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Negative correlation between poly-ADP-ribosylation of spleen cell histone proteins and initial duration of dimethylnitrosamine exposure to mice in vivo measured by Western blot immunoprobe assay: a possible biomarker for cancer detection

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Abstract

Improved cancer detection involving suitable biomarkers with easy applicability is a challenge to our fight against cancer. Poly-ADP-ribosylation (PAR) of proteins is a likely candidate biomarker for this purpose because it meets the criterion well. This report is a step towards testing suitability of PAR as a biomarker for cancer detection. Swiss albino mice were exposed to hepatocarcinogen, dimethylnitrosamine (DMN), at a chronic dose, which is known to induce carcinogenesis in liver. PAR was monitored by a Western blot immunoprobe assay in spleen, a lymphoid organ, to find a correlation between PAR of spleen histone proteins and duration of DMN exposure. A negative, non-linear correlation was found for most histone proteins. The inhibition of PAR of histones was significant from 4 weeks onwards until the end of the observation. The inhibition was potentiated when 3-aminobenzamide was simultaneously administered. The results open up the possibility of PAR of cellular proteins being used as biomarker for cancer detection.

Keywords: Cancer biomarker; Poly-ADP-ribosylation; Dimethylnitrosamine; Spleen; Western blot immunoprobe assay; Histone proteins; Mice

1. Introduction

Cancer detection continues to remain a challenge to scientists even today. It is estimated that with improved screening and consequent early detection, different cancer deaths could be reduced up to 35% besides reducing cancer morbidity [1,2]. Several laboratory-based tests as well as computed tomography procedures are under use for diagnosis of different types of cancers [2]. Common problems with laboratory-based tests are false-positive results and over- or under-diagnosis. The computed tomography procedures are relatively more accurate but can be applied only to limited types of cancers. They are also expensive, requiring highly skilled personnel and sophisticated set-ups. Development of new and sensitive biomarkers for early cancer detection, therefore, is in the center stage of continuing efforts to improve diagnostic capabilities in our fight against cancer [3]. Assay of suitable biomarkers, which are common to different cancers, will be desirable to ensure that less costly cancer detection tests are available to populations even in underdeveloped countries where diagnostic and other medical infrastructure are poor. Poly-ADP-ribosylation (PAR) of cellular proteins is one such likely candidate biomarker for cancer detection.

PAR is a ubiquitous and reversible enzyme catalyzed metabolic reaction causing post-translational modification of mainly chromosomal proteins [4]. PAR of proteins is

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strongly enhanced by DNA strand breaks [5] or by different external stimuli [6]. Implications of PAR of chromosomal proteins, especially histones, have been shown in genome organization and its functionality [7,8]. Thus, PAR is involved in a variety of cellular functions, including carcinogenesis [8,9]. The hallmarks of carcinogenesis include altered genome organization, modified gene expression profile, expression of neogenes and shut down of differentiation genes [8,10]. Such changes in the functionality of genome during carcinogenesis are likely to be mediated or, at least influenced by PAR of histone proteins, which has profound influence on genome organization [7–9]. Thus, PAR of DNA binding proteins, particularly histones, may possibly serve as a clinical parameter for detecting and monitoring carcinogenesis since even small changes in the level of PAR has significant effect on molecular processes associate with chromatin [9].

Using conventional radioisotopic assay of PAR [6,11] we have earlier monitored changes in total cellular PAR as well as that of histones and high mobility group proteins in liver, bone barrow and spleen tissues under the influence of arecoline [7,12], aqueous extract of betel nut [13] or dimethylnitrosamine (DMN) [13,14] in mice. However, we also noted possible technical shortcomings in the assay, which were likely to give erroneous measure of PAR [6]. Therefore, we developed and standardized an enzyme-linked immunosorbent assay (ELISA) based immunoprobe assay of PAR, which is simple, sensitive and specific [15]. The novel Western blot immunoprobe assay of PAR has been employed in this study to monitor the metabolic PAR following dimethylnitrosamine induced transformation in Swiss albino mice to understand the possible involvement of PAR in the critical and irreversible initiation stage of cellular transformation [10]. The investigation attempted to find a correlation between the time of DMN exposure to mice and PAR of spleen cell proteins of exposed mice. An inhibitor of enzyme poly-ADP-ribose polymerase and PAR, 3-amino-benzamide (3-AB) [4], was also used to see the influence of inhibition of PAR on carcinogenesis.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and were used without further purification. Solutions were prepared in double distilled water.

