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Quantification of PARP activity in human tissues: *ex vivo* assays in blood cells, and immunohistochemistry in human biopsies

Eszter M Horvath, M.D. Ph.D.[1], **Zsuzsanna K. Zsengellér, M.D., Ph.D.**[2], and **Csaba Szabo, M.D., Ph.D.**[3,*]

[1]Semmelweis University, Institute of Human Physiology and Clinical Experimental Research, Budapest, Hungary

[2]Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA

[3]Department of Anesthesiology, University of Texas Medical Branch, Galveston, TX 77555, USA

Summary

Poly (ADP-ribosyl)ation of proteins is a post-translational modification mediated by poly (ADP-ribose) polymerases (PARPs), that uses 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. Pharmacological inhibitors of PARP move toward clinical testing for a variety of indications, including cardioprotection and malignant tumors. Some of the compounds are already in clinical trials. 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.

Keywords

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

[*]Corresponding author: Csaba Szabo M.D., Ph.D., Department of Anesthesiology, The University of Texas Medical Branch, 601 Harborside Drive, Galveston, TX 77555, USA, szabocsaba@aol.com.

(irradiation, alkylation, etc.). This increase of polymer synthesis is usually transient and is followed by a rapid degradation phase with 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 has been ascribed to high activity of the polymer catabolizing enzyme poly(ADP-ribose) glycohydrolyase (PARG). Therefore, the level of intracellular PAR content depends on the activity of the two enzymes: PARP and PARG (1,2,3).

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), 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 over-activation 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 is activation has detrimental effects in various inflammatory processes, cardiac and cerebral ischemia, diabetes mellitus where over-activation 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 are 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 pathological 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 provides further opportunity to separate debris and different cell types, Western-blot and cell-based assay does not have this advantage.

2. Materials

Isolation of circulating leukocytes

1. Blood collection tube with heparin 10 ml (BD)
2. Histopaque-1077 (Sigma-Aldrich)

3. Dulbecco's PBS (Invitrogen)
4. SuperFrost Ultra Plus slides (Menzel-Glazer, Braunschweig, Germany)
5. Smear fixation: Methanol 100%, 50%

Immunohistochemistry

1. Xylene
2. Ethanol, anhydrous denatured, histological grade (100% and 95%)
3. Deionized water (dH_2O)
4. 10X PBS pH 7.4

14.4 gms	Sodium Phosphate dibasic
2.4 gms	Sodium Phosphate Monobasic
80.00 gms	Sodium Chloride
1000 ml	dH2O

   Adjust pH to 7.4

5. PBS pH 7.4

   900ml dH2O

   100ml 10 X PBS

   Adjust pH to 7.4

6. TPBS: 1X PBS with Triton X-100

2–3 ml	Triton X-100
1 L	1 X PBS

7. Blocking serum: 150 μl Normal horse serum in 10 ml TPBS in VECTASTAIN Elite
8. ABC Kit (Mouse IgG) (Vector Laboratories)
9. Antigen Unmasking: 10 mM Sodium Citrate Buffer pH6

2.94 gms	sodium citrate trisodium salt dehydrate
1 L	dH_2O.

   Adjust pH to 6.0.

   OR

   200 mM Citrate buffer pH3

   34.8 gms citric acid

   4 gms NaOH

   Adjust pH to 3.0.

10. 3% Hydrogen Peroxide

10 ml	30% H_2O_2
90 ml	dH_2O

11. Primary Antiserum: Anti- Poly (ADP-Ribose) Polymer Clone 10H (Tulip Biolabs or Calbiochem) Antibody dilutions: 1:1000, 1:1200

12. Secondary Antiserum: Biotinylated Horse Anti-Mouse IgG Antibody in VECTASTAIN Elite ABC Kit (Mouse IgG) (Vector Laboratories)

    50 µl antibody in 10 ml blocking serum.

13. Visualization: DAB Peroxidase Substrate Kit, 3,3′-diaminobenzidine (Vector Laboratories) Prepare as follows:

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

    OR

14. Tris Buffer pH 7.6

12.11 gms	Tris (Sigma 7–9)
18.00 gms	Sodium Chloride

    Dissolve in 1 liter of distilled water. Adjust the pH to 7.6 with concentrated hydrochloric acid. Bring the volume to two liters with distilled water.

15. 0.1M Acetate Buffer pH 6.0

16.40 gms	Sodium Acetate Anhydrous

    Dissolve in 1 liter of distilled water. Adjust the pH to 6.0 and bring the volume to two liters with distilled water.

16. Tris-Cobalt pH 7.2

1.2 gms	Tris (Sigma 7–9)
1.0 gm	Cobalt Chloride
180.0 ml	Distilled Water

    Combine all the ingredients in 180 ml of distilled water. Adjust the pH to 7.2 with 0.1M HCl and bring to a volume of 200 ml. **See** Note 1.

17. Counterstain: 0.1% Nuclear Fast Red in 5% Aluminum Sulfate

[1]You may use this solution for a period of two weeks, if used every day. It should be made fresh when it turns a dark black color.)

0.1 gm	Nuclear Fast Red
5.0 gms	Aluminum Sulfate
100.0 ml	Distilled Water

Dissolve the nuclear fast red in 5% aluminum sulfate with the aid of heat. Cool, filter, and add a few grains of thymol as a preservative. Filter before use.

