Chapter 2

Quantification of PARP Activity in Human Tissues: Ex Vivo Assays in Blood Cells and Immunohistochemistry in Human Biopsies

Eszter M. Horvath, Zsuzsanna K. Zsengellér, and Csaba Szabo

Abstract

Poly(ADP-ribosyl)ation of proteins is a posttranslational modification mediated by poly(ADP-ribose) polymerases (PARPs) that use NAD^+ as substrate to form the negatively charged polymer of poly(ADP-ribose) (PAR). After DNA damage, PARP-1 is responsible for approximately 90% of the total cellular PARylation activity. Numerous studies showed activation of PARP-1 in various conditions associated with oxidative and nitrosative stress, such as ischemia-reperfusion injury, diabetes mellitus, and inflammation, and also proved the beneficial effects of PARP inhibitors. Several pharmacological inhibitors of PARP moved toward clinical testing for a variety of indications, including cardioprotection and malignant tumors, and in late 2014, olaparib became the first PARP inhibitor approved for human use for the therapy of ovarian cancer. These advances necessitate the detection of PARP activation in human tissues. In the present chapter, we review specific methods used to detect PARP activation in human circulating leukocytes and in human tissue sections.

Key words Poly(ADP-ribose), PARP, Immunohistochemistry, DAB, Circulating leukocytes, Biopsy

1 Introduction

PARP-1 is an abundant nuclear enzyme present in all eukaryotic cells with the exemption of neutrophil granulocytes. It is a molecular sensor of DNA damage; its catalytic activity is stimulated more than 500-fold on binding to DNA breaks. Poly(ADP-ribose) or PAR is a polymer synthesized by PARP using NAD+ as substrate. PARP catalyzes the formation of the polymer PAR, with chain lengths ranging from 2 to 300 residues, containing approximately 2% branching in the chain. PAR becomes attached to nuclear proteins and to PARP itself (automodification). Under normal conditions, cells display low basal level of PAR polymer, which can dramatically increase in cells exposed to DNA-damaging agents (irradiation, alkylation, etc.). This increase of polymer synthesis is usually transient and is followed by a rapid degradation phase with

Alexei V. Tulin (ed.), *Poly(ADP-Ribose) Polymerase: Methods and Protocols*, Methods in Molecular Biology, vol. 1608,
DOI 10.1007/978-1-4939-6993-7_2,

a short half-life which can be less than 1 min. The low endogenous level of polymer in unstimulated cells and its rapid catabolism during DNA damage have been ascribed to high activity of the polymer-catabolizing enzyme poly(ADP-ribose) glycohydrolase (PARG). Therefore, the level of intracellular PAR content depends on the activity of the two enzymes: PARP and PARG [1–3].

The resulting PAR production has three major roles: (1) PARylation of histone H1 and H2B contributes to the relaxation of chromatin and increases the access to breaks, (2) indication of the occurrence and severity of DNA damage (consequent DNA repair or cell death), and (3) recruitment of single-strand break repair (SSBR)/base excision repair (BER) factors to the damaged site [1].

Concerning the consequences of PARP-1 activity, cells exposed to DNA-damaging agents can enter three pathways based on the intensity of stimuli. In case of mild DNA damage, PARP-1 activation results in relaxation of chromatin structure, recruitment of repair enzymes, and consequent DNA repair [1]. More severe DNA damage induces apoptotic cell death, during which caspases inactivate PARP-1 by cleaving it into two fragments [4]. Extensive DNA breakage usually triggered by a massive oxidative or nitrosative stress leads to the overactivation of PARP-1 that results in the rapid depletion of cellular NAD+ and consequently APT, leading to energy depletion and necrotic cell death [3].

PARP-1 is important in the survival and genomic integrity of cells; however, its activation has detrimental effects in various inflammatory processes, cardiac and cerebral ischemia, and diabetes mellitus where overactivation of PARP-1 can be observed [1–3, 5–7].

There are various methods that can be used for the estimation of PARP-1 activity in tissues and cells (Table 1). In case of human studies, the availability of tissues is mostly limited to small biopsy samples. Another possibility is to measure the PARP activity of isolated circulating leukocytes that may reflect the level of systemic PARP activation and correlate with the pathophysiological condition and the effect of therapeutic agents given to the patients [8, 9].

As neutrophils and eosinophils do not have PARP-1, in all methods, isolating mononuclear cells provides increased accuracy. (The distribution of white blood cells does not alter the results.) However, this isolation is usually based on gradient centrifugation: Especially in pathological conditions where the size and density of cells change, the resulting cell suspension contains not only mononuclear cells but other cell types and debris. While immunohistochemistry and flow cytometry provide further opportunity to separate debris and different cell types, Western blot and cell-based assay do not have this advantage.

