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1α,25-Dihydroxyvitamin D_3 Inhibits Rat Liver Ultrastructural Changes in Diethylnitrosamine-Initiated and Phenobarbital Promoted Rat Hepatocarcinogenesis

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Abstract The active metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3[1,25$(OH)_2D_3$] has been receiving increasing attention and has come to the forefront of cancer chemoprevention research as being a regulator of cellular growth, differentiation and death. In the present study, attempts have been made to investigate the in vivo chemopreventive effect of 1,25$(OH)_2D_3$ in two-stage rat liver carcinogenesis. Hepatocarcinogenesis was initiated with a single intraperitoneal injection of diethylnitrosamine [DEN] (200 mg/kg b. wt.) at week 4. After a brief recovery period of 2 weeks, all the DEN-treated rats were given phenobarbital (0.05%) in the basal diet and continued thereafter till the completion of the experiment. The results of our experiment showed that the rats which received 1,25$(OH)_2D_3$ for 14 weeks (0.3 μg/100 μL propylene glycol, *per os*, twice a week), starting the treatment 4 weeks prior to DEN injection, exhibited maximum protective effect in maintaining the normal cellular architecture of the hepatocytes than the group of rats which received this micronutrient for only 9 weeks. Moreover, continuous supplementation of 1,25$(OH)_2D_3$ maintains the concentration of hepatic microsomal cytochrome P-450 like that of normal vehicle control. Thus, long-term supplementation of 1,25$(OH)_2D_3$ significantly ($P < 0.001$) inhibits hepatic cytosolic lipid peroxidation, thereby protecting the cell membranes from free-radical mediated damage. These results suggest that 1,25$(OH)_2D_3$ is useful in the inhibition of rat liver carcinogenesis. J. Cell. Biochem. 81:357–367, 2001. © 2001 Wiley-Liss, Inc.

Key words: 1α,25-dihydroxyvitamin D_3; cytochrome P-450; lipid peroxidation; ultrastructure; rat liver; diethylnitrosamine; phenobarbital

Study of the cellular changes that precede the development of liver cancer in animals exposed to chemical hepatocarcinogens has led most investigators over the last 20 years to the conclusion that hepatocellular carcinomas arise by dedifferentiation of adult liver cells. These studies have concentrated on a series of lesions called "foci" and "nodules" which have been designated "premalignant" [Farber and Sarma, 1987]. Xenobiotics such as phenobarbital (PB), polychlorinated biphenyls and many other compounds that induce hepatic biotransformation enzymes, promote experimental hepatocarcinogenesis in rodents previously exposed to initiating carcinogens [Goldsworthy and Pitot, 1985]. Several proposed biological mechanisms for liver tumor promotion by PB and other inducing xenobiotics have been supported by experimental data [Kolaja et al., 1996].

The biologically active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is an important regulator of cellular growth, differentiation and death. 1,25$(OH)_2D_3$ is known to mediate its cellular action through an intracellular receptor, the vitamin D receptor (VDR), having molecular properties similar to those of the superfamily of steroid receptors. 1,25$(OH)_2D_3$ binds to VDR that acts as a ligand-inducible transcription factor. The resulting genomic effects include partial arrest in G_0/G_1 of the cell cycle and induction of differentiation [Campbell et al., 1997]. Besides its action on calcium homeostasis, 1,25$(OH)_2D_3$

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can influence the expression of several genes [Demay et al., 1990; Lemay et al., 1995], as well as the proliferation and differentiation of normal and neoplastic cells in vitro [Manolagas and Deftos, 1984; Abe et al., 1986].

Although the liver is the site of the C25-hydroxylation of vitamin D, it has been shown to have a very low proportion of VDRs (vitamin D receptors) and, consequently, is not considered a target site of vitamin D action. However, studies have demonstrated that calcium and/or vitamin D deficiency has significant effect on liver cell physiology [Bilodeau et al., 1995]. In addition, experimental evidence indicates that dietary calcium supplementation can protect against early hepatic changes due to choline deficiency [Ghoshal et al., 1987], whereas both calcium and vitamin D supplementation have been shown to reduce the growth of 7,12-dimethylbenz(a)anthracene-induced mammary tumors [Carroll et al., 1991]. In our recently published article we have shown that 1,25 $(OH)_2D_3$ in combination with vanadium can effectively inhibit DEN-induced rat liver carcinogenesis [Basak et al., 2000].

Although vitamin D has been shown to promote the differentiation of cancer cells and cell lines in vitro, the protective effect of $1,25(OH)_2D_3$ status against an insult known to induce neoplastic growth in vivo has not been investigated. The purpose of the present study is, therefore, to investigate, in vivo, the influence of $1,25(OH)_2D_3$ status on the response of a rat liver to a chemical insult known to induce morphological and functional changes leading to the appearance of a rapidly proliferating, pluripotent, "stem" cell compartment, the oval cells, which are able to differentiate into hepatocytes, ductular intestinal-like or neoplastic cells [Nagy et al., 1994; Golding et al., 1995].

MATERIALS AND METHODS

Chemicals

All the reagents and biochemicals, unless otherwise mentioned, were obtained from Sigma Chemical Co. (St. Louis, MO).

Treatment(s) of Rats

Male Sprague-Dawley rats (4 weeks old) obtained from the Indian Institute of Chemical Biology (CSIR), Calcutta, weighing 80–100 g were used for experimentation. The animals (4 rats/cage) were acclimatized to standard laboratory conditions for 1 week before the commencement of the experiment. During this period, the rats were maintained on a semi-purified basal diet (Lipton, Calcutta, India) and water *ad libitum*. All rats received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985).

