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Detection and quantification of poly-ADP-ribosylated cellular proteins of spleen and liver tissues of mice *in vivo* by slot and Western blot immunoprobing using polyclonal antibody against mouse ADP-ribose polymer

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Abstract

Poly-ADP-ribosylation (PAR) of cellular proteins has been shown to have decisive roles in diverse cellular functions including carcinogenesis. There are indications that metabolic level of poly-ADP-ribosylated cellular proteins might indicate carcinogenesis and, therefore, could be potentially used in cancer screening program. Keeping in mind the limitations of currently available assays of cellular PAR, a new assay is being reported that measures the metabolic level of poly-ADP-ribosylated cellular proteins. The ELISA based slot and Western blot immunoassay used polyclonal antibody against natural, heterogeneous ADP-ribose polymers. It could be successfully employed to qualitatively and quantitatively assay metabolic levels of poly-ADP-ribosylated proteins of spleen and liver tissues of normal mice or mice exposed to dimethylnitrosamine for up to 8 weeks; potentially PAR of cellular proteins could be assayed in any tissue or biopsy. Implications of the results in cancer screening program have been discussed. (Mol Cell Biochem **278:** 213–221, 2005)

Key words: immunoprobing, polyclonal antibody, poly-ADP-ribosylation (PAR), slot- and Western blots, dimethylnitrosamine (DMN), Swiss albino mice

Abbreviations: ELISA, enzyme linked immunosorbent assay; PAR, poly-ADP-ribosylation; PARP, poly-ADP-ribose polymerase; PARG, poly-ADP-ribose glycohydrolase; ^{32}P-NAD^{+}, ^{32}P labelled nicotinamide adenine dinucleotide; Ab, antibody; MAb, monoclonal antibody; PAb, polyclonal antibody; DMN, dimethylnitrosamine; NCM, nitrocellulose membrane; IgG, immunoglobulin protein; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; SDS, sodium dodecyl sulphate; PBS, phosphate buffered saline; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; RT, room temperature; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; SC-H, isolated histone proteins from spleen cells; SC-WH, spleen cell whole homogenate; L-WH, liver whole homogenate.

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Introduction

Poly-ADP-ribosylation (PAR), a ubiquitous phenomenon in eukaryotes, refers to a reversible post-translational modification of cellular proteins [reviewed in 1–5]. PAR modifies target proteins enzymatically by adding successive ADP-ribose units from a donor, NAD^+, in form of a branched or unbranched polymer. The main biosynthetic enzyme for PAR is poly-ADP-ribose polymerase (PARP) while that for its breakdown is poly-ADP-ribose glycohydrolase (PARG). The most preferred target proteins for PAR are nuclear or chromosomal proteins which include histones, endonuclease, topoisomerase I, DNA ligase II, PARP, etc. [2–4, 6–9]. The polymer of ADP-ribose being charged causes alterations in the net charge and conformation of the target protein [3, 10]. The altered charge and conformational states of modified chromosomal proteins, in turn, alter their interactions with DNA [3, 10–13]. This alteration, thus, changes the conformational state of chromatin and affects its functional status [10, 13]. Thus, involvement of PAR modification of proteins has been suggested in diverse cellular functions including carcinogenesis, gene expression, repair of DNA damages, cell division, etc. [10–18].

The assay of PAR of cellular proteins has been made using several diverse approaches [reviewed in 19]. The widely used and conventional radio-isotopic assay used ^{32}P-NAD^+ [20] or other radiolabelled NAD^+. Since NAD^+ is not permeable across cell membrane, it was necessary to permeabilize cells or nuclei to internalize the hot-NAD^+. Thus, the hot-NAD^+ could label the target proteins by poly-ADP-ribosylation reaction. The radioactivity on target proteins could now be monitored by either autoradiography or liquid scintillation counting (LSC). This gave the measure of PAR of cellular proteins [13, 16, 21–23]. In this assay, the tissue in which PAR has to be assayed must be made into single cell suspension or nuclei for uniform internalization of labelled-NAD^+, by trypsinization, agitation, homogenization and centrifugation. All these methodological interventions have been shown to induce *per se* unphysiologically high levels of cellular PAR [21]. Therefore, the level of cellular PAR assessed by the radio-isotopic method may not necessarily reflect the exact *in vivo* or metabolic status of PAR [21]. The assay is also limited to tissues, which can be easily converted into single cell suspension or nuclei, thereby, limiting application of the assay. Besides, the experimentalist is exposed to hazards of radiation. Limited improvement in the assay was made by either using fluorescent nucleoside ribosyl adenosine [24] or radiolabeling of cellular NAD^+ pool by ^{14}C or ^{3}H-adenine. Indirect assay of PAR has also been done by measuring PARP enzyme activity [25] or cellular NAD^+ pool [26]. Unphysiological conditions, lack of sensitivity or being time consuming have limited the use of these methods for assay of metabolic PAR of cellular proteins. Considering these limitations, it was recommended that several assays of PAR be performed to get a realistic assessment of PAR [19].