2.2. Administration of DMN and 3-AB

Young (8–12 weeks) Swiss albino mice (Balb/c) were exposed to DMN [16], at a dose rate of 10 mg kg^{-1} body weight in drinking water in a chronic oral administration protocol over a period of 8 weeks. The administration of 3-AB was through an acute weekly i.p. injection at a dose rate of 5.45 mg kg^{-1} body weight (equivalent to 2 mM of 3-AB per injection). 3-AB was administered to either a group of normal mice or a group of mice being chronically exposed to DMN. Mice were sacrificed for analysis at 2, 4, 6 and 8 weeks (exposed groups of mice). Unexposed and age-matched mice served as controls.

2.3. Preparation of antibody

Polyclonal antibody against mouse ADP-ribose was raised in rabbit [15]. ADP-ribose was isolated from mouse spleen cells as described earlier [15] and used as the antigen. The blood serum of immunized rabbit was partially purified to get polyclonal anti-ADP-ribose immunoglobulin, which was lyophilized and used for all experiments.

2.4. Isolation of histone proteins

Total cellular histone proteins were isolated from 60×10^{-6} spleen cells following method described earlier [12].

2.5. Sample preparation

Spleen cells were recovered from the unexposed (control) and exposed (DMN, 3-AB or DMN + 3-AB) groups of mice after sacrificing them by cervical dislocation. All cells from the spleen were homogenized using ice cold PBS and centrifuged for 15 min at 10,000 × *g*. The supernatant of the whole homogenate thus prepared as well as the histone preparation were used for Western blotting.

2.6. SDS–polyacrylamide gel (PAGE) and Western blotting

Samples for analysis were loaded on a 12% (w/v) SDS–polyacrylamide gel for electrophoresis in a mini electrophoresis gel apparatus (25 V cm^{-1} constant, 60 min). The resolved proteins on the gel were electroblotted on nitrocellulose membrane (NCM) at 10 °C in a Biorad transblot apparatus (100 V constant, 60 min) using Towbin buffer (25 mM Tris–Cl buffer, pH 8.3, 192 mM glycine and 20% methanol). The Western blotted NCM was either stained with India ink (0.2%, 3–4 h) to visualize total proteins or immunoprobed to visualize poly-ADP-ribosylated proteins.

2.7. Immunoprobe assay of PAR

PAR of individual cellular proteins was detected by Western blot immunoprobe assay as described earlier [15]. Briefly, the Western blots were incubated with, in sequence, 5% non-fat dry milk at 37 °C for 45 min, polyclonal anti-ADP ribose antibody (1:500) at 37 °C for 45 min and anti-rabbit IgG-alkaline phosphatase conjugate (1:15,000) at 37 °C for 45 min. Each incubation step was punctuated by

appropriate washing step(s). The bands on NCM were color developed with NBT/BCIP color developer.

2.8. Analysis of Western blots

An electrophoresis documentation and analysis system, KDS1D software, using a Kodak digital camera was used for quantification and analysis of the data. Mean intensity of independent replicates (three sets each with two mice) was taken as the measure of PAR of cellular proteins (intensity $\propto$ PAR of proteins). Statistical calculations and plotting of graphs were done using Origin and Excel programs, respectively. P values <0.05 were taken as significant.

