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POLY(ADP-RIBOSE) POLYMERASE ACTIVITY IN VARIOUS U937 CELL SUBCLONES WITH DIFFERENT SUSCEPTIBILITY TO HIV-1 INFECTION : ITS DRAMATIC DECREASE FOLLOWING PERSISTENT VIRUS INFECTION*

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Abstract : Poly(ADP-ribose) polymerase, a nuclear enzyme, is suggested to be involved in apoptotic cell death. It is also known that apoptotic cell death following HIV-1 infection is the most important feature of AIDS pathogenesis. Thus, to evaluate the relations between the enzyme and HIV-1 infection, we examined the enzyme activity of several subclones of human promonocytic cell line U937, which showed different susceptibility to HIV-1 infection. The nuclear extracts of two "high type clones" (possessing high susceptibility to HIV-1 infection) contained approximately 4 to 7-fold less enzyme than two low type clones when assayed under a full activation of enzyme. Parent clone, possessing an intermediate susceptibility to HIV-1, showed an intermediate enzyme level, suggesting that low level of this enzyme in cells is important for an effective infection of HIV-1. Furthermore, when these U937 subclones persistently infected with HIV-1 were examined, a dramatic decrease of the enzyme activity, reaching 2 to 16% of uninfected cells, was observed in all of these clones. The levels of poly(ADP-ribose) glycohydrolase in these clones were relativity unchanged. Activity gel analysis and immunoblotting of the enzyme in the clones revealed that the low enzyme activities observed in uninfected "high type clones" and all HIV-1-infected clones were due to a marked decrease of the enzyme protein itself. All of these results suggest that HIV-1 infection involves some mechanism to downregulate cellular poly(ADP-ribose) polymerase and that a lower level of the enzyme may be essential for an effective production of the virus and/or for a stable virus / host interaction.

Human immunodeficiency virus type 1 (HIV-1) infection causes immunodysfunction, as indicated by a reduction in the number of CD4+ cells including T lymphocytes and monocyte / macrophage: Although the mechanism of HIV-induced cell decline is not fully understood, syncytium formation, intracellular accumulations of unintegrated viral DNA and of viral

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Abbreviations used are: PARP, poly(ADP-ribose) polymerase; HIV, human immunodeficiency virus; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethylether)- N, N, N',N'-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate.

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envelope proteins, and destruction of cell membrane integrity caused by the release of virus particle were suggested to be the cause of the cell death (1). Accumulating evidence, however, suggests that apoptotic cell death of uninfected CD4+ T cells, induced by a concerted effect of various HIV-infected cells including macrophage and monocyte, may be the most important feature of AIDS pathogenesis (2).

On the other hand, recent findings on biological function of poly(ADP-ribose) polymerase (PARP : see reviews 3,4) have shown that this enzyme is involved in some types, at least, of apoptotic cell death although the exact role of the enzyme in the process has not been fully understood yet: several reports suggested that catalytic activity of PARP was required for a process of apoptosis of cells (5 - 8), while others suggested that degradation of PARP protein by an ICE-like protease (9, 10) was relevant to apoptotic pathway.

Recently, we isolated several subclones from human promonocytic cell line U937 and classified them into three types, high-, middle-, and low-types, in terms of HIV-1 replication kinetics such as appearance of intracellular HIV-1 antigens and extracellular reverse transcriptase activity (11). In order to examine the relation between HIV-1 infection and PARP we measured PARP activity in these clones and found that PARP activity was inversely correlated with the HIV-1 susceptibility and, more interestingly, persistent infection of HIV-1 dramatically decreased the enzyme of these clones, suggesting that the decrease of PARP might be quite important for the virus production and/or for a stable virus / host interaction.

MATERIALS AND METHODS

Materials [Adenosine-2,8-^{3}H]NAD^{+} and [adenylate-^{32}P]NAD^{+} were purchased from ICN Biochemicals Inc., and New England Nuclear Dupont respectively. Calf thymus PARP and nicked DNA were prepared according to previous methods (12, 13). ^{3}H-Labeled poly(ADP-ribose) and polyclonal antibody against calf thymus PARP was prepared as described previously (14, 15).

Cell lines Four U937 subclones, 5H, 17H, 15L, and 26L were established from U937 cells based on a difference in their susceptibility to HIV-1 (LAI strain) infection. Details of the properties of these clones will be described elsewhere (11). These cells were maintained in suspension culture in RPMI1640 medium supplemented with 10 % (V/V) fetal bovine serum at 37℃ in a humidified atmosphere of 5% CO_2.

