

RESEARCH ARTICLE

Dimethylnitrosamine-Induced Reduction in the Level of Poly-ADP-Ribosylation of Histone Proteins of Blood Lymphocytes - a Sensitive and Reliable Biomarker for Early Detection of Cancer

Lakhan Kma^{1*}, Rajeshwar Nath Sharan²

Abstract

Poly-ADP-ribosylation (PAR) is a post-translational modification of mainly chromosomal proteins. It is known to be strongly involved in several molecular events, including nucleosome-remodelling and carcinogenesis. In this investigation, it was attempted to evaluate PAR level as a reliable biomarker for early detection of cancer in blood lymphocyte histones. PAR of isolated histone proteins was monitored in normal and dimethylnitrosamine (DMN)-exposed mice tissues using a novel ELISA-based immuno-probe assay developed in our laboratory. An inverse relationship was found between the level of PAR and period of DMN exposure in various histone proteins of blood lymphocytes and spleen cells. With the increase in the DMN exposure period, there was reduction in the PAR level of individual histones in both cases. It was also observed that the decrease in the level of PAR of histones resulted in progressive relaxation of genomic DNA, perhaps triggering activation of genes that are involved in initiation of transformation. The observed effect of carcinogen on the PAR of blood lymphocyte histones provided us with a handy tool for monitoring biochemical or physiological status of individuals exposed to carcinogens without obtaining biopsies of cancerous tissues, which involves several medical and ethical issues. Obtaining blood from any patient and separating blood lymphocytes are routine medical practices involving virtually no medical intervention, post-procedure medical care or trauma to a patient. Moreover, the immuno-probe assay is very simple, sensitive, reliable and cost-effective. Therefore, combined with the ease of preparation of blood lymphocytes and the simplicity of the technique, immuno-probe assay of PAR has the potential to be applied for mass screening of cancer. It appears to be a promising step in the ultimate goal of making cancer detection simple, sensitive and reliable in the near future.

Keywords: Poly-ADP-ribosylation - carcinogenesis - DMN - immuno-probe - blood lymphocytes - histones

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Introduction

The fundamental objective of science is to make the human life qualitatively better, free of diseases and sufferings. Despite the fact that modern industrialized world has successfully eliminated infectious diseases, the major cause of death of last couple of centuries, fear of cancer as a killer disease has not subsided. Even in the new millennium with all encompassing developments in science and technology related to medical field including publication of human genome sequence, the epoch making feat of human endeavors, cancer continues to be a most deadly disease threatening mankind today. The frequency and lethality of cancer underlie the persistent public concern about this group of diseases. Hence, there is great interest in any news of progress in cancer therapy as well as early detection of cancer. Research aimed directly at cancer prevention and promoting use of available

knowledge for cancer prevention is highly desirable in the present state of cancer control. It is to be noted that search for appropriate molecular and biochemical markers for early cancer diagnosis has great relevance to the present approach of our fight against cancer since by early diagnosis cancer mortality and morbidity are likely to reduce significantly (Khan et al., 2012; Luna et al., 2012; van der Bilt et al. 2012; Bao et al., 2013; Hensing and Salgia, 2013; Suradej et al., 2013; Wang et al., 2013; Behrens et al., 2014; Sigari et al., 2014). It is also to be noted that study of cancer cases by looking into the proteomics profiles of patients has yielded valuable clinical correlation between them (Yang et al., 2012; Marzinke et al., 2013; Soond et al., 2013; Taguchi and Hanash, 2013; Ueda et al., 2013; Alvarez-Chaver et al., 2014; Harbeck et al., 2014; Huang et al., 2014; Humphries et al., 2014; Kondo, 2014).

