Characterization of Human Poly(ADP-ribos)e Polymerase with Autoantibodies*

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The addition of poly(ADP-ribose) chains to nuclear proteins has been reported to affect DNA repair and DNA synthesis in mammalian cells. The enzyme that mediates this reaction, poly(ADP-ribose) polymerase, requires DNA for catalytic activity and is activated by DNA with strand breaks. Because the catalytic activity of poly(ADP-ribose) polymerase does not necessarily reflect enzyme quantity, little is known about the total cellular poly(ADP-ribose) polymerase content and the rate of its synthesis and degradation. In the present experiments, specific human autoantibodies to poly(ADP-ribose) polymerase and a sensitive immunoblotting technique were used to determine the cellular content of poly(ADP-ribose) polymerase in human lymphocytes. Resting peripheral blood lymphocytes contained $0.5 \times 10^8$ enzyme copies per cell. After stimulation of the cells by phytohemagglutinin, the poly(ADP-ribose) polymerase content increased before DNA synthesis. During balanced growth, the T lymphoblastoid cell line CEM contained approximately $2 \times 10^8$ poly(ADP-ribose) polymerase molecules per cell. This value did not vary by more than 2-fold during the cell growth cycle. Similarly, mRNA encoding poly(ADP-ribose) polymerase was detectable throughout S phase. Poly(ADP-ribose) polymerase turnover over at a rate equivalent to the average of total cellular proteins. Neither the cellular content nor the turnover rate of poly(ADP-ribose) polymerase changed after the introduction of DNA strand breaks by gamma irradiation. The results showed that in lymphoblastoid cells poly(ADP-ribose) polymerase is an abundant nuclear protein that turns over relatively slowly and suggest that most of the enzyme may exist in a catalytically inactive state.

Poly(ADP-ribose) polymerase is a nuclear enzyme that catalyzes the transfer of ADP-ribose from NAD to nuclear proteins, including the enzyme itself (see Refs. 1 and 2). This post-translational modification reaction has been postulated to influence DNA repair (3, 4), DNA synthesis (5), cell differentiation (6), and cell transformation (7) by modulating the interaction between structural proteins and DNA (8) or by regulating the activities of nuclear enzymes (9). However, the amounts of poly(ADP-ribose) polymerase in intact cells and the regulation of enzyme synthesis and degradation have been difficult to measure precisely. The catalytic activity of poly(ADP-ribose) polymerase absolutely requires DNA and is stimulated strongly by DNA with strand breaks (10). Furthermore, NAD poorly penetrates cell membranes and is also metabolized by routes other than poly(ADP-ribosylation). For all these reasons, the relation between cellular levels of poly(ADP-ribose) polymerase and the catalytic activity of the enzyme has not been clearly established.

Poly(ADP-ribose) polymerase has been purified from several sources and characterized extensively. Polyclonal or monoclonal antibodies have been raised against the purified calf enzyme (11, 12) and the human enzyme (13). Although the catalytic properties of poly(ADP-ribose) polymerase are well conserved among mammals, the antigenic specificities differ from species to species (13). Thus, previous reports showed that polyclonal rabbit antibodies against calf thymus poly(ADP-ribose) polymerase reacted only weakly with the human enzyme (13).

Recently, we have identified specific human autoantibodies to poly(ADP-ribose) polymerase in the sera of patients with rheumatic disorders (14). The human autoantibodies recognized multiple determinants on the protein and reacted indistinguishably with the calf and human enzymes. These high titer and high affinity human autoantibodies allowed us to isolate poly(ADP-ribose) polymerase-specific cDNA from a human placental expression library. In this study, we have used the anti-poly(ADP-ribose) polymerase autoantibodies to quantitate the immunoreactive levels of poly(ADP-ribose) polymerase in various human cells under changing culture conditions.