Experimental Setup

The rats were divided randomly into five experimental groups (Fig. 1) according to our previous experimental regimen [Bishayee and Chatterjee, 1995]. Group A rats were the *normal vehicle control* (received normal saline once and $1,25(OH)_2D_3$ vehicle for 14 weeks). Group B, C and D rats received a single intraperitoneal (i.p.) injection of DEN at the rate of 200 mg/kg body weight at 9 weeks of age. After a brief recovery of 2 weeks, all the DEN-treated rats were given PB at 0.05% daily in the basal diet till week 14. Group B was *carcinogen control*. Treatment of $1,25(OH)_2D_3$ (0.3 μg/100 μL propylene glycol *per os* twice a week) [Sardar et al., 1996] in group C rats was started 4 weeks prior to DEN injection and continued thereafter till week 14 (*Long-term continuous study*). In group D, $1,25(OH)_2D_3$ treatment at the same dose mentioned above was started 1 week after DEN injection and continued thereafter till the completion of the experiment (*Promotion study*). Group E was $1,25(OH)_2D_3$ *control* (received 0.3 μg/100 μL propylene glycol *per os* twice a week for the entire period of the study). All the treatments were withdrawn at week 14 and the rats were sacrificed under proper ether anesthesia at week 15 to carry out different experiments.

Morphology and Morphometry of Liver

Soon after the sacrifice, livers were promptly excised from all the treated and control rats, weighed and examined on the surface for subcapsular macroscopic lesions (hyperplastic nodules, HNs). The nodules with approximate spheres were measured in two perpendicular directions to the nearest millimeter into three categories viz, ≥ 3, <3 to >1, and ≤ 1 mm according to the published criteria of Moreno et al. [1991].

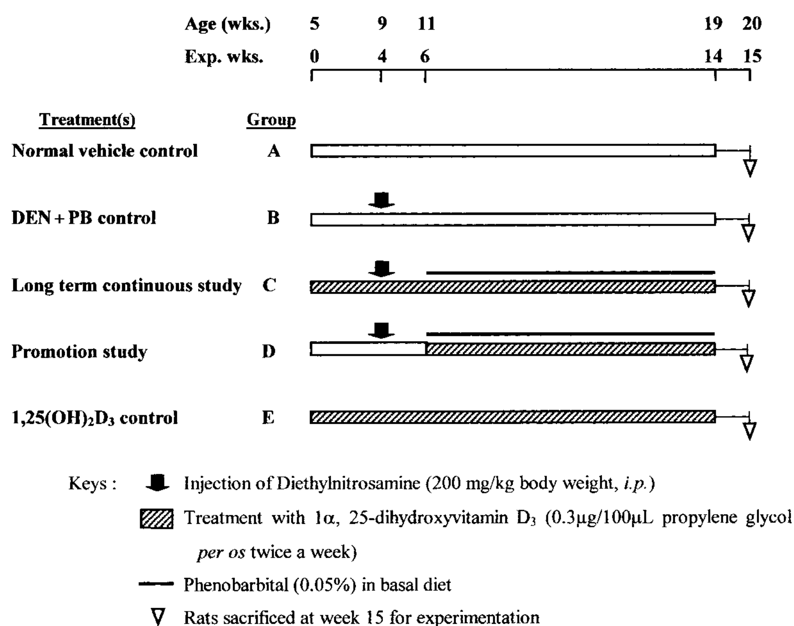

Fig. 1. Basic experimental regimen.

Transmission Electron Microscopy (TEM) of Hepatic Tissue

Hepatic tissue was quickly excised, fixed in 3% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.4) at 4°C for 1 h. After fixation with glutaradehyde, the tissue was washed thoroughly and fixed in 1% osmium tetroxide (in phosphate buffer) at room temperature for 1 h. The tissue was then washed in water, dehydrated in graded ethyl alcohol and embedded in ERL-4602 medium. Ultrathin sections of about 500–600 Å were cut with a glass knife by LKB ultramicrotome (Ultratome 48044). Sections were then stained with uranyl acetate and lead acetate [Epstein and Holt, 1963; Reynolds, 1963].

Preparation of Liver Fraction

The animals were sacrificed with proper anesthesia. Liver of either lobes were excised, minced and homogenized with ice-cold 1.15% (w/v) KCl solution (pH 7.4) in a teflon coated glass homogenizer to make a 10% (w/v) homogenate. The homogenate was then subjected to the differential centrifugation. First, the homogenate was centrifuged at 9,000*g* for 30 min and the resultant supernatant fraction was centrifuged at 1,05,000*g* for 90 min in a Sorval-OTD-50B Ultracentrifuge. The cytosolic fraction was represented by the supernatant of the ultracentrifugation and was stored at −20°C until further use. The pellet of 1,05,000*g* was resuspended in one-tenth volume of homogenizing buffer that served as the microsomal fraction. All operations were performed at 0–4°C.

Enzyme Assays

Total hepatic cytochrome P-450 (Cyt. P-450) in the liver microsomal fraction was measured according to the method of Omura and Sato [1964]. Hepatic cytosolic enzymatic lipid peroxidation was estimated according to the method of Okhawa et al. [1979] which is based on the formation of malondialdehyde (MDA). Total hepatic cytosolic and microsomal protein were estimated by the method of Lowry et al. [1951] using bovine serum albumin as the standard.

Statistics

All the data were analyzed by Student's *t*-test and expressed as mean ± standard deviation (SD). Protection percentage was calculated by applying the equation {(mean control−mean treatment) ÷ Mean control} × 100.