Preparation of nuclear extract Cells (3 - 4 x 10^{7}) were harvested by a low speed centrifugation (1,500 rpm for 4 min), washed twice with 30 ml of cold Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS(-)), and homogenized with 4 ml of a nuclei preparation buffer (20 mM Hepes, pH 7.4, 0.25 M sucrose, 5mM $MgCl_2$, 2mM DTT, 1mM EGTA, 1mM PMSF, 5 μg /ml of leupeptin, 1mM benzamidine HCl, 1mM NaN_3, and 0.2% Nonidet P40) in a Dounce homogenizer. The homogenates were centrifuged for 10 min at 600g and 4 ℃. The resulting nuclear pellets were washed once again with the same buffer. The nuclei were extracted with 1 ml of an extraction buffer (25 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine HCl, 10 μg/ml leupeptin, and 10 % glycerol) containing 0.35 M NaCl. The sample was centrifuged at 140,000 g for 1 h, and the supernatant was used as nuclear extract.

Assay for PARP activity Assay was principally carried out according to our previous method (12). An appropriate amount of nuclear extracts was incubated in a reaction mixture (25 mM Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2$, 0.5 mM DTT, 20 μM [^{3}H]NAD^{+} (25 cpm/ pmol), and 0.2 μg of nicked DNA) in a total volume of 0.2 ml. After incubation at 25 ℃ for

10 min, the reaction was terminated by the addition of 1 ml of cold 5 % trichloroacetic acid (TCA), and the acid-insoluble radioactivity was measured as described previously (12).

Assay for poly(ADP-ribose) glycohydrolase The enzyme activity of nuclear extract was measured as follows. An appropriate amount of nuclear extract was mixed with a reaction mixture containing 50 mM potassium phosphate buffer, pH 7.4, 10 mM 2-mercapto-ethanol, 0.1 mM PMSF , 50 μg/ml of bovine serum albumin, and 25 μM [3H]poly (ADP-ribose) (2,730 cpm/nmol ADP-ribose) in a total volume of 0.2 ml. The reaction was carried out at 37 ℃ for 10 min, and terminated by the addition of 1 ml of cold 5% TCA. The enzyme activity was expressed as acid-soluble ADP-ribose formed per 10 min.

Activity gel analysis for PARP The activity gel analysis of PARP in nuclear extract was carried out according to the procedure described by Scovassi *et al.*(16).

Immunoblotting The analysis of PARP of nuclear extracts by immunoblotting with rabbit polyclonal antibody raised against purified calf thymus PARP was carried out as described previously (15).

Other procedures Protein concentration of nuclear extract was measured by using Bio Rad Protein Assay Kit. Analysis of autoradiogram was carried out by ATTO densitograph system.

RESULTS

PARP activity of U937 subclones. Recently we established several U937 subclones, which were different in the susceptibility to HIV-1 infection from parent U937 cells.

When HIV-1 infection was monitored by indirect immunofluorescence (IF) test with the use of the serum from an HIV-1-seropositive patient, 100% of the cells of high susceptible clones ("high clones"), 5H and 17H, were IF-positive at day 12 after HIV-1 infection, whereas less than 0.1% of "low clones", 15 L and 26 L, were IF-positive by the time. All of these clones expressed almost the same level of CD4 as the parent U937 cells. Details of the procedure for the cell cloning and other properties of these U937 cell subclones will be described elsewhere (11).

In an attempt to elucidate the basis for different susceptibility of these clones to HIV-1 infection , we isolated cell nuclei and examined PARP activity of the nuclear extracts. As shown in Table 1 , total PARP activity , when assayed under a fully activated condition using excess nicked DNA (17), showed a marked difference between " high clones " and " low clones " : PARP activities of 5H and 17H clones were approximately 5-fold less than those of 15L and 26L clones. Parent U937 cells, which possessed intermediate susceptibility to HIV-1 infection, showed an intermediate level of PARP activity, suggesting that the enzyme levels in these cells are inversely correlated with their HIV-1 susceptibilities.

Poly (ADP-ribose) glycohydrolase activity was almost the same among these clones except that 5H showed approximately 2-fold higher activity than other 3 clones (Table 2), suggesting that the low PARP activities in 5H and 17H clones are not the result of the underestimation of the PARP activity caused by contaminating poly(ADP-ribose) glycohydrolase.