EXPERIMENTAL PROCEDURES AND RESULTS

DISCUSSION

These experiments have analyzed for the first time the content of poly(ADP-ribose) polymerase in human cells using specific immunologic methods. The poly(ADP-ribose) polymerase levels were determined by a sensitive immunoblotting procedure with high affinity human autoantibodies. Prior to immunoblotting, cells were rapidly solubilized by boiling in Laemmli's sample buffer containing 2% SDS. Under these conditions, poly(ADP-ribose) polymerase was released from

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2 Portions of this paper (including "Experimental Procedures," "Results," and Figs. 1-7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
3 The abbreviations used are: SDS, sodium dodecyl sulfate; PBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; kb, kilobase pairs.

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the nuclear matrix, and enzyme proteolysis was minimal. This result is consistent with earlier findings that demonstrated the complete release of proteins from the nuclear matrix following SDS extraction (15). Furthermore, the rapid solubilization procedure minimized DNA damage and thereby prevented the attachment of high molecular weight ADP-ribose polymers to the poly(ADP-ribose) polymerase protein. For these reasons, the immunoblotting technique was preferable to immunoprecipitation or enzyme-linked immunosorbent assay for the quantitation of enzyme protein in crude extracts. However, immunoprecipitation was useful for the comparative measurements of poly(ADP-ribose) polymerase turnover under changing culture conditions. With these methods, we were able to show that 1) poly(ADP-ribose) polymerase is an abundant nuclear protein, 2) the total immunoreactive cellular content of poly(ADP-ribose) polymerase varies by not more than 2-fold during the growth cycle of proliferating cells, and 3) the turnover rate of poly(ADP-ribose) polymerase is approximately equivalent to the average turnover rate of total cellular protein.

Previous experiments have measured changes in the cellular content of poly(ADP-ribose) chains during the cell growth cycle. These experiments showed that levels of the polymer reached a maximum during G2/M phase (1, 15-19). However, the rate of poly(ADP-ribose) synthesis does not necessarily reflect poly(ADP-ribose) polymerase content, but also depends upon the state of enzyme activation by DNA. The activity of poly(ADP-ribose) polymerase in permeabilized cells following DNase I treatment has been used to estimate the total intracellular poly(ADP-ribose) polymerase, because nicked DNA highly stimulates enzyme activity (3). We also observed a significant correlation between the amounts of poly(ADP-ribose) polymerase determined by immunoblotting and the DNase I inducible poly(ADP-ribose) polymerase activity. However, the changes in DNase I activatable poly(ADP-ribose) polymerase during the cell growth cycle were approximately 2-fold more than the changes in the total immunoreactive enzyme. Recent reports have suggested that some poly(ADP-ribose) polymerase molecules may be tightly bound to the nuclear matrix (20, 21). Changes in the structure and composition of chromatin during the cell cycle would be expected to influence poly(ADP-ribose) polymerase activity. Thus, it may be the interaction of poly(ADP-ribose) polymerase with DNA, rather than the levels of enzyme, that principally regulates poly(ADP-ribose) synthesis during the cell growth cycle.

The amounts of poly(ADP-ribose) polymerase per cell increased to a much greater degree following stimulation of peripheral blood lymphocytes with phytohemagglutinin than during the passage of cultured CEM or WI-L2 lymphoblastoid cells from G1 to G2/M phase. Moreover, the poly(ADP-ribose) polymerase content in the mitogen-stimulated lymphocytes increased to a much greater degree following stimulation of HeLa cells has been reported to be 78,000 molecules/s/cell (27), which is equal to 7.7 pmol of NAD/min/10^6 cells. Thus, it appears that the pace of NAD turnover in human cells is only about 1% of the maximal rate of poly(ADP-ribose) synthesis. The relative abundance of cellular poly(ADP-ribose) polymerase is in accord with the slow turnover rate of the enzyme and with the apparent stability of poly(ADP-ribose) polymerase following activation of the enzyme by irradiation of DNA. During balanced growth, cultured lymphoblasts contain more than enough poly(ADP-ribose) polymerase than that which is required for poly(ADP-ribose) synthesis.

