Influence of Orotic Acid on Multistage Hepatocarcinogenesis in the Rat: Resistance of Hepatocytes from Nodules to the Mitoinhibitory Effects of Orotic Acid

(43511D)

S. MANJESHWAR, E. LACONI, P. M. RAO, S. RAJALAKSHMI, AND D. S. R. SARMA[1]
Department of Pathology, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Abstract. **This study was designed to determine whether hepatocytes from hepatic nodules are resistant to the mitoinhibitory effects of orotic acid. Hepatic nodules were initiated in Fischer 344 male rats with 1,2-dimethylhydrazine.2HCl (100 mg/kg ip) given 16 hr after two thirds partial hepatectomy and promoted by feeding a diet containing 1% orotic acid. Eight to 9 months later, when persistent nodules had developed, the rats were taken off the orotic acid diet and maintained on a semisynthetic basal diet for 2 to 5 weeks. The effect of orotic acid on the DNA synthesis in the hepatocytes isolated from hepatic nodules and from the surrounding nonnodular liver and from the age- and sex-matched control rats was studied. The results indicated that a dose of orotic acid (120 μM) that almost completely inhibited the transforming growth factor-α-induced DNA synthesis in hepatocytes from nonnodular surrounding liver and from age- and sex-matched control liver could not inhibit the DNA synthesis in hepatocytes from hepatic nodules. These results are consistent with the postulate that orotic acid may promote liver carcinogenesis by a differential mitoinhibition of normal hepatocytes while permitting the initiated hepatocytes to respond to growth stimuli and form hepatic nodules. However, it needs to be determined whether differential mitoinhibition of normal hepatocytes is the mechanism by which orotic acid promotes liver carcinogenesis.**

[P.S.E.B.M. 1993, Vol 202]

Orotic acid is a precursor of pyrimidine nucleotide biosynthesis. However, when given in excess, it results in an imbalance in nucleotide pools characterized by an increase in uridine nucleotides and a decrease in adenosine nucleotides (1–3). Since nucleotides are precursors of nucleic acids, an imbalance in nucleotide pools can cause perturbations in DNA repair and replication. Similarly, since most of the sugars are transferred as nucleotide sugars, an imbalance in nucleotide pools can also influence the glycosylation of lipids and proteins, including that of membranes (4). Not too surprisingly, the influence of orotic acid on tumorigenesis has attracted attention (5–7).

Using the recently developed initiation and promotion protocols, the effect of orotic acid on multistage hepatocarcinogenesis in the rat was studied (8–12). It appears that orotic acid is not an initiator of liver carcinogenesis, i.e., it did not induce initiated hepatocytes that can be promoted to form foci or nodules of enzyme-altered hepatocytes by a resistant hepatocyte model, by phenobarbital, or by a diet deficient in choline (13). Orotic acid is also not a hepatocarcinogen, as exposure of noninitiated rats to orotic acid for 1 year did not result in hepatocellular carcinomas (10). However, orotic acid promoted liver carcinogenesis in rats initiated with a number of liver carcinogens (8–12).

Promotion may be defined operationally as clonal expansion of initiated cells to form foci/nodules, polyps, or papillomas, one or more of which form precur-

[1] To whom requests for reprints should be addressed at Department of Pathology, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada M5S 1A8.

P.S.E.B.M. 1993, Vol 202: 25–29

0037-9727/93/2021-0025$3.00/0
Copyright © 1993 by the Society for Experimental Biology and Medicine

sor lesions for the development of cancers (14). Any hypothesis on tumor promotion, therefore, should account for the differential growth of initiated cells. The differential can be at the level of initiated cell (Fig. 1), at the level of the promoting regimen (Fig. 2), or both. At the initiated cell level, initiated hepatocytes may exhibit a low threshold to growth stimuli or they may escape from the negative regulation. This would allow a selective expansion of initiated hepatocytes. As shown in the Figure 2, at the level of the promoter, the promoter can selectively act on initiated cells and amplify the initiated cell population (Mode A). In this instance, the promoter is a mitogen. Alternatively, the promoter can differentially mitoinhibit the noninitiated surrounding cells while permitting the initiated cells to respond to the growth stimuli and form foci/nodules (Mode B). In this instance, the promoter is a mitoinhibitor and the initiated cells should be relatively resistant

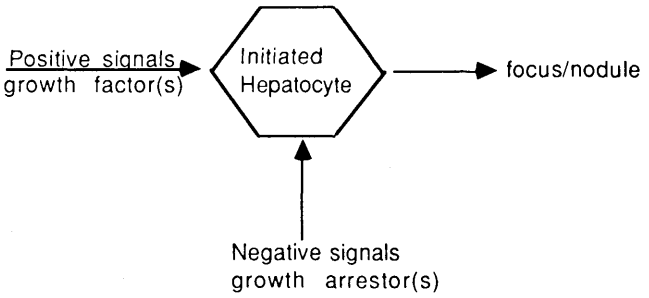

Figure 1. Schematic representation of how an initiated hepatocyte may be selectively amplified to form a focus/nodule. Initiated hepatocytes may have a low threshold for growth stimuli and/or a decreased response to negative growth regulators, i.e., growth arrestors. If initiated hepatocytes have these properties, they are at an advantage over noninitiated hepatocytes for their growth.

