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Molecular Basis of Vanadium-Mediated Inhibition of Hepatocellular Preneoplasia During Experimental Hepatocarcinogenesis in Rats

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Abstract Carcinogen-induced early DNA lesions and metallothionein (MT) over-expression have been implicated in cell proliferation and thereby subsequent expression of premalignant phenotype of the cell. We have therefore investigated the chemopreventive potential of vanadium in a multi-biomarker approach, viz. 8-hydroxy-2′-deoxyguanosines (8-OHdGs), DNA single-strand breaks (SSBs), DNA-protein crosslinks (DPCs), chromosomal aberrations (CAs), in situ MT expression, and cell proliferation in rat liver preneoplasia. Hepatocarcinogenesis was induced in male Sprague–Dawley rats with a single, necrogenic, intraperitoneal (i.p.) injection of diethylnitrosamine (DEN) (200 mg/Kg body weight) at week 4 of the experimental protocol followed by promotion with phenobarbital (PB) (0.05% in basal diet), on and from week 8 and continued till 32 weeks in a long-term regimen. There was a significant and steady elevation of modified DNA bases 8-OHdGs ($P < 0.0001$; 90.69%) along with substantial increments of the extent of SSBs ($P < 0.001$) and CAs ($P < 0.001$) following DEN exposure. Supplementation of vanadium at a dose of 0.5 ppm abated the formations of 8-OHdGs (80.63%; $P < 0.0001$), SS-DNAs ($P < 0.001$) and SSBs/DNA unit ($P < 0.01$; 56.39%), DPCs (59.26%; $P < 0.0001$) and CAs (71.52%; $P < 0.001$) in preneoplastic rat liver studied at various time points. Low dose of vanadium treatment further reduced liver-MT immunoreactivity ($P < 0.05$) and BrdU-labeling index ($P < 0.02$) and a significant positive correlation ($r = 0.92$; $r^2 = 0.85$; $P = 0.0001$) was noted between them. Continuous vanadium administration also decreased nodular incidence (66.67%) and nodule multiplicity (62.12%; $P < 0.001$) along with substantial improvement in the altered hepatocellular phenotype when compared to DEN + PB treatment alone. The study indicates that vanadium-mediated suppression of cell proliferation and resulting premalignant expression might be due to the observed reductions in hepatic 8-OHdGs, SSBs, DPCs, CAs, and MT immunoreactivity. Vanadium is chemopreventive for DEN-induced hepatocellular preneoplasia in rats. J. Cell. Biochem. 101: 244–258, 2007.

Key words: vanadium; hepatocarcinogenesis; preneoplasia; DNA damage; 8-OHdGs; DNA strand-breaks; DNA-protein crosslinks; chromosomal aberrations; metallothionein; cell proliferation

Vanadium, a dietary micronutrient is generally present in most of the human diet, including grain, cereals, fish, fruits and vegetables and is required in small amount for normal cell metabolism as well as for proper growth and development of mammals [Hopkins and Mohr, 1971; Nielsen and Uthus, 1980; French and Jones, 1993]. Vanadium compounds have been found to be potentially effective against murine leukemia, fluid and solid Ehrlich ascites tumor [Kopf-Maier and Kopf, 1988], human lung, breast, gastrointestinal tract, and nasopharyngeal carcinomas [Köpf-Maier, 1994; Sakurai et al., 1995], and human leukemia cells, multiple myeloma cells and a number of solid tumors derived from cancer patients [Navara et al., 2001; D'Cruz and Uckun, 2002; Scrivens et al., 2003]. This nutritional element is involved in

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various DNA maintenance reactions and thereby may prevent genomic instability leading to cancers [French and Jones, 1993; Stern et al., 1993; Evangelou, 2002].

A series of studies from our laboratory has shown that supplementation of 0.5 ppm vanadium in drinking water was quite effective in suppressing chemically induced hepatocarcinogenesis in rats without any toxic manifestations [Bishayee et al., 1999; Basak and Chatterjee, 2000; Basak et al., 2000; Chakraborty et al., 2005]. The 0.5 ppm (4.27 μmol/L) concentration of vanadium was chosen by dose-response studies made in our laboratory much earlier [Bishayee and Chatterjee, 1995]. This particular low dose of vanadium has been found to be non-toxic and well tolerated with adequate growth responsive effect.

We extend our study further to have insights into the molecular events associated with vanadium-mediated inhibition of rat liver preneoplasia. Carcinogen-induced DNA damage has been implicated as one of the early steps in chemical carcinogenesis [Lindahl, 1993]. It has been shown that 8-hydroxy-2′-deoxyguanosine (8-OHdG), the most sensitive and potential marker of oxidative DNA damage is closely associated with certain diseases, including cancer, and is produced in various experimental models of chemical carcinogenesis [Kasai, 1997; Nakae et al., 1997; Shen et al., 2003]. Thus, studying the pattern of changes in the levels of tissue-specific 8-OHdGs following carcinogen assaults could be quite relevant in understanding the initiation event of carcinogenesis. Besides 8-OHdGs, the magnitudes of DNA single-strand breaks (SSBs) and DNA-protein crosslinks (DPCs) are the measures of genotoxicity following carcinogen exposure. The inability of cells to repair such damage adequately is a putative causal event in chemical carcinogenesis [Jia et al., 2002; Shen et al., 2003]. Chromosomal aberrations (CAs), which occur with greatest frequency in cells and involved in the origin, progression, and diversification of cancers are considered to be good somatic markers as well [Land et al., 1983]. We have further studied the role of liver metallothionein (MT) expression on hepatic cell proliferation, as several reports indicate that over-expression and elevated level of MT is associated with increased cell proliferation in various tumors [Oyama et al., 1996; Jayasurya et al., 2000].

The present study was designed with two basic objectives: first, to assess the roles of 8-OHdGs, SSBs, DPCs, CAs, and MT on hepatic cell proliferation and thereby preneoplastic development following carcinogen assaults; and second, to find out the chemopreventive potential of vanadium in limiting preneoplasia in a defined rodent model of experimental hepatocarcinogenesis. The diethylnitrosamine (DEN)-phenobarbital (PB) rat model provides a potential tool for studying molecular and cellular changes resulting from the administration of the carcinogen to the development of premalignant phenotype of the cell, mechanisms of cell growth, differentiation, and cell death [Farber, 1984a]. This study is an attempt to focus on the interaction of the dietary micronutrient vanadium with the critical molecule like DNA and inhibitions of early DNA and chromosomal damages by this trace metal at low dose.

MATERIALS AND METHODS

Materials and Maintenance of Animals

All the reagents and biochemicals, unless otherwise mentioned were obtained from Sigma Chemicals Co. (St. Louis, MO), USA and E. Merck, (Darmstadt) Germany.