RESULTS

Effect of $1,25(OH)_2D_3$ on Hepatic Nodulogenesis

A significant increase in the incidence of hepatic HNs was observed in DEN+PB-treated rats (Group B) (Table I). Long-term treatment

TABLE I. Effect of Long-Term Supplementation of 1α,25-Dihydroxyvitamin D_3 on Hepatic Nodulogenesis

Group	Treatment(s)	No. of rats with nodules/ total no. of rats	Nodule incidence (%)	Total no. of nodules	Nodules relative to size (% of total no.)			Avg. no. of nodules/ nodule bearing liver
					≥ 3 mm	< 3 to > 1 mm	≤ 1 mm	
B	DEN+PB	8/8	100	118	50 (42.37)	30 (25.42)	38 (32.20)	14.75 ± 2.81[a]
C	DEN+PB+ $1,25(OH)_2D_3$ (Long term)	4/8	50	43	10 (23.25)	23 (53.49)	10 (23.25)	5.37 ± 5.83[b]
D	DEN+PB+ $1,25(OH)_2D_3$ (Promotion)	6/8	75	61	26 (42.62)	19 (31.15)	16 (26.23)	7.62 ± 4.95[c]

[a]Values are mean ± SD.
[b]$P < 0.002$, significantly different from DEN+PB control (Group B) by Student's *t*-test.
[c]$P < 0.01$, significantly different from DEN+PB control (Group B) by Student's *t*-test.

of rats with $1,25(OH)_2D_3$ for 14 weeks (Group C) not only significantly ($P < 0.002$) abated the nodule multiplicity, but also reduced the total number of nodules when compared with group B rats. Moreover, in group C rats the number of ≥ 3 mm sized nodules were appeared to be reduced more than group D rats which received $1,25(OH)_2D_3$ only for 9 weeks (promotion study). In group D rats, the nodule multiplicity, though inhibited compared to group B, was not as significant ($P < 0.01$) as group C.

Effect of $1,25(OH)_2D_3$ on DEN-PB Induced Changes in Rat Hepatic Ultrastructure

The structure and organization of the internal organelles in normal vehicle control (Group A) (Fig. 2) and $1,25(OH)_2D_3$ control (Group E) rats appeared normal with no detectable changes. Rats initiated with DEN at week 4 and promoted by PB for 8 continuous weeks (Group B) showed abnormal hepatic architecture (Fig. 3). The most striking obser-

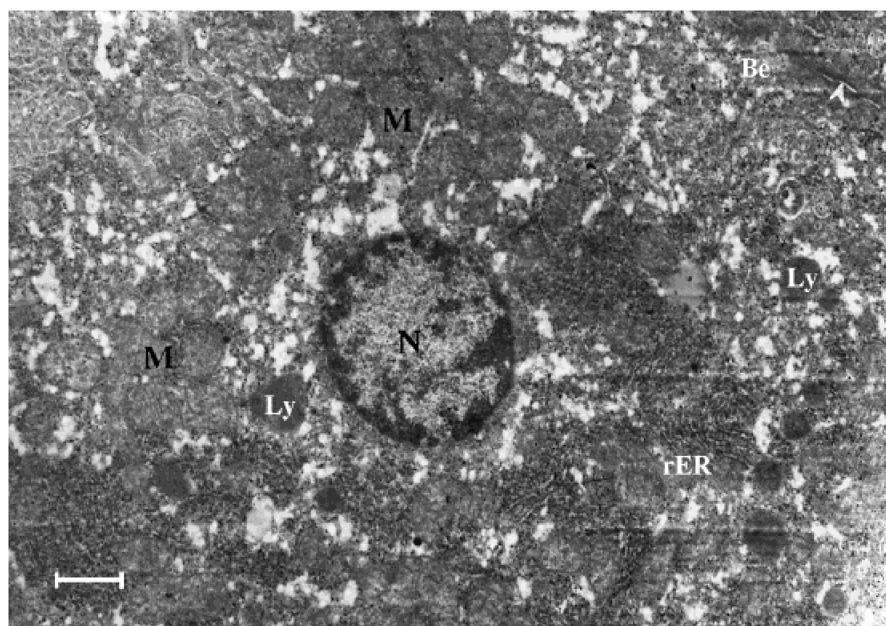

Fig. 2. Electron micrograph of normal vehicle control rat hepatocyte showing a round small nucleus (N) and many mitochondria (M). Presence of lysosomes (Ly) and rough surfaced endoplasmic reticulum (rER) are also seen. Size and shape of the bile canal (Bc) and basement membrane (arrowheads) appear normal.

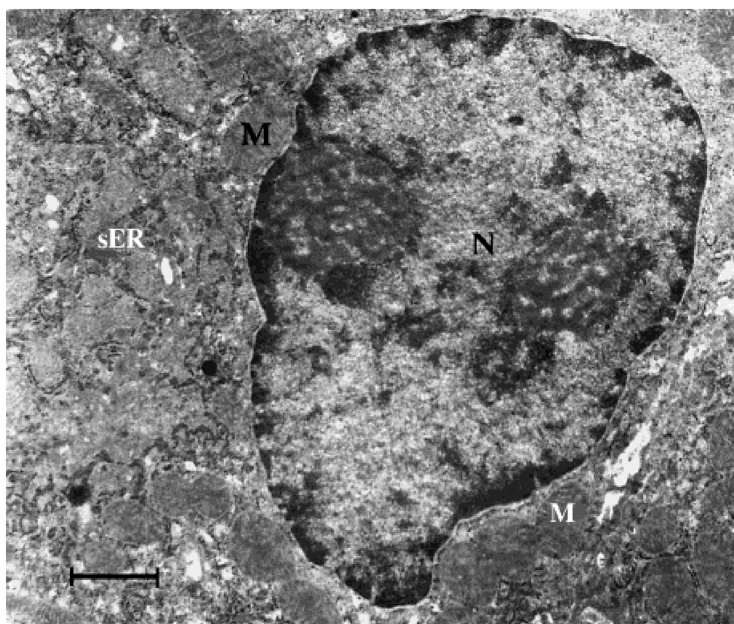

Fig. 3. Electron micrograph of 14 weeks old DEN+PB-treated rat liver cell. One shrunken nucleus (N) with peripheral chromatin bodies and a nucleolus are seen. Fewer but swollen and rounded mitochondria (M) and scattered smooth endoplasmic reticulum (sER) are also present.

