at 30°C for 2 hours in the presence or absence of 50 μM Ac-DEVD-CHO (Bachem, Switzerland). Proteolytic cleavage of 35S-labeled PARP and PARPD214A was analyzed by SDS-PAGE and autoradiography. PARP and PARPD214A were fused in-frame to the N-terminus of GFP and the cleavage of GFP-fused PARP and PARPD214A expressed in HeLa cells was examined by western blot analysis using monoclonal antibody against GFP.

**Electron microscopy**
Cells expressing GFP-fusion proteins were collected by FACS, treated with 100 J/m2 UV, and the cleavage of GFP-PARP was analyzed by western blot analysis using monoclonal antibody against GFP following UV irradiation. While GFP-PARP was cleaved into a C-terminal 89 kDa, ~50 kDa, ~40 kDa and ~35 kDa of molecular mass. PARPD214A was not cleaved by caspase-3 (Fig. 1B). To determine the mode of cell death mediated by UV in cells expressing PARPD214A, HeLa cells expressing GFP-PARP D214A was examined by western blot analysis using monoclonal antibody against GFP following UV irradiation. While GFP-PARP was cleaved into a C-terminal 89 kDa, ~50 kDa, ~40 kDa and ~35 kDa of molecular mass. PARPD214A was fused in-frame to GFP was transfected into HeLa cells. Cells expressing GFP-fusion proteins were collected by FACS, treated with 100 J/m2 UV, and the cleavage of GFP-PARP D214A in transfected HeLa cells was analyzed by western blot analysis using monoclonal antibody against GFP following UV irradiation. While GFP-PARP was cleaved into a C-terminal 89 kDa fragment and an N-terminal 56 kDa fragment containing GFP (an N-terminal 25 kDa fragment fused to ~30 kDa GFP) during apoptosis, GFP-PARP D214A was cleaved into smaller fragments in response to UV irradiation. At least four degradation products of GFP-PARP D214A were detected at 70 kDa, ~50 kDa, ~40 kDa and ~35 kDa of molecular mass. Data also indicate that the cleavage of GFP-PARP D214A was mediated by caspase-3 since the cleavage was completely inhibited by Ac-DEVD-CHO, a specific caspase-3 inhibitor (Fig. 1C). The degradation of GFP-PARP D214A coincides with the PARP degradation observed during necrotic cell death in HL60 cells (Shah et al., 1996). Consequently, it was necessary to determine the mode of cell death mediated by UV in cells expressing PARPD214A.

**Type of cell death induced by UV in HeLa cells expressing PARPD214A**
The type of cell death induced by 100 J/m2 UV irradiation in HeLa cells was documented using FACS Cell Quest software (Beckton-Dickinson, USA) after 10 μM propidium iodine (PI) staining for 30 minutes as described (Shimizu et al., 1996).

**RESULTS**

**Expression of caspase-resistant PARPD214A in HeLa cells**
The roles of PARP cleavage in apoptosis were examined using caspase-resistant PARP in which Asp214 at P1 position was substituted with Ala (PARPD214A) (Fig. 1A). Fig. 1B shows that in vitro translated [35S]PARP was cleaved by the recombinant caspase-3, whereas in vitro translated [35S]PARPD214A was not cleaved by caspase-3 (Fig. 1B). To examine the cleavage of PARP and PARPD214A in UV treated HeLa cells, vector constructs in which PARP or PARPD214A was fused in-frame to GFP were transfected into HeLa cells. Cells expressing GFP-fusion proteins were collected by FACS, treated with 100 J/m2 UV, and the cleavage of GFP-PARP and GFP-PARP D214A in transfected HeLa cells was analyzed by western blot analysis using monoclonal antibody against GFP following UV irradiation. While GFP-PARP was cleaved into a C-terminal 89 kDa fragment and an N-terminal 56 kDa fragment containing GFP (an N-terminal 25 kDa fragment fused to ~30 kDa GFP) during apoptosis, GFP-PARP D214A was cleaved into smaller fragments in response to UV irradiation. At least four degradation products of GFP-PARP D214A were detected at 70 kDa, ~50 kDa, ~40 kDa and ~35 kDa of molecular mass. Data also indicate that the cleavage of GFP-PARP D214A was mediated by caspase-3 since the cleavage was completely inhibited by Ac-DEVD-CHO, a specific caspase-3 inhibitor (Fig. 1C). The degradation of GFP-PARP D214A coincides with the PARP degradation observed during necrotic cell death in HL60 cells (Shah et al., 1996). Consequently, it was necessary to determine the mode of cell death mediated by UV in cells expressing PARPD214A.