Male Sprague–Dawley rats obtained from the Indian Institute of Chemical Biology (CSIR), Kolkata, India weighing 80–100 g at the beginning of the experiments were used throughout the study. The animals were acclimatized to standard laboratory conditions (temperature $24 \pm 1^{\circ}C$, relative humidity $55 \pm 5\%$ and a 12 h photoperiod) in Tarson Cages (four to five rats per cage) for 1 week before the commencement of the experiment. During the entire period of study, the rats were supplied with a semi-purified basal diet (Lipton India Ltd., Mumbai, India) and water ad libitum. The recommendations of Jadavpur University's "Institutional Animal Ethics Committee" ["Committee for the Purpose of Control and Supervision of Experiment on Animals" (CPCSEA Regn. No. 0367/01/C/CPCSEA) INDIA] for the care and use of laboratory animals were strictly followed throughout the study and the particular project was approved by the Chairman of the Committee.

Experimental Regimen

Rats were randomly divided into four experimental groups for carrying out various

experiments. In short-term experimental protocol, groups C and D rats were the DEN-treated groups that received a single, necrogenic, intraperitoneal (i.p.) injection of DEN (200 mg/kg body weight in 0.9% saline) at 9 weeks of age, that is, at week 4 of experimentation. Group A rats were the normal control; whereas, Group B (vanadium treatment) and Group D (vanadium + DEN) rats received 0.5 ppm (4.27 μmol/L) vanadium (w/v) as ammonium metavanadate (NH_4VO_3, +V oxidation state) in drinking water, ad libitum starting 4 weeks prior to DEN initiation and stopped at week 4. Solutions of vanadium (pH 7.0) were renewed every 2–3 days. Daily food and water intakes were noted and the body weights of the animals from each group were recorded every second day. All the rats were sacrificed by decapitation between 09:00 and 11:00 h under proper light ether anesthesia after week 4 at various time points to carry out experimentations. All the animals were fasted overnight before sacrifice. For the estimation of 8-OHdGs and DPCs, rats were sacrificed after 3, 6, 12, 18, 24, and 48 h; for SSBs after 18–20 h; and for CAs study after 5, 30, 60, and 90 days of DEN challenge, livers were promptly excised, chromosomes and hepatic DNA were isolated.

For the assessment of MT immunoreactivity, cell proliferation index and histomorphometry of preneoplastic lesions, rats comprising of four groups [group E (normal control); group F (vanadium treatment); group G (DEN + PB treatment); and group H (vanadium + DEN + PB)] were sacrificed after 33 weeks, starting the experiment at week 0 (long-term experimental protocol). All the DEN-initiated rats (groups G and H) at week 4 were administered PB as the carcinogenic promoter in basal diet (0.05%), once daily for 5 days a week after a 3 weeks of recovery from DEN initiation, that is, after week 7 and continued thereafter till 32 weeks. Vanadium at the same concentration as given in the short-term treatment was supplemented throughout the study, that is, for 32 consecutive weeks. At least 1–2 h prior to killing, the rats used for cell proliferation study were injected with 5-bromo-2′-deoxyuridine (BrdU) at a dose of 100 mg/kg body weight, intraperitoneally for the assessment of hepatic BrdU-labeling index.

Measurement of 8-OHdGs

DNA was extracted from perfused livers of different groups of rats with chloroform only (because phenol exposure would artificially increase oxidative base concentration in DNA samples) following the protocol of Dahlaus and Appel [1993] with minor modifications. Briefly, one volume of nuclear fraction obtained from liver homogenate by centrifugation was mixed with eight volumes of extraction buffer [1 M NaCl, 10 mM Tris-HCl, 1 mM ethylenediamine tetra-acetic acid (EDTA), 2% sodium dodecyl sulfate (SDS), pH 7.4] and one volume of chloroform: isoamyl alcohol (12:1 v/v). After vigorous shaking, the aqueous phase was separated by centrifugation and DNA in TE buffer was incubated with a mixture of ribonucleases (RNase T1 and RNase A). Finally, DNA was extracted again and precipitated with chilled ethanol. DNA concentration was estimated spectrophotometrically using 20 A_{260} U/mg.

Two hundred micrograms of DNA (4–5 A_{260} U/200 μl) in 40 mM Tris, pH 8.5, containing 10 mM $MgCl_2$ was first denatured by heating at 95°C for 3 min and then cooled on ice. The extracted DNA was digested into deoxynucleosides by incubation for 2 h at 37°C with a mixture of DNase I (from bovine pancreas; 200 U/mg DNA), spleen exonuclease (0.01 U/mg), snake venom exonuclease (0.5 U/mg), and *E. coli* alkaline phosphatase (10 U/mg) [Park et al., 1989]. The incubation was terminated with acetone to precipitate proteins and DNA was dissolved in distilled, deionized water.

The content of 8-OHdGs in the digested DNA was measured by an electrochemical detector (ECD) coupled with high performance liquid chromatography (HPLC-ECD system) [Shen et al., 1995] which consisted of a Waters 600E pump, a Whatman Partisphere-5 C_{18} column and a UV detector (Hewlett-Packard 1050, 254 nm) connected to an ECD (Hewlett-Packard 1049A) in series for monitoring dGs and 8-OHdGs, respectively. The mobile phase consisted of 10 mM $NH_4H_2PO_4$, 10 mM KCl, 1 mM EDTA, and 10% aqueous methanol (pH 4.7) and the flow rate was set at 1.0 ml/min. The content of 8-OHdGs in each DNA sample was expressed as the molar ratio of 8-OHdG $\times 10^5$ to total dG based on the peak height of authentic 8-OHdGs with EC detector and the UV absorbance at A_{254} of dGs [Shen et al., 1995]. On prolonged incubation of the DNA sample with *E. coli* alkaline phosphatase under the conditions described above, the content of 8-OHdG increased at the rate of 0.4 ± 0.1 residue/10^5

dG/h. Therefore, this blank value for 8-OHdGs was subtracted from the observed data.

Assay of DNA Unwinding

The principle of Fluorimetric Analysis of DNA Unwinding (FADU) is that the fluorescent dye ethidium bromide (EtBr) binds selectively to double-stranded DNA (DS-DNA) in the presence of single-stranded DNA (SS-DNA) when short duplex regions in SS-DNA molecules are destabilized by alkali treatment [Sarkar et al., 1997]. After isolation of DNA from the frozen rat liver by a modification of the published criteria [Gupta, 1984], DNA solution was divided equally among three sets of tubes [Sarkar et al., 1997]. The contribution to fluorescence by components other than DS-DNA (including free dye) is estimated from a blank sample (B) in which the DNA sample is first sonicated highly and then treated with alkali under conditions, which cause complete unwinding of low molecular weight DS-DNA. A second sample is used for estimating the total fluorescence (T), that is, fluorescence due to the presence of DS-DNA with contaminants. The difference (T–B) provides an estimate of the amount of DS-DNA in the DNA pool. A third sample (P) is exposed to alkaline conditions sufficient to permit partial unwinding of the DNA, the degree of unwinding being related to the size of DNA.

$$\text{Percent D (DS-DNA\%)} = \frac{(P - B)}{(T - B)} \times 100$$

Estimation of SSBs

It is assumed that the distribution of SSBs in the DNA population follows a simple Poisson's Law. Under this circumstance, it is possible to make an approximate estimate of the average number of SSBs (n) per DNA fragment from the following equation [Basak, 1996]:

$$e^{-n} = \frac{D}{S} + D$$

S = percentage DNA that remains single-stranded after alkali treatment, D = percentage entage remaining as DS-DNA. D/S + D represents the fraction (f_o) of the molecules without strand-breaks. The values of "n" corresponding to different DNA solutions isolated from different groups were then estimated.