3. Results

3.1. Total proteins on Western blots

Fig. 1 shows the ink stained Western blots of isolated histone proteins (panel A) and cellular proteins of the whole homogenate (panel B) from spleen cells representing total proteins transferred onto NCM from SDS–PAGE gel. The results show that quantity of proteins blotted from control (lane 0) and 2-, 4-, 6- and 8-week treatment groups (lanes 2, 4, 6 and 8, respectively) were similar. Western blot of isolated histone proteins (panel A) helped identification of histone protein bands among other cellular proteins of spleen cell whole homogenate (panel B). Compared to SDS–PAGE gel stained with coomassie brilliant blue R 250 (not shown), the transfer of protein on NCM by Western blotting was almost total.

3.2. Poly-ADP-ribosylated proteins on Western blots

Fig. 2 shows the immunoprobed Western blots representing the poly-ADP-ribosylated proteins of spleen cells in control (lane 0), and 2-, 4-, 6- and 8-week treatment groups (lanes 2, 4, 6 and 8, respectively). Panel A shows the immunoprobed Western blots of spleen cell histone proteins isolated from mice exposed to DMN (top row), 3-AB (middle row) and DMN + 3-AB (bottom row) while panel B shows the corresponding immunoprobed Western blots of whole homogenates of spleen cells of mice. Thus, protein bands visible in Fig. 2A are poly-ADP-ribosylated histone proteins while that in Fig. 2B are the poly-ADP-ribosylated cellular proteins including histones. The results reveal that histones were the main target proteins of PAR in mouse spleen cells. Among the histone proteins, core histone proteins (arrow) were clearly more modified than histone H1 (also see Fig. 1). Some other proteins, particularly high molecular weight non-histone proteins, were also poly-ADP-ribosylated (open arrow, Fig. 2B). The overall extent of poly-ADP-ribosylation was higher in the controls (lane 0), which exhibited progressive reduction in the level of PAR of different proteins with progression of period of exposure to DMN, 3-AB or DMN + 3-AB (lanes 2, 4, 6 and 8).

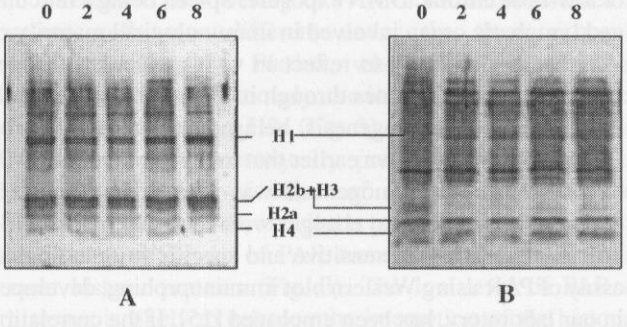

Fig. 1. India ink stained Western blots of spleen protein of unexposed (control) and chronically DMN exposed mice for a period up to eight weeks. Panel A—isolated histone proteins from mice spleen cells; panel B—whole homogenate of mice spleen cells; lanes from left to right: 0 (control), 2, 4, 6 and 8 weeks of DMN exposure, respectively.