3. Methods

3.1 Leukocyte isolation

1. Pipet 3 ml of room temperature Histopaque-1077 into 15ml conical tubes.
2. Layer 3ml of heparinized whole blood onto the Histopaque-1077 layer. (3ml Syringe with 20G needle)
3. Centrifuge at 400g at room temperature for 30 min.
4. Carefully aspirate the middle opaque layer (500μl) and place it into a new 15ml tube.
5. Add 10ml of DPBS and mix by gentle aspiration
6. Centrifuge at 250 × g for 10 minutes.
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 minutes.
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 1min and methanol 50% for 2min.
15. Dry the smears.
16. Store them on 4°C.

See Note 2.

3.2 Immunohistochemistry

3.2.1 Deparaffinize/Hydrate Sections

1. 3 changes of xylene for 5 minutes each.
2. 2 changes of 100% EtOH for 5 minutes each.
3. 2 changes of 95% EtOH for 5 minutes each.

[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 occurs by time. On the other hand leaving blood samples on the bench for 1.5 hours is an excellent way to make positive controls.

4. 1 change of 70% EtOH for 5 minutes each.
5. Clear in PBS pH 7.4 for 5 minutes. (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” 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 4 slides per jar!).
5. Microwave on HI for 7 minutes. (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 minutes. (To simmer.) Do this at 3×5 min. increments checking the citrate buffer after each 5 minutes. Add dH_2O if necessary.
8. Remove the reservoir from the microwave. Remove the coplin jars from the reservoir and cool on the countertop for 15 minutes.
9. Rinse in distilled water ×2 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 90 ml of methyl alcohol and 10 ml of 30% hydrogen peroxide.
2. Incubate at room temperature for 15 minutes.
3. Clear in PBS for 5 minutes.

3.2.4 Blocking of Non-Specific Binding

1. Place slides in a bath containing blocking serum or overlay (at least 100 μl/slide) the slides with the blocking serum and cover with glass or plastic cover slip. See Note 4.
    1. Incubate slides for 1–2 hours at room temperature. See Note 5.
    2. 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]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]Prepared blocking serum can be stored at 4°C for a month and can be reused several times.
[5]If you decide overlaying blocking serum, place slides into a humified chamber during incubation.

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 humified chamber at 4°C.
3. Wash slides in a coplin jar ×5 for 5 minutes 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 100μl/slide) the slides with the secondary antibody and cover with glass or plastic cover slip.
2. Incubate slides in for 30 minutes at room temperature on a shaker or in a humified chamber.
3. Wash slides in a coplin jar ×5 for 5 minutes each with TPBS on a shaker at room temperature.

3.2.7 Application of the Avidin-Biotin Complex Reagent

1 Dilute the avidin-biotin complex according to the directions in the Vector Elite Kit using TPBS. (It must be prepared at least 30 minutes prior to use.)

2 Place slides in a bath containing ABC reagent or overlay (at least 100 μl/slide) the slides with the ABC reagent and cover with glass or plastic cover slip.

4 Incubate slides in for 30 minutes at room temperature on a shaker or in a humified chamber.

3 Wash slides in a coplin jar ×5 for 5 minutes each with TPBS on a shaker at room temperature.

3.2.8 Development of Colored Reaction Product Using Diaminobenzidine (DAB)

1 Apply DAB Peroxidase Substrate Kit, 3,3′-diaminobenzidine according to the manufacturer's instructions.

OR

5 Incubate the slides in NiDAB for 4 minutes on a shaker at room temperature.

6 Rinse briefly in Tris/Saline pH 7.6.

7 Incubate in Tris/Cobalt for 4 minutes at room temperature.

8 Wash briefly in distilled water.

9 Counterstain in 0.1% Nuclear Fast Red in 5% Aluminum Sulfate for 2 minutes.

10 Wash in running water.

3.2.9 Dehydration and Mounting

1. 1 change of 70% EtOH – 10 dips.
2. 2 changes of 95% EtOH – 10 dips each.
3. 3 changes of 100% EtOH – 10 dips each.
4. 3 changes of Xylene—clear – 10–15 dips each.
5. Coverslip 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. (Figure 1.) See Note 6 and Note 7 for evaluation and interpretation notes.

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[6]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.

[7]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.

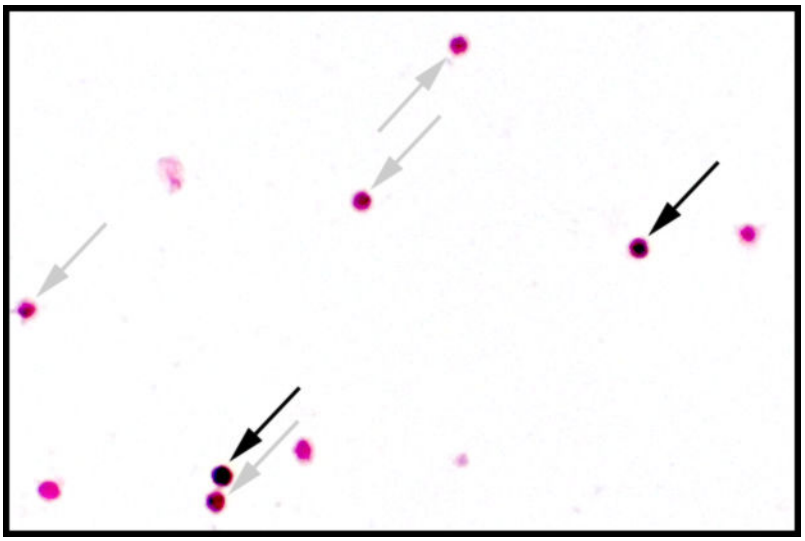

Figure 1. Representative PAR staining of leukocytes smears

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.

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)