Poly-ADP-ribosylation (PAR) is a post-translational

¹Cancer and Radiation Countermeasures Unit, ²Radiation and Molecular Biology Unit, Department of Biochemistry, North-Eastern Hill University, Meghalaya, India *For correspondence: lakhonkma@gmail.com

modification of mainly chromosomal proteins (Althaus and Richter, 1987). PAR is an enzymatic and reversible cellular reaction. It involves the transfer of ADP-ribose moieties from endogenous nicotinamide adenine dinucleotide (NAD⁺), the substrate for the reaction, to target proteins creating a linear or branched polymer of ADP-ribose moieties. ADP-ribose moieties are covalently bound to target proteins. A multitude of cellular functions and molecular events, including carcinogenesis, angiogenesis, nucleosome-remodelling, cell death, etc. are known to have strong involvement of PAR (Devi and Sharan 2006; Kma and Sharan, 2006; Sala et al., 2008; Binu et al., 2012; Ozaki et al., 2012; Chiou et al., 2013; Donà et al., 2013; Kalmar-Nagy et al., 2013; Long et al., 2013; Yuan et al., 2013).

In the present backdrop, this piece of work was designed to explore and extend ways and means to make cancer detection possible with applied potentials to implement it for screening human population, particularly in developing or under-developed countries like India. The biological end-point chosen for the investigation was status of poly ADP-ribosylation (PAR) of histones following exposure of mice to a specific hepatocarcinogen carcinogen, dimethylnitrosamine (DMN), and the resultant influence of PAR of nuclear proteins on local alterations in the architecture of genomic DNA.

Materials and Methods

Chemicals

All chemicals were of analytical grade or of highest purity grade. Solutions used were prepared in Millipore water. The slot and Western blot apparatus were from Bio-Rad, USA.

Experimental animals

Swiss albino mice (BALB/c) aged 6-8 weeks old, maintained in a well ventilated animal room under controlled temperature (25°C), were used in all experiments. They were provided with standard mouse diet (Pranav Agro Industries Ltd., India) and drinking water ad libitum and 12 h light and 12 h dark cycle.

Administration of dimethylnitrosamine (DMN)

Mice were exposed to DMN for up to 4 weeks at a dose of 10 mg kg⁻¹ body weight through drinking water. The analysis was performed after 0 (control), 1, 2, 3 and 4 weeks of DMN administration.

Isolation of histones

Histone proteins were isolated from blood lymphocytes (BL) and spleen cells (SC), which also served as positive controls. Control or treated mice were sacrificed by cervical dislocation. Spleens were excised out. Simultaneously, blood was drawn from mouse heart and blood lymphocytes prepared as described earlier (Kma and Sharan, 2006). Spleen cell and blood lymphocyte cell suspensions were made in phosphate buffered saline (PBS), pH 7.4 and were counted. Thirty million (30 x 10⁶) spleen cells, 12 x 10⁶ lymphocyte cells in 4 ml PBS was used for the isolation. The methods of West and Bonner

(1980) were used. The isolate, spleen cell histones (SC-H) and blood lymphocyte histones (BL-H), was used for the assay of PAR.

Immunoprobe assay of poly-ADP-ribosylation

PAR was assayed following slot and Western blotting of histone samples. The assay has been described in detail in our previous publication (Sharan et al., 2005). Briefly, samples were slot blotted on activated polyvinylidene difluoride (PVDF) membranes or subjected to 12% SDS-PAGE and subsequently transferred the resolved proteins on activated PVDF by Western blotting. One set of slot and Western blotted membranes were used for PAR immunoprobe using polyclonal antibody (PAb) against PAR. A replica of the slot and Western blotted PVDF was stained with India ink for total protein visualization (Kma and Sharan, 2006).

Preparation of genomic DNA

Genomic DNA was prepared from mice spleen using a standard method for mammalian tissue (Asubel et al., 1995) with some modifications. Control and 1, 2, 3, or 4-week treatment groups of mice were killed by cervical dislocation. Spleens were taken out, weighed and frozen at -80°C. Nine hundred mg of the tissue was ground to powder with pre-chilled mortar and pestle. To this digestion buffer (100mM NaCl, 10mM Tris-HCl, pH 8.0, 25mM EDTA, pH 8.0, 0.5% (w/v) SDS, 0.1 mg/ml proteinase K. The proteinase K being labile was added fresh for every use) was added at a volume of 1.2 ml for 100 mg of tissue. The sample was transferred to tightly capped tubes and incubated for 12 to 18 h at 50°C with frequent shaking. The samples were extracted with extraction solution (Phenol:chloroform:isoamyl alcohol (25:24:1) for few minutes. This was then centrifuged for 10 min at 1700g. The top (aqueous) layer was transferred to a new tube and the volume was measured. To this, half the volume of 7.5 M ammonium acetate and two volume of 100% ethanol were added. It was then centrifuged for 2 min at 1700 g. The pellet, genomic DNA was collect in eppendorf tubes and washed once with 70% ethanol. The pellet was air dried in oven at 37°C for 15 min. The DNA was quantified by diphenylamine test (Burton, 1968) with some modifications. The DNA was then resuspended in TE buffer (10mM Tris-Cl and 1mM EDTA, pH 8.0) at approximately 1mg ml⁻¹.