Isolation and Quantitation of DPCs

DPCs were measured as described by Zhitkovich and Costa [1992]. Liver tissue was homogenized in 0.5 ml of 2% SDS and 1 mM phenyl-methyl-sulfonyl fluoride (PMSF) in 20 mM Tris-HCl (pH 7.5) in a total volume of 1.5 ml. The homogenate was incubated at 37°C with ribonuclease A (RNase A, 200 μg/ml) for 1 h. To initiate isolation of DPC, DNA was sheared by passage of the lysate (20–25 times) through a 24-gauge needle using a hypodermic syringe. After a further addition of 0.5 ml of 100 mM KCl containing 20 mM Tris-HCl, pH 7.5 (Solution A), the mixture was vigorously vortexed and heated at 65°C for 10 min, inverted and then placed on ice for 5 min to form the KCl-SDS-Protein-DNA precipitates which was collected by centrifugation at 5,000*g* for 6 min at 4°C. The pellet was resuspended in Solution A and the washed precipitate was then incubated with 0.2 mg/ml proteinase K at 50°C for 3 h containing 0.1 M KCl and 10 mM EDTA in 20 mM Tris-HCl (pH 7.5). Released SDS was removed by cooling the sample in presence of bovine serum albumin. The amount of DNA in the supernatant was determined using a fluorescent dye (Hoechst 33258) in a spectrofluorimeter (LS-45, Perkin Elmer, USA) with excitation and emission wavelengths of 365 and 460 nm, respectively. Total hepatic DNA was determined by measuring the free DNA in the supernatants during several washing steps. The DNA-protein crosslinking coefficient (DPC coefficient) was expressed as a ratio of the percentage of SDS-precipitable DNA in treated sample to the percentage of SDS-precipitable DNA in untreated control sample.

Rat Liver Chromosome Preparation by Partial Hepatectomy

Pretreatment was performed by injecting (i.p.) colchicine in 0.9% sodium chloride at 2 mg/kg body weight 3 h before killing. Hepatocytes were isolated following the procedure of Horiuchi et al. [1984] that involved incubation of finely minced rat liver slices (about 1 mm^3) with 0.05% collagenase type IV (Sigma) solution for 30 min. The supernatant was then carefully removed and 10 ml of Hanks' solution (Ca^{2+} and Mg^{2+} free) was added to the tissue. The hepatocyte suspension was obtained by gently pipetting the tissue up and down and then allowing it to stand for 5 min. The supernatant

was then subjected to centrifugation at 3,200*g* for 5 min. Pellets of isolated hepatocytes were resuspended in 0.075 M KCl and kept at 37°C for 25 min. The cells were then fixed with a fixative (methanol-glacial acetic acid, 3:1) that was changed three times. After the third fixation, chromosome slides were prepared by spreading the fixed cells over chilled (in 50% methanol) and grease free slides and put through a flame. The slides were kept overnight at room temperature and stained with 3% Giemsa (pH 5.9) for 30 min for scoring chromosomal anomalies.

Scoring of CAs

Metaphase cells with one or more types of CAs were scored blind from 50 well-spread metaphase plates per rat (i.e., 250 metaphase plates/group, $n = 5$) and the frequency of CAs was expressed as the percentage of total aberrant metaphase plates and as the average number of total aberrations.

Immunostaining of MT and Cell Proliferation Assay

The hepatic cells that had incorporated BrdU in the hours prior to the animals being killed were next detected by the method of Lanier et al. [1989]. The deparaffinized tissue sections were hydrolyzed briefly with 0.1 N HCl for 10 min followed by several washings in Tris-NaCl. Immunolocalization of MT protein and BrdU labeling in separate liver sections were performed by the streptavidin-avidin-biotin-peroxidase-complex method [Dragon et al., 1994; Jin et al., 2002]. Briefly, endogenous peroxidase activity was blocked with 1% H_2O_2. After incubation with 5% normal goat serum, sections were incubated overnight at 4°C with rabbit anti-rat MT-1 (1:50) and mouse anti-BrdU clone (1:500). Sections were then incubated with the respective biotinylated secondary antibodies, goat anti-rabbit IgG (1:200) and rabbit anti-mouse IgG (1:500) followed by incubation with streptavidin peroxidase (1:200) and subsequent chromagen development with 0.5% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.33% H_2O_2 as the substrate. MT immunoreactivity was expressed as percentage of immunopositive cells. The number of +ve cells was determined in relation to the total number of cells in 10 high power fields [Jin et al., 2002]. BrdU-labeling index (BrdU-LI) as an indicator of cell proliferation was determined as the number of hepatocyte nuclei exhibiting BrdU incorporation per 1,000 hepatic nuclei [Dragon et al., 1994].

Histomorphometry of Liver Tissue

For the rats sacrificed at 33 weeks, the livers were promptly excised, blotted, and weighed. The livers were then examined macroscopically on the surface as well as in 3-mm cross sections for gross visible persistent nodules (PNs), which represent focal proliferating, hepatic lesions with a low tendency for spontaneous regression [Farber, 1984b]. The PNs were easily identified from the reddish-brown non-nodular surrounding parenchyma (NNSP) by their grayish-white color which clearly differentiated them from the adjacent liver tissue. The PNs that approximated spheres were measured in two perpendicular directions to the nearest millimeter to obtain an average diameter of each nodule. The PNs were divided into three categories, with respect to their diameter and total area of liver parenchyma occupied, namely, ≥ 3, $<3->1$, and ≤ 1 mm [Moreno et al., 1991].

After draining the blood, liver slices were taken from each lobe of the liver. The tissue slices were at once immersed in 10% buffered formalin solution for fixation, dehydrated with graded ethanol solutions from 50 to 100%, and then embedded in paraffin. Sections of 5 μm in thickness were cut and stained with hematoxylin and eosin [Stewart et al., 1980]. The histopathological slides were observed under an ADCON 5591 (ADCON, Cleveland, USA) photomicroscope. All the slides were examined without prior knowledge of the treatment given to the animals from which the tissue samples under investigation were taken.

Statistical Analysis

The data were analyzed using the GraphPad Prism software package, Version 4.01. Student's *t*-test was performed to compare sample means and the results were expressed as mean ± SE. One-way ANOVA followed by Tukey–Kramer multi-comparison test was also performed to evaluate the changes among different time intervals within a variable using the error calculated from ANOVA. Pearson's correlation was used to analyze the relationship between variables. Statistical significance was set at $P < 0.05$ for all the values.