The high immunogenic property of PAR lead to development of an immunological assay of PAR by employing antibody (Ab) against ADP-ribose polymers. By far this approach has been the most satisfactory [reviewed in 27]. The assay is direct, sensitive and specific and does not involve use of a radiolabelled substrate [27, 28]. Attempt has been made to use monoclonal antibody (MAb) for the assay. However, it was found that different MAb were needed to detect linear and branched PAR of cellular proteins [29]. Polyclonal antibody (PAb) has been successfully employed in ELISA-based approach to assay cellular PAR *in vitro* and *in vivo* [27, 28]. However, in these investigations only different types of cells in culture were used. To the best of our knowledge no attempt has been made to assay metabolic level of cellular PAR in different tissues of mouse *in vivo* using the ELISA-based immunoassay of PAR.

This study was undertaken to design and develop a sensitive assay to measure the metabolic level of PAR of cellular proteins in different tissues of a mouse. The assay uses PAb raised against naturally occurring heterogeneous ADP-ribose polymer isolated from normal mouse spleen cells. ELISA based immunoprobing of slot- and Western blots were employed to determine the endogenous metabolic levels of PAR of cellular proteins in liver and spleen tissues. The PAR of proteins was also quantified. For comparison, tissues from dimethylnitrosamine (DMN), a hepatocarcinogen, exposed mice were also subjected to the assay of cellular PAR. Qualitative and quantitative analyses were performed.

Materials and methods

Chemicals

All chemicals were of analytical grade or of highest purity grade available. Polyclonal antibody (PAb) specific against heterogeneous ADP-ribose polymers was raised in a rabbit in the laboratory. Alkaline phosphatase conjugated goat anti-rabbit IgG (whole molecule), BCIP/NBT buffered substrate tablets, Tween 20 and DMN were obtained from Sigma Chemical Co., USA. Hybond-C extra supported 0.45 μm nitrocellulose membrane (NCM) was procured from Amersham, UK.

Experimental animals

Swiss albino mice (Balb/C) aged 8–12 weeks, maintained in communal cages in a well-ventilated animal room under controlled temperature ($22 \pm 2\,^{\circ}$C), were used in all experiments. They were provided with standard mouse diet (Pranav Agro Industries Ltd., Delhi) and drinking water *ad libitum* and 12 h

light and 12 h dark cycle. For immunization, a healthy and young Russian chinchilla strain rabbit was used.

Administration of dimethylnitrosamine (DMN)

Healthy mice were chronically exposed to DMN for up to 8 weeks at a dose of 10 mg kg^{-1} body weight through drinking water. The analysis was performed after 0 (control or C), 2, 4, 6 and 8 weeks of DMN administration.

Isolation of naturally occurring, heterogeneous ADP-ribose polymers

Heterogeneous ADP-ribose polymers were isolated from spleen cells of young and healthy mice following the method described earlier [30]. Briefly, spleen cell suspension in pre-chilled PBS (1 ml) was homogenized and centrifuged at 1300 $\times$ *g* for 15 min at 4 °C. The pellet was resuspended in 10% TCA and incubated on ice for 30 min before centrifugation at 20,000 $\times$ *g* for 15 min. The TCA pellet was washed with distilled water, resuspended in 0.1 M Tris-glycine buffer, pH 10.5 and incubated at 37 °C for 60 min with regular gentle shaking. It was again centrifuged at 20,000 $\times$ *g* for 60 min. The resulting supernatant containing heterogeneous polymers of ADP-ribose was desalted on Sephadex G-25 gel by elution with 0.03 M tris-barbiturate buffer, pH 7.2 containing 6 M urea and 0.1% SDS at a flow rate of 1 ml/5 min. The eluent fractions of the peak were pooled and dialyzed extensively for about 48 h in double distilled water with several changes. The dialysate was frozen, lyophilized and stored for use as antigen (Ag).