**Measurements of cellular NAD+ and ATP levels**
Cells expressing GFP-fusion proteins were collected by FACS and incubated for 12 hours. After induction of apoptosis by 100 J/m2 UV irradiation, NAD+ content in the cells (105) was determined by the method previously described (Jacobson and Jacobson, 1976). Cellular ATP level was measured using Bioluminescent Somatic Cell Detection Kit (Sigma, USA).
nucleus, chromatin condensation, and membrane blebbing (Fig. 2A, left). In contrast, the cell death mediated by UV in cells expressing GFP-PARP<sup>D214A</sup> was characterized as necrosis based on the enlarged cell size, swelling of subcellular organelles, disruption of the cytoplasmic membrane, and large cytosolic vacuoles with intact nucleus (Fig. 2A, middle). However, co-expression of GFP-PARP<sup>D214A</sup> with the GFP-fused N-terminal fragment of PARP, GFP-N214, resulted in apoptosis instead of necrosis following UV treatment, suggesting that the N-terminal fragment of PARP stimulates UV-induced apoptosis but prevents necrosis (Fig. 2A, right).

DNA-binding activity of the N-terminal fragment of PARP stimulates apoptosis but prevents necrosis

In an effort to gain an insight into the biological function of the N-terminal fragment of PARP in apoptosis, point mutations were introduced into zinc fingers of the N-terminal fragment. Cells were transfected with a mutated N-terminal fragment in which the basic Arg138 in the putative α helix of the second zinc finger was altered (GFP-N214<sup>R138I</sup>), or a double mutant in which both Cys25 and Cys125 involved in zinc finger coordinations were replaced by Gly (GFP-N214<sup>C21G+C125G</sup>) (Fig. 3A). To confirm if the N-terminal fragment and the mutated N-terminal fragments were equally expressed in transfected HeLa cells, equal amounts of cell extracts were subjected to immunoblot analysis using monoclonal antibody against GFP. The immunoblot analysis revealed that GFP-fused N-terminal fragment and its mutated derivatives were equally expressed in transfected cells (Fig. 3B).

Our data demonstrate that the DNA-binding ability of the N-terminal fragment of PARP is important for the stimulation of UV-induced apoptosis. The expression of the N-terminal fragment stimulated UV-induced apoptosis. However, the stimulation of necrosis by the N-terminal fragment was directly related to its DNA-binding ability. Data in Fig. 3C demonstrate that the expression of GFP-N214<sup>R138I</sup> or GFP-N214<sup>C21G+C125G</sup> in HeLa cells failed to stimulate UV-induced apoptosis. Furthermore, these mutated N-terminal fragments that lack the DNA-binding ability also failed to prevent necrosis following UV irradiation in cells expressing uncleavable PARP<sup>D214A</sup> (Fig. 3D).
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DNA-binding activity of the N-terminal fragment of PARP inhibits the catalytic PARP activity in UV treated HeLa cells

Expression of GFP-PARPDD214A resulted in a marked elevation of the cellular PARP activity in permeabilized HeLa cells. Trans-dominant inhibition of the cellular PARP activity by the N-terminal fragment of PARP was seen in transfected cells overexpressing GFP-N214. However, the mutated N-terminal fragments that lack the DNA-binding ability (GFP-N214R138I and GFP-N214C25G+C125G) failed to inhibit the cellular PARP activity in cells expressing GFP-PARPDD214A (Table 1).