Table 1
Basic characteristics of methods for PARP activity measurement

Method	Measured parameter	Evaluation	Sample preparation
Immunohistochemistry	PAR content of cells	Computer based Opportunity to separate cell types Objective	Methanol-fixed smears (long tenability)
Flow cytometry	PAR content of cells	Computer based Opportunity to separate cell types Objective	Requires fresh blood
Western blot	Auto-PARylation	Densitometry Objective	Frozen isolated cells (long tenability)
Cell-based activity measurement	PARP activity	Colorimetry Objective	Frozen isolated cells (long tenability)

2 Materials

2.1 Isolation of Circulating Leukocytes

1. Blood collection tube with heparin 10 mL.
2. Histopaque-1077 (Sigma-Aldrich).
3. Dulbecco's PBS (DPBS).
4. SuperFrost Ultra Plus slides.
5. Smear fixation: methanol 100% and 50%.

2.2 Immuno-histochemistry

1. Xylene.
2. Ethanol, anhydrous denatured, and histological grade (100% and 95%).
3. Deionized water (dH_2O).
4. Distilled water (ddH_2O).
5. Phosphate-buffered saline (PBS) 0.05 M, pH 7.4 (premade solutions are available).
   Alternative recipe for PBS: Make a tenfold dilution of the stock solution below in ddH_2O, and adjust pH to 7.4 with HCl or NaOH.
   10× PBS stock solution: Dissolve 14.4 g of sodium phosphate dibasic, 2.4 g of sodium phosphate monobasic, and 80.00 g of sodium chloride in ddH_2O to have a final volume of 1 L.

6. TPBS: 1× PBS with Triton X-100: Dissolve 2–3 mL of Triton X-100 in PBS solution.
7. Blocking serum: 2.5% Normal Horse Serum in TPBS.
8. *Antigen unmasking*: Antigen Unmasking Solution, citric acid based (premade solutions are available).
   Alternative unmasking solutions.
   10 mM sodium citrate buffer, pH 6: Dissolve 2.94 g of sodium citrate trisodium salt dihydrate in ddH_2O to have a final volume of 1 L. Adjust pH to 6.0.

   or

   200 mM citrate buffer, pH 3: Dissolve 34.8 g of citric acid and 4 g of NaOH in ddH_2O to have the final volume of 1 L. Adjust pH to 3.0.
9. 0.5% hydrogen peroxide in methanol: Mix 1 mL of 30% H_2O_2 with 60 mL of methanol (100%) or 3% hydrogen peroxide in ddH_2O. Mix 10 mL of 30% H_2O_2 with 90 mL of ddH_2O.
10. Primary antiserum: Anti-Poly(ADP-ribose) polymer clone 10H (Tulip Biolabs or Calbiochem); antibody dilutions, 1:1000 and 1:1200.
11. Secondary antiserum: HRP-conjugated anti-mouse IgG polyclonal antibody (e.g., ImmPRESS HRP Anti-Mouse IgG (Peroxidase) Polymer Detection Kit, made in Horse (Vector Laboratories), per manufacturer instruction).
12. Visualization: Nickel-enhanced DAB (NiDAB) solution (ready to use kits are available, such as Peroxidase (HRP) Substrate Kit (with nickel), 3,3'-diaminobenzidine (Vector Laboratories). Use per manufacturer instruction).

    Self-made solutions needed for visualization with NiDAB.

    NiDAB solution: Dissolve 95 mg of DAB (handle DAB with care; it is a possible carcinogen), 1.6 g of sodium chloride, and 2 g of nickel sulfate in 200 mL of 0.1 M acetate buffer, pH 6.0. Just prior to use, filter the NiDAB solution using Whatman #1 or, equivalent, filter paper. Add 25 μL of 30% hydrogen peroxide to the NiDAB solution, and pour into a staining dish.

    Tris Buffer, pH 7.6: Dissolve 12.11 g of Tris and 18.00 g sodium chloride in 1 L of ddH_2O. Adjust the pH to 7.6 with concentrated hydrochloric acid. Bring the volume to 2 L with distilled water.

    M Acetate Buffer, pH 6.0: Dissolve 16.40 g of sodium acetate anhydrous in 1 L of ddH_2O. Adjust the pH to 6.0 and bring the volume to 2 L with distilled water.

    Tris-cobalt pH 7.2: Dissolve 1.2 g of Tris and 1.0 g of cobalt chloride in 180.0 mL of ddH_2O. Adjust the pH to 7.2 with 0.1 M HCl and bring to a volume of 200 mL.

13. Counterstain: Nuclear Fast Red (NFR) (premade solutions are available).
    Alternative NFR recipe.

    0.1% Nuclear Fast Red in 5% aluminum sulfate: Dissolve 5.0 g of aluminum sulfate in 100.0 mL ddH_2O, and then dissolve 0.1 g of Nuclear Fast Red in this solution with the aid of heat. Cool, filter, and add a few grains of thymol as a preservative. Filter before use (*see* **Note 1**).