vation was the substantial increase in the quantity of agranular or smooth endoplasmic reticulum (sER), in comparison with control hepatocytes (Group A). An accompanying decrease in glycogen stores was also observed, the glycogen areas being smaller and fewer in number. The granular or rough endoplasmic reticulum (rER) appeared to be reduced in quantity in the hepatocytes of DEN-treated and PB-promoted rats. The Golgi zones were situated, as in normal hepatocytes, near the bile canaliculi. However, pronounced hypertrophy of the Golgi apparatus was observed in the majority of hepatocytes. The bile canaliculi were often enlarged and somewhat distorted. Treatment of rats with $1,25(OH)_2D_3$ for 14 (Group C) and 9 continuous weeks (Group D) offered protection against DEN+PB-induced changes to the hepatocytes. But this protection was seemed to be more when $1,25(OH)_2D_3$ was started 4 weeks prior to DEN. The shape and structure of nucleus as well as nucleolus and nuclear membrane looked almost normal like that of normal control hepatocytes (Group A) (Fig. 4, 5). No detectable hypertrophy of the Golgi apparatus was observed in this group (Group C). However, a mild dilation and distortion of the Golgi cisternae and bile canaliculi was observed in group D rats which received $1,25(OH)_2D_3$ for 9 weeks only, i.e., started 1 week after DEN injection and continued thereafter for 9 continuous weeks (Figs. 6, 7). The mitochondria in these groups (Group C and D) were generally normal in form although in group D rats there was a tendency for them to be elongated. Though an increase in glycogen content was observed in group C rats, but this appears to be less than what was observed in normal vehicle (Group A) and $1,25(OH)_2D_3$ control (Group E) hepatocytes.

Effect of $1,25(OH)_2D_3$ on Hepatic Microsomal Cytochrome P-450 Concentration

A significant decrease ($P < 0.001$) in the concentration of hepatic microsomal Cyt. P-450 was observed in DEN-initiated and PB-promoted rats (Group B) (Table II) when compared with the normal vehicle control (Group A). Treatment with $1,25(OH)_2D_3$ for 14 (Group C) and 9 weeks (Group D) significantly increases the concentration of Cyt. P-450 when compared with DEN+PB control. It was observed that, when $1,25(OH)_2D_3$ supplementation was started 4 weeks before initiation with DEN (Group C) the percentage increase was higher ($P < 0.001$, 60%) than when given after DEN (Group D) ($P < 0.005$, 42.4%).

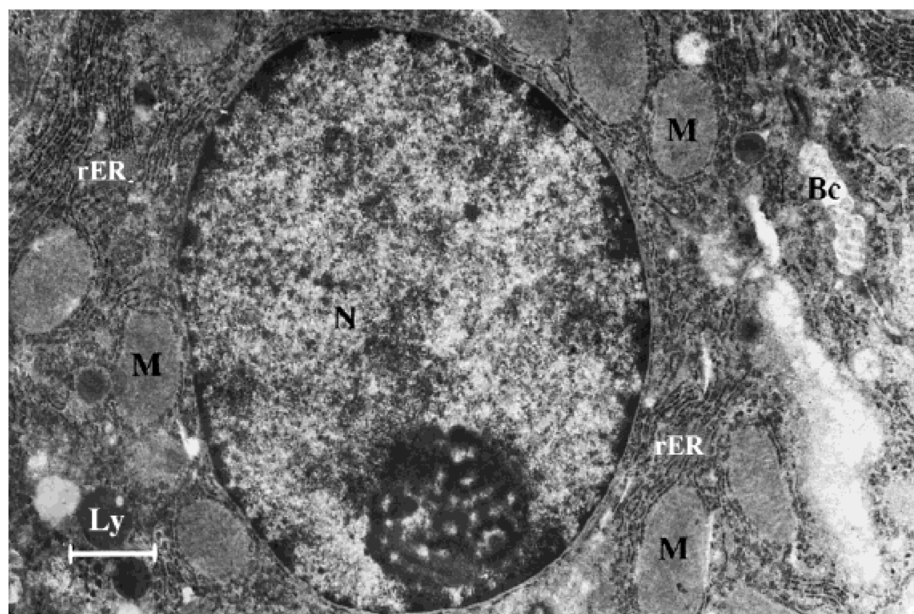

Fig. 4. Electron micrograph of rat liver treated with DEN+PB+ $1,25(OH)_2D_3$ (Long term continuous). Round nucleus (N) with a clear nucleolus, longer and parallely arranged rough endoplasmic reticulum (rER) and elongated but higher numbers of mitochondria (M) with clear cell membranes are demonstrated. The size of the bile canal (Bc) appears normal and lysosomes (Ly) are present near the nucleus.

Effect of $1,25(OH)_2D_3$ on Hepatic Cytosolic Lipid Peroxidation

Table II depicts the effect of $1,25(OH)_2D_3$ on hepatic cytosolic lipid peroxidation in different groups of rats treated with DEN and promoted by PB. A significant increase ($P < 0.001$) in the total content of MDA was observed in DEN+PB-treated rats (Group B) when compared with the normal vehicle control (Group A). Continuous treatment of rats with $1,25(OH)_2D_3$ for 14 (Group C) and 9 weeks (Group D) significantly