DNase I digestion of genomic DNA

Genomic DNA, isolated from spleen cells of control and treated group mice, were digested with DNase I enzyme to find out the structural alterations in the genomic DNA (Margison and O'Conner, 1979). Sterilized conditions were maintained throughout the experiment. Five μ l (28 μ g) of DNA sample was taken in an eppendorf tube and 8 μ l of digestion buffer (85mM KCl, 1mM CaCl₂, 5mM HEPES, pH 7.5 and 5% sucrose) was added. To this, 3.5 μ l (7.5 U) of DNase I was added. It was spun mildly and incubated for 3 min at 37°C. Immediately after incubation, 3.5 μ l of stop buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 15mM EDTA and 0.3% SDS) was added to stop the reaction.

Agarose gel electrophoresis

Agarose gel (0.6%) was prepared in TAE buffer (40mM Tris-acetate, pH 8.42, 1mM EDTA) and allowed to polymerize for 15-20min at room temperature. The samples (3 μ l, equivalent to 3.8 μ g of DNA) containing equivalent volume of loading buffer (40% sucrose and 0.25% bromophenol blue) were loaded in the TAE buffer-submerged wells. Electrophoresis was carried out at 100 V (constant) for 60 min in a mini gel electrophoresis system (Mupid, Japan). The gel was stained in 0.7 μ g ml⁻¹ of ethidium bromide for 15 min, destained and photographed after placing on a UV transilluminator.

Pulse-field gel electrophoresis

Agarose gel (1.2%) was prepared by dissolving 1.3g of agarose in 110ml of TBE (0.05 M Tris base, boric acid and EDTA, pH 8.3). The gel was allowed to polymerize in the gel casting kit on the horizontal gel platform. Samples (3 μ l, equivalent to 3.8 μ g of DNA) were loaded in wells after adding equivalent volume of loading buffer (40% sucrose and 0.25% bromophenol blue). Electrophoresis was carried out for 4h at 200 volts (constant) with pulse time of 25 sec at 8°C using gene navigator® system-pulse field gel electrophoresis system (Pharmacia Biotech, Sweden). After electrophoresis the gel was stained in 0.7 μ g ml⁻¹ ethidium bromide for 25 min, destained and then photographed on a UV transilluminator.

Quantification and analysis

The band intensities of the poly-ADP-ribosylated histones on immunoprobed slot and Western blots were quantified in terms of total pixel intensity of the band (Sharan et al., 2005; Kma and Sharan, 2008). Bio-Rad imaging densitometer and molecular analyst 1D software were used for capturing the images, quantification and analysis of the data. Data obtained from poly-ADP-ribosylated histones were expressed as per cent of control. Excel software was used for statistical analysis involving calculation of mean \pm SEM and t-test for statistical significance of data. Graph was plotted using SigmaPlot 9.0 software. Data were considered as statistical significant at a p-value of ≤ 0.05 .

Results

Influence of DMN on PAR of total histones

Histone proteins were prepared from SC and BL of normal and DMN treated mice. The samples were slot blotted on PVDF membrane. One of the slot blotted membrane was stained with India ink for total protein content, while replica of it was immunoprobed for PAR (Figure 1).

There was reduction in the PAR signal of the treated histone samples in comparison to the controls (Figure 1, immunoprobed panel), especially in 3rd and 4th week of DMN treatment, while total proteins slotted were quite identical (Figure 1, ink stained panel). Densitometric quantification of the immunoprobed slot blotted membranes corroborated the visual observation. The statistical significance of difference between control and DMN exposed groups was calculated at $p \leq 0.05$. (Figure 2)

showed that there was a negative correlation between the level of PAR of SC-H and BL-H and the period of DMN treatment. Although the reduction was very significant in both the cases, SC-H showed a relatively better response than BL-H at the corresponding time period vs. the respective controls (Figure 2). In the 4-week treatment groups, the reduction was up to 70% of the control in case of SC-H, whereas in case of BL-H, the reduction in PAR was more than 60% of the control at the corresponding period (Figure 2).

