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CHRONIC EXPOSURE OF MICE TO AQUEOUS EXTRACT OF BETEL NUTS (AEBN) INHIBITS POLY-ADP-RIBOSYLATION (PAR) OF TOTAL CELLULAR PROTEINS AS WELL AS HISTONE H1 PROTEIN

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ABSTRACT

Molecular events involved in carcinogenesis are greatly influenced by poly-ADP-ribosylation (PAR) of chromosomal protein as the modification alters chromosomal superstructure and, consequently, its functional status. In this investigation we have attempted to find the correlation between exposure to aqueous extract of betel nuts (AEBN) and the PAR of total cellular and histone H1 proteins vis-à-vis initiation stage of AEBN induced carcinogenesis in a mouse model. Progressive inhibition of PAR of total cellular and histone H1 proteins were observed in bone marrow cells (BMC), spleen cells (SC) and peripheral blood lymphocytes (BL) of mice exposed to chronic, low-dose AEBN. Among the three tissues used in the investigation, BMC exhibited maximum inhibition of PAR, followed by SC and BL. However, the inhibition of PAR was statistically significant even in BL after 3 weeks of exposure to AEBN. The results suggest that PAR could be a promising candidate as a reliable biomarker for early detection of cancer. The slot blot immunoprobe assay of PAR using polyclonal antibody employed in the investigation can be potentially used for early cancer detection and in cancer screening programs.

Keywords: Aqueous extract of betel nut (AEBN), Poly-ADP-ribosylation (PAR), carcinogenesis, histone H1 protein, immunoprobe assay of PAR, mice.

INTRODUCTION

Poly-ADP-ribosylation (PAR) is a post-translational modification of chromosomal proteins in which ADP-ribosyl moieties are covalently attached to target proteins (Althaus and Richter, (1987)). The endogenous nicotinamide adenine dinucleotide (NAD^+) donates ADP-ribose residues for the reaction. The process is catalyzed by poly-ADP-ribose polymerase (PARP) enzyme, which is itself also a target protein for PAR (automodification) (Althaus and Richter, (1987)). The target proteins are numerous, majority of which are chromosomal proteins (Althaus and Richter, (1987); Poirier and Moreau, (1992); Sharan *et al.* (1998)). PAR is reported to be involved with various cellular processes including carcinogenesis (Althaus and Richter, (1987); Sharan *et al.* (1998); Miwa and Sugimura, (1990); Shall, (1994)). Carcinogenesis is a complex multi-step process involving mutations, structural alterations, gene rearrangements, gene amplifications, altered and neo-gene expressions, etc. (Pitot, (1986); Pitot *et al.* (1991)). All these molecular events are also greatly influenced by PAR of chromosomal protein as the modification alters chromosomal superstructure (Althaus and Richter, (1987); Poirier and Moreau, (1992); Miwa and Sugimura, (1990); Shall, (1994)).

Carcinogenesis is directly or indirectly initiated or induced by chemically synthesized compounds such as dimethylnitrosamine (DMN), diethylnitrosamine (DEN), benzopyrene, *etc.* or by natural constituents of plants etc. (OSHA, (1991)). Betel nut (*Areca catechu*. L.) has been one of such plants which have been reported to show strong etiological association with human carcinogenesis (IARC, (1985); Sharan, (1996); Stich *et al.* (1983); Pariat and Sharan, (1998); Wary and Sharan, (1988)). Betel nut (BN) consumption is very common in various tribes inhabiting South and Southeast Asia, including India, and Pacific Ocean islands such as Papua-New Guinea (Sharan, (1996); Majumdar *et al.* (1979)). Betel quid, one of the various forms of consumption of BN, consisting of lime and betel leaf, has long been suspected to be associated with various cancers of the oral cavity and upper digestive tracts (IARC, (1985); Sharan, (1996)). Earlier research has convincingly shown that the alkaloids such as arecoline, arecaidine, guvacoline, muscarine, *etc.* present in the BN extract affects various metabolic functions (Sharan, (1996); Majumdar *et al.* (1979); Sharan and Wary, (1992)) and are potential carcinogens (IARC, (1985); Sharan, (1996); Pariat and Sharan, (1998); Wary and Sharan, (1988); Balachandran and Sharan, (1995)). The aqueous extract of betel nuts (AEBN) has been subject of much investigation as a general carcinogen (Sharan, (1996); Wary and Sharan, (1988); Sharan and Wary, (1992); Balachandran and Sharan, (1995); Dave *et al.* (1992); Ashby *et al.* (1979); Saikia *et al.* (1999); Rao and Das, (1989); Pariat and Sharan, (2002)). It was shown to induce high degree of mutation in Ames test (Balachandran and Sharan, (1995)). AEBN and the predominant alkaloid of BN, arecoline, have also