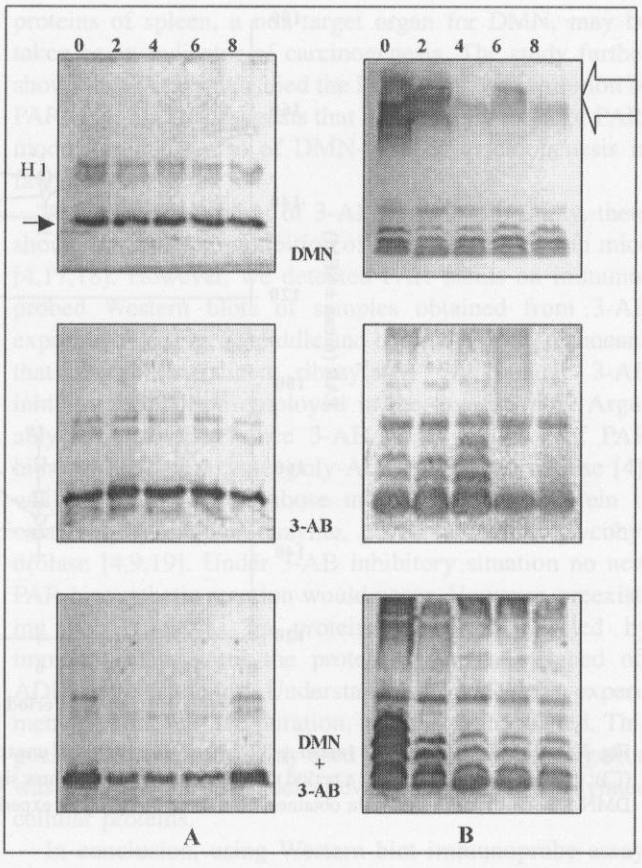

Fig. 2. Immunoprobed Western blots of spleen cell proteins of unexposed (control) and DMN (top row), 3-AB (middle row) or DMN + 3-AB (bottom row) exposed groups of mice for a period up to 8 weeks. Panel A—isolated histone proteins from mice spleen cells; panel B—whole homogenate of mice spleen cell. Closed arrow—core histone proteins; open arrow—high molecular weight non-histone proteins; H1—histone protein H1; lanes from left to right: 0 (control), 2, 4, 6 and 8 weeks of DMN exposure, respectively.

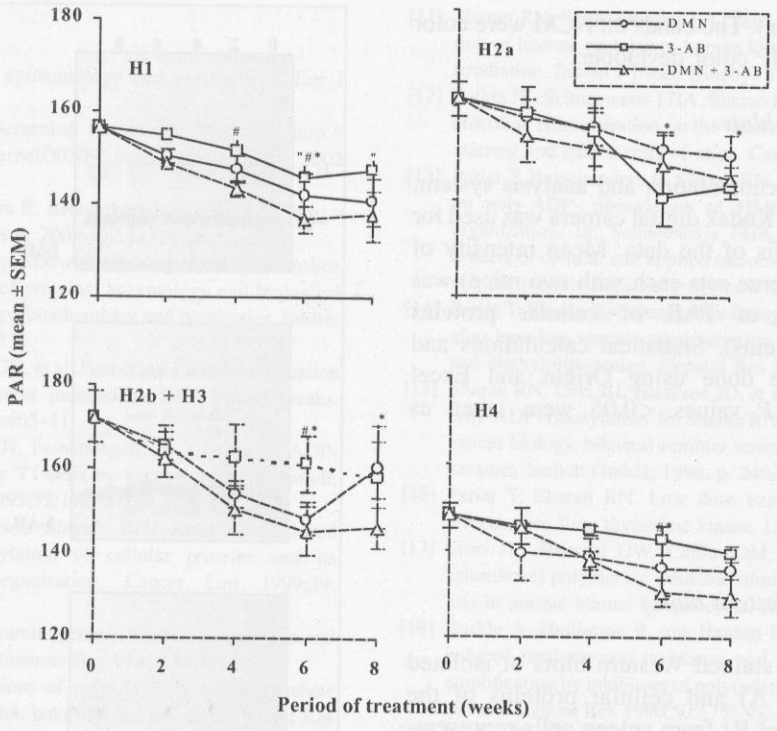

Fig. 3. PAR of histone proteins (mean ± S.E.M.) of spleen cells of unexposed (control) mice and those under different exposure regimes (DMN (○); 3-AB (□); or DMN + 3-AB: (△)) for a period up to 8 weeks. Notations show significant ($p < 0.05$) decrease in PAR after DMN exposure (′); after 3-AB (*) or after DMN + 3-AB exposure (#); data obtained from three independent experimental sets, each with two replicates of Western blots.

