Characterization of the necrotic cleavage of poly(ADP-ribose) polymerase (PARP-1): implication of lysosomal proteases

S Gobeil^{1,2}, CC Boucher^{1,2}, D Nadeau¹ and GG Poirier^{*1}

¹ Health and Environment Unit, Laval University Medical Research Center, CHUQ, and Faculty of Medicine, Laval University, Québec, Canada.

² Both authors contributed equally

* Corresponding author: GG Poirier, Unité Santé et Environnement, Local 9700, Centre de Recherche du CHUL, Ste-Foy, Québec, Canada, G1V 4G2.
Tel: (418)-654-2267; Fax: (418)-654-2159;
E-mail: guy.poirier@crchul.ulaval.ca

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Abstract

The poly(ADP-ribose) polymerase (PARP-1), a 113 kDa nuclear enzyme, is cleaved in fragments of 89 and 24 kDa during apoptosis. This cleavage has become a useful hallmark of apoptosis and has been shown to be done by DEVD-ase caspases, a family of proteases activated during apoptosis. Interestingly, PARP-1 is also processed during necrosis but a major fragment of 50 kDa is observed. This event is not inhibited by zVAD-fmk, a broad spectrum caspase inhibitor, suggesting that these proteases are not implicated in the necrotic cleavage of PARP-1. Since lysosomes release their content into the cytosol during necrosis, the proteases liberated could produce the cleavage of PARP-1. We therefore isolated lysosomal rich-fractions from Jurkat T cells. Our results reveal that the *in vitro* lysosomal proteolytic cleavage of affinity purified bovine PARP-1 is composed of fragments corresponding, in apparent molecular weight and function, to those found in Jurkat T cells treated with necrotic inducers like 0.1% H₂O₂, 10% EtOH or 100 μ M HgCl₂. Moreover, we used purified lysosomal proteases (cathepsins B, D and G) in an *in vitro* cleavage assay and found that cathepsins B and G cleaved PARP-1 in fragments also found with the lysosomal rich-fractions. These findings suggest that the necrotic cleavage of PARP-1 is caused in part or in totality by lysosomal proteases released during necrosis. *Cell Death and Differentiation* (2001) 8, 588–594.

Keywords: PARP-1; necrosis; lysosomal proteases; Jurkat T cells

Abbreviations: β -NAG, β -N-acetyl-D-glucosaminidase; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid buffer; DEVD-pNa, acetyl-asp-glu-val-asp-p-Nitroanilide; EtOH, ethanol; HgCl₂, mercuric chloride; H₂O₂, hydrogen peroxide; NLS, nuclear localization signal; PARP-1, poly(ADP-ribose) polymerase-1; PBS, phosphate

buffer saline; SDS, sodium dodecylsulfate; Stau, staurosporine; UBF, human RNA polymerase I upstream binding factor; Vp-16, etoposide; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

Introduction

Poly(ADP-ribose) polymerase (PARP-1) is a nuclear enzyme that catalyzes the transfer of ADP-ribose polymers onto itself and other nuclear proteins in response to DNA strand breaks (Figure 1).¹ During apoptosis, cleavage of PARP-1 in fragments of 89 and 24 kDa has become a useful hallmark of this type of cell death.^{2,3} This cleavage is well studied and is generated by the caspases 3 and 7, proteases activated during apoptosis.^{4–6}

Recently, Shah and associates (1996) have shown that PARP-1 is processed to give a major fragment of 50 kDa during cytochalasin B induced necrosis in HL-60 human promyelocytic leukemia cells.⁷ Moreover, Casiano and associates (1998) have also shown in human leukemia Jurkat T cells the differential cleavage of some nuclear proteins like PARP-1, topoisomerase I and UBF with the necrotic inducers EtOH, H₂O₂, HgCl₂ and heat treatment as compared to their apoptotic cleavage pattern.⁸ A major fragment of 50 kDa was also found in the case of PARP-1.

The necrotic signature of PARP-1 is not inhibited by zVAD-fmk, a broad spectrum caspases inhibitor, suggesting that the known apoptotic proteases may not be implicated in this type of cell death.⁸ No proteases have been clearly implicated in the necrotic degradation of PARP-1, however it has been shown that during the course of necrosis, the content of lysosomes is released into the cytosol.⁹ This event might allow the proteases liberated to access to cytosolic and nuclear proteins like PARP-1 and process them.