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been reported to cause induction of cell transformation (Sharan, (1996); Pariat and Sharan, (1998); Sharan. and Wary, (1992); Saikia *et al.* (1999)) and strand breaks in DNA of mouse kidney cells *in vitro* (Wary and Sharan, (1988)). In this investigation we have attempted to find out a correlation between exposure to AEBN and the PAR of total cellular and histone H1 proteins vis-à-vis initiation stage of AEBN induced carcinogenesis in a mouse model.

MATERIALS AND METHODS

Chemicals: All chemicals were of analytical grade and were used without further purification. All required solutions were prepared in double distilled water.

Carcinogen preparation: Aqueous extract of betel nuts (AEBN) was prepared using protocol describe earlier (Pariat and Sharan, 1998)). Briefly, BN were peeled, coarsely grounded and suspended in double-distilled water overnight. The suspension was then filtered through filter paper and lyophilized to powdered form.

Animals: Swiss albino mice (Balb/C) were used in this investigation. The mice were maintained on standard mouse pellets (Pranav Agro Products, Delhi) and drinking water at 25°C in a controlled animal room of day-night cycle of 12 h duration.

Administration of AEBN: AEBN lyophilized powder was dissolved in distilled water at a concentration of 0.5 mg ml^{-1}. It was administered to young (6-8 weeks old) mice in chronic oral administration protocol reported earlier (Pariat and Sharan, (2002)). Mice were sacrificed for analysis by cervical dislocation at 0 (control), 1, 2, 3 and 4 weeks after initiation of the treatment.

Sample preparation: Whole homogenates (WH) of spleen cells (SC), bone marrow cells (BMC) and peripheral blood lymphocytes (BL) were prepared as described earlier (Kma and Sharan, (2003); Kma and Sharan, (2006)). BL was isolated using Biocoll based centrifugation method (Kma and Sharan, (2003); Kma and Sharan, (2006)) from freshly drawn blood from the heart of the untreated (control) and AEBN treated mice after sacrificing them. Protein estimation was done by Branford's method (Bradford, (1976)) using BSA as a standard.

Immunoprobe assay of poly-ADP-ribosylation: PAR was assayed using a polyclonal antibody (PAb) against ADP-ribose polymers as detailed in a recently published paper (Sharan *et al.* (2005)). Briefly, samples were slot blotted on activated polyvinyldifluoride (PVDF) membranes or subjected to 12% SDS-PAGE and subsequently transferred the resolved proteins on activated PVDF by Western blotting. Slot and Western blotted PVDF were immunoprobed with PAb against ADP-ribose polymers. Following this, a second Ab~ALP conjugate was used in combination with BCIP/NBT color developer to visualize poly-ADP-ribosylated proteins as detailed earlier (Sharan *et al.* (1998); Kma and Sharan, (2003); Kma and Sharan, (2006); Sharan *et al.* (2005)). A replica of the slot and Western blotted PVDF were stained with India ink (0.2 %, 3-4 h) to visualize total proteins.

Analysis of immunoprobed slot- and Western blots: The band intensities of the poly-ADP-ribosylated proteins were quantified in terms of total pixel intensity of the band (Kma and Sharan, (2003); Kma and Sharan, (2006); Sharan *et al.* (2005)). 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 protein were expressed as per cent of control. Statistical analysis involving calculation of mean ± SEM and t-test for statistical significance of data were performed using Excel software. Graph was plotted using Origin software. P-values of ≤ 0.05 were taken as biologically significant.