Marked decrease of PARP in cells persistently infected with HIV-1 . When U937 subclone cells were infected with HIV-1 and maintained, after transient cytopathogenicity, for over 6 months, all of the clones restored an exponential growth under a state of the persistent infection of HIV-1 (11). When these cells were examined, PARP activities in

Table 1. Poly(ADP-ribose) polymerase activity of uninfected and HIV-1-infected U937 subclones.

subclone	total PARP activity* (nmol/mg protein)	
	uninfected	HIV-1-persistently infected
5 H	7.6	0.4
17 H	9.9	0.6
15 L	58.4	9.7
26 L	32.3	0.7
parent	20.0	—

*About 5 μg protein of nuclear extracts was assayed for PARP activity as described under "Materials and Methods".

nuclear extracts from all of the four subclones tested were strikingly decreased (Table 1). The percent ratio of the enzyme activity of infected cells to that of the uninfected cells were 5.3 (5H), 6.1 (17H), 16.6 (15L), and 2.2% (26L), respectively.

Activity gel analysis . To avoid possible intervention of poly(ADP-ribose) degrading activity and some PARP degrading enzyme during the PARP activity assay with a conventional method, we carried out activity gel analysis according to the method by Scovassi *et al.* (16). As shown in Fig. 1, the enzyme activities in all clones detected by this method were in fair agreement with those assayed by conventional method (Table 1), indicating that lower PARP activities in uninfected 5H and 17H clones, and all HIV-1-infected clones were due to the decrease of active PARP molecule itself.

Table 2. Poly(ADP-ribose) glycohydrolase activity of U937 subclones

subclone	Poly(ADP-ribose) glycohydrolase activity* (nmol/mg protein)	
	HIV-1-uninfected	HIV-1-persistently infected
5 H	153.4	143.1
17 H	86.2	149.0
15 L	91.1	160.0
26 L	81.2	159.0

* Ten μg protein of nuclear extracts was assayed for poly(ADP-ribose) glycohydrolase activity as described under "Materials and Methods".

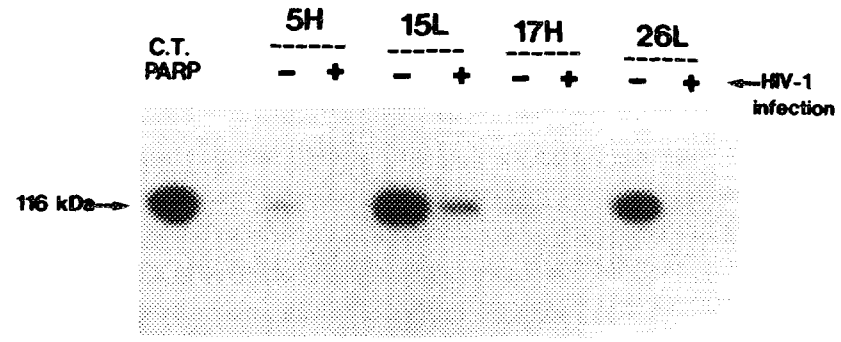

Fig.1. Activity gel analysis of PARP in nuclear extracts.
Nuclear extracts (containing 2.4 μg protein) from U937 subclones 5H, 15L, 17H, and 26L with (+) and without (-) HIV-1 infection were electrophoresed at 60V for 3h on SDS polyacrylamide gel (7.5% gel). After the electrophoresis, the gel was subjected to an activity gel analysis according to the method described by Scovassi et al. (16). Purified calf thymus PARP (C.T. PARP, 55ng) was used as a control. The position of purified C.T. PARP (116 kDa) is indicated by an arrow.

Immunoblot analysis . In order to determine whether the marked difference of PARP activity among various U937 subclones with or without HIV-1 infection is due to the decrease of PARP protein or not, we carried out immunoblot analysis using polyclonal antibody raised against calf thymus PARP. As shown in Fig. 2, the density of PARP bands at 116 kDa in 8 samples from U937 subclones with and without HIV-1 infection approximately paralleled the enzyme activities of these clones shown in Table I, indicating that the decrease of PARP in 5H, 17H, and HIV-1-infected subclones was mainly due to the decrease of PARP molecule itself rather than due to the presence of some inhibitory activity or some modification of the enzyme.

DISCUSSION

Present results clearly demonstrated : (1) the susceptibility of U937 cell clones to HIV-1 infection is inversely correlated with their PARP activity, (2) persistent HIV-1 infection

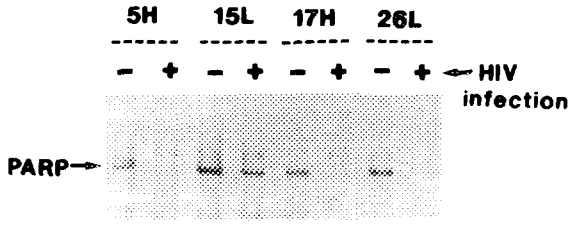

Fig. 2. Immunoblotting of PARP in nuclear extracts.
Nuclear extracts (1.5 μg protein) of U937 subclones with (+) and without (-) HIV-1 infection were analyzed by immunoblotting as described under " Materials and Methods". The arrow indicates the position of calf thymus PARP (116 kDa).

results in a dramatic decrease of the enzyme activity in all U937 subclones tested, and (3) the decrease is mainly due to the decrease of the enzyme molecule itself rather than due to modifications suppressing the catalytic activity such as phosphorylation of the enzyme (18).

