Poly(ADP-ribose) synthesis: a marker of cellular stress

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Summary
Poly(ADP-ribosylation) is a post-translational modification of proteins that is implicated in many cellular processes, such as DNA repair, transcription and cell death. The investigation of the synthesis and accumulation of poly(ADP-ribose) (PAR) as a consequence of PARP overactivation in response to DNA damage/stress can provide the direct evidence for an involvement of poly(ADP-ribosylation) in damage response. In this respect, HeLa and HL60 cells have been stressed with different drugs, i.e. etoposide, actinomycin D, bleomycin and H_2O_2, or by growth factor deprivation, and the consequent accumulation of PAR was monitored. The detection of poly(ADP-ribose) within the cell could be a marker of apoptosis and of DNA damage/stress.

Key words: Apoptosis, cellular stress, multiparametric immunofluorescence, DNA damage, poly(ADP-ribosylation).

Introduction

Poly(ADP-ribosylation) is a post-translational modification of proteins playing a multifunctional role in cellular processes, such as DNA repair, transcription and cell death. The best known poly(ADP-ribose) polymerase, PARP-1, is a DNA nick sensor and uses β-NAD^+ to form polymers of ADP-ribose (PAR) onto nuclear protein acceptors. The transient nature of poly(ADP-ribosylation) is ensured by the concerted action of PARP and poly(ADP-ribose) glycohydrolase (PARG), which is responsible for most poly(ADP-ribose) degradation. Polymers of ADP-ribose are rapidly removed by PARG, which catalyzes the hydrolysis of the ribosyl-ribose glycosidic bonds of linear and branched polymers to produce free ADP-ribose residues. The general scheme of poly(ADP-ribosylation) is shown in Figure 1, which details the three different steps of the reaction, i.e. initiation, elongation and degradation (Giansanti *et al.*, 2010).

To monitor *in situ* PAR synthesis, a monoclonal antibody against PAR has been produced in Japan (Kawamitsu *et al.*, 1984). This reagent was widely used in many laboratories and still represents an invaluable tool for monitoring PAR synthesis. One of the first applications of the antibody was made by Alexander Bürkle on living cells treated with alkylating agents (Bürkle *et al.*, 1993; Küpper *et al.*, 1996; van Gool *et al.*, 1997). In this way, it has been possible to follow the synthesis and accumulation of PAR as a consequence of PARP overactivation in response to DNA damage, and to provide the direct evidence for an involvement of poly(ADP-ribosylation) in damage response. However, it has to be noticed that 10H antibody recognizes polymers of ADP-ribose longer than 10 units, thus being unable to detect short oligomers (Kawamitsu *et al.*, 1984).

In collaboration with Alexander Bürkle, we focused on PAR induction under stress conditions able to drive cells to apoptosis. We developed a multiparametric procedure (the so-called "tricolour" procedure) to visualize apoptotic cells by detecting chromatin condensation with Hoechst staining, DNA degradation by TUNEL assay and PAR synthesis by means of a specific monoclonal antibody (Negri *et al.*, 1997; Donzelli *et al.*, 1997). This new protocol allowed the simultaneous analysis of three apoptotic parameters and was further exploited to couple the visualization of PAR synthesis with the detection of the destiny of PARP-1 during apoptosis (Soldani *et al.*, 2001). In

fact, during apoptosis PARP-1 is degraded by caspases which cleave it into two inactive fragments, p24 and p89, the latter being extruded from the nucleus to the cytoplasmic blebs (Soldani *et al.*, 2001; Soldani and Scovassi, 2002).

Materials and Methods

Cell culture and treatments

HeLa cells were grown as monolayer in Dulbecco's modified Eagle's medium (D-MEM). HL60 cells were grown in suspension in RPMI medium. Media were supplemented with 10% foetal bovine serum (FBS), 4 mM glutamine, 2 mM sodium pyruvate, 100 U/mL penicillin and 0.1 mg/mL streptomycin (all reagents were from Celbio). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

For immunofluorescence experiments, either 1×10^5 HeLa or 5×10^5 HL60 cells were seeded on coverslips, and allowed to grow for 24 h. Then, actinomycin D, etoposide, bleomycin and H_2O_2 were added to the cells under the conditions reported in the legend of Figure 2. For long-term culture experiments, HeLa cells were maintained in culture in the same medium for up to 9 days. All the reagents were from Sigma-Aldrich. Untreated cells were processed in parallel.

Immunodetection of poly(ADP-ribose)

The multiparametric tricolour assay was performed according to Donzelli *et al.* (1997) and Negri *et al.* (1997). Briefly, DNA apoptotic fragments were detected using the apoptosis detection system (Promega) based on the TUNEL (TdT-mediated dUTP nick-end labeling) assay, followed by the immunofluorescence detection of PAR by means of the incubation with the monoclonal antibody 10H against PAR, and then with the FITC-conjugated anti-mouse secondary antibody. Finally, chromatin condensation and nuclear morphology were visualized by staining DNA with 0.1 μg/mL Hoechst 33258 (Sigma).