3 Methods

3.1 Leukocyte Isolation

1. Pipet 5 mL of room temperature Histopaque-1077 into 15 mL of conical tubes.
2. Layer 5 mL of heparinized whole blood onto the Histopaque-1077 layer (5 mL syringe with 20G needle).
3. Centrifuge at 400 × *g* at room temperature for 30 min.
4. Carefully aspirate the middle opaque layer (1000 μL) and place it into a new 15 mL tube.
5. Add 10 mL of DPBS and mix by gentle aspiration.
6. Centrifuge at 250 × *g* for 10 min.
7. Aspirate the supernatant and discard.
8. Resuspend cell pellet with 5.0 mL DPBS and mix by gentle aspiration.
9. Centrifuge at 250 × *g* for 10 min.
10. Repeat washing step.
11. Reconstitute pellet with 500 μL DPBS.
12. Pipet 50 μL of cell suspension on one microscope slide. Smear with a cover slip.
13. Dry the smears.
14. Fix them in methanol 100% for 1 min and methanol 50% for 2 min.
15. Dry the smears.
16. Store them on 4 °C (*see* **Note 2**).

3.2 Immuno-histochemistry

3.2.1 Deparaffinize/ Hydrate Sections

1. Three changes of xylene for 5 min each.
2. Two changes of 100% EtOH for 5 min each.
3. Two changes of 95% EtOH for 5 min each.
4. One change of 70% EtOH for 5 min each.
5. Clear in ddH_2O for 5 min (in case of leukocyte smears, start here).

3.2.2 Antigen Unmasking

1. Fill plastic Coplin jars with the citrate buffer (working solution).
2. Add tap water to plastic reservoir dish to a level of 1–2 in. to create a sink.
3. Place Coplin jars in reservoir.
4. You can place 3–4 slides in each Coplin jar (do not put more than four slides per jar!).
5. Microwave on HI for 7 min (to boil).
6. Check fluid level in Coplin jars and add dH_2O if level has evaporated significantly.
7. Microwave at 60% power for 15 min. (to simmer) Do this at 3 × 5 min. increments checking the citrate buffer after each 5 min. Add ddH_2O if necessary.
8. Remove the reservoir from the microwave. Remove the Coplin jars from the reservoir and cool on the countertop for 15 min.
9. Rinse in distilled water in two quick changes. Rinse in PBS bath for 5 min.
10. Continue with immunostaining as per your protocol (*see* **Note 3**).

3.2.3 Treatment for Removal of Endogenous Peroxidase Activity

1. Place slides in a bath containing 60 mL of methyl alcohol and 1 mL of 30% hydrogen peroxide or 90 mL of ddH_2O and 10 mL of 30% hydrogen peroxide.
2. Incubate at room temperature for 15 min.
3. Clear in 1× ddH_2O and 1× PBS for 5 min each.

3.2.4 Blocking of Nonspecific Binding

1. Place slides in a bath containing blocking serum or overlay (at least 200 μL/slide) the slides with the blocking serum and cover with glass or plastic cover slip.
2. Incubate slides for 1 h at room temperature (*see* **Note 4**).
3. Drain slides before incubating in the appropriate primary antiserum dilution. Do *not* rinse the slides in PBS. Do *not* let slides dry at any point in this procedure.

3.2.5 Application of the Primary Antiserum

1. Dilute the primary antiserum in blocking serum at the appropriate predetermined dilution.
2. Overlay the slides with the antiserum, cover with glass or plastic cover slip, and incubate overnight in a humified chamber at 4 °C.
3. Wash slides in a Coplin jar 3× for 5 min each with TBPS on a shaker at room temperature.

3.2.6 Application of the Secondary Antibody

1. Place slides in a bath containing secondary antibody, or overlay (at least 200 μL/slide) the slides with the secondary antibody and cover with glass or plastic cover slip.
2. Incubate slides for 30 min at room temperature on a shaker or in a humified chamber.
3. Wash slides in a Coplin jar 3× for 5 min each with TPBS on a shaker at room temperature.

3.2.7 Development of Colored Reaction Product Using Diaminobenzidine (DAB)

1. Apply DAB Peroxidase Substrate Kit, 3,3′-diaminobenzidine according to the manufacturer's instructions.
   Or
2. Incubate the slides in NiDAB solution for 4 min on a shaker at room temperature.
3. Rinse briefly in Tris Buffer (pH 7.6).
4. Incubate in Tris-cobalt, pH 7.2, for 4 min at room temperature.
5. Wash briefly in distilled water.