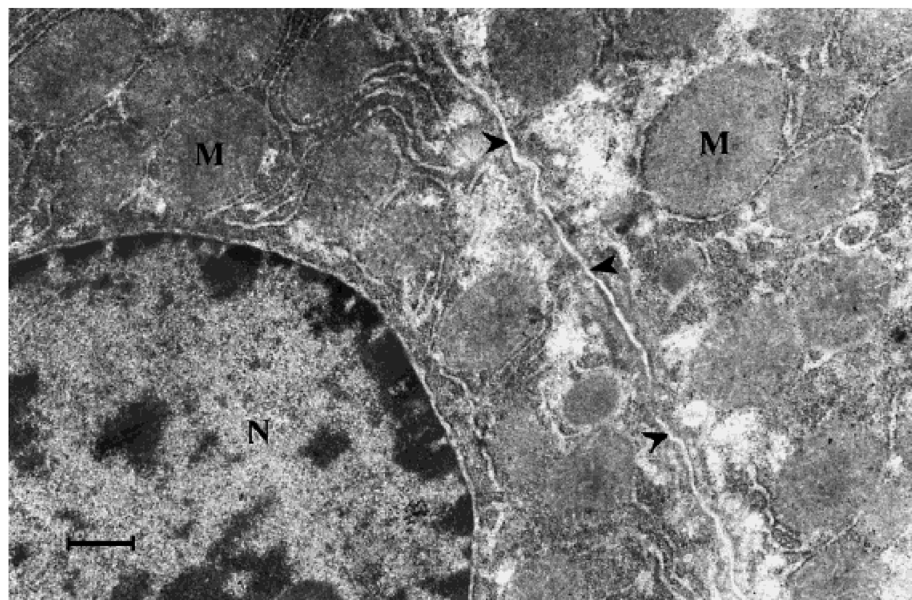

Fig. 5. Electron micrograph of DEN+PB+$1,25(OH)_2D_3$ (Long term continuous)-treated rat liver showing a part of the nucleus (N) and large, round numerous mitochondria (M). The basement membranes of the two hepatocytes (arrowheads) look almost normal.

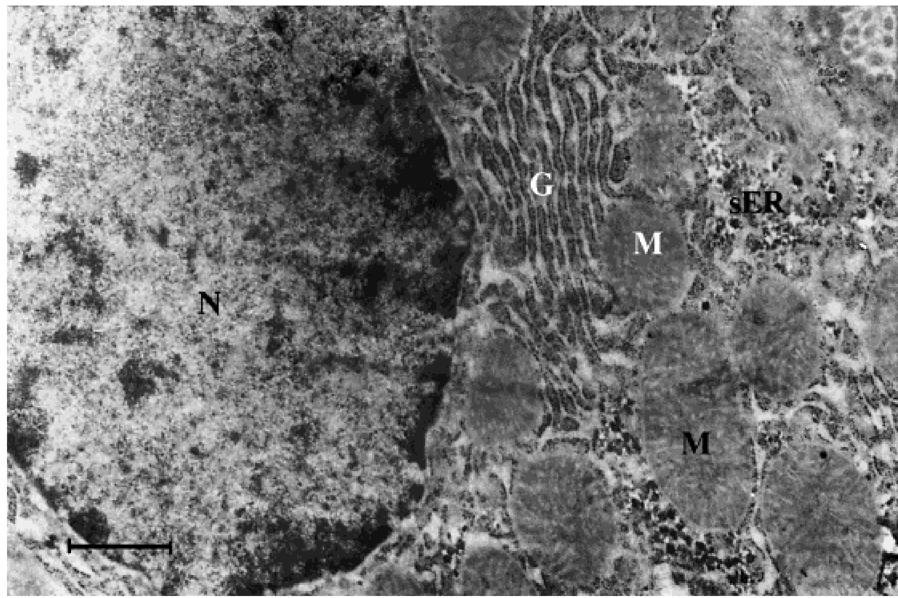

Fig. 6. Electron micrograph of DEN+PB+$1,25(OH)_2D_3$ (Promotion)-treated rat liver. Micrograph shows basement membrane (arrowheads), few lysosomes (arrow) and round nucleus (N). Presence of smooth endoplasmic reticulum (sER) and a Golgi zone (G) are also seen.

($P < 0.001$ and $P < 0.002$, respectively) reduced this elevation compared to DEN+PB control (Group B). It was also observed that supplementation of $1,25(OH)_2D_3$ which started 4 weeks before initiation with DEN and continued thereafter for another 10 weeks (total 14 weeks) offered better protection (53.04%) than group D in which $1,25(OH)_2D_3$ was supplemented for only 9 weeks, starting the treatment 1 week after DEN initiation (38.27%). Supplementation of $1,25(OH)_2D_3$ only in group E rats though increased the lipid peroxidation compared to group A, but that was statistically insignificant.

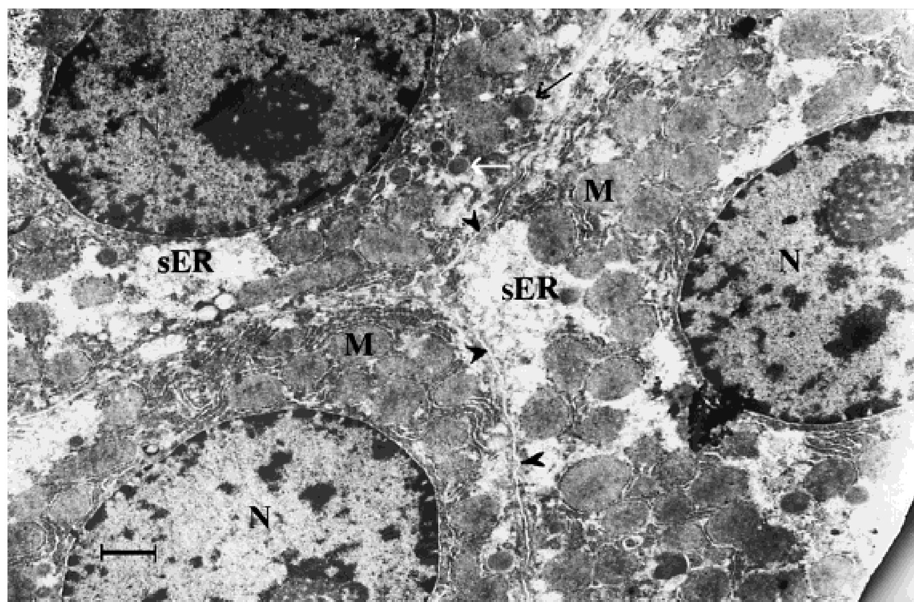

Fig. 7. Electron micrograph of DEN+PB+$1,25(OH)_2D_3$ (Promotion)-treated rat liver showing slightly irregular surfaced nucleus (N), round and elongated shaped mitochondria (M). Proliferation of Golgi zones (G) and presence of smooth endoplasmic reticulum (sER) are also seen.

