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Poly-ADP-ribosylation of histone proteins of human kidney T1-cells *in vitro* following γ-irradiation

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Poly-ADP-ribosylation of cellular proteins is involved with radiation induced damage and its repair. It has been observed that suspension of human kidney T1-cells *in vitro* attained elevated levels of poly-ADP-ribosylation due to experimental manipulations necessary for preparation of single cell suspension from monolayer cell cultures. These cells in suspension were exposed to various doses of γ-rays with or without subsequent repair incubation. The PADPR of histones H3, H1 and H2B increased with increasing dose of radiation and decreased after 90 min of repair incubation. Concomitant with these changes, the affinity of histones to DNA in chromatin reduced immediately after irradiation. Normal affinity was reestablished after post-irradiation repair incubation. The results indicate that induction of poly-ADP-ribosylation of histone proteins by radiation and by manipulations to prepare single cell suspension involved different cellular components.

Poly-ADP-ribosylation (PADPR), a post-translational modification of proteins, involves successive condensation of ADP-ribosyl moieties from NAD^+ on various chromosomal proteins by poly-ADP-ribose polymerase (PADPRP)[1-3]. The precise physiological role of this reaction is not understood and several possibilities have been suggested[2,4-7]. PADPR is enhanced endogenously[8], or by radiation induced DNA strand breaks[2] or during DNA excision repair[4]. The dependence of PADPR on DNA strand breaks or nicks have been emphasized[4,8-13]. Though PADPRP is the main target of PADPR (automodification), reversible relaxation and condensation of chromatin[14,15] by hyper- and hypo-poly-ADP-ribosylation of histones, respectively, points out that these proteins are also targets of PADPR reactions (heteromodification)[16]. Recently developed model of Althaus[16] suggests that the automodification cycle shuttles dissociation-reassociation of histones with DNA. This influences unfolding-folding of chromatin and guides specific proteins to the DNA damage site for excision repair. Other models by Satoh and Lindahl[17] and de Murcia and de Murcia[8] for repair of DNA strand breaks suggest that automodification of the PADPRP causes a transient block of DNA replication and causes temporary cell cycle arrest. Consequently, the damaged DNA sites become available for repair. In this, the automodified PADPRP dissociates from DNA and makes the site of damage conspicuous. Simultaneously, heteromodification of histones leads to decondensation of chromatin providing accessibility of repair enzymes to the site of damage. These facilitate repair of DNA strand break. Schneeweiss *et al.*[18] have shown that T1-cells in suspension contained an elevated level of total cellular PADPR as compared to the basal metabolic level in a cell monolayer. Thus, when cell suspensions are γ-irradiated the resulting PADPR levels represent PADPR induced by manipulations necessary for preparation of cell suspension as well as by radiation. Because PADPR is enhanced by both interventions it is necessary to find out the genuine

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contribution of radiation induced PADPR that has bearing on DNA damage and repair[16]. In this report, PADPR of histone proteins of human kidney T1-cells *in vitro* has been studied in cell suspension exposed to ^{137}Cs γ-radiation on ice with and without repair incubation following irradiation.

Materials and Methods

Chemicals

All chemicals were of analytical grade and were used without further purification. [^{32}P]-NAD^+ (0.37 - 185 TBq/m*M*) was from NEN, UK.

Cell culture

Human kidney T1-cells were grown as described earlier[9]. The monolayer culture was washed with phosphate buffered saline (PBS), trypsinized (0.25% at 37°C for 10 min) and agitated to prepare single cell suspension of T1-cells in complete medium. The cells were stored on ice for the experiments.

Irradiation and repair incubation

^{137}Cs γ-radiation source (1.1 Gy/min) was used to irradiate T1-cell suspensions in complete medium on ice to 1.8, 30, 120, 240 and 486 cGy. Incubation of cells in complete medium at 37°C for 90 min has been defined as repair incubation.