RESULTS

During the entire period of study, no differences in food and water consumption were observed among the various groups of animals. The final body weight of DEN + PB-treated rats (group G) was significantly less (258.75 g; $P < 0.001$) than that of the normal control (group E, 354.12 g) (Table not shown). Treatment with vanadium significantly increased (335.53 g; $P < 0.001$) the final body weight of group H rats compared to the carcinogen-treated group (group G). There was no significant difference among the groups in their liver weights (group E, 6.43 g; group F, 6.61 g; group G, 8.94 g; group H, 6.78 g). On the other hand, the relative liver weight (RLW) of group G rats was found to be increased significantly (3.76; $P < 0.001$) compared to that of group E (1.76). Vanadium treatment reduced the RLW in group H rats (2.19; $P < 0.001$) when compared with group G. Moreover, no statistically significant differences in body weights, liver weights, and RLWs of the normal control (group E) and vanadium-treated (group F) rats were noticed till 32 weeks, suggesting that 0.5 ppm of vanadium supplement had practically no adverse effects on the growth responses of the rats; otherwise, growth retardation or premature death would have occurred. The dose has also been found to be well tolerated under the experimental condition studied and revealed no gross changes or histological abnormalities in the tissues like liver, kidney, stomach, etc.

Effect of Vanadium on the Levels of Formation of 8-OHdGs in Rat Liver Induced by DEN at Sequential Time Points

The formation of 8-OHdGs in DNA was significant and rapid and the time dependency of hepatic DNA base lesion to reach a maximal level ranged from 3 to 18 h following DEN exposure (90.69%; $P < 0.0001$) (Fig. 1) when compared to group A. After that, there was a steady maintenance of oxidative adducts in the DEN-treated group (group C). On the other hand, there was a significant reduction in 8-OHdG levels in vanadium-supplemented rat liver (group D) at 18 h (28.14%; $P < 0.02$), 24 h (49.37%; $P < 0.001$), and 48 h (80.63%; $P < 0.0001$) when compared to group C. One-way ANOVA showed significant changes in the pattern of 8-OHdG levels [$P < 0.0001$;

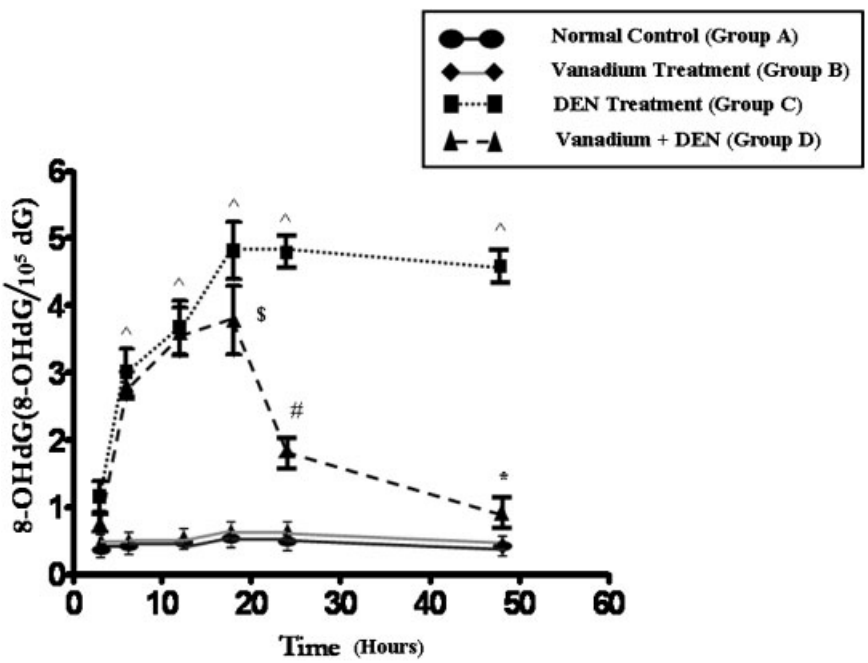

Fig. 1. Levels of formation and persistence of oxidative DNA bases 8-hydroxy-2′-deoxyguanosines (8-OHdGs) in rat liver at sequential time points in presence (Group D) or absence (Group C) of 0.5 ppm vanadium supplementation following a single, necrogenic dose (200 mg/kg body weight) of diethylnitrosamine (DEN) injection. Each *bar* indicates mean ± SE (n = 15). $^{\wedge}P < 0.0001$ when compared with normal control (Group A); $^{\$}P < 0.02$, $^{\#}P < 0.001$, and $^{*}P < 0.0001$, respectively, at 18, 24, and 48 h after DEN injection when compared with DEN treatment (Group C).

Computed F is much greater than the Critical $F_{0.05\ (2.32)}$ which amounts to 55.17 (group C) and 232.29 (group D); Table not shown] during six sequential time points, which suggests a prominent role of 8-OHdG in carcinogenesis and its modulation by vanadium. In Figure 1, background 8-OHdG levels (0.8– 1.2 residue/10^5 dG) in control DNA samples were subtracted.

Effect of Vanadium on the Generation of SS-DNAs and SSBs in Rat Liver following 18–20 h of DEN Injection

A significant rise (64.33%; $P < 0.001$) in the total percentage of hepatic SS-DNAs could be observed in group C rats when compared with normal control rats (group A) (4.44%) (Table not shown). The percentage of native DS-DNAs in DEN-treated (group C) rats (35.67%) was found to be almost 3-fold less than in normal control rats (95.56%), where as the total aberrant single-stranded regions in group C rats were almost 16-fold higher than that of group A control. This explains that the potent hepatocarcinogen DEN exerts a direct damaging effect on DNA. Treatment with vanadium in group D rats strictly abated (32.14%; $P < 0.001$) the generation of SS-DNAs following carcinogen challenge when compared to DEN treatment alone (Group C). Moreover, the native DS-DNAs in group D rats (59.03%) were more than 1.5 fold higher than in group C rats (35.67%). In group C, a significant increase ($P < 0.001$) in the number of SSBs/DNA could be observed when compared with that of the normal control (group A). Treatment with 0.5 ppm vanadium throughout the study, showed a significant decrease (56.39 %; $P < 0.01$) in the number of SSBs/DNA in group D rats when compared to group C. However, there was no statistical difference in the amount of SS-DNAs between normal control (group A) and vanadium-treated (group B) rats, suggesting that supplementation had no side effects in vivo.

Effect of Vanadium on the Formation of Hepatic DPCs (DPC Coefficient)

Figure 2 shows DPC coefficients in different groups of rats treated with DEN and vanadium. It is evident that, there was a significant induction of DPCs in DEN-treated (group C)