Preparation of polyclonal antibody (PAb) against the isolated antigen (Ag)

The lyophilized Ag was dissolved in PBS, pH 7.4, at a concentration of 0.4 μg μl^{-1}. Emulsion of the diluted Ag was prepared with equal volume of Freund's complete adjuvant (FCA) by vigorous homogeneous mixing. The emulsion in aliquots of 500 μl (100 μg of Ag) was intradermally administered at multiple sites on the back of a healthy rabbit after cleaning the immunization area with 70% ethanol. Booster immunization was administered 4 weeks later with Ag emulsified in Freund's incomplete adjuvant (FIA) in a ratio of 1:1. Blood was collected between 4^{th} and 6^{th} day after the booster immunization from the lateral ear vein of the rabbit in a clean glass tube washed with 0.9% normal saline. Repeated bleeding was performed on the same rabbit after 4 to 6 days following every booster immunization.

The tube containing blood was allowed to stand tilted for 8–10 h at room temperature (RT). The serum was collected by a micropipette in a sterile tube. It was centrifuged at 10,000 $\times$ *g* for 10 min at 4 °C to remove any remaining cell debris. The clear serum, containing PAb, was concentrated by precipitating the immunoglobulin proteins with saturated ammonium sulfate. The precipitate, predominantly immunoglobulin protein, was lyophilized and stored refrigerated for use as PAb.

Sample preparation

For the assay of cellular PAR, liver and spleen tissues of mice were used. Tissues were excised from the untreated (control) and DMN-treated mice after sacrificing them by cervical dislocation. Liver was chopped into small pieces while all spleen cells were recovered from the spleen. Whole homogenate (WH) of liver (0.15 g in 1.5 ml) or all spleen cells recovered from one spleen in 1 ml were prepared in ice-cold PBS, pH 7.4, using a motorized tissue homogenizer. Supernatants of the WH after centrifugation at 10,000 $\times$ *g* for 15 min were used for the assay of PAR. The protein content of the WH was determined by Bradford method [31].

Histone preparation

Histone proteins were isolated from spleen cells following the method described earlier [32]. Briefly, 6×10^6 spleen cells in 50 ml of minimal essential medium was mixed with 0.5 ml of SPC buffer (1 mM potassium phosphate buffer, pH 6.8 containing 0.32 M sucrose and 1 mM $CaCl_2$), 20 ml of HCl and 12 ml of mercaptoethanol. The mixture was kept on ice for 60 min. After centrifugation at 12,000 $\times$ *g* for 5 min, 0.75 g ml^{-1} urea, 5 ml of 0.2% O-phenolphthalein and 0.05 $\times$ volume of 1 M DTT were added to the supernatant. After addition of 50 ml of NH_3 and 5 min incubation at RT, glacial acetic acid was added to a final concentration of 1 M. The isolate, spleen cell histones (SC-H), was used for the assay of PAR.

Slot blotting

Samples were first heat inactivated by boiling the sample tube in a water bath for 5 min in order to inactivate endogenous alkaline phosphatase enzyme activity [30]. Prior to loading the heat inactivated samples, NCM was soaked in double distilled water for 15 min. Samples (2 μg protein in 100 μl) were loaded onto the slots of a Bio-dot microfiltration apparatus (Bio-Rad) connected to a vacuum pump. The process of blotting usually lasted about 20 min.

SDS-PAGE and Western blotting

Samples for Western blotting were first subjected to 12% SDS-PAGE in a Protean II mini-gel electrophoresis apparatus

(Bio-Rad) at 25 V cm^{-1} (constant) for 60 min [33]. Trans-blot electrophoretic transfer cell (Bio-Rad) was used for Western blotting using NCM pre-activated for 15 min in Towbin buffer (25 mM tris-Cl, pH 8.3; 192 mM glycine; 20% methanol). The resolved proteins on the gel were Western blotted onto the activated NCM using pre-cooled and continuously stirred Towbin buffer at 100 V (constant) for 60 min at about 10 °C.

Staining of trans-blotted proteins on NCM with India ink

Following slot and Western blotting, the trans-blotted NCM was stained for 3-4 h in 0.2% India ink prepared in PBS containing 0.3% Tween 20. This developed all blotted proteins on the NCM.

Immunoprobing poly-ADP-ribosylated proteins trans-blotted on NCM

PAR of cellular proteins was detected by immunoprobing the blotted NCM. The slot blot gave the measure of total PAR of all blotted cellular proteins while PAR of individual proteins was assayed by immunoprobing the Western blots. The protocol of immunoprobe assay included, in sequence, the following steps of incubation at 37 °C for 45 min each:

(i) Blocking solution (5% non-fat dry milk in PBS).
(ii) PAb (1:500).
(iii) Alkaline phosphatase conjugated second-Ab (1:15,000).

Each of the above steps was followed by washing with TBS (20 mM tris-Cl buffer, pH 7.5, 500 mM NaCl) and TTBS (TBS containing 0.05% Tween 20) for 2 × 5 min each at RT. After thorough final wash, the color on the NCM was developed by incubating the NCM in BCIP/NBT color developer (7–15 min). The membrane was then washed with doubled distilled water and air-dried.