DNA-binding activity of the N-terminal fragment of PARP contributes to the maintenance of cellular energy

The hypothesis that the prevention of necrosis by the expression of the N-terminal fragment of PARP in transfected cells expressing PARPDD214A was attributable to the maintenance of the basal level of the intracellular energy was examined in Fig. 4. The intracellular levels of NAD⁺ and ATP in cells expressing

### Table 1. PARP activity in UV treated HeLa cells expressing PARP mutant

<table>
<thead>
<tr>
<th>Transfection</th>
<th>³H transfer from [³H]NAD⁺ (³H dpm x10³)⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEGFP-C1</td>
<td>2848±59</td>
</tr>
<tr>
<td>pEGFP-C1-N214</td>
<td>1131±64</td>
</tr>
<tr>
<td>pEGFP-C1-N214R138I</td>
<td>2257±188</td>
</tr>
<tr>
<td>pEGFP-C1-N214C25G+C125G</td>
<td>2712±210</td>
</tr>
<tr>
<td>pEGFP-C1-PARPDD214A</td>
<td>4259±142</td>
</tr>
<tr>
<td>pEGFP-C1-PARPDD214A+pEGFP-C1-N214</td>
<td>2292±91</td>
</tr>
<tr>
<td>pEGFP-C1-PARPDD214A+pEGFP-C1-N214R138I</td>
<td>3929±201</td>
</tr>
</tbody>
</table>

*The catalytic activities of PARP in transfected cells expressing PARP mutants were assessed in a cell-free system. Cells were irradiated with 100 J/m² of UV, incubated for 6 hours, and permeabilized as described in Materials and Methods. Permeabilized cells (10⁵) were incubated in a reaction buffer containing 0.3 μCi/ml of [³H]NAD⁺ at 30°C for 30 minutes. Acid-precipitable materials were collected and the transfer of [³H] from [³H]NAD⁺ was radiometrically assessed in a liquid scintillation counter. The values are expressed as the mean and standard deviation for three experiments.
Stimulation of apoptosis by N-terminal fragment of PARP

PARP<sub>D214A</sub> were reduced to 25% and 10% of untreated control values, respectively, by UV irradiation. Data also show that co-expression of GFP-PARP<sub>D214A</sub> with GFP-N214 significantly elevated both NAD<sup>+</sup> and ATP levels in UV treated HeLa cells. However, the expression of the mutated N-terminal fragments that lack the DNA-binding ability failed to replenish cellular energy, suggesting that the DNA-binding ability is crucial for the biochemical function of the N-terminal fragment of PARP in the maintenance of the basal level of the intracellular energy during the course of apoptosis.

**DISCUSSION**

There have been studies suggesting that the intracellular ATP concentration determines the type of cell death, either necrosis or apoptosis (Leist et al., 1997; Eguchi et al., 1997). These studies have proposed that apoptosis is an energy requiring process, therefore, cells may not commit apoptotic suicide if the cellular ATP is exhausted. In order for the progression of apoptosis, there must exist a biochemical mechanism by which a certain level of cellular ATP is maintained during apoptosis.

Activated PARP reduces the cellular level of NAD<sup>+</sup> and results in a depletion of the cellular energy source. Palomba et al. (1996) have reported that the treatment of human leukemia cells with a chemical inhibitor of PARP, 3-aminobenzamide (3AB), prevented necrosis but triggered apoptosis. This observation implies that the maintenance of an adequate level of NAD<sup>+</sup> by inhibiting PARP activity is important for the progression of apoptosis and for the prevention of necrosis.
The depletion of NAD⁺ by PARP activation causes exhaustion of the cellular ATP pool, failure of oxidative respiration, and a change in mitochondrial and cellular permeability, resulting in necrotic cell death (Kroemer et al., 1997). The stable presence of the four degradation products of PARP in cells expressing PARPD214A, a mutant PARP that is resistant to caspase-3, in response to UV irradiation suggest that the degradation of GFP-fused PARPD214A in transfected cells is likely the result of the specific proteolytic activity (Fig. 1C). The generation of small degradation products in apoptotic cells expressing GFP-PARPΔ214A was coincident with the necrosis-specific degradation of PARP observed in HL60 cells (Shah et al., 1996). Further studies are required in order to identify the protease(s) uniquely activated in cells expressing PARPD214A.