3.3. Quantitative analysis of Western blot data

Different histone protein bands (H1, H2a, H2b + H3 and H4) under three exposure regimes (DMN, 3-AB and DMN + 3-AB) on immunoprobed Western blots (Fig. 2) were identified based on Fig. 1. The PAR of individual histone proteins was quantified and plotted (Fig. 3). In the presence of 3-AB, the PAR of different histone proteins showed a trend of decline with progression of 3-AB exposure period. Exposure to DMN also exhibited similar but more pronounced lowering of PAR with duration of exposure. This inhibitory effect of DMN was further accentuated especially for histone proteins H1, H2b + H3 and H4 after 4 weeks of simultaneous exposure to DMN + 3-AB. The inhibition was generally significant after 4 week but not before (Fig. 3; see pointers of test of significance).

4. Discussion

Currently several biochemical and direct or aided visual (morphological) tests are employed to detect cancer in humans. Many of the tests require surgical intervention to obtain biopsies. Though attempts are made to minimize the intervention, trauma and follow-up complications very often become serious impedance to cancer detection and screening programs. Consequently, the usefulness of currently available tests is limited. Thus, there is an urgent need to look for appropriately sensitive tests, which could be applied on patients with minimum intervention and trauma. In that sense, tests involving blood cells will perhaps be most desirable provided it can indicate carcinogenesis. With this in view, we have initiated research to formulate a convenient avenue to detect cancer in Swiss albino mice. In the first step, we have chosen for this study a liver specific chemical carcinogen DMN, which has been used in several of our previous studies [13,14,16]. PAR of chromosomal proteins was chosen as the biomarker for the assay since involvement of PAR in carcinogenesis has been recognized [7–9]. PAR is also likely to be a common biomarker for different cancers since it affects functionality of chromosome, which is altered during carcinogenesis [8,10]. We have monitored the status of PAR in mouse spleen cells during the first 8 weeks of low-dose chronic DMN exposure. Spleen being a reticular and lymphatic organ involved in immunological maturity of lymphocytes is likely to reflect in vivo molecular changes since the blood circulates through it. Therefore, spleen cells may indicate carcinogenesis being initiated in liver by DMN. It has been shown earlier that in this protocol of DMN exposure, hepatocarcinogenesis was initiated in mice [16] and several biochemical changes were recorded in liver [13–16]. A novel, simple, sensitive and specific immunological assay of PAR using Western blot immunoprobing, developed in our laboratory, has been employed [15]. If the correlation between the onset of carcinogenesis in liver by DMN and PAR of spleen cell proteins is significant, this may form a basis for predicting cancer by examination of biopsies of any lymphoid organ, including blood lymphocytes.

The immunoblot assay has been shown to be a specific assay of PAR of cellular proteins [15]. The Western blot, after immunoprobing, very selectively developed bands of cellular proteins that were poly-ADP-ribosylated (Fig. 2). Therefore, even though a large number of cellular protein bands present on the Western blot were visible following India ink staining (Fig. 1), only few bands were stained after immunoprobing of a replicum Western blot (Fig. 2). Histone proteins are known to be the primary targets of PAR [6,7,11] and the results in Fig. 2B supports it. To further confirm it, in a separate experiment, histone proteins were isolated from spleen cells. They were resolved on PAGE gel, Western blotted (Fig. 1A) and immunoprobed (Fig. 2A). The pattern of PAR of histone proteins, as detected by the immunoprobe assay (Fig. 2A), was quite similar to that of the whole homogenate (Fig. 2B).