Assay of poly-ADP-ribosylation

The method, described previously[9,18], was followed. In brief, $3\text{-}4\times10^6$ T1-cells as a suspension in complete medium, with or without irradiation, were collected by centrifugation (500×*g*, 2 min, 4°C) and suspended in a permeation (P) buffer (10 m*M* tris-HCl, *p*H 7.8/4°C; 1 m*M* EDTA; 4 m*M* $MgCl_2$ and 30 m*M* 2-mercaptoethanol) to obtain 2×10^7 cells/ml by permeation for 15 min on ice. The P buffer was removed by centrifugation and the cell pellet (50 µl) was added to 25 µl of a reaction (R) buffer (80 m*M* tris-HCl, *p*H 7.8/37°C and 1.2 m*M* [^{32}P]NAD^+ containing 111 kBq ^{32}P activity). The reaction was for 15 min at 37°C and terminated by the addition of 300 µl of denaturation buffer (final: 10 m*M* potassium phosphate buffer, *p*H 7; 2%; sodium dodecyl sulphate; 0.1 *M* dithiothreitol; 20% glycerol and 0.006% (w/v) bromophenol blue) and immersion of the tube containing the sample in a boiling water bath for 2 min.

Chromatin isolation and histone chromatography

Chromatin was isolated from T1-cell suspension by the method of Bonner *et al.*[19] with minor modification. Briefly, 3.5×10^8 cells in complete medium were homogenized in a glass homogenizer. The pellet collected by centrifugation (3,500×*g* for 30 min) was suspended in 5 ml of 0.85 *M* sucrose and rehomogenized. The homogenate was then centrifuged at 70,000×*g* for 60 min. The pellet was suspended in 10 ml saline EDTA (75 m*M* NaCl and 24 m*M* EDTA, *p*H 7.9) and washed twice by centrifugation (3,500×*g* for 15 min). The resulting pellet was suspended in 2 ml of 5 m*M* Tris-HCl buffer (*p*H 7.9), gently poured over 11 ml of 1.7 *M* sucrose and centrifuged (80,000×*g* for 180 min) after mildly disturbing the interphase. The chromatin pellet was suspended in 2 ml of TE buffer (10 m*M* Tris-HCl, *p*H 8 and 1 m*M* EDTA) and dialyzed overnight in 2 litres of 100 m*M* potassium phosphate buffer (*p*H 6.7) containing 0.63 *M* NaCl. Purity of chromatin preparation was determined by measurements of absorbance at 260 and 280 nm. Histone proteins were fractionated from this preparation on a 25 ml hydroxyapatite column following the method of Simon and Felsenfeld[20]. Accordingly, H1 eluted in the first, H2A + H2B in the second and H3 + H4 in the third major peak with 50 ml each of 100 m*M* potassium phosphate buffers (*p*H 6.7) containing 0.63 *M*, 0.93 *M* and 2.0 *M* NaCl, respectively. Each peak fraction was pooled, lyophilized, suspended in water and dialyzed extensively in water. The dialyzed samples were lyophilized and dissolved in TE buffer. The entire experiment was carried out at 4°C.

Gel Electrophoresis

A polyacrylamide gel (10 - 25% continuous gradient) was prepared following the method of Laemmli[21]. After electrophoresis the gel was fixed in 20% trichloroacetic acid, stained by coomassie brilliant blue and dried overnight under vacuum.

Autoradiography

The dried gel was used to expose Kodak scientific imaging film (X-Omatic) for 2 to 3 hr at

- 80°C in an autoradiography cassette with two regular intensifying screens. The optical densities of the exposed bands on the film were measured by a video densitometer (Bio-Rad; model 620 using 1-D Analyst software).

