Influence of the Vitamin D Status on the Early Hepatic Response to Carcinogen Exposure in Rats

RU-KUN HE and MARIELLE GASCON-BARRE
Centre de Recherche Clinique André-Viallet, Hôpital Saint-Luc (R.-K.H., M.G.-B.), and Département de Pharmacologie, Faculté de Médecine, Université de Montréal (M.G.-B.), Montréal, Canada
Accepted for publication December 16, 1996

ABSTRACT
Although 1,25-dihydroxyvitamin D₃ has been shown to promote the differentiation of cancer cells and cell lines in vitro, its protective effect against a chemical insult known to induce neoplastic growth in vivo has not been evaluated. The aim of this study was to investigate, in vivo, the influence of the vitamin D status on the early response to an insult known to induce morphological and functional changes leading to hepatocarcinogenesis. The influence of vitamin D status on the susceptibility of rat liver to carcinogenesis was studied after the administration of diethylnitrosamine and 2-acetylaminofluorene, in association with a partial hepatectomy (Solt-Farber protocol), to normal or vitamin D-depleted rats. Preneoplastic foci (γ-glutamyltranspeptidase-positive and glucose-6-phosphatase-negative) appeared in both groups of animals as early as 1 week after 2-acetylaminofluorene withdrawal and continued to increase during the subsequent weeks. Livers from vitamin D-depleted rats exhibited a significant increase in the number of foci over that observed in normal rats at weeks 1 and 5 after 2-acetylaminofluorene withdrawal. However, the main effect of vitamin D depletion was on focus size, which was found to be significantly greater in vitamin D-depleted rat livers at weeks 2 to 6; focus area (volume fraction) was also found to be consistently larger in livers of vitamin D-depleted rats than in those of normal rats. Labeling of oval cells, a cell compartment possibly associated with the repopulation of the liver parenchyma, was significantly reduced by vitamin D depletion. Control rat livers of both groups showed normal liver histology, and no foci, nodules or oval cells were detected in either group. The present data suggest that vitamin D depletion leads to increased in vivo susceptibility to chemicals known to induce hepatocarcinogenesis. Long-term studies must be conducted to evaluate the effect of vitamin D status on the evolution of preneoplastic foci into frank hepatocellular carcinoma.

Received for publication May 20, 1996.

1 These studies were supported by the Medical Research Council of Canada.

ABBREVIATIONS: 2-AAF, 2-acetylaminofluorene; DEN, diethylnitrosamine; γ-GT, γ-glutamyltranspeptidase; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃.
of colon, breast and prostate cancer in humans (Yang and Newmark, 1987; Gorham et al., 1989; Garland et al., 1990; Bostick et al., 1993; Feldman et al., 1995), but the evidence establishing a cause and effect relationship between the in vivo vitamin D status and neoplastic growth remains tenuous. The purpose of the present study was, therefore, to investigate, in vivo, the influence of the vitamin D status on the response of 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 (Faris et al., 1991; Pack et al., 1993; Nagy et al., 1994; Factor et al., 1994; Golding et al., 1995). In this model, a process of hepatocarcinogenesis develops over a period of several months to 1 year, but preneoplastic foci are known to appear early after the end of the cancer-inducing protocol. We now report that a state of hypocalcemic vitamin D depletion increases the susceptibility of rat liver to the development of preneoplastic foci early after exposure to a potent hepatic carcinogen.

Materials and Methods

Animals. All experiments were carried out in male Sprague-Dawley rats (Charles River, St. Constant, Canada) housed in stainless steel wire cages, with a 12-hr light/dark cycle. Normal rats (9–10 weeks of age) were fed a regular rat chow diet containing 2115 IU/kg vitamin D3, and 0.97% calcium (Purina Rat Chow; Ralston Purina Inc., Mississauga, Canada). They were acclimatized for a period of 5 days before being randomly assigned to the experimental protocol. Vitamin D depletion was performed by feeding a semisynthetic vitamin D-deficient diet containing 0.9% elemental calcium (with normal phosphorus content to avoid the development of rickets) and demineralized water to nursing females; the diet was continued for a period of 6 weeks after weaning, as already described (Haddad et al., 1986; Éthier et al., 1990). Vitamin D depletion was judged by the presence of hypocalcemia, which in these animals was accompanied by low to undetectable serum 25-hydroxyvitamin D and low 1,25(OH)2D concentrations, as reported previously (Haddad et al., 1986; Éthier et al., 1990, 1993; Beaulieu et al., 1993). All animals (four or five/group) were fed ad libitum throughout the experimental period. All protocols were carried out according to the Standard of Ethics for Animal Experimentation of the Canadian Council on Animal Care and were approved by the local animal ethics committee.