Primary antibody 10H (diluted 1:100) was an Alexis product; anti-mouse secondary antibody (diluted 1:50) was from Jackson Immunores. Measurements of fluorescence intensity were obtained with a Leitz Orthoplan microscope equipped with a 50X objective. The following filter combinations for fluorescence, chromatic beam splitter and emission were used: 450-490 nm, 500 nm and 515 nm for FITC fluorescence; 340-380 nm, 400 nm and 420 nm for Hoechst; 530-580 nm, 560 nm and 630 nm for Texas red.

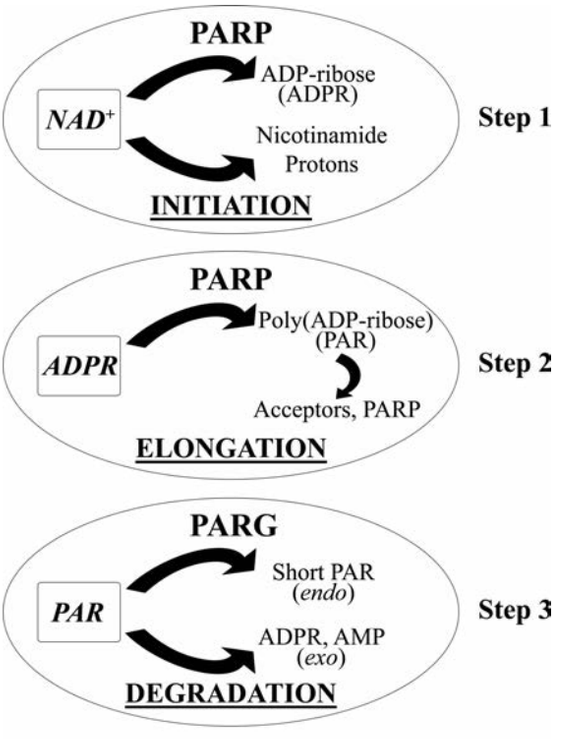

Figure 1. Poly(ADP-ribosylation) reaction. Step 1: Initiation. Step 2: Elongation. Step 3: Degradation. PARP, poly(ADP-ribose) polymerase; ADPR, ADP-ribose; PAR: poly(ADP-ribose); PARG, poly(ADP-ribose) glycohydrolase.

DNA ladder analysis

Control and treated cells (2.5×10^6) were rinsed twice in cold PBS containing 5 mM EDTA. Genomic DNA was extracted and analyzed by agarose gel electrophoresis as reported in Donzelli *et al.* (1999).

Western blot

Cells were washed twice with ice-cold PBS and resuspended at the concentration of 10^7/mL in a denaturing buffer, according to Donzelli *et al.* (1999). After sonication on ice for 30 s (60 W) and heating for 15 min at 65°C, samples were electrophoresed as reported in Soldani *et al.* (2001) and transferred onto nitrocellulose membrane. PARP-1 expression was monitored with the monoclonal antibody C2-10 (Alexis) diluted 1:1000. PAR synthesis was detected by the above described 10H