In an attempt to verify the implication of lysosomal proteases in the necrotic cleavage of PARP-1, we isolated lysosomal rich-fractions from Jurkat T cells and performed an *in vitro* cleavage assay with affinity purified bovine PARP-1. We also characterized the *in vitro* and *in vivo* necrotic cleavage of PARP-1 with the Western and Activity Western blot techniques. The Activity Western blot is a reliable technique to map the C-terminal portion of PARP-1 and the integrity of the catalytic domain.¹⁰

In this study, we show that the fragments obtained with *in vitro* assays containing Jurkat T cells lysosomal-rich fractions and affinity purified bovine PARP-1 are similar, in apparent molecular weight and function, to those found in

Figure 1 Schematic representation of the structure of poly(ADP-ribose) polymerase (PARP-1). PARP-1, a 113 kDa DNA repair enzyme, is a protein divided in three functional domains. These domains were determined by partial proteolysis with papain and α -chymotrypsin. The DNA-binding domain contains two zinc fingers that bind to DNA single and double-strand breaks. In response to DNA damage, the catalytic domain synthesizes polymers of ADP-ribose which can be accepted by PARP-1 itself, principally on the automodification domain, or by other proteins. During apoptosis, PARP-1 is cleaved in its NLS by caspases to give fragments of 89 and 24 kDa. The polymers can be monitored by the mouse monoclonal 10H antibody

Jurkat T cells treated with necrotic inducers like H_2O_2 (0.1%), EtOH (10%), $HgCl_2$ (100 μ M). With the *in vitro* cathepsins B and G digestion (lysosomal proteases) PARP-1 fragments observed were comparable with those obtained with the lysosomal-rich fractions. These findings suggest that the necrotic cleavage of PARP-1 is produced in part or in totality by lysosomal proteases released during necrosis.

Results

No detectable caspase activation following necrotic treatment of Jurkat T cells

Jurkat T cells were treated with apoptotic and necrotic inducers for 3, 6 or 12 h at a density of 500 000 cells/ml. The apoptotic treatments, 150 μ M Vp-16 and 150 nM staurosporine (Stau), did not provoke any major intake of trypan blue before 12 h. In contrast, all the necrotic treatments induced a rapid loss of membrane integrity (within 3 h). The most efficient necrosis inducer was H_2O_2 at a concentration of 0.1% which provoke trypan blue uptake in more than 90% of the cells after only 3 h of treatment (data not shown).

In order to analyze caspase activation, we performed DEVD-ase assays on apoptotic and necrotic extracts. As expected, the Stau and Vp-16 treatments activated DEVD-ase caspases (Figure 2). In contrast, none of the necrotic treatments caused a detectable caspase activation at the times tested.

Different cleavage pattern of PARP-1 in Jurkat T cells following apoptotic and necrotic treatments

We used the combination of Western and Activity Western blots in order to map more precisely the cleavage of PARP-1

Figure 2 DEVD-ase activity after apoptotic and necrotic treatments of Jurkat cells. DEVD-ase activity of the apoptotic and necrotic treatments as monitored by cleavage of DEVD-pNa substrate. Twenty μ g of cellular extracts were incubated with 50 μ M of DEVD-pNa and the appearance of pNa was monitored at 405 nM. Results are from three different experiments, bars represent S.E.M

during necrosis. As shown in Figure 3, a 6 h treatment with Vp-16 or Stau provoked the usual PARP-1 apoptotic signature consisting in the appearance of an 89 kDa fragment.³

As expected, the necrotic inducers provoked the appearance of multiple bands (Figure 3). The main active fragment, monitored by Activity Western blot, was at 55 kDa (Figure 3A) which corresponds to the entire catalytic domain of the protein (Figure 1).¹¹ With the C-2-10 antibody which maps to the N-terminal part of PARP-1, the major fragment obtained was at 62 kDa (Figure 3B). The EtOH treatment also produced enzymatically active fragments of 42 and 72 kDa suggesting an intact C-terminal

Figure 3 Western and Activity Western blot of PARP-1 after apoptotic and necrotic treatments of Jurkat T cells. Jurkat T cells were treated with apoptotic (150 nM Stau; 150 μ M Vp-16) and necrotic (0.1% H_2O_2 ; 10% EtOH; 100 μ M $HgCl_2$) inducers for 6 h. Following treatments, 20 μ g of proteins were resolved on a 10% SDS-PAGE and analyzed by immunoblotting for PARP-1. (A) Activity Western blot of PARP-1 with the monoclonal antibody 10H which detects polymer of ADP-ribose. (B) Western blot of PARP-1 with the monoclonal antibody C-2-10

catalytic activity since the Asp-992 which is present at the end of the C-terminal is necessary for the catalytic function.¹² A 42 kDa fragment can also be seen with the other necrotic inducers but in a weaker manner (Figure 3A). It is also possible to notice with the necrotic inducers some fragments migrating around 89 kDa as observed by Shah and associates (1996) and Casiano and associates (1998).^{7,8}