Poly(ADP-ribose) polymerase has been postulated to function as an emergency enzyme that is stimulated specifically by DNA damage (28). The activation of the enzyme has been shown to influence DNA-histone interactions. A sudden increase in poly(ADP-ribose) synthesis can also lead to the rapid consumption of NAD and ATP, and thereby can prevent or delay cell proliferation (28, 29). Perhaps the large storage pool of poly(ADP-ribose) polymerase, which is maintained in lymphoblasts throughout the cell cycle, facilitates the rapid activation of the enzyme at individual sites of DNA strand break formation. Considering the relatively large amounts of poly(ADP-ribose) polymerase in the nucleus, it is also possible that the enzyme plays a structural role in the formation of chromatin and that the poly(ADP-ribose)ylation reaction modifies chromatin structure by changing the interaction of the protein with DNA.

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Supplemental Material to
Characterization of human poly(ADP-ribose)polymerase with antibodies.

M. D. Dibner, A. N. Battey, J. F. Towbin, H. Staehelin, T., and Gordon, J. (1976) Biochemistry 15, 1215-1220

Methods: 1. Homogenate of cultured cells. 2. Cell lysates. 3. In vitro translation. 4. In vivo labeling. 5. In situ hybridization.

Results: 1. Homogenate of cultured cells. 2. Cell lysates. 3. In vitro translation. 4. In vivo labeling. 5. In situ hybridization.

Conclusions: 1. Homogenate of cultured cells. 2. Cell lysates. 3. In vitro translation. 4. In vivo labeling. 5. In situ hybridization.

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Legends: 1. Homogenate of cultured cells. 2. Cell lysates. 3. In vitro translation. 4. In vivo labeling. 5. In situ hybridization.

Additional notes: 1. Homogenate of cultured cells. 2. Cell lysates. 3. In vitro translation. 4. In vivo labeling. 5. In situ hybridization.

Appendix: 1. Homogenate of cultured cells. 2. Cell lysates. 3. In vitro translation. 4. In vivo labeling. 5. In situ hybridization.
Autoantibody-reactive Human Poly(ADP-ribose) Polymerase

**RESULTS**

Distribution of Poly(ADP-ribose) polymerase in HeLa Cells. Before estimating the cellular content of poly(ADP-ribose) polymerase (PARP), we determined the amount of the enzyme by using a rocket immunoelectrophoresis assay, since previous reports have indicated that PARP binds tightly to nuclear proteins (13). The sedimentation of cells directly in Lammel’s sample buffer, which contains 7 M urea and 2 M guanidinium, followed by brief sonication, yielded recoveries of poly(ADP-ribose) polymerase and less proteinase, even after repeated freezing and thawing, or homogenization combined with phase 1 digestion and high-speed ultracentrifugation (data not shown). Thus, the former method was used to release poly(ADP-ribose) polymerase in subsequent immunoblotting experiments.

Poly(ADP-ribose) polymerase purified from cells of human cytoplasm (Figure 1) and human lymphoid cell lines. Human serine/threonine poly(ADP-ribose) polymerase reacted equally with both stains (data not shown).

**Fig. 1:** Purification of Poly(ADP-ribose) polymerase from HeLa Cells. After each step of purification, protein was fractionated on SDS-polyacrylamide gels and transferred to nitrocellulose paper. The autoradiographs were then scanned using a laser densitometer (LKB Ultrascan XL). The electrophoretic mobilities of the major bands were compared with those of markers (kDa) and with those of markers (kDa).

**Fig. 2:** Identification of Poly(ADP-ribose) polymerase in human peripheral blood lymphocytes. A standard curve for poly(ADP-ribose) polymerase was established based on the densitometric determination of purified human peripheral blood lymphocytes. Poly(ADP-ribose) polymerase catalytic activity, as well as the synthesis of DNA and RNA, were estimated at the same time points. As shown in Figure 3, the amount of poly(ADP-ribose) polymerase per cell increased approximately four-fold after the addition of 1 nM of DNA polymerase (Panel A). The amounts of polymerase that were incorporated into DNA and RNA in the presence of 1 nM of DNA polymerase were used as a substrate for further experiments. After 24 h incubation, the amount of polymerase that was incorporated into DNA and RNA increased approximately 300-fold (Panel B). Interestingly, the increase in poly(ADP-ribose) polymerase content proceeded at the rate of 2 nM per hour (Panel C).