Influence of DMN on PAR of individual histone proteins

In order to assess the effect of DMN treatment on the PAR level of specific histone proteins, the isolated histones from SC and BL of normal and DMN treated mice

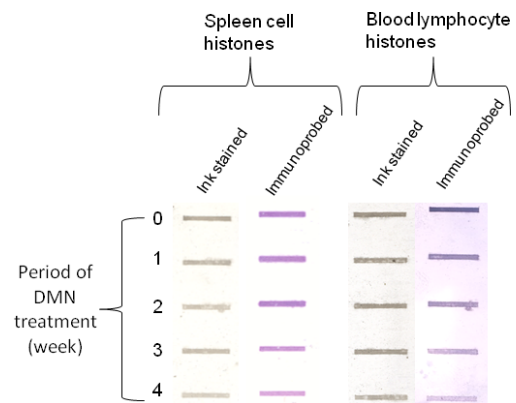


Figure 1. Slot Blots of Total Histones Isolated from Spleen Cells (First Two Panels) and Blood Lymphocytes (Last Two Panels). One of the slot blotted membranes were stained with India ink for total protein content, and its replica immunoprobed for PAR. Rows: 0: Control; 1, 2, 3 and 4: DMN treatment period in week, respectively

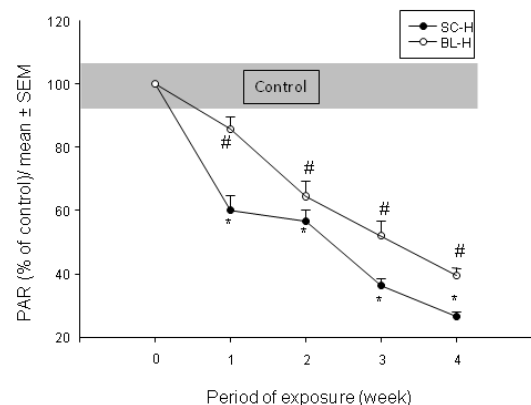


Figure 2. Plot of Poly-ADP-Ribosylation (PAR) of Histones Isolated from Spleen Cells (SC-H) and Blood Lymphocytes (BL-H) in Control and DMN Treated Mice. The data were obtained by densitometric quantification of the immunoprobed slot in figure 1. The mean pixel density of the ADP-ribosylated histones are expressed as % of control in the form of mean \pm SEM. 0: Control; 1, 2, 3 and 4: DMN treatment period in week. '# and "*" denotes statistically significant ($p \leq 0.05$) reduction in the level of PAR at different treatment period vs. the respective control

were further subjected to SDS-PAGE and the resolved proteins were transferred to PVDF membrane by Western blotting. One of the Western blotted membranes was immunoprobed for PAR and a replica of it was stained with India ink for total protein content (Figure 3).

A reduction in the band intensity, which represents a fall in the level of PAR of various SC-H and BL-H, was observed in the DMN treated groups when compared with the control (Figure 3, left panels in A & B). Significant reduction in the level of PAR was observed in the 3rd and 4th week of treatment in both the cases. No noticeable variation in the total protein content was seen in control and treated groups (Figure 3, right panel in A & B). Densitometric quantification of the immunoprobed individual histones was also carried out. The statistical significance of difference in the level of PAR of individual histones between control and DMN exposed groups was calculated at $p \leq 0.05$. Upon plotting the data in a graph, a negative correlation, similar to the slot blotted data (Figure 2), was observed between the level of PAR of different SC-H and period of DMN treatment (Figure 4A), particularly after 3 and 4 weeks of DMN treatment. The reduction was over 65%, over 70% and about 80% of control for histones H1, H3/H2B and H2A, respectively, in the 4-weeks treatment groups (Figure 4A).