Quantification and analysis

The colored bands on trans-blotted NCM (slot as well as Western blots) were captured and digitized immediately by a digital camera (Kodak) or an imaging densitometer (Bio-Rad). Quantification and analysis of the mean intensities of bands were done using an electrophoresis documentation and analysis software, KDS-1D (Kodak). Mean ± S.E.M. of the data were calculated and subjected to Student's *t*-test for statistical evaluation.

Ouchterlony immunodiffusion assay

On 1% agarose (w/v) gel on a clean glass slide, an equimolar quantity of the PAb was loaded in the central well while the Ag and BSA were loaded in the two outer wells. The gel slides were kept in a humid chamber at 37 °C for about 36 h. After the appearance of precipitin line, the gel was stained with coomassie brilliant blue, destained and photographed.

Degradation of ADP-ribose polymer by snake venom phosphodiesterase (SVP)

Isolated histone preparations (2 μg in 10 μl) in different tubes were treated with SVP (2 units) at 37 °C for increasing time periods (15, 20, 25, 30, 35 and 40 min) to selectively degrade the ADP-ribose polymer attached to histone proteins [1, 2]. SVP untreated and treated samples were slot blotted. The slot blots were immunoprobed.

Results

Immunogenicty and specificity of polyclonal antibody (PAb) against cellular PAR

The heterogeneous ADP-ribose polymer of mouse spleen cells elicited reasonably good immune response in rabbit. The resulting PAb was tested for its immune reactivity and specificity by Ouchterlony immunodiffusion assay. Figure 1 shows a distinct precipitin line (arrow) between the PAb and Ag wells. No precipitin line was observed between the PAb and BSA wells. To further assess the specificity, isolated histone proteins were treated with SVP enzyme for increasing time periods. SVP is known to specifically split the pyrophosphate bond of poly-ADP-ribose endonucleolytically yielding PR-AMP and 5′ AMP from the polymer terminus [1, 2]. Equal amounts of undigested and SVP digested samples were then slot blotted and the NCM was immunoprobed with the PAb (Fig. 2). The untreated control sample (slot 0) showed intense PAR signal after color development while the SVP digested

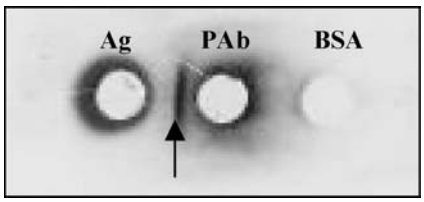

Fig. 1. Commassie brilliant blue stained precipitin line (arrow) between heterogeneous ADP-ribose polymers (Ag) and polyclonal antibody (PAb) wells punctured on a 1% agarose gel; the diffusion occurred in a moist environment at 37 °C for about 36 h. The right well contained BSA. Equimolar quantities of proteins were loaded in each well.

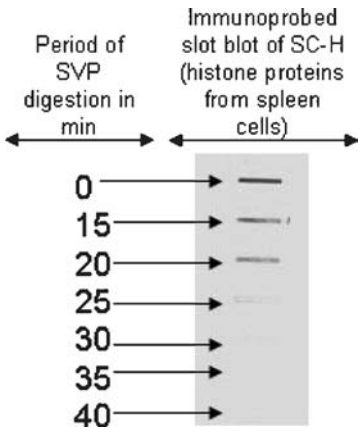

Fig. 2. Immunoprobed slot blot on nitrocellulose membrane (NCM) showing specificity of PAb interaction with ADP-ribose polymers. Samples of histone proteins isolated from spleen cells (SC-H) of mice were digested with snake venom phosphodiesterase (SVP) enzyme for O (control), 15, 20, 25, 30, 35 and 40 min at 37 °C. Following slot blotting, the NCM was immunoprobed with PAb (see text for details).

samples showed progressively weak signal. The PAR was undetectable after 30 min of SVP digestion (Fig. 2).

Qualitative and quantitative changes induced by DMN in PAR of total cellular proteins of liver and spleen cells as measured by slot-blot immunoprobing

Three different preparations, namely, isolated histone proteins from spleen cells (SC-H), whole homogenate of spleen cells (SC-WH) and whole homogenate of liver (L-WH) were used in these experiments. Equal amounts of heat inactivated samples from unexposed control (C) and DMN exposed mice (2, 4, 6 and 8 weeks) were used. Two identical slot blots were made for each set. One blot was stained by India ink to visualize all slotted proteins (left panels) while the replica blot was immunoprobed by PAb to assay poly-ADP-ribosylated proteins in the slots (right panels). Figure 3 shows the result. Three to five independent experiments, each in duplicate, were performed and the mean intensities of the slots were calculated using KDS-1D software. From these data mean ± SEM of total cellular PAR were calculated for SC-H, SC-WH and L-WH and plotted (Fig. 4). Significant ($p \leq 0.5$) lowering in the total PAR of cellular proteins was observed in all three cases at 6 and 8 weeks after DMN exposure.