In vitro cleavage of PARP-1 by lysosomal-rich extract and purified cathepsins B and G proteases

As shown in Figure 4, affinity purified bovine PARP-1 was not contaminated with degradation fragments. It is important to notice that the PARP-1 bovine sequence shares 98% homology with the human sequence and that the major cleavage sites are conserved.¹³

Affinity purified bovine PARP-1 incubated with human lysosomal-rich extracts was cleaved to give two major active fragments of 55 and 42 kDa (Figure 4A). The non-active fragments obtained were at 74 and 62 kDa (Figure 4B). We also noticed the presence of an 89 kDa fragment in Activity and Western blots.

The processing of PARP-1 by lysosomal purified proteases cathepsin B and D did give significant results at shorter times as compared with cathepsin G (Figure 4A and C). The main active PARP-1 fragments obtained

Figure 4 Western Blot and Activity Western blot of *in vitro* assays of PARP-1 cleavage. Conditions of the *in vitro* assay: 100 ng of purified bovine PARP-1 were incubated in a final volume of 100 μ l with lysosomal rich-fractions (80 μ l), cathepsin B, D or G (3.5 mU) for 1 h at 37°C. Samples were then diluted in reducing loading buffer and processed for Western and Activity Western blots as described in Materials and Methods. (A) Activity Western blot of *in vitro* PARP-1 cleavage. (B) Western blot of *in vitro* PARP-1 cleavage with C-2-10 antibody. (C) Activity Western blot of PARP-1 cleaved with cathepsins B and D

with cathepsins B and G were at 55 and 42 kDa (Figure 4A and C) whereas fragments of 74 and 62 kDa were detected by Western blot (Figure 4B). The cleavage pattern with cathepsins B and G was almost similar to the one obtained with lysosomal rich-extracts except for the 89 kDa fragment which was not generated in the case of cathepsin G processing (Figure 4B).

Sequencing of major PARP-1 fragments obtained by cathepsins B and G processing

The major fragments recovered by cathepsins B and G proteolysis were sequenced on a N-terminal protein sequencer (473A protein sequencer, Applied Biosystems) by automatic Edman degradation. The 55 and 42 kDa were analyzed as described in Materials and Methods. The sequences obtained are localized in very close proximity to the α -chymotrypsin sensitive sites (Figure 5).

Discussion

The first description of PARP-1 necrotic cleavage came from our laboratory in 1996 and described a different proteolysis of PARP-1 during necrosis induced by treatment of HL-60 cells with cytochalasin B as compared to apoptosis.⁷ No specific protease activity was suggested to be responsible for that necrotic cleavage and more work was necessary. In 1998, Casiano and associates published a study on different nuclear proteins cleaved during necrosis including PARP-1 cleavage but no documentation was given as the source of the necrotic proteases involved.⁸ In this paper, we report the

possible implication of lysosomal enzymes in the necrotic cleavage of PARP-1.

As expected, DEVD-ase caspases are activated during apoptosis induced by Vp-16 and Stau but, during necrosis induced by EtOH, HgCl₂ and H₂O₂, we failed to observe any activation of these caspases. The observations of Casciano and associates (1998) that the broad spectrum caspases inhibitor zVAD-fmk inhibit neither necrosis nor the necrotic cleavage of PARP-1 are in agreement with our findings.⁸ Taken together, these results lead to the conclusion that during necrosis, DEVD-ase caspases are not implicated in the execution phase nor in the degradation of PARP-1 and that other protease(s) may be involved in these functions.

Lysosomal proteases might be ideal candidates for the proteolytic degradation of proteins during necrosis since they are released from lysosomes into the cytosol during this type of cell death. The proteases liberated can therefore process the cytoplasmic and nuclear proteins. In this study, we show that human lysosomal-rich fractions contain an activity that can process purified bovine PARP-1 in a signature close to the one obtained *in vivo* with necrotic treatments.