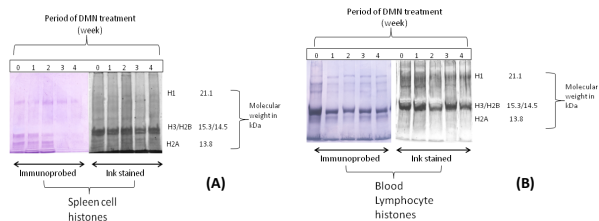


Figure 3. Western Blots of Isolated Histone Proteins of Mice After it were Subjected to SDS-PAGE. A) Spleen cell histones, and B) Blood lymphocyte histones. One of the Western blotted membranes was stained by India ink for total protein content (right blots) and a replica of it was immunoprobed for PAR (left blots). 0: Control; 1, 2, 3 and 4: DMN treatment period in week, respectively

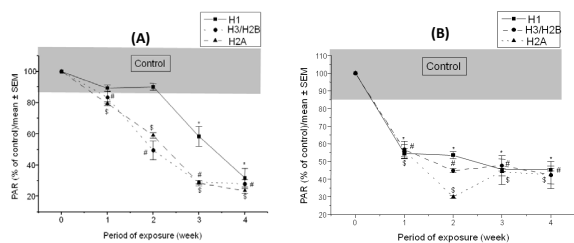


Figure 4. Plot of Poly-ADP-Ribosylation (PAR) of Specific Histones (H1, H3/H2B and H2A) Isolated from spleen cells A) and Blood Lymphocytes B) in Control and DMN Treated Mice. The data were obtained by densitometric quantification of the specific histones on immunoprobed Western blots in figure 3. The mean pixel density of the ADP-ribosylated histones are expressed as % of control in the form of mean \pm SEM. 0: Control; 1, 2, 3 and 4: DMN treatment period in week. ‘*’, ‘#’ and ‘\$’ denotes statistically significant ($p \leq 0.05$) reduction in the level of PAR of H1, H3/H2B and H2A respectively, at different treatment period vs. the respective control

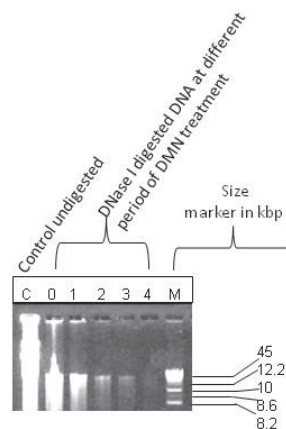


Figure 5. Agarose Gel Electrophoretogram of Genomic DNA Isolated from Spleen Cells of Mice Exposed to DMN Up to 4 Weeks and digested by DNase I. Lanes: C: Control undigested; 0: Control; 1, 2, 3 and 4: DMN treatment period in week; M: Size marker in kbp

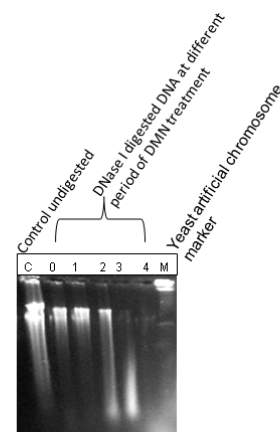


Figure 6. Agarose Gel Electrophoretogram of Genomic DNA Isolated from Spleen Cells of Mice Exposed to DMN Up to 4 Weeks and digested by DNase I. Lanes: C: Control undigested; 0: Control; 1, 2, 3 and 4: DMN treatment period in week; M: Yeast artificial chromosome marker

Similarly, BL-H demonstrated the characteristic negative correlation between the PAR of histone proteins and DMN exposure period (Figure 4B). There was up to 50% reduction in the PAR of H1, over 55% reduction in H3/H2B and H2A in 4-weeks treatment groups when compared with the control (Figure 4B). In general statistically significant reduction in the level of PAR in individual histones were in seen in SC-H and BL-H, particularly after the 3rd weeks of DMN exposure in comparison to the controls.

Effects of DMN on genomic DNA

Genomic DNA was isolated from spleen cells of normal and DMN exposed mice. The genomic DNA was subjected to DNase I digestion and loaded on a 0.6% agarose gel for electrophoresis. The genomic DNA was progressively more digested by DNase I with increase in the period of DMN exposure (Figure 5). Noticeable increase in the pattern of digestion was observed from 2-week of treatment onwards in the 3 and 4-weeks

treatment groups, drastic increase in the DNase I digestion was observed.