TABLE II. Effect of Long-Term Supplementation of 1α,25-Dihydroxyvitamin D_3 on Hepatic Microsomal Cytochrome P-450 Concentration and Cytosolic Lipid Peroxidation

Group	Treatment(s)	Total no. of rats	Cytochrome P-450 conc. (nmol/mg microsomal protein)	Lipid peroxidation (nmol MDA/mg protein)
A	Normal vehicle control	8	0.59 ± 0.04^{a}	0.83 ± 0.31
B	DEN+PB control	8	$0.33 \pm 0.04^{*}$	$20.25 \pm 4.5^{*}$
C	DEN+PB+1,25$(OH)_2D_3$ (Long term)	8	$0.53 \pm 0.05^{**}$	$9.51 \pm 2.64^{**}$
D	DEN+PB+1,25$(OH)_2D_3$ (Promotion)	8	$0.47 \pm 0.07^{***}$	$12.50 \pm 2.8^{****}$
E	1,25$(OH)_2D_3$ control	8	0.58 ± 0.06	2.78 ± 0.79

[a]Values are Mean ± SD.
*$P < 0.001$, significantly different from normal vehicle control (Group A) by Student's *t*-test.
**$P < 0.001$, significantly different from DEN+PB control (Group B) by Student's *t*-test.
***$P < 0.005$, significantly different from DEN+PB control (Group B) by Student's *t*-test.
****$P < 0.002$, significantly different from DEN+PB control (Group B) by Student's *t*-test.

DISCUSSION

Cancer is a complicated and a serious disease that can cause severe morbidity and mortality. It is actually an abnormal growth in any part of the body in which the growth rate exceeds the normal body growth rate and does not coordinate with the surrounding tissues. The enzyme system of the endoplasmic reticulum plays an important role in the detoxification of many drugs, chemical carcinogens, and other toxic agents, it is also responsible for catalyzing the metabolic activation of some substrates to highly reactive free-radicals, alkylating or arylating intermediates, which then react with critical cellular macromolecules to initiate toxic and carcinogenic events [Guengerich and Shimada, 1991]. A link between the expression of drug-metabolizing enzymes and the proliferation potential of preneoplastic cells has been drawn by Farber and his colleagues. They suggested that a decrease in monooxygenase activity combined with increased levels of detoxifying phase II enzymes will allow preneoplastic cells to escape from toxic environmental effects, thus leading to a selective proliferation of these cells [Farber, 1984; Roomi et al., 1985]. The selective outgrowth of Cyt. P-450-deficient lesions during hepatocarcinogenesis would then be related to alterations in regulatory systems which affect both the expression of the monooxygenases and the growth-controlling components of the preneoplastic cell population. Since chemical hepatocarcinogenesis proceeds through the early appearance of HNs, the manifestation of phenotype alteration in the nodules, especially alteration of Cyt. P-450 activity, is thought to contribute to the later stage of chemical carcinogenesis. In the present study we have found decreased activity of Cyt. P-450 enzyme in DEN+PB treated rats (Group B). This is in accordance with the results obtained by several workers [Åström et al., 1983; Buchmann et al., 1987; Tsuda et al., 1988]. In this case, the decrease in Cyt. P-450 level would not be an obligatory prerequisite for growth, but a phenotypical indication of alterations in the regulation of homeostasis within preneoplastic and neoplastic cells. The observed decrease in Cyt. P-450 expression during development of malignancy does not result from an irreversible block in enzyme synthesis, possibly due to the alterations in the Cyt. P-450 encoding structural genes, but may rather be related to changes in the constructive regulation of these enzymes. This decrease may further be involved in the control of plasma membrane Ca^{2+} permeability which may have some role in carcinogenesis as it is reported that a Cyt. P-450 may be the link between the intracellular calcium stores and plasma membrane Ca^{2+} channels [Alvarez et al., 1991]. The results obtained with 1,25$(OH)_2D_3$ (Group C and D) explain the stabilization of Cyt. P-450 in the rats. Again, Cyt. P-450 is a constituent of 25-hydroxyvitamin D_3-1α hydroxylase [Chen et al., 1993], the enzyme responsible for activation of 1,25$(OH)_2D_3$. It competes with the 25-hydroxyvitamin D_3-1α hydroxylase resulting in catabolic inactivation of 1,25$(OH)_2D_3$ and thereby prolonging the actions of the 1,25$(OH)_2D_3$. Thus, knowledge on the hepatic Cyt. P-450 content of a particular tumor type and a better understanding of the impact of 1,25$(OH)_2D_3$ on this enzyme will undoubtedly be of great use for designing therapies.

It was shown previously that 1,25$(OH)_2D_3$ inhibits the appearance of γ-glutamyltranspep-

tidase-positive and glucose 6-phosphate-negative foci in rats after treatment with DEN and 2-acetylaminofluorene [He and Gascon-Barre, 1997]. A striking observation of the present study was that the long-term treatment with $1,25(OH)_2D_3$ in group C rats has most significant effect ($P < 0.002$) in inhibiting nodule multiplicity as well as HNs of ≥ 3 mm size from the DEN+PB control (Group B). Although it is evident that not all the hepatocytes nodule become cancerous during the life span of the animals, numerous observations do support the concept that the nodules are the precursors of hepatic cancer [Farber, 1980]. In view of this, inhibition and regression of nodule growth by $1,25(OH)_2D_3$ treatment may be important for cancer prevention.

