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SHORT COMMUNICATION

Detection of poly(ADP-ribose) polymerase and its reaction product poly(ADP-ribose) by immunocytochemistry

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Summary

Poly(ADP-ribose) polymerase catalyses the formation of ADP-ribose polymers covalently attached to various nuclear proteins, using NAD^+ as substrate. The activity of this enzyme is strongly stimulated upon binding to DNA single or double strand breaks. Poly(ADP-ribosyl)ation is an immediate cellular response to DNA damage and is thought to be involved in DNA repair, genetic recombination, apoptosis and other processes during which DNA strand breaks are formed. In recent years we and others have established cell culture systems with altered poly(ADP-ribose) polymerase activity. Here we describe immunocytochemistry protocols based on the use of antibodies against the DNA-binding domain of human poly(ADP-ribose) polymerase and against its reaction product poly(ADP-ribose). These protocols allow for the convenient mass screening of cell transfectants with overexpression of poly(ADP-ribose) polymerase or of a dominant-negative mutant for this enzyme, i.e. the DNA-binding domain. In addition, the immunocytochemical detection of poly(ADP-ribose) allows screening for cells with altered enzyme activity.

Introduction

Among the early responses of eukaryotic cells to DNA damage is the poly(ADP-ribosyl)ation of proteins, which is catalysed by the nuclear enzyme poly(ADP-ribose) polymerase (EC 2.4.2.30) with NAD^+ serving as substrate (for a review, see Althaus & Richter, 1987). Binding of the amino-terminal DNA-binding domain of this enzyme to DNA strand breaks causes enzyme activation, which leads to the formation of ADP-ribose polymers covalently linked with glutamate or aspartate residues of proteins. While the enzyme itself is the main acceptor of poly(ADP-ribose) in cells, modification of other chromosomal proteins has also been shown. Poly(ADP-ribose) synthesis can be stimulated by treating cells with, for example, ionizing radiation, alkylating agents, or active oxygen. High concentrations of poly(ADP-ribose) stimulate the catalytic activity of poly(ADP-ribose) glycohydrolase, resulting in rapid polymer turnover.

Previous work with low-molecular-weight inhibitors of poly(ADP-ribose) polymerase (reviewed in Boulikas, 1991) and, more recently, with molecular genetic tools, has led to the view that poly(ADP-ribosyl)ation plays a role in DNA repair, genetic recombination, and in other cellular responses to DNA damage (for a review, see de Murcia & Ménissier-de Murcia, 1994). Recently, a CED-3/interleukin-1β-converting enzyme-related protease called Yama or CPP32 was cloned that specifically and quantitatively cleaves poly(ADP-ribose) polymerase during apoptosis (Nicholson *et al.*, 1995; Tewari *et al.*, 1995).

By making use of antibodies raised against the DNA-binding domain of human poly(ADP-ribose) polymerase and against its reaction product poly (ADP-ribose), respectively, we previously developed an immunofluorescence protocol for the simultaneous detection of the DNA-binding domain of the over-expressed enzyme and of poly(ADP-ribose) synthesized

in living cells (Küpper *et al.*, 1990; 1995). Here we describe immunocytochemical protocols for these two antigens based on the peroxidase reaction, which allow for the convenient mass screening of cells with altered enzyme expression, and of cells in which poly(ADP-ribosyl)ation is activated or repressed.

Materials and methods

Cell culture

The cell lines CV-1 (African green monkey kidney) and CO60 (SV40-transformed embryonic hamster cells) were maintained as monolayers in Dulbecco's MEM (DMEM) (Gibco, Karlsruhe, Germany) supplemented with L-glutamine (1 mM), penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}), and 10% fetal calf serum (Gibco).

Electrotransfection

To overexpress poly(ADP-ribose) polymerase transiently in cells, electrotransfection was carried out with plasmid pPARP31 comprising the open reading frame of human poly(ADP-ribose) polymerase under the control of the human cytomegalovirus promoter/enhancer (van Gool *et al.*, manuscript in preparation). Exponentially growing CO60 cells were trypsinized, and $5x10^6$ cells in 140 µl Dulbecco's phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4) were combined with 20 µg of supercoiled plasmid DNA in 60 µl 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. This mixture was incubated for 5 min at room temperature. Then electrotransfection (Gene Pulser and capacitance extender, Bio-Rad Laboratories, Richmond, CA) was done at 120 V and 960 µF in cuvettes with an electrode gap of 0.2 cm (Bio-Rad). Ten minutes later, cells were resuspended in medium and dispensed on microtiter plates, or on coverslips placed in Petri dishes, respectively.