Chemical induction of liver morphological changes. The influence of vitamin D status on liver susceptibility to carcinogenesis was studied after the administration of DEN and 2-AAF according to the Solt-Farber protocol (Solt and Farber, 1976; Evarts et al., 1989). Briefly, a single dose of DEN (Sigma Chemical Co., St. Louis, MO) was administered i.p. (150 mg/kg b.wt. in 0.9% normal saline). All animals were returned to their quarters and left untreated for a period of 2 weeks. 2-AAF (Sigma) was then administered by gavage (1.5 mg/animal, dissolved in carboxymethylcellulose) for a period of 4 days, after which animals were subjected to two-thirds partial hepatectomy (Éthier et al., 1990). 2-AAF administration was continued for the next 4 days. Beginning 24 hr before the end of the 2-AAF regimen, rats were injected daily with 0.5 µg of 3H thymidine (specific activity, 75 Ci/mmol; DuPont Chemicals, Boston, MA), for a period of 4 days. Animals were killed under ether anesthesia, after an overnight fast, 1, 2, 3, 4, 5 or 6 weeks after the last dose of 2-AAF. Blood was drawn from the abdominal aorta, and the liver was thoroughly washed with normal saline and processed as described below. Control rats were subjected to a similar protocol but received only normal saline i.p. and carboxymethylcellulose by gavage. They were also subjected to two-thirds partial hepatectomy and [3H]thymidine injection.

Morphological studies. Liver samples were taken from the right and caudate lobes and either immediately frozen in liquid nitrogen for γ-GT and glucose-6-phosphatase evaluation or fixed in 10% neutral formalin, embedded in paraffin blocks and stained (4-µm sections) with hematoxylin-phloxine-saffron. Each hematoxylin-phloxine-saffron-stained section was evaluated by light microscopy for the detection of preneoplastic foci, nodules and oval cells. Oval cells were determined as cells, in the perportal area of the hepatic acinus, with an oval nucleus and the capacity to incorporate [3H]thymidine in the presence of the cytotstatic agent 2-AAF (Alison et al., 1993). Autoradiography was performed as already described (Éthier et al., 1990), on a total of 4000 cells taken at random in each liver section. All evaluations were carried out in a double-blind manner.

Results

Serum ionized calcium was found to be significantly influenced by the vitamin D status, with values of 0.78 ± 0.04 and 1.27 ± 0.03 mM in vitamin D-depleted and normal rats, respectively (P < .0001), at the beginning of the experiment. Circulating ionized calcium did not change in either group during the course of the studies, with values of 0.85 ± 0.04 and 1.27 ± 0.01 mM, respectively (P < .0001), at the time of euthanasia.

Figure 1 illustrates the histological appearance of the liver in both groups of animals after the hepatocarcinogenesis protocol; the complete time course for the morphological data is presented in figure 2. As illustrated, preneoplastic foci (γ-GT-positive and glucose-6-phosphatase-negative) were already present in both groups of animals 1 week after 2-AAF withdrawal and continued to increase during the subsequent weeks. Livers from vitamin D-depleted rats exhibited a significant increase, over that observed in normal rats, in the number (fig. 2A) of foci at weeks 1 and 5, but the number of foci was significantly lower than in normal rats at week 3 after 2-AAF withdrawal. Focus size (fig. 2B), however, was found to be significantly higher in vitamin D-depleted rat livers at weeks 2, 3, 5 and 6. Finally, due to the increase in focus size, focus area (volume fraction) (fig. 2C) was consistently higher in vitamin D-depleted livers than in normal rat livers. Control rat livers of both groups had normal liver histology, and no foci, nodules or oval cells were detected in either group.