RESULTS

In this investigation experimental sets of 6 mice each of same sex and age groups were exposed chronically to AEBN in drinking water (0.5 mg ml^{-1}) for periods up to four weeks. The exposed mice were sacrifice at intervals of 1, 2, 3 and 4 weeks of treatments. Sex and age matched group of untreated mice served as control. WH of SC, BMC and BL were prepared. The samples were slot or Western blotted on PVDF as described (Sharan *et al.* (1998); Kma and Sharan, (2003); Kma and Sharan, (2006); Sharan *et al.* (2005)). For slot blotting, 2-4 µg protein in 100 µl volume was used. For Western blotting, 35-40 µg protein in 25 µl was loaded in each well on SDS-PAGE. Figure 1 shows the slot blots of BMC, SC and BL. For each of the tissues the left panel represents the immunoprobed slots and the right panel is that of India-ink stained (Fig. 1). The gradual reduction in PAR band intensity of treated samples can be seen when it is compared with control (Fig. 1; left panels) for approximately the same quantity of slotted protein (Fig. 1; right panels). The reduction was visually significant in all tissues examined after 3rd and 4th week of exposure to AEBN. Upon quantification of the bands, the data were plotted in a graph (Fig. 2). This graph confirms the observed trend. About 60-80% reduction in total cellular PAR was observed after 4 weeks of AEBN treatment (Fig. 2). Maximum reduction in total cellular PAR was observed in BMC which was followed, in sequence, by SC and BL (Figs. 1 and 2). The immunoprobed slot blot reflects the net effect of AEBN exposure on total cellular PAR; it does not show which specific protein was poly-ADP-ribosylated. This information was provided by the immunoprobed Western blots. Because of important role

of histone H1 in chromatin superstructure and its easy accessibility for PAR, histone H1 is known to be a preferred target protein for PAR (Althaus and Richter, (1987); Miwa and Sugimura, (1990)). This investigation was designed to ascertain contribution of PAR of histone H1 protein in the observed total cellular PAR (Figs. 1 and 2). The immunoprobed Western blots (Fig. 3) showed high degree of PAR of a 20 kDa protein, which is very likely to be histone protein H1 (average mol. wt. 21.1 kDa), responding negatively to AEBN exposure. In case of BMC and SC (Fig. 3A, B), the decrease in PAR of H1 was observed from 2nd week of treatment (Fig. 4). However, BL (Fig. 3C) showed significant reduction in PAR only after 3 weeks of treatment (Fig. 4). While BMC and SC exhibited about 80% reduction in PAR of H1 after 4 weeks of AEBN exposure, BL showed about 60%

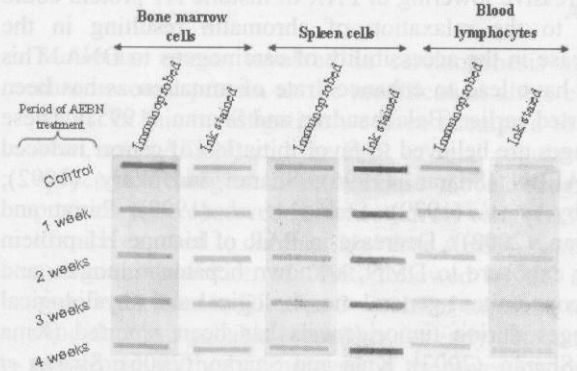

Figure 1: Slot blots of whole homogenates of mouse bone marrow cells, spleen cells and blood lymphocytes from control or aqueous extract of betel nut (AEBN) exposed mice. The blots were immunoprobed to visualize total cellular poly-ADP-ribosylation (PAR) (left panels) or stained with India ink for visualization of total proteins (right panels).

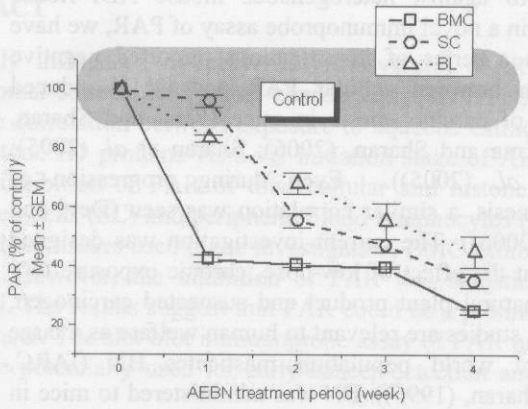

Figure 2: Plot of poly-ADP-ribosylation (PAR) of total cellular protein of bone marrow cells (BMC) (—□—), spleen cells (SC) (---o---) and blood lymphocytes (BL) (---Δ---) in control and aqueous extract of betel nut (AEBN) exposed mice. The data were obtained by densitometric quantification of the immunoprobed slots in figure 1. The mean pixel density of the ADP-ribosylated protein bands are expressed as % of control in form of mean ± SEM.