Indirect immunofluorescence

For the detection of the overexpressed enzyme, electrotransfected CO60 cells grown on coverslips were fixed with absolute ethanol at –20°C for 10 min, followed by air-drying. Then, cell ghosts were rehydrated in phosphate-buffered saline. Immunofluorescence with anti-FII serum raised against the DNA-binding domain of the enzyme was performed exactly as described previously (Küpper *et al.*, 1990; for antibody sources and dilutions see Table 1).

For the immunostaining of poly(ADP-ribose) two different protocols were used (Küpper *et al.*, 1990). For the detection of poly(ADP-ribose) synthesized in ethanol-fixed cells, medium was removed, and cells grown on coverslips were washed with PBS and fixed with absolute ethanol at –20°C for 10 min. Thereafter, air-dried cells were rehydrated with PBS. To allow for poly(ADP-ribose) polymerase activity, cell ghosts were incubated in 100 mM Tris-HCl (pH 8.0) containing 10 mM magnesium chloride, 1 mM dithiothreitol and 400 µM NAD^+ for 2 h at 30°C. Thereafter, cells were incubated with poly(ADP-ribose)-specific monoclonal antibody 10H (concentrated 10-fold by ultrafiltration of hybridoma supernatant), and immunofluorescence was performed as described previously (Küpper *et al.*, 1990; Bürkle *et al.*, 1993).

Table 1. Antibodies used for the detection of poly(ADP-ribose) polymerase and poly(ADP-ribose)

*Antibody**	*Donor/species/dilution**	*Reference/supplier*
Anti-FII	Polyclonal rabbit antibody, 1:2000 for IC, 1:200 for IF,	Simonin *et al.*, 1991
10H	Mouse monoclonal antibody, 10× concentrate for IC and IF	Kawamitsu *et al.*, 1984
Anti-rabbit peroxidase	Polyclonal goat antibody, 1:1000 for IC	Dianova, Hamburg Germany
Anti-mouse peroxidase	Polyclonal goat antibody, 1:1000 for IC	Dianova
Anti-mouse FITC	Polyclonal goat antibody, 1:50 for IF	Bio-Yeda, Rehovot, Israel
Anti-rabbit TRITC	Polyclonal goat antibody, 1:200 for IF	Dianova

*Abbreviations: IC, immunocytochemistry; IF, immunofluorescence; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate

For the detection of poly(ADP-ribose) synthesized in living cells as a consequence of carcinogen treatment, cells growing on coverslips were treated with 50 µM *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Serva, Heidelberg, Germany) in complete medium for 10 min at 37°C. Thereafter, cells were washed with PBS containing 1 mM calcium chloride and fixed with 10% ice-cold trichloroacetic acid. Poly(ADP-ribose)-specific immunofluorescence was performed exactly as described previously (Küpper *et al.*, 1990; Bürkle *et al.*, 1993) .

Immunoperoxidase labelling

Cells were seeded into 96-well microtiter plates (Nunc, Wiesbaden, Germany). Depending on the antigen of interest, three different protocols were used. For the detection of poly(ADP-ribose) polymerase, cells were fixed with 5% formaldehyde in PBS (200 µl) for 30 min at room temperature. Fixed cells were then incubated at room temperature with 200 µl of 100 mM glycine in PBS for at least one minute, followed by a three-minute incubation in 200 µl 0.4% Triton X-100. Cell ghosts were washed twice with PBS and blocked with PBS containing 5% dry milk (Merck, Darmstadt, Germany) and 0.3% Tween-20 (blocking solution) for 1 h at room temperature. Then, anti-FII serum diluted in blocking solution was applied overnight at 4°C (for sources and dilutions of antibodies see Table 1). Thereafter, cells were washed four times with PBS containing 0.3% Tween-20 at room temperature, by mounting the microtiter plates on a Thermomixer (Eppendorf, Hamburg, Germany) which was set at 500 r.p.m. After washing, cells were incubated with peroxidase-conjugated anti-rabbit immunoglobulins in blocking solution for 1 h at room temperature. Cells were washed again four times with PBS containing 0.3% Tween-20, and once with PBS only. The peroxidase reaction was carried out at room temperature in PBS contain-

ing 0.6 mg ml^{-1} diaminobenzidine (Sigma), 0.028% nickel sulphate, and 0.03% hydrogen peroxide until signals became detectable (5–10 min).