Figure 5 Sequence of PARP-1 major fragments obtained by cathepsins B and G proteolysis. Two μ g of purified bovine PARP-1 were incubated with 70 mU of cathepsin G and 40 mU of cathepsin B for 2 h. Products of proteolysis were migrated on a 10% SDS-PAGE and transferred onto a PVDF membrane. After Coomassie Blue staining major bands were analyzed on a 473A protein sequencer (Applied Biosystems)

The *in vivo* necrotic signature observed with Western and Activity Western blot includes two major PARP-1 fragments: a C-terminal 55 kDa active and a N-terminal 62 kDa inactive fragment. With a cytochalasin B necrotic treatment of HL-60 cells, Shah and associates (1996) obtained a major PARP-1 fragment of 50 kDa.⁷ The mapping was done with the monoclonal antibody C-2-10, meaning that the fragment obtained must be located in the N-terminal part of the protein. With other necrotic inducers and the C-2-10 mapping, we failed to observe any fragment around 50 kDa in the Jurkat T cells. The only band recovered near this molecular weight with C-2-10 was at 62 kDa. The differences between these results are difficult to explain but may arise from the type of necrotic inducer and/or the cell line used.

Moreover, with human autoimmune antibodies, Casiano *et al*, (1998) also demonstrated the appearance of a major PARP-1 fragment around 50 kDa in necrotic Jurkat T cells.⁸ They also found a band at 62 kDa with EtOH treatment. As they used polyclonal antibodies, they did not determinate the exact mapping of their fragments but their findings are apparently in agreement with our data as we also found a C-terminal 55 kDa and a N-terminal 62 kDa fragment after necrotic treatments of Jurkat T cells. The fact that they obtained a 62 kDa fragment with the EtOH treatment may be related to a possible destruction or denaturation of the epitopes (recognized by their polyclonal antibodies) by the other necrotic inducers.

In an attempt to better characterize the activity implicated in the proteolysis of PARP-1 in Jurkat T cells, we postulate that a cathepsin B or a cathepsin B-like activity is implicated in the lysosomal degradation of PARP-1 as the degradation fragments obtained with cathepsin B were similar to the pattern observed with lysosomal-rich fractions. Indeed, no 89 kDa fragment was produced with purified cathepsin B, D or G. However, we cannot exclude a more efficient degradation of this fragment by the purified cathepsins B or D.

As reported before, the partial digestion of human PARP-1 by α -chymotrypsin leads to the formation of fragments at 40, 54, 62 and 76 kDa.¹⁴⁻¹⁶ We obtained the same fragments, in length and orientation, with the cathepsins B and G proteolytic cleavage of bovine PARP-1.

Then, in order to compare the cleavage sites of cathepsins B and G to those of α -chymotrypsin, we sequenced the major fragments obtained by cathepsins B and G proteolysis. The fragments sequenced have an apparent molecular weight of 55 and 42 kDa. The bovine PARP-1 55 kDa cleavage sequence is similar (for cathepsin G and differ from one amino acid with cathepsin B) to the one with α -chymotrypsin (Figure 5). In the case of the 42 kDa fragment, the cleavage sequence differs as chymotrypsin cleaves 2 amino acids after the amino acids selected by cathepsin G (Figure 5). We have also done this experiment with cathepsin B (a lysosomal enzyme) which cleaves within one or three amino acids near the sites cleaved by cathepsin G and α -chymotrypsin. These two proteolytic cleavage sites may represent fragile sites in the PARP-1 structure and one could suggest that these sites

are cleaved during pathological conditions or purification process.

Effectively, during bovine PARP-1 purification, after the DNA cellulose step which keeps proteins interacting with DNA, some degradation fragments at 72 and 62 kDa appear. These fragments correspond to those obtained with the necrotic treatments applied in this study as well as with the proteolytic cleavages by cathepsins B and G and by α -chymotrypsin. This would be in accordance that PARP-1 contains fragile sites which could be challenged by many proteases including lysosomal proteases. We have found a significant amount of cathepsin B in Jurkat cells by Western blot analysis.

In summary, we have shown that lysosomal proteases are implicated in PARP-1 necrotic cleavage. Moreover, purified cathepsins B and G cleave PARP-1 *in vitro* with a pattern similar to the one obtained in *in vivo* necrosis. This would suggest that a cathepsin B-like activity could be a major factor in the *in vivo* cleavage of PARP-1 during necrosis. Further work will be necessary to identify more precisely the proteolytic activity implicated in the degradation of proteins in necrosis and to find out if the necrotic cleavage of PARP-1 is universally conserved. If conserved, the cleavage pattern of PARP-1 could help investigators to better discriminate between necrosis and apoptosis when biochemical markers of cell death are necessary.

Materials and Methods

Unless specified, all materials were from Sigma-Aldrich. HgCl₂ was from Fisher. Peroxidase-conjugated affinitypure goat anti-mouse IgG were from Jackson Immuno Research Laboratories. Molecular weight markers were from BioRad. C-2-10, a monoclonal antibody mapping the N-terminal part of PARP-1 is produced in our laboratory and the 10H monoclonal antibody mapping poly-ADP ribose is a gift from Dr. Alexander Burkle. A monoclonal antibody IM27F directed against cathepsin B was purchased from Pharmingen.