Pulse-field gel electrophoresis (PFGE) of DMN treated genomic DNA after DNase I digestion

The isolated genomic DNA from SC of normal and DMN exposed mice were further analyzed following digestion by DNase I using PFGE. Similar pattern of progressive increase in the digestion of the genomic DNA was obtained with increase in DMN exposure period. (Figure 6). However, a better resolution was achieved by PFGE compared to agarose gel electrophoresis.

Discussion

Early detection of cancer continues to be a formidable challenge to the scientific community and search for a suitable biomarker for use in mass screening of population is of utmost importance in our fight against cancer (Khan et al., 2012; Luna et al., 2012; van der Bilt et al. 2012; Bao et al., 2013; Hensing and Salgia, 2013; Suradej et al., 2013; Wang et al., 2013; Behrens et al., 2014; Sigari et al., 2014). Constant efforts are being made worldwide to find an effective and reliable biomarker and a convenient assay for early detection of cancers, especially in its initiation stage, which is when a cell gets committed to transformation. In this work attempts has been made to explore the possibility of poly ADP-ribosylation (PAR) of histone proteins as a biomarker for early detection of cancer. Correlations between the cellular PAR and different cellular and molecular events including carcinogenesis have been observed in our laboratory and elsewhere (Sharan et al., 2005; Devi and Sharan, 2006; Kma and Sharan, 2006; Sala et al., 2008; Binu et al., 2012; Ozaki et al., 2012; Chiou et al., 2013; Donà et al., 2013; Kalmar-Nagy et al., 2013; Long et al., 2013; Yuan et al., 2013). PAR was monitored by ELISA based immuno-probe assay, developed in our laboratory (Sharan et al., 2005). This assay was chosen because of its sensitivity, reproducibility and the ease with which it could be used (Kma and Sharan, 2006; Kma and Sharan, 2008).

It has also been shown earlier that initiation of carcinogenesis in mice generally occurs within 4 weeks or so (Pariat and Sharan, 2002; Tago et al., 2013). Therefore, the period of investigation in this piece of work was limited to 4 weeks. The choices of SC and blood lymphocytes for monitoring PAR of proteins were made to test applicability of the assay in diverse tissues of mice with different physiological states. Additionally, SC also served as a positive control. Blood lymphocytes were chosen for the work for an additional important reason. From the viewpoint of possible applied use of results in screening of population for early detection of cancer, blood lymphocyte is a very convenient tissue. Drawing of blood from a subject requires minimal medical intervention and causes virtually no trauma. If blood lymphocytes mirror biochemical and physiological status of a subject, use of biopsies could be totally avoided in the future. As we know, present medical practices heavily depend on analysis of biopsies especially in cancer diagnosis. There is not only surgical intervention and trauma involved in the

process of obtaining biopsies; there are ethical questions too associated with it. Therefore, investigation using blood lymphocytes assumes further significance.

A negative correlation exists between the level of PAR of SC-H and BL-H and the period of DMN exposure (Figures 1 & 2). It is also evident from (Figure 3) that most histones of SC and BL were poly ADP-ribosylated and showed similar trend of lowering of PAR with period of DMN exposure. Quantitative plot of PAR for SC (Figure 4A) and BL (Figure 4B) reaffirm the observation. The isolated histone proteins were identified and, for the purpose of quantitative analysis, grouped into three categories, namely H1, H3/H2B and H2A in this study. This was done based on their migration on PAGE gel and convenience of quantification (Figure 3A & B). While the PAR of histone H1 reduced to about 50% of control in the 4th week of DMN treatment in blood lymphocytes, SC showed relatively more effect reducing nearly 65% of the control. Histones H3/H2B also follows the same general pattern of diminution of PAR showing less effect in blood lymphocytes and more effect in SC. Histone H2A also repeated the pattern exhibiting differential PAR in the two tissues examined. The plots of these data are given in Figures 4A & 4B for SC and BL, respectively. The general conclusion that may be arrived at is that PAR differentially modifies different histone proteins in different tissues. This is in line with earlier reports of the same nature (Kma and Sharan, 2006, Kma and Sharan, 2008). The histone proteins of SC seemed to be relatively more modified under the influence of DMN while BL showed lower extent of PAR of its histone proteins under the influence of DMN. There will be need of further investigation to clarify the reason for this differential effect of DMN. Nonetheless, it can be concluded that DMN induced changes in histone proteins by way of reducing their extents of poly ADP-ribosylation during the initiation stage of DMN induced carcinogenesis. The PAR of histone proteins observed in our study confirms the earlier report of histones being the major target of cellular PAR (Kma and Sharan, 2006; Fontan-Lozano et al., 2010; Redon et al., 2010), making histones the most preferred target proteins for PAR.