To detect poly(ADP-ribose) synthesized in ethanol-fixed cells, medium was removed, cells were washed with PBS, fixed with absolute ethanol at –20°C for 10 min, and incubated in NAD^+ buffer, as described above for immunofluorescence. For the detection of poly(ADP-ribose), the primary antibody incubation was performed with monoclonal antibody 10H for 1 h at room temperature. Cells were washed four times with PBS containing 0.3% Tween-20 by rotational shaking as described above, and then incubated with peroxidase-conjugated anti-mouse immunoglobulin diluted in blocking solution. Cells were washed again four times with PBS containing 0.3% Tween-20 and once with PBS alone, and signals were developed exactly as described above for the detection of overexpressed poly(ADP-ribose) polymerase

For the detection of poly(ADP-ribose) synthesized in living cells, MNNG treatment and fixation were the same as described above for immunofluorescence. Prior to antibody incubation, air-dried cells were rehydrated with PBS. Incubation with primary antibody 10H was done overnight at room temperature. The further steps were as described in the preceding paragraph.

Results and discussion

Immunocytochemistry to detect overexpressed poly(ADP-ribose) polymerase

Although poly(ADP-ribose) polymerase is an abundantly expressed nuclear protein, it cannot be readily detected by the immunocytochemistry protocol described here, as holds true for the immunofluorescence protocol described earlier (Küpper *et al.*, 1990). This permits the convenient detection of overexpression of the enzyme following cell transfection. In the experiment shown in Fig. 1A and B we electrotransfected the expression plasmid pPARP31 into CO60 hamster cells, in order to overexpress transiently the human enzyme. For immunocytochemical detection, formaldehyde fixation (see Materials and methods) turned out to be better suited than the ethanol or the trichloroacetic acid fixation protocols which we had described previously for the immunofluorescence detection of poly(ADP-ribose) polymerase (Küpper *et al.*, 1990). Since the activity of this enzyme can be reconstituted in ethanol-fixed cells (see below), DNA strand breaks must be formed during fixation which are recognized by the zinc fingers of the enzyme. We speculate that this reduces the number of free epitopes for the anti-FII serum, while there should be no DNA binding of the enzyme during formaldehyde fixation. The micrograph in Fig. 1A shows two cells with strong nuclear signals as compared with nontransfected neighbouring cells, indicative of overexpressed enzyme. Similar results were obtained with indirect immunofluorescence performed after the traditional ethanol fixation protocol (Fig. 1B). It is also possible to use anti-poly(ADP-ribose) polymerase immunofluorescence in combination with formaldehyde fixation, which results in increased sensitivity (data not shown).

Immunocytochemistry to detect poly(ADP-ribose)

In many situations it is useful to visualize the enzyme reaction product, poly(ADP-ribose), *in situ*. We have employed two different procedures: first, enzyme activity was reconstituted in ethanol-fixed CV-1 cells by incubating the cell ghosts with NAD^+. Detection of ADP-ribose polymer produced in cell ghosts is shown in Fig. 1E and F by immunoperoxidase and immunofluorescence, respectively. By contrast, when NAD^+ was omitted during incubation of cell ghosts, no polymer-specific staining was detectable, as shown in Fig. 1C and D. Second, we treated living CV-1 cells with the alkylating agent MNNG to introduce DNA strand breaks and then fixed the cells with trichloroacetic acid for the subsequent *in situ* detection of polymer accumulated in living cells (Küpper *et al.*, 1990; Bürkle *et al.*, 1993). Detection of poly(ADP-ribose) synthesized in living cells after treatment with 50 μM MNNG is shown in Fig. 1G and H. If MNNG treatment was omitted, no polymer-specific staining was detectable (data not shown). We found that the sensitivity of immunofluorescence and immunoperoxidase labelling was comparable, the lowest MNNG dose for the induction of detectable amounts of polymer being about 10 μM in these cells for both methods (data not shown).

Ding *et al.* (1992) described a protocol for immunocytochemical detection of poly(ADP-ribose) polymerase using the biotin/streptavidin system in combination with alkaline phosphatase. Surprisingly, we have not been able to detect the overexpressed enzyme with a secondary antibody directly coupled with alkaline phosphatase, while it was clearly detectable by a secondary antibody directly coupled with peroxidase (Fig. 1). Poly(ADP-ribose) polymerase and its reaction product, poly(ADP-ribose), are nuclear antigens. It is conceivable that under the conditions used here, phosphatase-coupled antibodies do not have good access to the nuclear compartment(s) of these antigens, in contrast to peroxidase antibody conjugates. This may possibly be related to the smaller size of peroxidase.