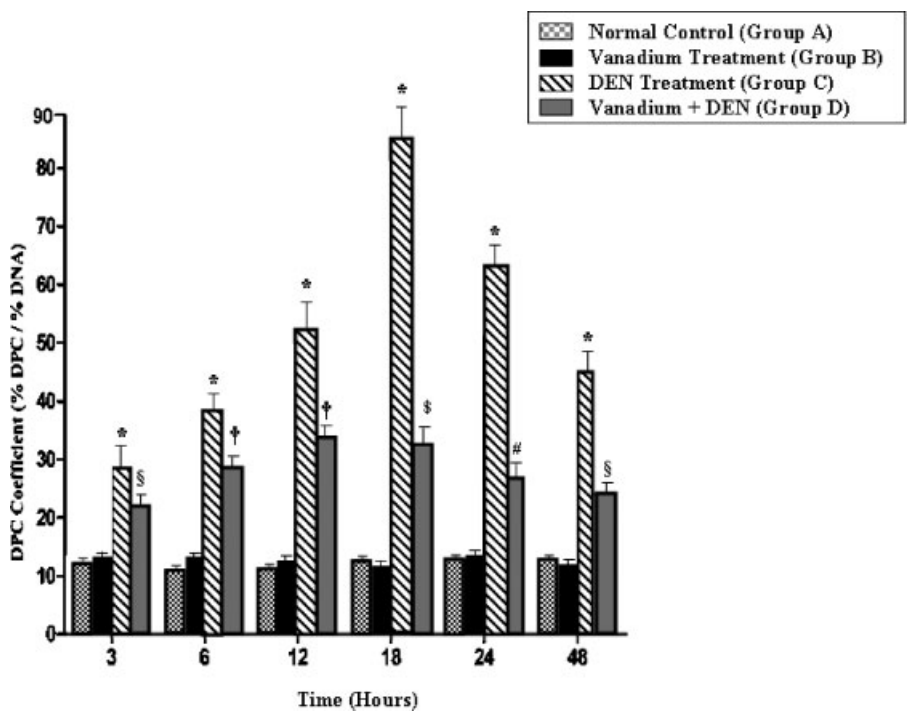

Fig. 2. Effect of 0.5 ppm vanadium supplementation on the levels of formation of DNA-protein crosslinks (DNA-protein crosslinking coefficients) in rat liver at sequential time points, that is, after 3, 6, 12, 18, 24, and 48 h of a single, necrogenic dose (200 mg/kg body weight) of diethylnitrosamine (DEN) injection. Each *column* and *bar* indicates mean ± SE (n = 15). $^{*}P < 0.0001$ when compared to normal control (Group A) at all the time points; $^{\S}P < 0.01$ at 3 and 48 h, $^{\dagger}P < 0.05$ at 6 and 12 h, $^{\$}P < 0.0001$ at 18 h, and $^{\#}P < 0.001$ at 24 h after DEN injection when compared with DEN treatment (Group C).

rats at all the specified time intervals (i.e., after 3, 6, 12, 18, 24, and 48 h of DEN injection) but the amount of DPCs formation was maximum (76.98%; $P < 0.0001$) after 18 h of DEN exposure when compared to normal control rats (group A). Vanadium supplementation significantly reduced ($P < 0.0001$) the formation of DPC in group D rats. There was a gradual reduction in the DPC coefficients (i.e., 25.32% at 6 hours... 59.26% at 18 h) in group D rats when compared to group C. Furthermore, one-way ANOVA showed significant changes in the pattern of DPC levels during six sequential time points studied herein in the group C rats [$P < 0.0001$; Computed F is much more greater than the Critical $F_{0.05\ (2.48)}$ which amounts to 13.11; Table not shown].

Effect of Vanadium on DEN-Induced CAs in Rat Liver

Table I summarizes the effect of vanadium on the frequency distribution of CAs in hepatocytes of rats following 5, 30, 60, and 90 days of DEN treatment. The results showed that a single, (i.p.) injection of DEN (group C) induced a significant ($P < 0.001$) number of aberrant metaphase chromosomes in hepatocytes observed at 5 (11.60%), 30 (71.20%), 60 (105.20%), and 90 days (120.80%) after DEN injection when compared with that of normal control (group A). There were further substantial inductions of all types of CAs (i.e., structural 63.60%, numerical 26.80%, physiological 30.40%) in hepatocytes that took peak at 90 days in group C rats. After that, there was no significant alteration in the pattern of chromosomal karyotype observed during the longer time-period interval (i.e., beyond 90 days) as was confirmed by the time-response studies carried out by us (Data not shown). Also, the amount of physiological aberrations (30.40%) took upper hand from numerical type (26.80%) in DEN-treated rats (group C) at day 90, which suggests that the effect of lethality on chromosomes increased as the duration rose following DEN threat. A significant decrease in the mean number of total aberrations following vanadium treatment could be observed in group D rats after 30 (35.39%; $P < 0.01$); 60 (47.53 %; $P < 0.001$), and 90 days (71.52%; $P < 0.001$) of DEN injection when compared to the group C. Furthermore, vanadium treatment alone (group B) demonstrated no clastogenic effect when compared to the normal control (group A) throughout the study.

Effect of Vanadium on MT Expression and Cell Proliferation

In situ immunolocalizations of MT protein (Fig. 3) and BrdU-immunopositive hepatocytes (Fig. 4) were observed in DEN-initiated and PB-promoted liver tissue (group G) depicting a strong immunoreactivity in comparison to that of normal control (group E). Generally,

TABLE I. Effect of 0.5 ppm Vanadium on the Frequency Distribution of Diethylnitrosamine-Induced Chromosomal Aberrations in Rat Hepatocytes (250 Plates/Group)

			Structural CAs		Numerical CAs		Physiological CAs		Total aberrations			
Time (days)	Group	Treatment (s)	No.	%	No.	%	No.	%	No.	%	Mean ± SE (n = 5)	Inhibition (%)
05	A	Normal cont.	01	0.40	00	0.00	00	0.00	01	0.40	0.20 ± 0.58	—
	B	V treat.	01	0.40	00	0.00	00	0.00	01	0.40	0.20 ± 0.39	—
	C	DEN treat.	19	7.60	08	3.20	02	0.80	29	11.60	5.80 ± 1.09*	—
	D	V + DEN	12	4.80	07	2.80	02	0.80	21	8.40	4.20 ± 1.12	27.58
30	A	Normal cont.	01	0.40	01	0.40	00	0.00	02	0.80	0.40 ± 0.63	—
	B	V treat.	01	0.40	00	0.00	00	0.00	01	0.40	0.20 ± 0.45	—
	C	DEN treat.	90	36.00	53	21.20	35	14.00	178	71.20	35.60 ± 2.84*	—
	D	V + DEN	68	27.20	25	10.00	22	8.80	115	46.00	23.00 ± 1.68**	35.39
60	A	Normal cont.	02	0.80	01	0.40	00	0.00	03	1.20	0.60 ± 0.51	—
	B	V treat.	01	0.40	01	0.40	00	0.00	02	0.80	0.40 ± 0.49	—
	C	DEN treat.	137	54.80	65	26.00	61	24.40	263	105.20	52.60 ± 2.28*	—
	D	V + DEN	77	30.80	34	13.60	27	10.80	138	55.20	27.60 ± 1.06***	47.53
90	A	Normal cont.	02	0.80	01	0.40	01	0.40	04	1.60	0.80 ± 0.51	—
	B	V treat.	01	0.40	01	0.40	00	0.00	02	0.80	0.40 ± 0.68	—
	C	DEN treat.	159	63.60	67	26.80	76	30.40	302	120.80	60.40 ± 2.49*	—
	D	V + DEN	105	42.00	29	11.60	33	13.20	167	66.80	17.20 ± 1.12***	71.52

CAs, chromosomal aberrations; Cont., control; DEN, diethylnitrosamine; Treat., treatment; V, vanadium.
*$P < 0.001$ when compared to normal control (Group A).
**$P < 0.01$ and ***$P < 0.001$ when compared to DEN Treatment (Group C).