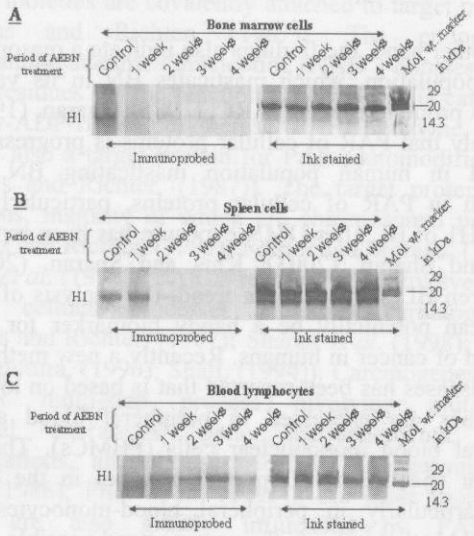

Figure 3: Western blots of whole homogenate of bone marrow cells (panel A), spleen cells (panel B) and blood lymphocytes (panel C). One of the blots was immunoprobed to visualize poly-ADP-ribosylation (PAR) of individual proteins (left blots) while the replica was stained with India ink to visualize total proteins (right blots). Poly-ADP-ribosylated histone H1 protein is marked 'H1' on the immunoprobed Western blot.

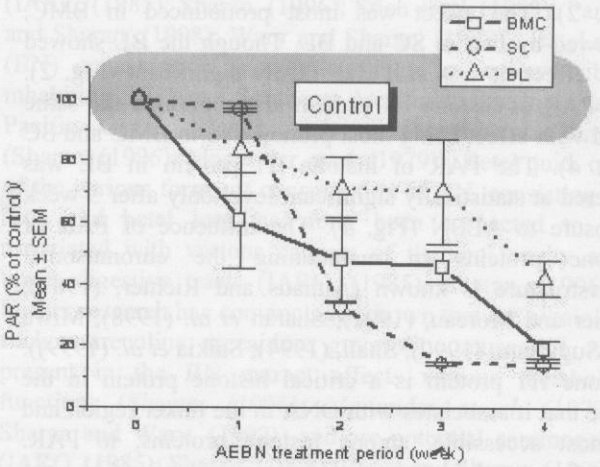

Figure 4: Plot of poly-ADP-ribosylation (PAR) of histone H1 protein of bone marrow cells (BMC) (—□—), spleen cells (SC) (---o---) and blood lymphocytes (BL) (---Δ---) in control and aqueous extract of betel nut (AEBN) exposed mice. The data were obtained by densitometric quantification of the histone H1 band on the immunoprobed Western blots in figure 3. The mean pixel density of the ADP-ribosylated H1 proteins are expressed as % of control in form of mean ± SEM.

reduction during the same period of treatment (Fig. 4). A general trend of negative relationship between initial period of AEBN exposure and level of PAR, as revealed by immunoprobe assay, seems to be hallmark of early cellular response in all the samples undertaken for the study (Figs. 2 and 4).