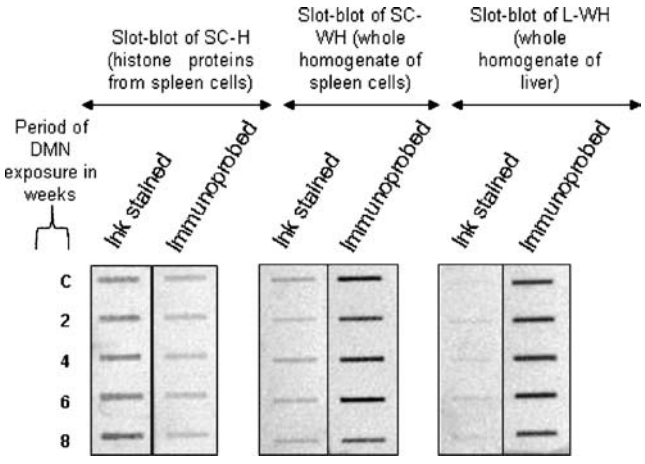

Fig. 3. Replica slot blotted nitrocellulose membrane (NCM) stained with India ink and immunoprobed with PAb to visualize all slotted proteins and poly-ADP-ribosylated proteins in the slots, respectively. Samples of isolated histone proteins from spleen cells (SC-H), spleen cell whole homogenate (SC-WH) and liver whole homogenate (L-WH) of unexposed (C) and dimethylnitrosamine (DMN) exposed mice for 2, 4, 6 and 8 weeks were used (see text for details).

Qualitative and quantitative changes induced by DMN in PAR of individual histone proteins of liver and spleen cells as measured by Western-blot immunoprobing

Identical samples were also subjected to PAGE followed by Western blotting. While one blot was stained with India ink to visualize all Western blotted proteins, its replica was immunoprobed using PAb to visualize poly-ADP-ribosylated proteins. The results are shown in Fig. 5. It is obvious that most of the poly-ADP-ribosylated cellular proteins belonged to histone class of proteins as only these protein bands were colored after immunoprobing (Fig. 5, right panels). It is known that histone proteins are the most preferred target proteins for cellular PAR reaction [36]. The histone proteins were identified based on their molecular weights and our earlier work [35], and quantified into four groups, namely, H1, H2a, H2b + H3 and H4. Three to five independent experiments, each in duplicate, were performed and the mean band intensities of these histone proteins were calculated using KDS-1D software. From these data, mean ± SEM of PAR of histone proteins were calculated and plotted for SC-H, SC-WH and L-WH (Fig. 6). Different histone proteins showed different extents of lowering of their PAR with progression of DMN exposure. The lowering of PAR was significant ($p \leq 0.5$) for all histone proteins except H2a after 6 or 8 weeks of DMN exposure in case of SC-H and after 4, 6 or 8 weeks of DMN exposure in case of L-WH. In case of SC-WH, the lowering of PAR was found to be significant only after 8 weeks of DMN exposure (Fig. 6).

Discussion

Due to predicted involvement of poly-ADP-ribosylation of chromosomal proteins in a variety of cellular functions, including carcinogenesis, proper assay of this post-translational modification in different tissues assumes significance. This has the potentials to become a tool for early diagnosis of