Results and Discussion

Enhancement of PADPR is dependent on, among others, DNA strand breaks[4,16]. Consequently, γ-radiation that is known to induce DNA strand breaks stimulated PADPR in T1-cells. Fig. 1A shows the autoradiographic pattern of PADPR of T1-cells in suspension before (lane 1) and after exposure to different doses of γ-radiation in full medium on ice (lanes 2 through 6). After a post-irradiation repair incubation PADPR attained significantly low levels (Fig. 1B; lanes 2 through 6). The non-irradiated T1-cell suspension (Fig. 1A; lane 1) after repair incubation revealed practically no PADPR (Fig. 1B; lane 1). It has been shown that interventions necessary to prepare single cell suspension from a monolayer culture enhanced cellular PADPR[18]. Therefore, the PADPR of T1-cell suspension after exposure to γ-rays (Fig. 1A, lane 2 through 6) represents a level that is a summation of PADPR induced by manipulations for preparation of cell suspension and by γ-radiation.

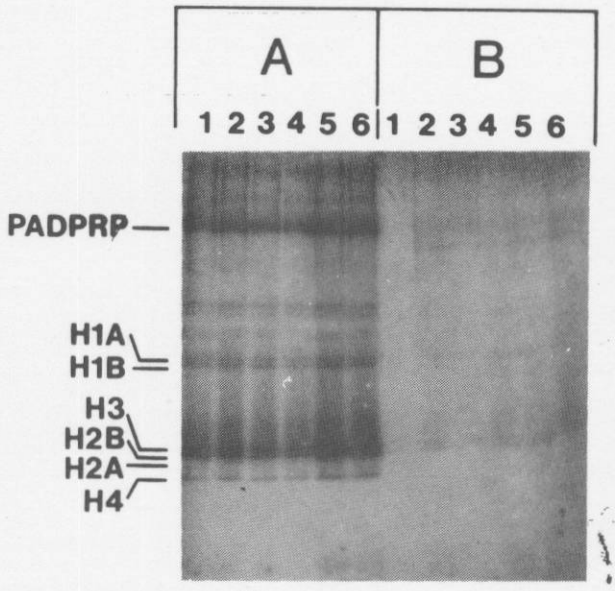


Fig. 1—Autoradiogram of poly-ADP-ribosylated T1-cell proteins [Panel A shows pattern of PADPR in non-irradiated T1-cell suspension (lane 1) and that after exposure of T1-cell suspensions to 1.8, 30, 120, 240 and 486 cGy of γ-radiation (lanes 2 through 6) respectively. Panel B depicts PADPR of the same samples after a post-irradiation repair incubation of 90 min at 37°C in complete medium. The main poly-ADP-ribosylated proteins have been identified]

The non-irradiated control cells (Fig. 1A; lane 1) and cells exposed to varying doses of radiation (Fig. 1A; lanes 2 through 6) showed high levels of PADPR. Upon repair incubation, the levels of PADPR reduced differently in the non-irradiated and irradiated cells. Unlike the non-irradiated control that was subjected to repair incubation (Fig. 1B; lane 1), a detectable level of PADPR persisted in all γ-irradiated T1-cell suspensions after repair incubation (Fig. 1B; lanes 2 through 6). This points out that there was qualitative difference in the trigger of PADPR initiated by manipulations for preparation of cell suspension and by γ-rays. In the former, repair incubation abolished the elevated level of PADPR completely while in the latter this was not so. The residual level of PADPR after post-irradiation repair incubation is indicative of continuance of part of trigger connected to radiation induced PADPR. The involvement of membrane components in PADPR response to physical interventions has been suggested[18,22] and association of chromatin (DNA) with radiation induced cellular PADPR reaction is known[4,9]. Because PADPR affects chromatin organization[14,15], the persistent low levels of PADPR after irradiation and subsequent repair incubation (Fig. 1B; lanes 2 through 6) show that radiation exposed chromatin remained in a relaxed state as compared to the non-exposed chromatin (Fig. 1B; lane 1)[23].