DISCUSSION

Using PAb against heterogeneous mouse ADP-ribose polymers in a novel immunoprobe assay of PAR, we have earlier, in a series of investigations, reported negative correlation between cellular PAR and DMN induced initiation of carcinogenesis in mice (Kma and Sharan, (2003); Kma and Sharan, (2006); Sharan *et al.* (2005); Devi *et al.* (2005)). Even during progression of carcinogenesis, a similar correlation was seen (Devi and Sharan, (2006)). The current investigation was designed to find out the effect of low-dose, chronic exposure to a general, natural plant product and suspected carcinogen, BN. Such studies are relevant to human welfare as a large section of world population masticates BN (IARC, (1985); Sharan, (1996)). BN was administered to mice in form of AEBN in drinking water in a low-dose, chronic exposure protocol. In general, the exposure resulted in gradual, exposure period dependent decrease in the level of PAR of total cellar proteins (Fig. 1) as well as of histone H1 protein (Fig. 3) in all three tissues examined in this investigation. Upon quantification, the trend was clearer (Figs. 2, 4). The total cellular PAR was significantly inhibited after 2 weeks of exposure to AEBN (Fig. 2). The effect was most pronounced in BMC, followed by that in SC and BL. Though the BL showed least effect, it was still statistically significant (Fig. 2). The PAR of histone H1 protein also exhibited the same trend with effect being most pronounced in BMC and SC (Fig. 4). The PAR of histone H1 protein in BL was lowered at statistically significant level only after 3-week exposure to AEBN (Fig. 4). The influence of PAR of histone proteins in maintaining the chromosomal superstructure is known (Althaus and Richter, (1987); Poirier and Moreau, (1992); Sharan *et al.* (1998); Miwa and Sugimura, (1990); Shall, (1994); Saikia *et al.* (1999)). Histone H1 protein is a critical histone protein in the sense that it associates with DNA in the linker region and is most accessible, among histone proteins, to PAR. Therefore, modification of this proteins is bound to have effect on the nature of its interaction with the DNA and, consequently, to chromatin functions (Saikia *et al.* (1999)). Our finding is also supported by an earlier study where it was shown that PAR of HMG proteins of liver was modified after AEBN treatment (Pariat and Sharan, (1998)). HMG proteins are known to play key role in the organization and function of chromatin (Pariat and Sharan, (1998); Saikia *et al.* (1999)). Therefore, it appears that reduction in the level of total cellular PAR and/or PAR of histone H1 protein would also alter gene expression pattern. As AEBN is a known carcinogen (IARC, (1985); Sharan, (1996); Stich *et al.* (1983); Pariat and Sharan, (1998); Wary and Sharan, (1988); Majumdar *et al.* (1979); Sharan and Wary, (1992); Balachandran and Sharan, (1995); Dave *et al.* (1992); Ashby *et al.* (1979); Saikia *et al.* (1999); Rao and Das, (1989); Pariat and Sharan, (2002)), it would be reasonable to assume that progressive lowering of PAR upon exposure to AEBN is in favor of initiation of carcinogenesis in mice. Progressive lowering of PAR of histone H1 protein could lead to the relaxation of chromatin resulting in the increase in the accessibility of carcinogens to DNA. This may have lead to enhanced rate of mutation as has been reported earlier (Balachandran and Sharan, (1995)). These changes are believed to favor initiation of cancer induced by AEBN (Sharan, (1996); Sharan and Wary, (1992); Ashby *et al.* (1979); Saikia *et al.* (1999); Pariat and Sharan, (2002)). Decrease in PAR of histone H1 protein after exposure to DMN, a known hepatocarcinogen, and the concomitant general morphological and physiological changes during tumorigenesis has been reported (Kma and Sharan, (2003); Kma and Sharan, (2006); Sharan *et al.* (2005); Devi *et al.* (2005); Devi and Sharan, (2006)). Since AEBN also replicates the same pattern, it appears that initiation of carcinogenesis either by a specific carcinogen (e. g., DMN) or a general carcinogen (e.g., AEBN) follows the same molecular path. This might suggests a similar mechanism of action of AEBN, DMN or any other carcinogen on PAR of total cellular proteins and/or histone proteins. More work will be required to substantiate this hypothesis.

On the other hand, the findings also indicate a reason why human population, which masticates BN in its various forms, is prone to cancer (IARC, (1985); Sharan, (1996)). It is likely that PAR of cellular proteins is progressively inhibited in human population masticating BN. The reduction in PAR of cellular proteins, particularly the histone H1 of BL after DMN exposure has been reported (Kma and Sharan, (2003); Kma and Sharan, (2006)). Since even BL shows similar trend, the analysis of PAR in BL can potentially be a handy biomarker for early detection of cancer in humans. Recently a new method to detect diseases has been reported that is based on looking how genes are expressed in peripheral blood and/or peripheral blood mononuclear cells (PBMCs). The test looks for specific transcriptional changes in the blood cells, particularly in peripheral blood-monocytes and lymphocytes that indicate the presence and possible status of disease elsewhere in the body. It was successfully used to detect renal cancer in 45 patients (Burczynski and Dorner, (2006)) and also to distinguish among different types of genitourinary cancers (Osman *et al.* (2006)). Difference in the peripheral blood profiles between patients with solid tumors and healthy controls was observed in above cases. Therefore, our investigation on BL becomes more relevant although our approach is

different. It will probably also reduce our dependence on human biopsies for detection of cancer to a certain extend. As the hunt for a reliable biomarker for early detection of cancers goes on, PAR appears promising. The slot blot immunoprobing of PAR using PAb has been developed, standardized and employed in our investigation is easy, reliable and cost effective. Currently, we are studying the PAR of total cellular proteins in BL and other tissues of cancer patients and normal humans with no history of cancers.

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