Since U937 subclones with a high susceptibility to HIV-1 infection had a tendency to contain a decreased level of PARP, a lower PARP level, may benefit the expression of the virus gene or the virus production in an early phase of the virus infection. On the other hand, a prolonged and sustained downregulation of PARP observed in all of the cells persistently infected with HIV-1 suggests that the cells with a markedly suppressed PARP level may have an advantage in surviving after HIV-1 infection and in maintaining a stable virus / host interaction, since the persistently infected U937 clones could continue a stable, exponential proliferation for over several months, expressing HIV-1 markers (11). Thus, a marked downregulation of cellular PARP seems to be related to prevention of the death of cells infected with HIV-1. This hypothesis is compatible with our recent observation that two HIV-1 sensitive clones, 5H and 17H, which possessed a low PARP activity, were much more resistant to TNF- α mediated apoptosis than the two low sensitive, 15L and 26L clones, while all of these clones expressed almost the same amount of TNF-α receptor (M. Kameoka, unpublished results). In addition to the above observation, there are several reports suggesting that PARP activity is required for a process of apoptosis and the inhibition of this enzyme blocks apoptosis (5 - 8), although other reports suggest that degradation of PARP itself is involved in apoptotic signalling pathway (9, 10). Thus, HIV-1 may have some mechanism to decrease PARP of host cells to protect them from apoptosis as a strategy for survival of the virus in persistently infected cells.

Concerning the biological significance of the presently observed persistent infection of HIV-1 in U937 cells of promonocytic origin, Schuitemaker *et al.* (19) have suggested a key role of the HIV-1 infection to monocytes/macrophage in AIDS pathogenesis by demonstrating that, in chimpanzee, which do not induce AIDS on HIV-1 infection in spite of the long term virus production, the monocytes were completely insusceptible to HIV-1 infection and possessed an intact antigen-presenting function even after a long term of the virus infection of the animals. Furthermore, accumulating evidence supports that apoptosis of uninfected CD4 positive T cells involving an inappropriate signal transduction by HIV-1-infected, antigen-presenting cells including monocytes is the major feature of AIDS pathogenesis(2, 20, 21).

Though the mechanism of downregulation of PARP in persistently infected cells with HIV-1 has not been fully studied yet, we have observed that HIV-1 infected clones, 15L and 26L cells, contained approximately 2- and 4-fold higher PARP degrading activity, respectively, than that of uninfected cells (Y. Tanaka, unpublished results). An increase of similar PARP degrading activity was also detected in uninfected, 15H and 17H clones. Although the precise characterization of this protease is under progress, the enzyme is of serine protease type, and seems to be different from a NFκB-hydrolyzing protease found by

Franzoso *et al.* (22), since the protease is increased in a U937 low type subclone, which replicates HIV-1 less efficiently.

Although the molecular mechanism of the correlation between the downregulation of PARP and HIV-1 infection is still unclear, following observations may be relevant to the presently observed change of cellular PARP : (1) The group of Taniguchi (23, 24) has demonstrated that a high level of cellular PARP strongly suppressed the IFN-γ-induced expression of MHC class II gene, suggesting a possibility that PARP decrease may be involved in the expression of some other genes involved in HIV-1-infection, and also, Mann *et al.*. (25) and Larcher *et al.* (26) have shown that, in U937 cells, HIV-1 infection induced MHC class II antigen; (2) Buki *et al.* (27) found that PARP inhibited reverse transcriptase *in vitro* by binding to RNA-DNA hybrid templates. Thus, PARP decrease may directly affect HIV-1 replication or it may affect expression of some cellular genes following HIV-1 infection.

Further characterization of the PARP cleaving enzyme, investigation of the exact metabolism of PARP in HIV-1-infected cells, and its biological meaning in HIV-1 infection and AIDS pathogenesis will be the subject of next coming communication. Recently, Ameisen *et al.* (2) suggested that one potential therapeutic strategy against AIDS may be to block the HIV-1-mediated apoptosis signal transduction pathway. Thus, our results may suggests PARP as one of the targets for this line of AIDS study.

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