The amounts of immunoassay poly(ADP-ribose) polymerase were compared to the amounts of catalytically active enzyme in homogenates of HeLa cells, with or without treatment with brefeldin A. The poly(ADP-ribose) polymerase activity was then subjected to a poly(ADP-ribose) polymerase (PARP) assay using 1 nM of DNA polymerase (Panel A). As shown in Figure 4, the amount of immunoassay poly(ADP-ribose) polymerase in homogenates of HeLa cells treated with brefeldin A was approximately 1.5-fold greater than that of control cells (Panel B). This result is consistent with previous reports (1,13), the immunoassay PARP activity increased from 1.5-fold to 3.0-fold by 3 days after stimulation, at which time the synapses were also maximal.

**Fig. 3:** Poly(ADP-ribose) polymerase in Physiological Activated Peripheral Blood Lymphocytes. Panel A: Peripheral blood lymphocytes were stimulated with phytohemagglutinin (2.5 μg/ml), and every other day, 1 X 10⁶ cells were harvested for measurement of catalytically active enzyme (Panel A). Panel B: Immunofluorescence staining pattern of poly(ADP-ribose) polymerase in HeLa cells treated with brefeldin A and cycloheximide (upper panel), and with cycloheximide (lower panel). Panel C: Immunofluorescence staining pattern of poly(ADP-ribose) polymerase in HeLa cells treated with brefeldin A and cycloheximide (upper panel), and with cycloheximide (lower panel).

**Fig. 4:** Poly(ADP-ribose) polymerase in Synaptosomal Cell Lines. Human lymphoblastoid CM cells were incubated with 10 μM of phosphatidylcholine (Panel A). After 24 h incubation, the CM cell line was incubated with 10 μM of phosphatidylcholine (Panel B). The amount of poly(ADP-ribose) polymerase increased approximately 2.5-fold after 24 h incubation with 10 μM of phosphatidylcholine (Panel C). The amount of poly(ADP-ribose) polymerase increased approximately 2.5-fold after 24 h incubation with 10 μM of phosphatidylcholine (Panel D).

Flow Cytometric Analysis: To avoid any potential artifacts associated with cell synchronization, a flow cytometric analysis was developed for the detection of poly(ADP-ribose) polymerase in single cells. The amount of poly(ADP-ribose) polymerase during the cell cycle was determined by synchronization of the single-cell line by propidium iodide (PI) staining (Panel A). The amount of poly(ADP-ribose) polymerase in CM cells and lymphoblastoid cells was comparably constant during the cell cycle. To determine the amount of antibody fluorescence in CM cell lines, we used the cell synchronization (Panel B).
Autoantibody-reactive Human Poly(ADP-ribose) Polymerase

Turner et al. (1983) studied the effects of DNA damage on cellular poly(ADP-ribose) polymerase. They found that the enzyme was induced by DNA damage and that the induction was dose-dependent. The enzyme was also found to be induced by X-irradiation and by certain chemotherapeutic agents. The enzyme was shown to be involved in the repair of DNA damage and to be a key component of the cellular response to DNA damage.

Fig. 5: Flow Cytometric Analysis of Poly(ADP-ribose) polymerase. Contour plots of DNA, stained with propidium iodide, were analyzed by flow cytometry. The percentage of cells with an abnormal DNA content was determined.

Fig. 6: Synthesis and Turnover of Poly(ADP-ribose) polymerase. Panel A: Human T lymphoblastoid cells were incubated with [3H]nicotinamide for 0 (lane 1), 1 hr (lane 2), 4 hr (lane 3), and 24 hr (lane 4) and then lysed in NP-40 buffer. Panel B: Same cells were incubated for 20 hours, then chased with cold nicotinamide. After 5 hr (lane 1 and 2), 5 hr (lane 3), and 24 hr (lane 4), cells were lysed. In both experiments, equal amounts of protein from the cell extracts were immunoprecipitated with the patient's serum containing anti-poly(ADP-ribos e) polymerase antibodies (lanes 1-3) or with normal human serum (lane 4). The immunoreactive 115 kDa bands were detected by autoradiography and compared to the total acid precipitable radioactivity (C).