PARP cleavage during apoptosis was known to prevent the cellular energy depletion during apoptosis by inactivating the cellular PARP activity (Kaufmann et al., 1993; Simbulan-Rosenthal et al., 1998). In support of this, cells expressing PARPD214A underwent necrosis in response to UV irradiation (Fig. 2). The cellular PARP activity in UV treated HeLa cells expressing the transfected uncleavable PARPΔ214A was much higher than in UV treated control cells expressing GFP only (Table 1).

Expression of the N-terminal fragment of PARP containing the DNA-binding domain has been known to inhibit poly(ADP-ribosylation) in transfected cells. The trans-dominant inhibition of the resident PARP by the transfected N-terminal fragment was reported in cultured mammalian cells (Kupper et al., 1990; Molinete et al., 1993; Schreiber et al., 1995). Works by Molinete et al. (1993) have suggested a critical function for the DNA-binding capacity of the N-terminal fragment of PARP on the trans-dominant inhibition of the resident PARP activity. They found that the DNA-binding ability was greatly altered if the metal binding ability of the second zinc finger was affected by point mutations. Our data in Fig. 3 imply that the DNA-binding ability of the N-terminal fragment of PARP is a determinant of the type of cell death – necrosis or apoptosis (Fig. 3). The trans-dominant inhibition of the catalytic activity of the resident PARP by the N-terminal fragment might explain the positive role of the N-terminal fragment on apoptosis. The mutated N-terminal fragments of PARP, PAPRΔ138I and PARPΔC25G+C215G, failed to inhibit the catalytic activity of the cellular PARP (Table 1). Both mutated N-terminal fragments also failed to maintain the minimal cellular energy required for the progression of apoptosis in UV treated cells expressing PARPD214A (Fig. 4).

Apoptosis and necrosis are considered conceptually and morphologically distinct forms of cell death. However, the two apparently opposite forms of cell death seem to share common initiation pathways since both forms of cell death can be elicited by the same stimuli, according to their intensities (Bonfoco et al., 1995; Hampton and Orrenius, 1997). There are a number of studies suggesting that the availability of caspase is the determinant in the choice between apoptosis and necrosis (Lemaire et al., 1998; Hirsch et al., 1997; Verschuren et al., 1998). The PARP cleavage catalyzed by caspase-3 during apoptosis has been proposed to inactivate PARP and prevent depletion of NAD and ATP, which is required for later events in apoptosis (Boulares et al., 1999). The present study proposes that the DNA-binding activity of the N-terminal fragment of PARP inactivates the cellular PARP activity and promotes the execution of apoptosis but prevents necrosis, by contributing to...
the maintenance of the minimal intracellular energy. PARP is catalytically activated by binding to DNA breaks, therefore, the DNA-binding activity of the N-terminal fragment may inhibit the cellular PARP activity by competing for the DNA breaks required for the activation of the cellular PARP. The DNA-binding activity of the N-terminal fragment early during the course of apoptosis may also inhibit DNA repair by limiting the access of DNA repair enzymes to the sites of DNA damage. The inhibition of DNA repair by the inactivation of the cellular PARP may accelerate the progression of apoptosis. The transdominant inhibition of cellular PARP activity by the N-terminal fragment may also activate some of the regulatory proteins involved in the execution of apoptosis through deribosylation.

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REFERENCES