Cell culture and treatments

Human leukemia Jurkat T cells were grown in RPMI 1640 (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum (Wisent), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco-BRL). Typically, 10⁷ cells were treated at concentration of 5 $\times$ 10⁵ cells/ml. For necrosis induction, 100 μ M HgCl₂, 10% EtOH or 0.1% H₂O₂ were used. For apoptosis induction, 150 μ M Vp-16 or 150 nM Staurosporine were used. Cells were harvested at 3, 6 or 12 h after treatment.

Western blotting

After drug treatments, cells were washed once with ice-cold PBS buffer pH 7.4 (140 mM NaCl; 3.7 mM KCl; 2.9 mM KH₂PO₄; 7.7 mM Na₂HPO₄) and resuspended in PBS. Aliquots were taken for protein determination by a modified Bradford assay.¹⁷ The remaining fraction was completed with 1 $\times$ reducing loading buffer (62.5 mM Tris-HCl, pH 6.8; 6 M urea; 10% glycerol; 2% SDS; 5% β -mercaptoethanol freshly added; 0.003% bromophenol blue). Western blots were done according to Duriez *et al*.¹⁸ For Western blot analysis of cathepsin B, 50 000–500 000 Jurkat

cells were used with a dilution of 1/400 of the anti cathepsin B antibody. The molecular weight of the fragments were determined using the AlphaEase™ Stand Alone Software from Alpha Innotech Corporation.

Activity Western blotting

After drug treatments, cells were processed as for Western blot. SDS-PAGE and blotting were done according to Shah and associates (1995) except that activated DNA was omitted in order to better identify cleaved PARP-1 fragments containing the automodification domain of PARP-1.¹⁰ This technique uses the ability of PARP-1 to synthesize poly (ADP-ribose) from NAD to verify if the C-terminal is intact. The polymer can thus be detected immunologically which can be followed with a second antibody detection using a more N-terminal of PARP-1 antibodies such as C-2–10. The molecular weight of the fragments were determined as described in the Western blotting section.

Purification of lysosomes rich-fractions

2×10^8 Jurkat T cells in 2 ml of TES buffer (10 mM triethanolamine; 1 mM EDTA; 0.25 M sucrose) were homogenized with a Dounce homogenizer and a tight-fitting pestle. The homogenate was spun at $250 \times g$ for 10 min at 4°C. The supernatant (0.7 ml) was loaded onto a 15 ml column of isoosmotic Percoll 20% and spun at $20\,000 \times g$ during 90 min at 4°C.¹⁹ Fractions of 0.5 ml were collected from the top according to Blige and associates (1994).²⁰ After sonication, the samples were tested for the lysosomal enzyme β -NAG as described by Affar and associates (1998).²¹

PARP-1 cleavage assays

Affinity purified PARP-1 was incubated with purified cathepsins (B, D or G) or lysosomal rich-fraction at 37°C during the indicated times.²² All reactions were carried out in PBS pH 7.4. Reactions were stopped by addition of an equal amount of 1 $\times$ reducing loading buffer and heated (15 min at 65°C). Samples were then processed for Western and Activity Western blots as for cell samples.

PARP-1 sequencing

Two μ g of affinity purified bovine PARP-1 were incubated with cathepsin B or G for 2 h in the conditions described before. The reaction was stopped by addition of an equal amount of 1 $\times$ reducing loading buffer. The samples were then loaded on a 10% SDS-PAGE minigel. Transfer was carried out 1 h at 100 V on a PVDF membrane (Applied Biosystem) in CAPS buffer. After transfer, the membrane was stained with Coomassie Blue and the bands of interest were cut and subjected to a N-terminal sequencing by automatic Edman degradation performed on an Applied Biosystem model 473A pulsed liquid protein sequencer.

DEVD-ase assay

Treated cells were washed with ice-cold PBS and lysed in ice-cold hypotonic buffer (25 mM HEPES, pH 7.4; 1 mM EGTA; 5 mM MgCl₂; 0.1% Triton X-100; 100 mM PMSF; 2 mM DTT; 1 $\times$ antiproteases cocktail tablet (Boehringer Mannheim)). The homogenates were centrifuged at $15\,000 \times g$ for 10 min at 4°C. DEVD-ase activity was determined in the supernatant using the DEVD-pNA (Biomol) as a substrate.²³

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