By using the protocols described here, mass screening of clones which have been transfected to overexpress either poly(ADP-ribose) polymerase or its DNA-binding domain can be conveniently performed. The immunocytochemical detection of poly(ADP-ribose) may be used to screen for DNA-damaging agents which stimulate poly(ADP-ribose) polymerase activity in cells, or as a functional assay, to screen for cell clones with up- or down-regulated poly(ADP-ribosyl)ation.

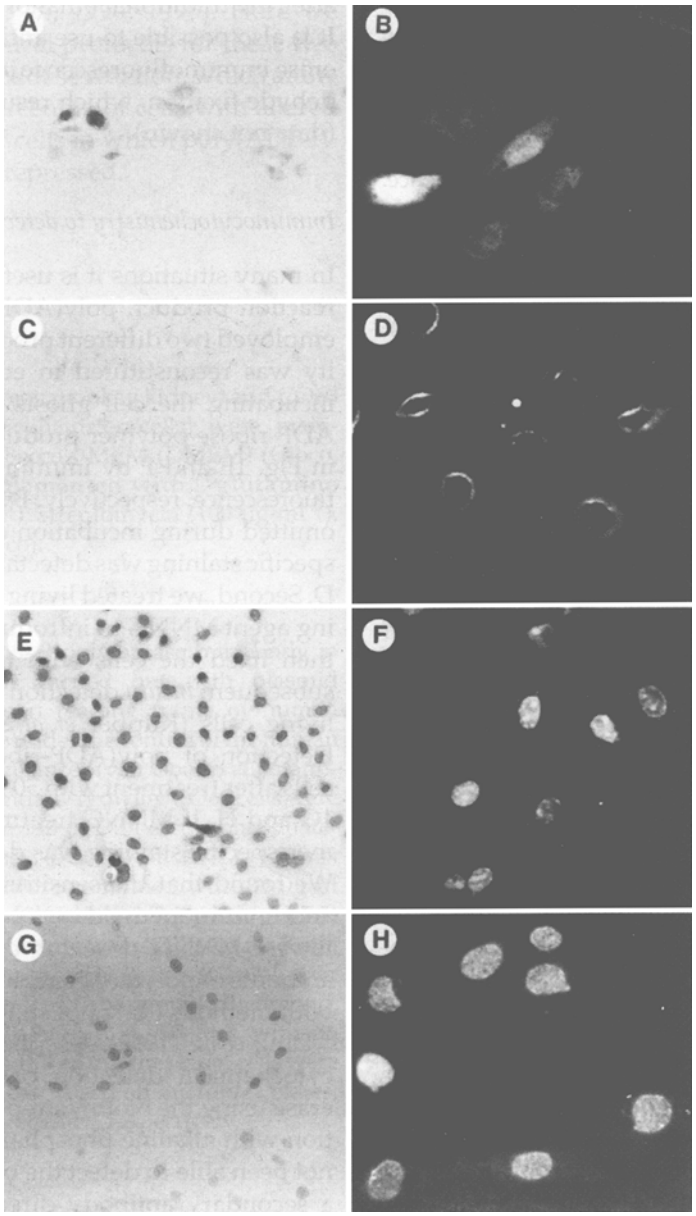

Fig. 1. Immunodetection of overexpressed poly(ADP-ribose) polymerase and of poly(ADP-ribose). Immunocytochemistry (left) and immunofluorescence analysis (right) was performed to detect the enzyme overexpressed in CO60 cells after transfection of pPARP31 (A, B), or to detect poly(ADP-ribose) either synthesized in (untransfected) ethanol-fixed CV-1 cells during NAD^+ incubation (E, F) or synthesized in living CV-1 cells after treatment with 50 μM MNNG (G, H). Ethanol-fixed control cells were incubated in buffer without NAD^+ (C, D). Detection of overexpressed enzyme was performed with anti-FII serum, and detection of poly(ADP-ribose) was performed with monoclonal antibody 10H. Peroxidase-conjugated immunoglobulins were used as secondary antibodies for immunocytochemistry. Immunofluorescence detection was performed with immunoglobulins conjugated with tetramethylrhodamine isothiocyanate (TRITC) (poly[ADP-ribose] polymerase detection) and immunoglobulins conjugated with Fluorescein isothiocyanate (poly[ADP-ribose] detection), respectively, as described in Materials and methods. (A, C, E and G, ×80; B, D, F and H, ×250)

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