The lipid peroxidation and membrane protein damage are common devastating consequences of oxygen-derived free-radicals, and the production of structural and functional deformities of tissues has frequently been suggested [Esterbauer, 1982]. Their exact mechanism of action is still obscure and ambiguous. MDA is most frequently used as a measure for the rate and the extent of lipid peroxidation in biological samples [Slater, 1982] and the metabolic fate of this aldehyde has been extensively studied [Siu and Draper, 1982]. Nevertheless, MDA, a product of lipid peroxidation of poly unsaturated fatty acid (PUFA) metabolism and degradation, has been reported to be carcinogenic and mutagenic [Lawrence and Melissa, 1980]. In our present experiment, we have found that long-term continuous treatment with $1,25(OH)_2D_3$ (Group C) rather than post-DEN treatment (Group D) effectively reduces the formation of MDA in the liver. Wiseman [1993] has shown the ability of vitamin D in inhibiting iron-dependent lipid peroxidation in liposomes and has suggested that this may be of importance in protecting the membranes of cells against free radical-induced oxidative damage, as it is possible that this highly lipophilic compound may accumulate in membranes to achieve the concentrations found to inhibit lipid peroxidation.

Electron microscopic observation revealed that a normal liver cell contains usual organelles with a particularly rich supply of mitochondria as well as rER. The Golgi complex is especially prominent. The endoplasmic reticulum is often arranged in parallel rows [Gartner and Hiatt, 1997]. There were evident differences between carcinogen control cell structure distributions and normal control cells, especially in the nuclear pattern, array of endoplasmic reticulum, mitochondrial distribution, existence of lipid droplets, Golgi apparatus, and lysosome distributions. In the present experiment, we have also observed severe liver damage in DEN+PB-treated cells (Group B). Enlarged and marginated nucleoli were found in metabolically active non-neoplastic cells while Golgi complex was poorly developed in the rapidly proliferating neoplastic cells. Fewer but enlarged mitochondria with considerable variation in the number and length of the cristae were present in the carcinogen control. Since many neoplastic cells depend upon anaerobic glycolysis for their energy requirements, large numbers of mitochondria were not needed to provide the energy necessary for rapid cell growth [Erlandson, 1994]. Supplementation of $1,25(OH)_2D_3$ reduced the severity of nuclear and mitochondrial damages, regulated the pattern and number of rER with clear cell membranes and less lipid droplets existed in the cells, eventually showing similar cell structure with normal control cells. But this protective effect of $1,25(OH)_2D_3$ seems to be less in group D rats where it was supplemented only for 9 weeks starting the treatment 1 week after initiation with DEN. Long-term, continuous supplementation of $1,25(OH)_2D_3$ in group C rats resulted in larger stacked segments of rER, less swollen mitochondria and higher numbers of mitochondria outside the nucleus (Figs. 4, 5) indicating its better chemotherapeutic effect. It was reported that the degree of irregularity of the nucleus is related to the degree of malignancy of the cells or to the extent of damage [Henderson et al., 1992]. Such nucleolar changes are indicative of active protein synthesis and rapid cell proliferation [Erlandson, 1994]. Liver cells of normal rats treated with $1,25(OH)_2D_3$ do not show any significant damage/change in the cells. These results strongly suggest that $1,25(OH)_2D_3$ does not have any toxic effect on the non-neoplastic cells.

Regardless of the mechanism, the evidence presented here shows that $1,25(OH)_2D_3$ is very much effective in preventing DEN+PB-induced changes to the hepatocytes possibly through stabilizing the drug-metabolizing enzyme as well as inhibiting lipid peroxidation.

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REFERENCES

Abe J, Moriya Y, Saito M, Sugawara Y, Suda T, Nishi T. 1986. Modulation of cell growth, differentiation, and production of interleukin-3 by 1α,25-dihydroxyvitamin D_3 in the murine myelomonocytic leukemia cell line WEHI-3. Cancer Res 46:6316–6321.

Alvarez J, Montero M, García-Sancho J. 1991. Cytochrome P-450 may link intracellular Ca^{2+} stores with plasma membrane Ca^{2+} influx. Biochem J 274:193–197.

Åström A, DePierre JW, Eriksson L. 1983. Characterization of drug-metabolizing systems in hyperplastic nodules from the livers of receiving 2-acetylaminofluorene in their diet. Carcinogenesis 4:577–581.

Basak R, Basu M, Chatterjee M. 2000. Combined supplementation of vanadium and 1α,25-dihydroxyvitamin D_3 inhibit diethylnitrosamine-induced rat liver carcinogenesis. Chem Biol Interact 128:1–18.

Bilodeau M, Provencher S, Néron S, Haddad P, Vallières S, Gascon-Barré M. 1995. Hypocalcemia decreases early and late responses to epidermal growth factor in rat hepatocytes. Hepatology 21:1576–1584.

Bishayee A, Chatterjee M. 1995. Inhibitory effect of vanadium on rat liver carcinogenesis initiated with diethylnitrosamine and promoted by phenobarbital. Br J Cancer 71:1214–1220.

Buchmann A, Schwarz M, Schmitt R, Wolf CR, Oesch F, Kunz W. 1987. Development of cytochrome P-450-altered preneoplastic and neoplastic lesions during nitrosamine-induced hepatocarcinogenesis in the rats. Cancer Res 47:2911–2918.

Campbell MJ, Reddy GS, Koeffler HP. 1997. Vitamin D_3 analogs and their 24-oxo metabolites equally inhibit clonal proliferation of a variety of cancer cells but have differing molecular effects. J Cell Biochem 66: 413–425.

Carroll KK, Jacobson EA, Eckel LA, Newmark HL. 1991. Calcium and carcinogenesis of the mammary gland. Am J Clin Nutr 54:206S–208S.

Chen ML, Boltz MA, Armbrecht HJ. 1993. Effects of 1,25-dihydroxyvitamin D_3 and phorbol ester on 25-hydroxyvitamin D_3 24-hydroxylase cytochrome P-450 messenger ribonucleic acid levels in primary cultures of rat renal cells. Endocrinology 132:1782–1788.