Structural architecture and integrity of chromosomes or genomic DNA is critical. The structural integrity of the genomic DNA can be conveniently monitored by DNase I induced fragmentation of DNA (Apostolov et al., 2009). DNase I randomly cleave DNA, which is directly proportional to accessibility of the DNA to DNase I (Villani et al., 2010; Stephan et al., 2014). Therefore, if the DNA is in a condensed state, access of DNase I to DNA is limited and vice-versa. This can be conveniently visualized after the DNase I fragmented genomic DNA is electrophoresed on an agarose gel. This approach has been utilized to follow the influence of DMN on genomic DNA of mice during initial 4 weeks of exposure. (Figure 5) shows that the DMN enhanced the susceptibility of genomic DNA isolated from spleen cells of mice toward DNase I digestion. An increasing degradation of genomic DNA was observed as DMN exposure increased. The genomic DNA isolated from SC of mice exposed to DMN for 4 weeks (Lane 6, Figure 5) was virtually totally degraded by DNase I under the conditions of experiment

used in this study. The genomic DNA is heterogeneous as evident in lane 2 (Figure 5). However, certain stretches of DNA in the genome of untreated mice are accessible to DNase I in normal case also. This is shown as the large genomic DNA got fragmented to pieces of DNA of approx. size range of 10 to 60 kDa (Lane 2, Figure 5). However, upon exposure to DMN, the genomic DNA seemed to undergo relaxation starting from 1st week itself and the process continued till the end of our observation period (Lanes 3 to 6, Figure 5). Thus, there was a general and consistent pattern of increase in the degree of degradation of DNA. These observations indicate that genomic DNA underwent relaxation almost immediately following exposure to DMN *in vivo*.

The introduction of pulse-field gel electrophoresis (PFGE) has revolutionized the way to analyze large DNA pieces, hallmark of eukaryotes, to get additional insights. PFGE can resolve DNA pieces on agarose gel in the size range of 30-50 kbp to well over 10 Mbp (Schwartz and Cantor, 1984; Cheung and Gale, 1990). This was also used in the investigation to further our understanding on the genomic DNA isolated from DMN groups of mice. Following DNase I fragmentation, the samples were loaded for PFGE. Lane 1 (Figure 6) shows two large sized bands of DNA. Exposure to DMN caused progressive degradation of both these bands, which was relatively stronger for the lower band (Lanes 4-6, Figure 6). In the 4th week, the entire genome was reduced to very small pieces of DNA by DNase I, which looked like a smear (Lane 6, Figure 6). The result is in conformation with the agarose gel electrophoresis result (Figure 5) and suggests that the smaller sized fragment of genomic DNA was the initial target of DMN interaction.

In our experiments, evidence has been recorded of DMN induced change in the superstructure of genomic DNA (Figures 5 and 6). It has been shown earlier that carcinogenesis is favored when genes shift to relatively higher state of transcription (active genes) (De Souza Setubal Destro et al., 2010; Hong et al., 2014). The results presented in this work also showed that DMN pushed genes to progressively active state (Figures 5 and 6). The dose of DMN used in this investigation is reported to cause hepatocarcinogenesis (Pariat and Sharan, 2002; Kma and Sharan, 2006) and in that situation the genomic DNA of liver was in a relaxed state (Pariat and Sharan, 2002). DMN has been also reported to be carcinogenic elsewhere (Chikan et al., 2012). The results (Figure 5-6) show that even spleen cell genomic DNA was moved into relaxed state under the influence of DMN. It is possible that several genes of spleen cells also, including proto-oncogenes (s), were activated. This may mean that initiation of transformation might have been made even in spleen cells after DMN exposure. DMN have been reported to be metabolically activated or changed from potential carcinogen to ultimate carcinogen in mice when administered *in vivo* (Moto et al., 2006).