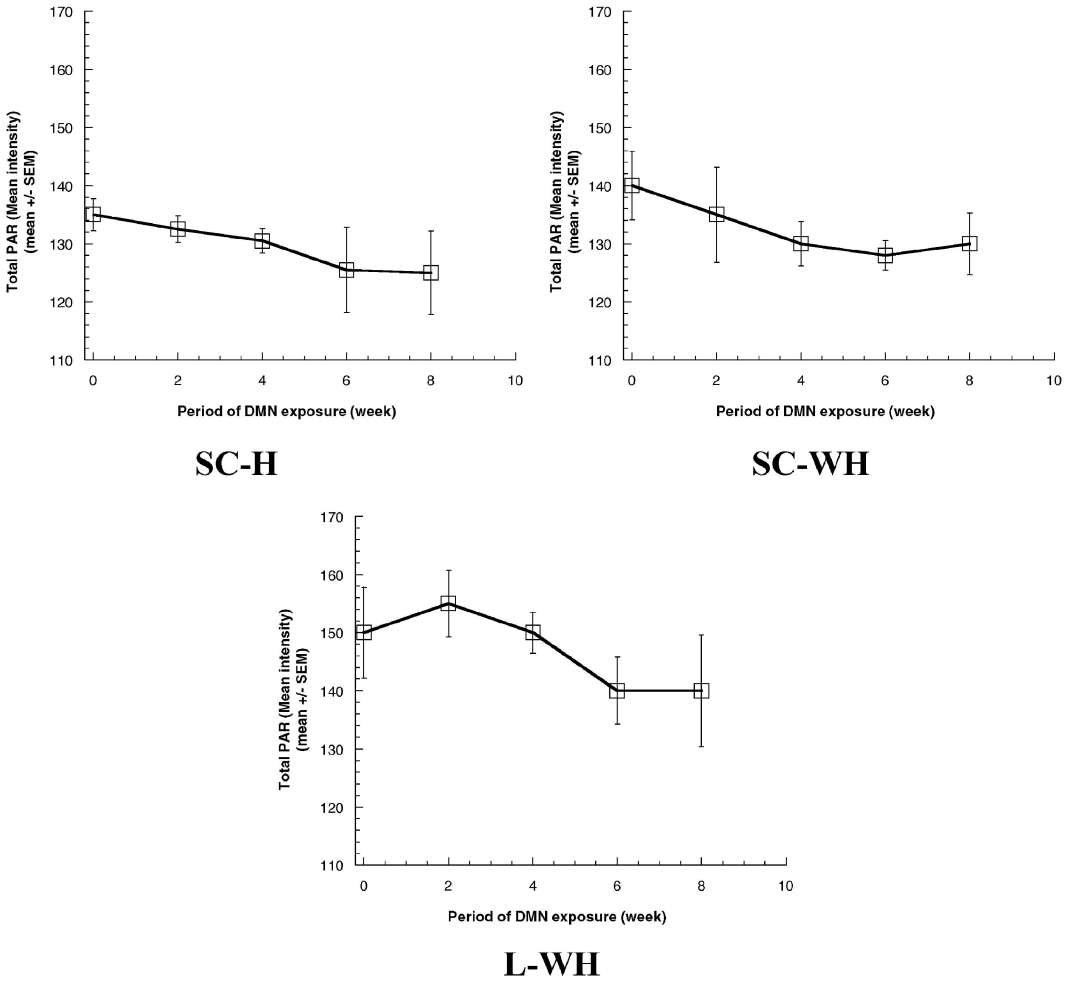

Fig. 4. Plots of total PAR of isolated histone proteins from spleen cells (SC-H), spleen cell whole homogenate (SC-WH) and liver whole homogenate (L-WH) of unexposed and dimethylnitrosamine (DMN) exposed mice. Data (mean intensity) was obtained from Fig. 3 using KDS-1D software and are mean ± S.E.M. of three to five independent experiments, each in duplicate.

cancer as well as in cancer therapy [10, 11, 30, 34, 35]. Exploiting the immunogenic property of poly-ADP-ribose, assay of PAR has been conveniently performed in different types of cells in culture [19]. However, assay of PAR has not been reported in tissues or biopsies of an experimental animal. With this in mind, the work as undertaken to develop a sensitive, quick and convenient assay to detect and quantify the metabolic level of PAR of total cellular proteins as well as of individual proteins from different tissues of mice. The assay employs ELISA based slot- and Western blot immunoprobing of ADP-ribose polymer attached to proteins. We report detection of metabolic level of poly-ADP-ribosylation of proteins in spleen cells and liver of normal and DMN exposed mice.

The first task was to raise specific Ab against ADP-ribose polymer. It is known that PAR modification of proteins involves formation of a highly variable and heterogeneous polymer of ADP-ribose units on different proteins [1–5]. Thus, it was important to raise PAb against the heterogeneous mixture of naturally occurring ADP-ribose polymers, the Ag, to ensure that all types of PAR modified proteins could be detected. It was especially important since different MAb were reported to be required for different types of PAR modification [29]. Perhaps due to this reason, in our preliminary work, MAb gave much fainter signals on slot blot than PAb for equal amounts of slotted proteins on NCM [30]. Therefore, all naturally occurring cellular ADP-ribose polymers from spleen cells of normal healthy mice were isolated for use as Ag. PAb was raised in high titer as evident from the Ouchterlony immunodiffusion assay (Fig. 1). A distinct precipitin line formation between the Ag, naturally occurring, heterogeneous ADP-ribose polymers and PAb shows this (arrow in Fig. 1). No nonspecific cross-reactivity was observed between the PAb and BSA pointing to specificity