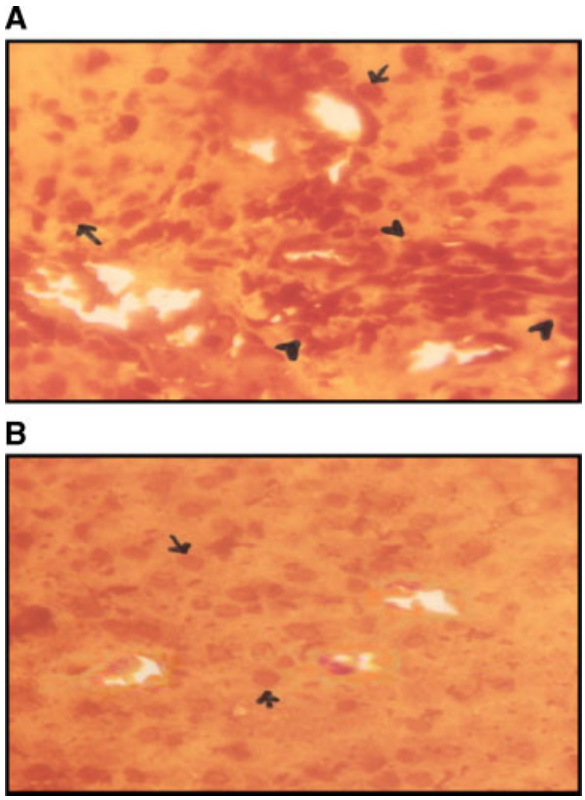

Fig. 3. Light micrographs of tissue sections from rat liver (after 32 weeks) showing immunostaining of metallothionein (MT) with anti-rat MT-1 antibody and 3,3′-diaminobenzidine tetrahydrochloride (DAB). **A**: Group G, DEN + PB treatment; (**B**) Group H, vanadium + DEN + PB. Arrow head (▲) indicates intense immunostaining of MT protein with prominent focal expression and isolated clusters of MT-positive cells. Arrow (⬆) indicates scattered/individual MT immunopositive cells. Magnification (A,B) 270×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

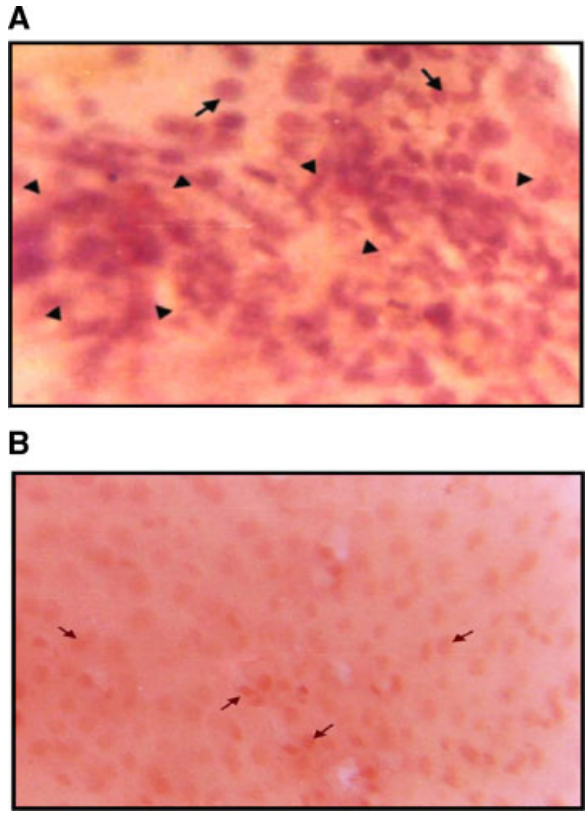

Fig. 4. Light micrographs of tissue sections from rat liver (after 32 weeks) showing 5′-bromo-2′deoxyuridine (BrdU) immunolabeling of hepatocyte nuclei with mouse anti-BrdU clone and 3,3′-diaminobenzidine tetrahydrochloride (DAB). **A**: Group G, DEN + PB treatment; (**B**) Group H, vanadium + DEN + PB. Arrow head (▲) indicates intense immunolabeling of BrdU with prominent focal areas. Arrow (⬆) indicates BrdU-immunopositive cells. Magnification (A,B) 270×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in sections (group G) with high MT immunopositivity, the immunopositive cells formed contiguous foci or sheets and on some occasions isolated clusters of positive hepatocytes were seen. Accordingly, an interesting feature of MT immunoexpression was noticed herein (Fig. 3A), which showed an intense staining of MT protein within the preneoplastic lesions indicating its prominent focal expression following DEN + PB treatment (group G) along with strong immunoreactivity (87.32%; $P < 0.0001$) when compared to normal control (group E) (Figure not shown). In comparison to DEN + PB treatment, sections from vanadium-treated rat liver (group H) showed a low MT immunoreactivity (58.69%; $P < 0.05$) with scattered positive cells (Fig. 3B).

Figure 4A showed an intense BrdU-immunolabeling of hepatocyte nuclei indicating focal proliferation as well as plenty of BrdU-positive cells throughout in group G rats. However, in vanadium-treated sections (group H) (Fig. 4B) no such focal labeling was noticed. In comparison to the non-focal labeling, focal index showed significant changes in both groups G and H. Furthermore, there was substantial reduction (2.78%; $P < 0.02$) (Figure not shown) in focal index in group H rats, indicating a prominent suppressive effect of vanadium on cell proliferation when compared with DEN + PB treatment alone (1.46%) in group G rats.

Relationship between MT Immunoreactivity and BrdU-LI

The percentage of MT-immunoreactivity and BrdU-focal labeling index (BrdU-LI) in the tissues of (i) DEN + PB-treated rats (group G)

and (ii) vanadium + DEN + PB-treated rats (group H) ranged from (i) 52.37 to 112.14% and 42.13 to 79.54%, respectively, and (ii) 0.78 to 3.96% and 0.45 to 1.84%, respectively, throughout the study. A significant positive correlation was observed between MT protein expression and BrdU-LI in both the groups G ($r = 0.88$; $r^2 = 0.77$; $P = 0.0007$) and H ($r = 0.92$; $r^2 = 0.85$; $P = 0.0001$) (Graphs not shown) suggesting that, MT expression might be associated with cell proliferation.

Hepatic Nodulogenesis and Histopathological Profile of Liver

There were no visible hepatocyte nodules in the livers of normal control (group E) and vanadium-treated (group F) rats. There was 100% nodular incidence in DEN + PB-treated rats (group G) (Table II). Supplementation of vanadium decreased nodular incidence (66.67%), total number of nodules (89.90%), and nodule multiplicity (62.12%; $P < 0.001$) in the DEN + PB-treated group H rats when compared to group G. There was a maximum occurrence of nodules ≥3 mm (51.37%) in group G rats, whereas there was no occurrence of this type of nodules in group H.