Reduction in the extent of poly-ADP-ribosylation of histone proteins has serious biological consequences. The first impact of this would be change in the structural organization of DNA since the charge status of histones shall be altered. Histones form an integral part in normal

organization of DNA to form chromatin. Qualitatively different extents of PAR of histones shall influence its weak-interactions with DNA to organize the chromatin superstructure (Osada et al., 2013). As is evident from DNase I degradation of genomic DNA, the superstructure underwent progressive relaxation (Figure 5-6) coinciding with decrease in the extent of PAR modification of different histone proteins. The alteration naturally shall have impact on the functional status of gene on chromosomes. Since it was shown that the genomic DNA underwent relaxation under similar conditions (Figure 5-6), it appears that lowering of total PAR of histone proteins may have facilitated the relaxation of the chromatin organization.

The level of PAR that has been assayed and quantified in this investigation is the result of the interplay of two main gene products involved in PAR metabolism, PARP and PARG (Fauzee et al., 2010; Feng and Koh, 2013). These genes are proposed to be located on chromosome 14 in mouse (Blake et al., 2014). The total genome of mouse underwent progressive relaxation (Figure 5-6). Thus, it can be proposed that at least selected segments of chromosome 14 had undergone alterations in a way that the expressions of PARP and PARG genes were altered. In general a lowering of PAR of histone proteins was observed in all cases. This may be caused by (a) low expression of PARP gene or (b) less active or inhibited PARP enzyme activity. Alternately, when (a) expression of PARG genes is more or (b) the PARG enzyme is more active then also the same will happen. This investigation did not address to these issues because one major purpose of this investigation was to look into the applied potential of PAR immuno-assay. However, the total outcome of any or all of these possibilities translated into lowering of extent of PAR of cellular proteins. This provides a valuable support to application of the results of the investigation for welfare of human kind.

Usually for detection of cancer a confirmatory test comes when the biopsies of cancerous tissues are investigated. Obtaining biopsies involves several medical and ethical issues and poses problems when a patient is subjected to surgical procedures to obtain biopsies. Further, obtaining biopsies of some tissues (e. g., brain tumor) is a serious medical intervention besides being traumatic to a patient. In this piece of work blood lymphocytes were used for the investigation after the mice was exposed to DMN *in vivo*. The aim was to find out if any correlation existed between the level of PAR of blood lymphocyte proteins and initiation of cancer. Simultaneously, the same was tested in spleen cells, which also served as positive controls for each other.

PAR of histone proteins was monitored using a novel ELISA-based immuno-probe assay developed in our laboratory. The assay is simple, sensitive, reliable, cost-effective and environment friendly since it does not involve use of any radioisotope. The observed effect of carcinogen on the PAR of blood lymphocyte histones provided us with a handy tool for monitoring biochemical or physiological status of individuals exposed to carcinogens without obtaining biopsies of cancerous tissues, which involves several medical and ethical issues. Simultaneously, spleen cells were also tested and

the same effect was observed in the PAR of spleen cell histones, which also served as positive controls for each other. Obtaining blood from any patient and separating blood lymphocytes are routine medical practices involving virtually no medical intervention, post-procedure medical care or trauma to a patient, and requires very basic and minimal medical infrastructures. The correlation between PAR of blood lymphocyte proteins and carcinogenesis does provide us with the prospect that medical screening in future might only involve drawing of blood from a patient for screening. This also makes it possible to handle a large number of patients at ordinary hospitals and primary health centers and even para-medics shall be able to do this. PAR, therefore, might possibly be a good biomarker for early detection of cancer or for monitoring progress of carcinogenesis since (1) it is a common post-translational modification associated with many proteins and (2) the assay, employed in this investigation, detects only the ADP-ribose moieties and not a particular protein. Combined with the ease of preparation of blood lymphocytes for the immuno-probe assay and the use of simple slot and Western blot techniques, immuno-probe assay of PAR has the potential to be applied for mass screening of cancer. It appears to be a promising step in the ultimate goal of making cancer detection simple, sensitive and reliable in the near future.

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