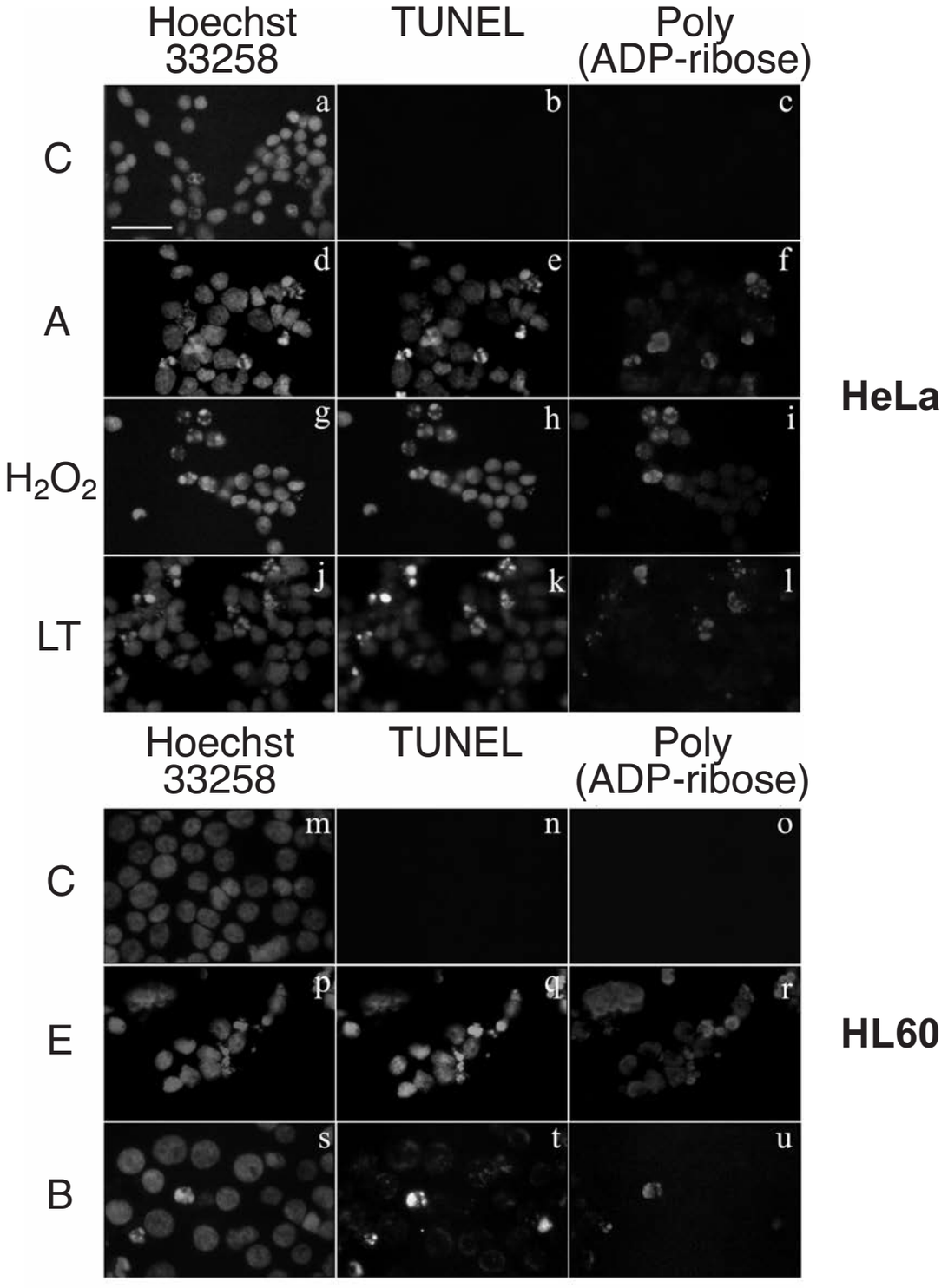

Figure 2. DNA fragmentation and poly(ADP-ribose) synthesis: *In situ* simultaneous detection in HeLa and HL60 cells. (Top) HeLa cells treated with 0.1 μg/mL actinomycin D for 12 h (A, d-f); 1 mM H_2O_2 for 30 min (H_2O_2, g-i); and maintained in culture in the same medium for up 9 days (LT, j-l). (Bottom) HL60 cells treated with 68 μM etoposide for 4 h (E, p-r); and with 250 μg/mL bleomycin for 24 h followed by 24 h of incubation in drug-free medium (B, s-u). In parallel to treated cells, untreated HeLa (C, a-c) and HL60 (C, m-o) cells were analyzed. Bar = 50 μm.

antibody diluted 1:200, and then with the HRP-conjugated anti-mouse secondary antibody (Jackson Immunores.) diluted 1:10000. Visualization of immunoreactive bands was achieved using a chemiluminescent substrate (Immun-Star™ WesternC™ Chemiluminescent Kit, Bio Rad).

Results and Discussion

We investigated the relevance of poly(ADP-ribosylation) process in the response to DNA damage/stress conditions possibly correlated to apoptosis occurrence. According to well-established protocols, human cancer cells have been stressed with different drugs, i.e. etoposide, a DNA topoisomerase II inhibitor (Torriglia *et al.*, 1999), actinomycin D, which interferes with RNA synthesis (Soldani *et al.*, 2001), bleomycin and H_2O_2, i.e. direct DNA-damaging agents (Donzelli *et al.*, 1997; Palomba *et al.*, 2001). In addition, a "physiological" stress caused by long-term culture in growth factor-deprived medium was applied (Torriglia *et al.*, 1999). These samples were analyzed by the immunofluorescence protocol that allows the simultaneous visualization of nuclear morphology (by Hoechst 33258 staining, blue fluorescence), DNA fragmentation (by TUNEL assay, green fluorescence) and PAR synthesis (by the use of a specific monoclonal antibody, red fluorescence). As illustrated in Figure 2, HeLa and HL60 untreated cells were characterized by a normal chromatin distribution (panels a and m, respectively) and by the absence of both TUNEL (b and n) and 10H fluorescence (c and o). Under stress conditions caused either by the administration of pro-apoptotic drugs (actinomycin D, etoposide) or by DNA damage induced by H_2O_2 and bleomycin, or growth impairment, we detected DNA fragmentation and PAR synthesis. In fact, cells with chromatin condensation and margination (panels d, g, j, p and s), exhibited a positive staining with TUNEL assay (panels e, h, k, q and t) and were also labelled by the anti-PAR antibody (panels f, i, l, r and u).