Both isolated histones and the whole homogenate of spleen cells visually exhibited that (a) histones were the main target proteins for PAR (Fig. 2B) and (b) the extent of PAR of histone proteins was progressively lowered under the influence of DMN (Fig. 2A and B). Upon quantification this trend was more apparent indicating a negative, non-linear correlation between PAR of spleen histone proteins and period of DMN exposure (Fig. 3). Among the histone proteins, the degree of PAR of core histones was generally higher than that of H1, the linker histone (Fig. 2). The PAR of all core histones was inhibited by DMN exposure, albeit with varying degrees. It is known that among the core histones, H2b may be one of the preferred targets for PAR though other core histones are also ribosylated [4,6,7,11]. Our results (Figs. 2 and 3) also support this. Some other proteins, especially high molecular weight non-histone proteins, were also modified as evident from the immunoprobed Western blot of whole homogenate (Fig. 2B, open arrow). One such likely protein is the enzyme poly-ADP-ribose polymerase (~120 kDa) that is known to be automodified to regulate its poly-ADP-ribosylation ability [4–6]. However, the contribution of its poly-ADP-ribosylation (automodification) seems to be minor in the total in vivo PAR as compared to the PAR of histones (Fig. 2B). Therefore, it has been ignored in this study.

The extent of lowering of PAR of different histones in spleen cells was different under the three exposure conditions (Fig. 3). Further, when DMN + 3-AB were administered simultaneously, the inhibition was maximized for all histones except H2a (Fig. 3). This also points out that not all histones were equally preferred targets for PAR reaction. In terms of effect of combined treatment of DMN + 3-AB on PAR of cellular proteins, spleen showed a significant inhibition of PAR from 4th week onwards for most histone proteins (Fig. 3). The low level of PAR of histones is likely to enhance the interaction of the histones with DNA, thereby, causing structural change in chromatin [4,7,8]. The dose and route of administration of DMN used in this study does eventually cause liver cancer in mice [16]. Therefore, the DMN-induced inhibition in PAR of histone proteins of spleen, a non-target organ for DMN, may be taken as an indicator of carcinogenesis. The study further shows that 3-AB intensified the DMN-induced inhibition of PAR (Fig. 3). This suggests that 3-AB, an inhibitor of PAR, modulates the course of DMN-induced carcinogenesis in favor of cancer.

At the concentration of 3-AB used in this study, there should be complete inhibition of PAR biosynthesis in mice [4,17,18]. However, we detected PAR bands on immunoprobed Western blots of samples obtained from 3-AB exposed mice (Fig. 2, middle and bottom rows). This means that protein remained ribosylated even under 3-AB inhibitory condition employed in the investigation. Arguably it is possible since 3-AB is an inhibitor of PAR biosynthesizing enzyme, poly-ADP-ribose polymerase [4], while degradation of ribose moieties from a protein is catalyzed by another enzyme, poly-ADP-ribose glycohydrolase [4,9,19]. Under 3-AB inhibitory situation no new PAR biosynthetic reaction would occur. However, preexisting PAR moieties on proteins shall be detected by immunoprobing until the protein is totally stripped off ADP-ribose moieties. Understandably, under the experimental conditions and duration, this has not happened. This goes to show that the assay used in the present investigation was sensitive and could detect even poorly ADP-ribosylated cellular proteins.

In conclusion, using Western blot immunoprobe assay, we have detected and quantified physiological level of PAR of mainly histone proteins of spleen. The hepatocarcinogen DMN, for which spleen is not the primary target organ, progressively inhibited the PAR of spleen cell proteins, which was significant for most histones from 4th week after exposure and until the end of the observation (8 weeks). The inhibition was more pronounced when 3-AB was simultaneously administered. These observations suggest that tissue other than the target organ might also indicate onset of chemical induced carcinogenesis. This opens the possibility of not necessarily depending on biopsies of cancerous tissue, for which surgical intervention is necessary, for cancer detection and screening. We are currently testing whether or not PAR of proteins of mouse blood lymphocytes shows any correlation with DMN induced onset of carcinogenesis in liver. This will make mass screening of cancer convenient for human population since the diagnosis shall involve drawing of blood from the person and no biopsies will be needed. Preliminary results in mice are very encouraging wherein blood lymphocytes also show negative and significant correlation (unpublished). More work is underway.

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