Demay MB, Gerardi JM, DeLuca HF, Kronenberg HM. 1990. DNA sequences in the rat osteocalcin gene that bind 1,25-dihydroxyvitamin D_3 receptor and confer responsiveness to 1,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA 87:369–373.

Epstein MA, Holt SJ. 1963. The localization by electron microscopy of HeLa cells surface enzymes splitting adenosine triphosphate. J Cell Biol 19:325–336.

Erlandson RA. 1994. Diagnostic transmission electron microscopy of tumors with clinicopathological, immunohistochemical and cytogenetic correlations. London: Raven Press Ltd. p 1–24, 69–137, 417–423.

Esterbauer H. 1982. Aldehyde products of lipid peroxidation. In: McBrien DCH, Slater TF, editors. Free radicals, lipid peroxidation and cancer. New York: Academic Press, p 101–128.

Farber E. 1980. The sequencial analysis of liver cancer induction. Biochim Biophys Acta 605:149–166.

Farber E. 1984. The biochemistry of preneoplastic liver: a common metabolic pattern in hepatocyte nodules. Can J Biochem Cell Biol 62:486–494.

Farber E, Sarma DSR. 1987. Hepatocarcinogenesis: a dynamic cellular perspectives. Lab Invest 56:4–22.

Gartner LP, Hiatt JL. 1997. Color textbook of histology. Philadelphia: WB Saunders Company, Pennsylvania, p 1057.

Ghoshal AK, Laconi E, Willemsen F, Ghoshal A, Rushmore TH, Farber E. 1987. Modulation of calcium by the carcinogenic process in the liver induced by a choline-deficient diet. Can J Physiol Pharmacol 65:478–482.

Golding M, Sarraf CE, Lalani EN, Anilkumar TV, Edwards RJ, Nagy P, Thorgeirsson SS, Alison MR. 1995. Oval cell differentiation into hepatocytes in the acetylaminofluorene-treated regenerating rat liver. Hepatology 22: 1243–1253.

Goldsworthy T, Pitot HC. 1985. An approach to the development of a short-term whole animal bioassay to distinguish initiating agents (incomplete carcinogens), promoting agents, complete carcinogens, and noncarcinogens in rat liver. J Toxicol Environ Health 16:389–402.

Guengerich FP, Shimada T. 1991. Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. Chem Res Toxicol 4:391–407.

He RK, Gascon-Barre M. 1997. Influence of vitamin D status on the early hepatic response to carcinogen exposure in rats. J Pharmacol Exp Ther 281:464–469.

Henderson BE, Ross RK, Shibata A, Paganini-Hill A, Yu MC. 1992. Environmental carcinogens and anticarcinogens. In: Lee W, Martin L, Charles BW, Gary K, editors. Cancer chemoprevention. New York: CRC Press p. 3–18.

Kolaja KL, Stevenson DE, Walborg EF, Klaunig JE. 1996. Dose dependence of phenobarbital promotion of preneoplastic hepatic lesions in F344 rats and B6C3F1 mice: effects on DNA synthesis and apoptosis. Carcinogenesis 17:947–954.

Lawrence JM, Melissa AT. 1980. Comparison of mutagenicities of malondialdehyde and the side products formed during its chemical synthesis. Cancer Res 40:276–282.

Lemay J, Demers C, Hendy GN, Delvin EE, Gascon-Barré M. 1995. Expression of the 1α,25-dihydroxyvitamin D_3-24-hydroxylase gene in rat intestine: response to calcium, vitamin D_3 and calcitriol administration in vivo. J Bone Miner Res 10:1148–1157.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275.

Manolagas SC, Deftos LJ. 1984. The vitamin D endocrine system and the hematolymphopoietic tissue. Ann Intern Med 100:144–146.

Moreno FS, Rizzi MBS, Dagli MLZ, Penteado MVC. 1991. Inhibitory effects of β-carotene on preneoplastic lesions

induced in Wistar rats by the resistance hepatocyte model. Cancer Res 12:1817–1822.
Nagy P, Bisgaard HC, Thorgeirsson SS. 1994. Expression of hepatic transcription factors during liver development and oval cell differentiation. J Cell Biol 126:223–233.
Okhawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358.
Omura T, Sato N. 1964. The carbon monoxide-binding pigment of liver microsomes. J Biol Chem 239:2370–2378.
Reynolds ES. 1963. The use of lead citrate at high pH as an electro-opaque stain in electron microscopy. J Cell Biol 17:208–213.
Roomi MW, Ho RK, Sarma DSR, Farber E. 1985. A common biochemical pattern in preneoplastic hepatocyte nodules generated in four different models in the rat. Cancer Res 45:564–571.
Sardar S, Chakraborty A, Chatterjee M. 1996. Comparative effectiveness of vitamin D_3 and dietary vitamin E on peroxidation of lipids and enzymes of the hepatic antioxidant system in Sprague-Dawley rats. Int J Vit Nutr Res 66:39–45.
Siu GM, Draper HH. 1982. Metabolism of malonaldehyde in vivo and in vitro. Lipids. 17:349–355.
Slater TF. 1982. Free radicals mechanisms in tissue injury. London:Pion.
Tsuda H, Moore MA, Asamoto M, Inoue T, Ito N, Satoh K, Ichihara A, Nakamura T, Amelized Z, Oesch F. 1988. Effect of modifying agent on the phenotypic expression of cytochrome P-450, glutathione S-transferase molecular forms, microsomal epoxide hydrolase, glucose 6-phosphate dehydrogenase and γ-glutamyltranspeptidase in the rat liver preneoplastic lesions. Carcinogenesis 9:547–555.
Wiseman H. 1993. Vitamin D is a membrane antioxidant. Ability to inhibit iron-dependent lipid peroxidation in liposomes compared to cholesterol, ergosterol and tamoxifen and relevance to anticancer action. FEBS Lett 326:285–288.