Phenotypically altered hepatocyte populations including PNs were found scattered in the livers of DEN-PB-treated groups (i.e., groups G and H); but no such alterations were noticeable in untreated normal control (group E; Fig. 5A) or in the vanadium treatment group (group F) (Figure not shown). In group G rats (Fig. 5B,C), a gross alteration in hepatocellular architecture was found and hepatocytes appeared oval or irregular in shape. The altered hepatocytes of foci and nodules were found consistently enlarged with more than one nucleus, which were moreover largely vesiculated with centrally located nucleoli. Some nuclei in the cells were large and hyperchromatic (basophilic), indicating prominent hyperbasophilic preneoplastic focal lesions around the portal vein that were clearly distinguishable from the surrounding non-nodular normal parenchyma. Extensive vacuolation was observed in the cytoplasm around the nucleus with masses of acidophilic (eosinophilic) material and a number of prominent clear cell foci. In contrast, the cellular architecture of hepatic lobules seemed to be almost like that of normal liver in group H (Fig. 5D) that received vanadium supplementation during the entire

TABLE II. Effect of 0.5 ppm Vanadium on the Development of Nodular Hyperplasia in Rat Liver Initiated With Diethylnitrosamine and Promoted by Phenobarbital After 32 Weeks

Group	Treatment(s)	No. of rats with nodules/total no. of rats	Nodule incidence (%)	Inhibition (%)	Total no. of nodules	Nodules relative to size (% of total number)			Nodule multiplicity[a] (mean ± SE)	Inhibition (%)
						≥3 mm	<3–>1 mm	≤1 mm		
G	DEN + PB	15/15	100.00	—	109	56 (51.37)	34 (31.19)	19 (17.43)	7.26 ± 1.13	—
H	V + DEN + PB	4/12	33.33	66.67	11	00 (0.00)	03 (27.27)	08 (72.72)	2.75 ± 0.28*	62.12

DEN, diethylnitrosamine; PB, phenobarbital; V, vanadium.
[a]Average number of nodules/nodule bearing liver.
*$P < 0.001$ when compared with DEN + PB treatment (Group G).

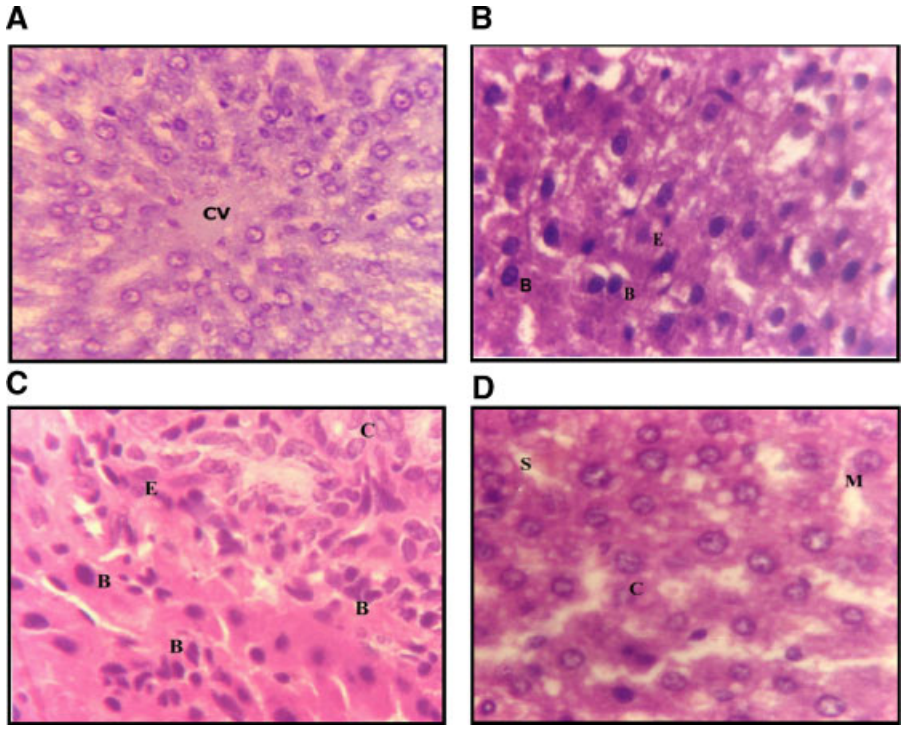

Fig. 5. Contiguous liver sections of rats being sacrificed after 32 weeks showing (**A**) normal hepatocellular architecture (Group E, normal control) depicting hepatocytes radiating from the central vein; (**B**,**C**) aberrant hepatocellular phenotype (Group G, DEN + PB treatment) with prominent hyperbasophilic preneoplastic focal lesions and the presence of eosinophilic and clear cell foci following a single, necrogenic, intraperitoneal injection of diethylnitrosamine (DEN) (200 mg/Kg body weight) at week 4 and application of phenobarbital (PB) (0.05% in basal diet) thereafter on and from week 8; and (**D**) almost normal hepatocellular architecture (Group H, vanadium + DEN + PB) following DEN + PB regimen along with continuous supplementation of 0.5 ppm vanadium starting the application 4 weeks before initiation and continued till 32 weeks. B, basophilic foci; CV, central vein; C, clear cell foci; E, eosinophilic cell; M, mixed cell; PV, portal vein; S, sinusoid. Magnification (A–D) H and E 450×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

period of study. Liver sections from this group presented only a few clear cell foci. The cells were generally filled with cytoplasmic material and were less vacuolated. The size of the nuclei was essentially the same as that of normal cells and cells with two nuclei were considerably fewer than in group G rats.

DISCUSSION

The present study reports the chemopreventive potential of vanadium in limiting oxidative DNA damages, MT expression and cell proliferation during the early stages of hepatocarcinogenesis in rats. Vanadium supplementation has been found to inhibit the formation of tissue-specific oxidative DNA bases 8-OHdGs, SSBs, DPCs, and CAs in hepatocytes. Vanadium effect was also noticed in decreasing nodulogenesis and in remodeling of preneoplastic liver tissue. The results thus indicate that, vanadium at the 0.5 ppm concentration, which is considered to be a "low dose" of vanadium, exerts potential anticarcinogenic effect on DEN-induced hepatocellular preneoplasia in rats.

Induction of significant and high steady levels of 8-OHdGs essentially plays a critical role for the activation of carcinogenic properties of cells [Floyd, 1990; Cattley and Glover, 1993; Shen et al., 1995]. Using the sensitive HPLC-ECD method, we detected here a substantial level of 8-OHdGs in hepatic DNA of rats treated with DEN over the vehicle control that supported this concept. The maximum level of modified bases was formed 18–20 h following DEN challenge. This indicates that 8-OHdGs may have a role in the initiation of DEN-carcinogenesis. Moreover, the longer maintenance of high levels of 8-OHdGs in liver DNA could be explained by the exhaustion and/or disturbance of DNA repair mechanisms leading to further DNA damages, such as strand-breaks [Kato et al., 1996; Nakae et al., 1997]. The present investigation showed a prominent suppressive

effect of vanadium at 0.5 ppm dose on the levels of 8-OHdGs in DEN-challenged rat liver. Thus, vanadium-mediated reduction of promutagenic lesions might be important in modulating the initiation event of hepatocarcinogenesis. In aqueous solutions, vanadium is found predominately as oxo-anions (e.g., VO_4^{3-}) and as such may exhibit nucleophilic character for the electrophilic agents to attack, thereby preventing DNA damage as per the "carcinogen interception mechanism" as proposed by Hamilton and Wilker [Hamilton et al., 2002, 2006; Hamilton and Wilker, 2004a,b]. The ability of vanadium to attenuate the formation of specific oxidative DNA lesions in liver may indicate its broad-spectrum potential to modulate the kinetics of adduct formation and removal in vivo [Hamilton and Wilker, 2004b; Hamilton et al., 2006]. There is a correlation between inhibition of carcinogenesis by trace metals and lowering of carcinogen binding to cells and DNA. These might involve molecular interactions between metal and carcinogen at different enzymatic and regulatory sites of target cells undergoing neoplastic transformation, as well as stimulation of the host immune system [Kasprzak and Waalkes, 1986].