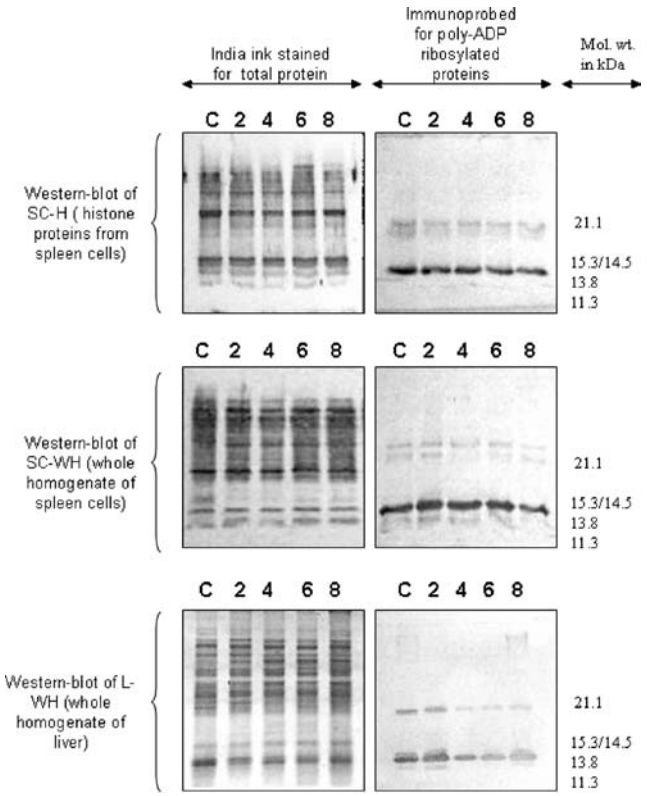

Fig. 5. Replica Western blotted nitrocellulose membrane (NCM) stained with India ink and immunoprobed with PAb to visualize all trans-blotted proteins and poly-ADP-ribosylated proteins, respectively. Samples of isolated histone proteins from spleen cells (SC-H), spleen cell whole homogenate (SC-WH) and liver whole homogenate (L-WH) of unexposed (C) and dimethylnitrosamine (DMN) exposed mice for 2, 4, 6 and 8 weeks were used (see text for details).