Fig. 2 graphs the mean of three quantitative analyses of main poly-ADP-ribosylated proteins after γ-irradiation (e. g., ^{32}P exposed bands on the autoradiograms in Fig. 1A) by a densitometer revealing γ-dose response curves for PADPR of histone proteins (heteromodification) and of PADPRP (automodification). The SD bars have not been plotted in Fig. 2 to avoid congestion and to highlight the trend. Among histones, H3 was the preferred target of PADPR followed by H1 and H2B (Fig. 2). This agrees with the observations of de Murcia *et al.*[8]. Because the PADPR is already at an elevated level in T1-cell suspension, any further, even marginal, increase of PADPR in

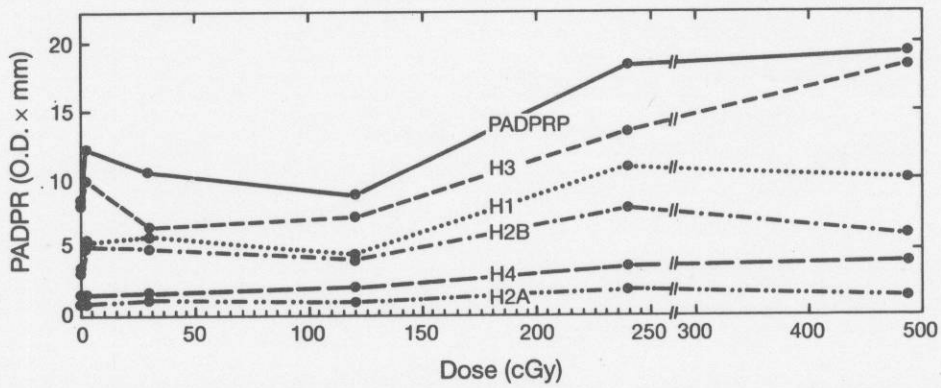
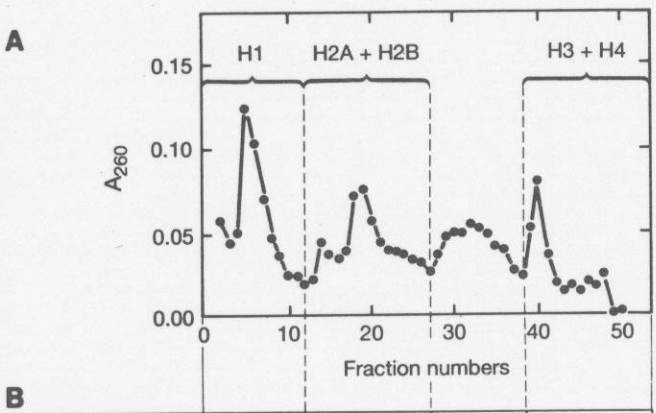


Fig. 2—Quantification of levels of poly-ADP-ribosylation (PADPR) of histone proteins and of poly-ADP-ribose polymerase (PADPRP) as a function of dose of γ-radiation.

response to γ-irradiation assumes significance. Therefore, small increase of PADPR in T1-cell suspension after γ-irradiation (Figs. 1-2) represents a significant change in cellular metabolism and, perhaps, repair. This interpretation is supported by several facts. At first, most optimal stimulation of PADPR is reported to enhance the level of cellular PADPR only up to 6-fold in eukaryotes[18,24]. This is in contrast to other known post-translational modifications of proteins. For instance, protein phosphorylation is reported to attain 63-fold higher level than its basal level upon stimulation[24]. Secondly, PADPR involves formation of polymers of rather large sized ADP-ribose moieties in contrast to other post-translational modification avenues. Thirdly, the turn-over rate of cellular PADPR reaction is extremely high (half-live = 1 min or less[25]). Therefore, it seems possible that even small changes in the level of PADPR may result in drastic change in the modified proteins[16] and, consequently, may exert significant metabolic influence[9].