The detection of apoptotic cells by this method is very convenient because it allows the identification of two biochemical apoptotic hallmarks *in situ*; the technique requires a limited number of cells and is easily manageable. To give an idea of the time- and material-saving features of the protocol, DNA degradation and poly(ADP-ribose) synthesis have been monitored by classical assays, i.e. DNA ladder visualization and western blot. As shown in Figure 3a, under conditions described for inducing apoptosis, agarose gel electrophoresis revealed the presence of DNA

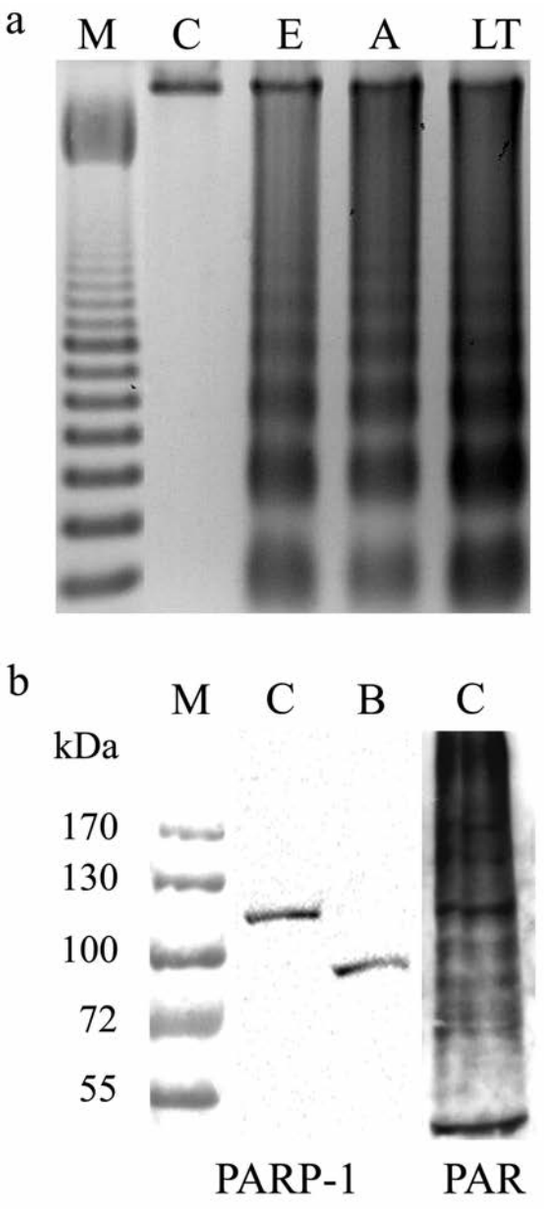

Figure 3. DNA fragmentation and poly(ADP-ribose) synthesis: Classical analyses. (a) Electrophoresis of DNA extracted from untreated HeLa cells (C) and from cells treated with etoposide (E), actinomycin D (A) or long-term cultured (LT). (M) DNA molecular marker (Fermentas). (b, left) Western blot analysis of PARP-1 in extracts from control (C) and bleomycin-treated HeLa cells (B). (b, right) Poly(ADP-ribose) (PAR) evaluated in control HeLa cells (C). M: protein markers (Fermentas).

ladder in apoptotic HeLa cells as an effect of the treatment with etoposide (E), actinomycin D (A) or long-term culture (LT). The typical DNA degradation was not detected in untreated cells (C). This analysis implies the use of at least 2.5×10^6 cells/sample and requires two days.

Analogously, the visualization of poly(ADP-ribose) synthesis by western blot (Figure 3b, right part), which reveals a series of bands corresponding to poly(ADP-ribosylated) proteins, is not as informative as the *in situ* analysis. Moreover, to identify PARP-1 as the major acceptor of PAR a parallel western blot is required (Figure 3b, left part, C: untreated HeLa cells with intact PARP-1 at 113 kDa; B: bleomycin-treated HeLa cells with cleaved fragment p89 of PARP-1).

In conclusion, we propose that the detection of poly(ADP-ribose) within the cell could be a marker not only of apoptosis but also of DNA damage and, more in general, of stress. This feature could be exploited to pinpoint stress-related condition due to Reactive Oxigen Species (ROS) accumulation, as it occurs during inflammation or in some diseases. Of note, it has been shown that the abnormal intracellular accumulation of poly(ADP-ribose) due to a massive activation of PARPs in response to continuous stress conditions could be deleterious, and may be counteracted by the use of PARP inhibitors (Giansanti *et al.*, 2010).

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