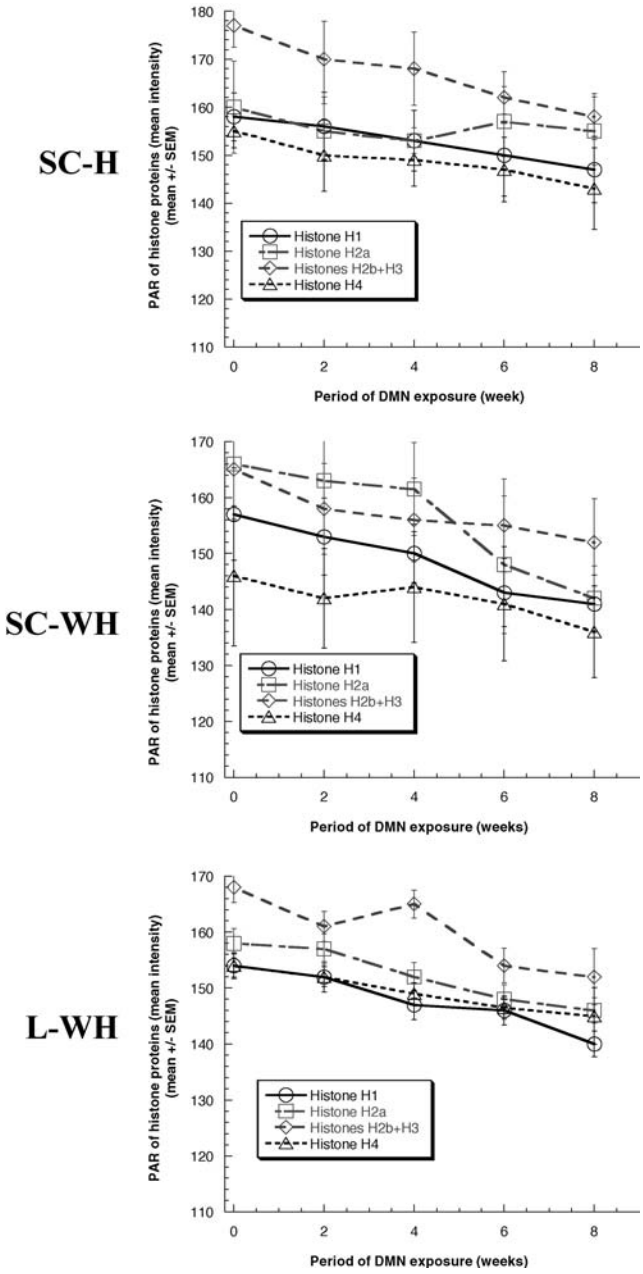

Fig. 6. Plots of PAR of individual histone proteins (H1, H2a, H2b + H3 and H4) resolved on Western blots. Samples of isolated histone proteins from spleen cells (SC-H), spleen cell whole homogenate (SC-WH) and liver whole homogenate (L-WH) of unexposed and dimethylnitrosamine (DMN) exposed mice were used. Data (mean intensity) was obtained from Fig. 5 using KDS-1D software and is mean ± S.E.M. of 3 to 5 independent experiments, each in duplicate (see text for details).

of the antigen (Fig. 1). Removal of the ADP-ribose polymer from histone proteins by SVP further indicates that the PAb was reacting very selectively with the polymers only (Fig. 2).

DMN was chosen for this experimental work since it is a known hepatocarcinogen [34, 35] and this as well as other carcinogens has been shown to affect PAR of cellular proteins [10, 11, 13, 30, 32]. The knowledge of metabolic level of PAR monitored by the immunoassay could reveal, to a certain extent, the biological link between PAR and carcinogenesis *in vivo*. Immunoprobing the slot blot gave the measure of the total PAR of cellular proteins from isolated histone proteins of spleen cells (SC-H), spleen cell whole homogenate (SC-WH) and liver whole homogenate (L-WH). Figure 3 shows the ink stained (left panels) and immunoprobed (right panels) proteins on NCM. India ink stained NCM represents the total proteins slotted while immunoprobed NCM shows the poly-ADP-ribosylated proteins that were slotted. Using KDS-1D software, it was possible to quantify the mean intensities of the slots/bands (Fig. 4). In general, negative correlations between total cellular PAR and period of DMN exposure were apparent in both spleen and liver tissues of mice (Fig. 4). However, the slot blot immunoprobing did not reveal the status of PAR of individual proteins. Therefore, Western blot immunoprobing was employed to assess the status of PAR of individual proteins.

Figure 5 shows the Western blots of SC-H, SC-WH and L-WH from unexposed (C) and mice exposed to DMN for 2, 4, 6 and 8 weeks. India ink stained NCM, show the total Western blotted proteins while the immunoprobed NCM represent the poly-ADP-ribosylated cellular proteins. It is evident from the immunoprobed NCM (Fig. 5, right panels) that histone proteins were the primary targets for poly-ADP-ribosylation. This is in line with earlier reports [3, 16, 21, 22, 23, 32, 35]. The identification of histone protein bands was done as reported elsewhere [35]. As expected, the core histone proteins were modified more than histone H1 (Fig. 5). The bands of immunoprobed NCM were quantified and plotted to reveal the PAR of histone H1, H2a, H2b + H3 and H4, proteins (Fig. 6). In general, the PAR of individual histone proteins also showed lowering with progression of DMN exposure. However, the lowering was variable for different histone proteins in the two tissues studied and significant for only some histone proteins primarily after 6 or 8 weeks of DMN exposure (Fig. 6). Using ascites tumorigenesis model, we have found that the reduction of PAR of core histone proteins was highly significant during progression stage of tumorigenesis (B. J. Devi and R. N. Sharan, unpublished results).

Notwithstanding the implications of these observations in the realm of carcinogenesis, which has been discussed in separate publications [34, 35], this report intended to show that the ELISA-based immunoprobe assay using PAb and slot and Western blotting was able to detect qualitative and quantitative changes in the total PAR of cellular proteins or PAR of individual proteins, respectively. The slot and Western blot immunoprobe assays were specific and sensitive. The assay could be applied to any tissue without involving tedious steps of sample preparations like those involved in conventional assays wherein preparation of cell suspension or nuclei, permeabilization, etc. were essential. Due to this, those assays were limited to cells in culture. In the immunoprobe assay reported here, potentially any tissue or biopsy could be homogenized and used for the assay of PAR immediately. This way, the effects of unphysiological conditions or methodological interventions on PAR metabolism [21] have been totally avoided. The assay, therefore, yields metabolic value of cellular PAR. Due to these reasons, we have hypothesized potential use of the assay as a predictive assay of carcinogenesis especially in mass screening programs [34, 35].

In conclusion, the report presents a modified immunoprobe assay of cellular PAR that can be applied to any tissue or biopsy. The method avoids unphysiological conditions thereby giving measure of metabolic level of PAR of proteins. While the slot blot assay gives a measure of total PAR of cellular proteins, the Western blot assay gives the measure of PAR of individual cellular protein.

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