DNA double-strand breaks (DSBs) are the potent inducers of genomic mutations and of cell death [Jackson, 2002]. Further, formation of DSBs from unrepaired SSBs may lead to progression of preneoplastic tissue towards carcinoma. In the present study, vanadium supplementation resulted in a substantial decrease in the amounts of SS-DNAs and SSBs produced by the DEN treatment. The formation of oxidative DNA adducts in conjunction with DNA strand-breaks thus indicates a positive correlation between these two molecular events, corroborating a previous report [Vu et al., 1985]. Besides SSBs, the enhanced formation of DPCs might also result in chromosomal and chromatid-type aberrations and therefore may be used as another important biomarker in chemical carcinogenesis [Sugiyama et al., 1986]. Intracellular thiol has been reported to be a critical factor in the formation of DPCs [Oleinick et al., 1987]. In this context, it is highly relevant to correlate our previous findings of DEN-induced glutathione (GSH)/glutathione S-transferase (GST) depletion with the enhanced formation of DPCs, as observed herein [Bishayee et al., 1999]. The time-response studies reveal a unique correlation pattern of 8-OHdGs and DPCs formations with respect to CAs and clastogenesis. They both followed the same trend, that is, there were significant inductions of DPCs and 8-OHdGs 18–20 h after DEN challenge which are the indicators of substantial genotoxicity and thereby initiation of the carcinogenic event. This suggests that formation of oxidative DNA adducts 8-OHdGs may be a critical event in initiating the process of carcinogenesis accompanied by increased SSBs and DPCs levels that might eventually result in clastogenesis.

We observed here a random increase of CAs in the carcinogen-treated rat hepatocytes at various time intervals during the early preneoplastic event. Most of the aberrations indicated a direct damaging effect on chromosomes, that is, structural type followed by numerical and physiological types. Again, structural aberrations have been associated with the initiation of premalignant phenotype of the cell [Grover and Fisher, 1971]. Thus, the ability of vanadium to suppress structural and numerical aberrations as observed here may reflect its potentials in limiting the initiation of hepatocarcinogenesis that corroborates our earlier observation [Bishayee et al., 1999].

Up-regulation of MT expression in rapidly proliferating tissues appears to suggest its critical role in normal and neoplastic cell growth. Immunohistological impressions clearly indicate the intense immunostaining of MT in preneoplastic liver tissue over normal control. Thus, localized expression of MT in areas of high proliferative activities in preneoplastic lesions and alteration of its expression might be an early event in the pathogenesis of hepatic neoplasia [Huang and Yang, 2002; Jin et al., 2002]. Cell proliferation, on the other hand, plays a pivotal role in all phases of carcinogenesis with multiple genetic changes. For altered hepatocyte foci (AHF) developing under conditions of initiator-promoter administration, an increase in focal cell proliferation relative to the normal-appearing, non-focal hepatocytes was noticed herein following carcinogen administration. Administration of promoting agents causes clonal expansion and selective enhancement of proliferation of the initiated cell populations over non-initiated cells in the target tissue resulting in the appearance of putative AHF [Schulte-Hermann et al., 1981, 1982]. Treatment with low dose of vanadium reduced MT immunoreactivity and

cell proliferation. The exact molecular mechanism of vanadium-mediated down-regulation of MT expression is not clear at present. Vanadium-mediated reductions of liver MT and hepatic cell proliferation in nodular lesions may reflect its antiproliferative potential and thus could be considered as an important step in delaying neoplastic progression.

Even though, not all the hepatocyte nodules become malignant during the life span of the animals, numerous observations support the concept that the hyperplastic/neoplastic nodules are the precursors of hepatocellular carcinoma [Farber and Cameron, 1980]. In view of this, inhibition of nodule incidence and enhancement of their regression by supplementary vanadium, as observed in our study may be important for cancer chemoprevention. This could be explained in the light of the fact that, although the precursor lesions were still present in the livers of vanadium treated rats, their growth rates slowed to such an extent that the appearance of visible PNs was delayed beyond the experimental end point owing to an increased latency period [Farber, 1990]. Histopathological findings further indicate that the hyperplastic nodular hepatocytes formed solid aggregates of one or more cells thick, the prominent "hyperbasophilic focal lesion" mainly around the portal vein in carcinogen-treated rats. The clear and acidophilic cells primarily form AHF, which are considered to be the small "preneoplastic focal lesions" that lead to malignant transformation in later stages of carcinogenesis with the formation of neoplastic nodules and ultimately hepatocellular carcinomas [Peraino et al., 1981]. Thus, the majority of the neoplastic nodules consist of a mixture of preneoplastic, truly neoplastic, and diverse intermediate cells. However, long-term vanadium treatment resulted in reduced hepatocyte aggregation and basophilicity with a reversal of heterogeneity towards normal cytology.

Results of this study clearly demonstrate that, continuous supplementation of vanadium inhibits early DNA damage and limits cell proliferation during the progression of hepatic preneoplasia in vivo. Our data suggest that, vanadium-mediated reduction in DNA and chromosomal damages might be attributed to decreasing MT expression and cell proliferation [Sgambato et al., 2001; De Flora and Ferguson, 2005; de Moura Espindola et al., 2005]. Furthermore, there exists a strong positive correlation between MT immunoreactivity and BrdU-LI in vanadium-treated rat liver, indicating that vanadium-mediated ablation of MT expression in preneoplastic lesions might be associated with suppression of cell proliferation, as also reported by several other workers [Oyama et al., 1996; Jayasurya et al., 2000; Jin et al., 2002]. In our previous report, we have shown that there were substantial decrements in both MT and Ki-67 proliferating antigen immunolabeling indices following vanadium supplementation and these limiting effects of vanadium on MT and Ki-67 expressions were significantly associated with each other [Chakraborty et al., 2005]. Therefore, all these data of our present and previous studies indicate that, vanadium-mediated inhibitions of cell proliferation and subsequent expression of premalignant phenotype might be due to the observed reductions in MT and Ki-67 immunoreactivities and associated DNA and chromosomal damages in rat liver. Thus, the potential efficacy of pharmacologically safe dose of orally administered vanadium may suggest its therapeutic use as a promising antineoplastic agent in the near future.

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