That the observed increase in PADPR of histone proteins after irradiation affects histone affinity to DNA was corroborated by direct measurement of histone association with DNA. For this, chromatin prepared from suspensions of (a) normal non-irradiated cells, (b) cells exposed to 8 Gy of γ-radiation and (c) cells subjected to repair incubation after 8 Gy of γ-irradiation, were loaded on hydroxyapatite columns. Fig. 3A shows a representative elution profile of three subclasses of histone (H1, H2A + H2B and H3 + H4) from the loaded chromatin preparations. The quantities of



B

NaCl in 0.1M K-phosphate	0.63 M NaCl	0.93 M NaCl	1.2 M NaCl	2.0 M NaCl
Control	100 %	100 %	–	100 %
γ-irradiation (486 cGy)	78 %	88 %	–	77 %
γ-irradiation (486 cGy) + repair	113 %	115 %	–	121 %

Fig. 3—Elution profile of histone proteins from chromatin of T1-cell suspension through a hydroxyapatite column (A). Panel B shows the relative amounts (percentage of control) of eluted subclasses of histones from non-irradiated controls, γ-irradiated group (486 cGy) and γ-irradiated group after repair incubation of 90 min at 37°C in complete medium.

collected histone subclasses were reduced after exposure to 486 cGy of γ-radiation as compared to the controls and regained the control level or overshot after repair incubation (Fig. 3B). A relaxed state of chromatin organization due to hyper-PADPR and a condensed state due to hypo-PADPR has been reported in literature[12,14-16]. It appears that γ-radiation induced PADPR caused relaxation of chromatin that lead to a partial loss of

histone proteins during the preparation of chromatin resulting in their reduced recovery from the chromatin after chromatography. In case of repair incubated group we suggest that due to repair of a set of damages, the level of PADPR went down making the chromatin more condensed than that of the controls. Therefore, the loss of histones from chromatin was relatively small during preparation of chromatin. This could be a reason of increased recovery of histone proteins by chromatography of chromatin of the repair incubation group. The values shown in Fig. 3B were reproducible, with biologically acceptable standard variation of about 10% or less, in several independent experiments. The reversible affinity of histones to DNA depending on the level of PADPR of histones after irradiation and after irradiation + repair incubation is in line with the histone shuttle model of Althaus[16]. However, the possibility of *de novo* synthesis of histone during repair incubation cannot be ruled out.

The results presented here bring to focus the cellular triggers associated with PADPR reactions. It is known that (i) PADPR reactions are triggered by strand breaks in DNA, (ii) γ-radiation causes strand breaks in DNA and (iii) repair incubation creates conditions for repair of damages to DNA. Thus, enhancement in the level of PADPR of various histone proteins following irradiation and its reduction following post-irradiation repair incubation (Figs. 1 & 2) may be attributed to DNA strand breaks and their subsequent repair, respectively. The trigger of PADPR after preparation of cell suspension, however, remains ambiguous. Manipulations for preparation of cell suspension may have caused some damages to DNA and enhanced cellular PADPR. These damages, however, differed qualitatively from those caused by γ-irradiation as repair incubation lead to complete abolition of cellular PADPR only in the former case (Fig. 1). In another possibility, cellular components other than DNA may also trigger PADPR. It is likely that manipulations (tripsinization and agitation) for preparation of cell suspension may have primarily affected membrane components[18]. Therefore, membrane component may also be a trigger of PADPR. This is in agreement with the observed effects of magnetic field exposure to monolayer cells in culture[18, 22]. Our unpublished data on the level of PADPR following exposure of cells to different membrane active substances also support this possibility.

Thus, results presented above show that T1-cells in suspension responded to γ-irradiation by a further increase of PADPR of histone proteins even when PADPR was already at an elevated level due to preparation of single cell suspension. This increase supports repair by inducing a relaxed conformation of chromatin rendering the damaged DNA more accessible to repair enzymes[12,14-16]. The different extents of repair in irradiated (damage by manipulations for preparation of cell suspension and γ-irradiation) and non-irradiated (damage only by manipulations for preparation of cell suspension) cell suspensions suggest that PADPR of histone proteins may be regulated not only by γ-radiation induced damage to DNA but also by damages to other cellular components like membrane[18,22]. Further conformatory work is under progress.

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