3.2.8 Counterstaining

1. Counterstain in Nuclear Fast Red for 2–10 min.
2. Wash in running water.

3.2.9 Dehydration and Mounting

1. One change of 70% EtOH—20 dips.
2. Two changes of 95% EtOH—20 dips each.
3. Three changes of 100% EtOH—20 dips each.
4. Three changes of xylene—clear—10–15 dips each.
5. Cover slip with Permount.

3.2.10 Evaluation

1. The objectivity of the evaluation of immunohistochemically stained leukocyte smears can be enhanced by using computer-based evaluation procedure. In case of PAR staining, the ratio of positive nuclei and the percent of positively stained area of nuclei are both good measures of staining intensity. (Fig. 1) (*see* **Notes 5** and **6** for evaluation and interpretation notes.)

4 Notes

1. You may use this solution for a period of 2 weeks, if used every day. It should be made fresh when it turns to a dark black color.
2. During leukocyte isolation, it is utmost important not to leave blood samples on room temperature for more than half an hour prior processing, because in whole-blood oxidative, nitrosative stress and consequent PARP activation occur by time. On the other hand, leaving blood samples on the bench for 1.5 h is an excellent way to make positive controls.

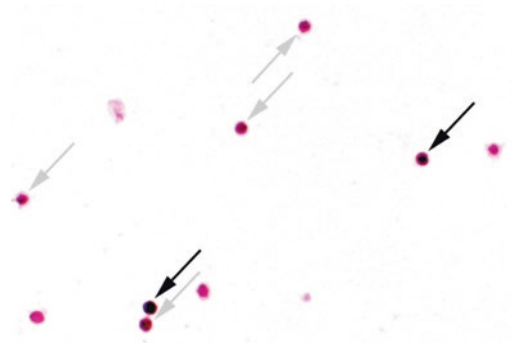

Fig. 1 Representative PAR staining of leukocytes smears. Black color represents Ni-DAB-labeled positive staining; red color is the Nuclear Fast Red counterstain. Black arrows show positive nuclei; grey arrows show partly positive nuclei

3. It is important to always check the fluid levels when you are microwaving. Fluid levels can go below the tissue level and cause high background staining.
4. If you decide overlaying the blocking serum, place slides into a humified chamber during incubation.
5. In case of small biopsy slides, be aware of edge effect. The ledge of slides may show more pronounced specific and unspecific staining. Always use negative control in each staining procedure.
6. PAR staining has usually but not necessary nuclear location. Low-intensity positive staining can be present in the cytoplasm. Do not discard these stainings if the negative control slide has no or slight staining.

References

1. Schreiber V, Dantzer F, Ame JC, de Murcia G (2006) Poly(ADP-ribose): novel functions for an old molecule. Nat Rev Mol Cell Biol 7:517–528
2. Jagtap P, Szabo C (2005) Poly(ADP-ribose) polymerase and the therapeutic effects of its inhibitors. Nat Rev Drug Discov 4:421–440
3. Virag L, Szabo C (2002) The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. Pharmacol Rev 54:375–429
4. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 371:346–347
5. Ame JC, Spenlehauer C, de Murcia G (2004) The PARP superfamily. Bioessays 26:882–893
6. de Murcia JM, Niedergang C, Trucco C, Ricoul M, Dutrillaux B, Mark M, Oliver FJ, Masson M, Dierich A, LeMeur M, Walztinger C, Chambon P, de Murcia G (1997) Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells. Proc Natl Acad Sci U S A 94:7303–7307
7. Pacher P, Liaudet L, Soriano FG, Mabley JG, Szabo E, Szabo C (2002) The role of poly(ADP-ribose) polymerase activation in the development of myocardial and endothelial dysfunction in diabetes. Diabetes 51:514–521
8. Tóth-Zsámboki E, Horváth E, Vargova K, Pankotai E, Murthy K, Zsengellér Z, Bárány T, Pék T, Fekete K, Kiss RG, Préda I, Lacza Z, Gerö D, Szabó C (2006) Activation of poly(ADP-ribose) polymerase by myocardial ischemia and coronary reperfusion in human circulating leukocytes. Mol Med 12:221–228
9. Horváth EM, Magenheim R, Kugler E, Vácz G, Szigethy A, Lévárdi F, Kollai M, Szabo C, Lacza Z (2009) Nitrative stress and poly(ADP-ribose) polymerase activation in healthy and gestational diabetic pregnancies. *Diabetologia*:1935–1943

http://www.springer.com/978-1-4939-6992-0

Poly(ADP-Ribose) Polymerase
Methods and Protocols
Tulin, A.V. (Ed.)
2017, XV, 528 p. 82 illus., 47 illus. in color. With online files/update., Hardcover
ISBN: 978-1-4939-6992-0
A product of Humana Press