The oval cell labeling is presented in figure 3. The labeling index was shown to decrease over time in both groups (P < .001), with indices of 7 ± 2.9 and 6 ± 1.8 cells/1000 cells in normal and vitamin D-depleted rat livers, respectively (not significant), 4 weeks after the end of the carcinogenesis induction protocol. However, evaluation of the effect of vitamin D depletion during the period of observation indicated that
Fig. 1. Representative photomicrographs of rat livers treated with DEN and 2-AAF. Animals were killed 3 weeks after the withdrawal of 2-AAF, as indicated in “Materials and Methods.” A (normal) and C (hypocalcemic, vitamin D-depleted), histological sections of rat liver specimens stained with hematoxylin-phloxine-saffron (magnification of ×32 for both sections); B (normal) and D (hypocalcemic, vitamin D-depleted), γ-GT-positive (and glucose-6-phosphatase-negative; data not shown) hepatic foci (magnification of ×20 for both sections).
the labeling index was consistently lower in livers of vitamin D-depleted rats than in those of normal animals (P < .001). Post hoc tests revealed that the significant decrease in oval cell number originated 1 week after 2-AAF withdrawal (P < .001), indicating that the main effect of vitamin D depletion was to impair the creation of oval cells early during the hepatocarcinogenesis protocol.

**Discussion**

In the present studies, the well characterized “resistant” hepatocyte model developed by Solt and co-workers (Solt and Farber, 1976; Solt et al., 1977) was used to evaluate the influence of vitamin D status on the early response to carcinogenesis. In this model, exposure to the genotoxic carcinogen DEN, followed by partial hepatectomy during treatment with the cytostatic agent 2-AAF, inhibits the replication of normal hepatocytes (resistant hepatocytes) and promotes the expansion of a novel, pluripotent, stem cell compartment (oval cells) originating in the portal triad. In the early stages of hepatocarcinogenesis, oval cells rapidly proliferate in the periportal area; they finally invade other areas of the liver acinus (Nagy et al., 1994). These cells show some common features with ductular cells in electron microscopy (Grisham, 1962; Evarts et al., 1987), but functionally they also express several phenotypic markers of normal hepatocytes, such as...
after bromobenzene intoxication (Haddad et al., 1987). Our observation suggests that oval cells may also be influenced by the in vivo vitamin D status.

The early morphological changes observed during this study also indicate that vitamin D depletion seems to promote the development of early putative preneoplastic foci. Indeed, although foci numbers were found to be quite similar in the two groups of animals, the size and volume fraction of the foci were significantly larger in livers of vitamin D-depleted rats than in those of normal rats. Collectively, these data suggest that vitamin D depletion in association with hypocalcemia leads to increased susceptibility to chemicals known to induce hepatocarcinogenesis with a reduction in oval cell number, possibly further inhibiting the normal repopulation of the liver parenchyma. Although the mechanism involved in the protective effect of normal vitamin D status on the early manifestation of tumor growth is not presently known, others have suggested that vitamin D and calcium may increase intracellular calcium bioavailability and reduce lipid peroxidation (Ghoshal et al., 1987). Such a mechanism could also be present in the model system, because a similar dietary regimen of vitamin D depletion accompanied by hypocalcemia has already been shown to reduce basal as well as stimulated intracellular calcium levels in hepatocytes (Gascon-Barré et al., 1994; Bilodeau et al., 1995). However, vitamin D alone [through the action of its active metabolite 1,25(OH)2D3] may also be the protective agent against the observed putative preneoplastic foci, because several nonhypocalcemic analogs of 1,25(OH)2D3 are known to be potent antitumor molecules (Kawa et al., 1996). Further studies will need to be conducted to investigate the longer-term protective effects of vitamin D on the genesis of hepatic tumors, as well as its influence on oval cells, because they are a common feature of several experimental models of hepatocarcinogenesis (Pack et al., 1993; Factor et al., 1994) and have also been observed in human hepatitis B virus-associated hepatocellular carcinoma (Hsia et al., 1992).

Acknowledgments

The authors are grateful to Manon Livernois for her excellent secretarial assistance.

References


Bostick, R. M., Potter, J. D., Selkirk, T. A., McKenzie, D. R., Kushl, L. H. and Folsom, A. R.: Relation of calcium, vitamin D, and dairy food intake to...


Send reprint requests to: Marielle Gascon-Barré, Ph.D., Centre de Recher- Che Clinique Andrie-Viallet, Hôpital Saint-Luc, 264 René-Lévesque Blvd. East, Montreal (Quebec), Canada H2X 1P1.