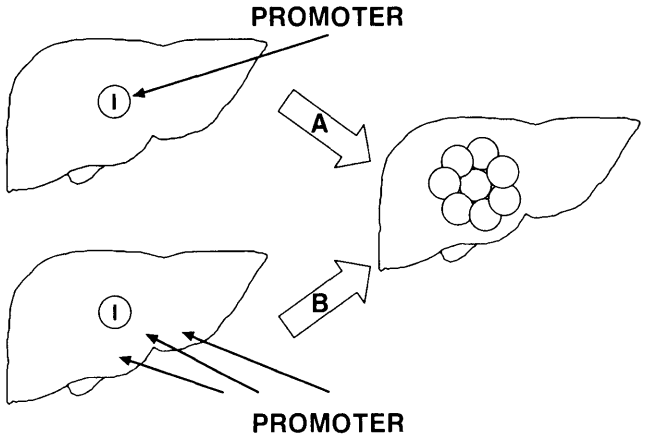

Figure 2. Schematic representation of how an initiated hepatocyte (I) may be selectively amplified to form a focus/nodule. In Mode A, the promoter is a selective mitogen and differentially amplifies the initiated hepatocyte to form a focus/nodule. In Mode B, the promoter is a selective mitoinhibitor to the noninitiated hepatocytes. Initiated hepatocytes, being relatively resistant to the mitoinhibitory effects of the promoter, have the advantage in responding to growth stimuli and in forming foci/nodules.

to the mitoinhibitory effects of the promoter (15). Orotic acid is not a mitogen to the liver (9). On the other hand, it inhibits DNA synthesis in hepatocytes both *in vivo* (16) and *in vitro* (17–21). These results suggest that orotic acid may promote liver carcinogenesis by the differential mitoinhibitory mode. One of the prerequisites for this postulate is that the initiated hepatocytes be relatively resistant to the mitoinhibitory effects of orotic acid. The results presented in this communication indicate that hepatocytes isolated from the hepatic nodules are indeed resistant to the mitoinhibitory effects of orotic acid.

Materials and Methods

Development of Hepatic Nodules. Hepatic nodules were generated by initiating male Fischer 344 rats, 110–120 g (fed a semisynthetic diet, No. 101; Dyets Inc., Bethlehem, PA) with 1,2-dimethylhydrazine.2HCl given intraperitoneally (100 mg/kg) 16 hr after a two thirds partial hepatectomy. Three weeks later, they were started on a basal diet containing 1% orotic acid (10). Eight to 9 months later, when persistent nodules had developed, the rats were taken off the orotic acid diet and maintained on the basal diet for 2 to 5 weeks before use in the experiment.

***In Vitro* Cell Culture Studies.** Hepatocytes (from hepatic nodules and nonnodular surrounding liver and age- and sex-matched control rat liver) were isolated by a collagenase perfusion technique as described previously (21). Briefly, 2×10^5 viable hepatocytes were cultured on 35-mm dishes coated with collagen (Vitrogen, 60 μg/dish; Collagen Corp., Palo Alto, CA) in modified William's E medium containing fetal bovine serum (10% v:v), insulin (20 units/liter), L-glutamine (2 m*M*), Hepes (10 m*M*), penicillin (100 units/ml), and streptomycin (100 μg/ml). After an attachment period of 3 hr at 37°C in air-CO_2 (95:5), the medium and nonattached cells were removed. At this time, medium was changed to serum-free modified William's E medium supplemented with L-proline (2 m*M*) and sodium pyruvate (10 m*M*). Appropriate dishes also contained epidermal growth factor or transforming growth factor (TGF)-α, orotic acid as orotic acid methyl ester (Sigma Chemical Co., St. Louis, MO), and [^{3}H] thymidine (5 μCi/dish). After 48 hr of incubation, the cells were washed in cold phosphate-buffered saline and then processed for DNA synthesis either by determining the labeling index or by determining acid-precipitable radioactivity (21). Eighty percent to 90% viability was observed in hepatocytes isolated from both hepatic nodules and from the surrounding nonnodular liver tissue.

Results and Discussion

Initial experiments were carried out to determine the optimum dose of TGF-α that induces DNA synthe-

sis in hepatocytes from nodules and surrounding nonnodular liver tissue. The results presented in Figure 3 demonstrate that increasing the dose of TGF-α resulted in an increase in DNA synthesis. Hepatocytes from nodules exhibited a higher basal labeling index compared with those from the surrounding liver. However, not all hepatocytes from the nodules responded to TGF-α, even at higher doses. It is intriguing that a significant fraction of nodule-hepatocytes do not respond to TGF-α. Similar patterns of results were obtained whether the DNA synthesis was monitored as labeling index or as acid-precipitable radioactivity.

The addition of orotic acid inhibited the DNA synthesis in the hepatocytes from the surrounding nonnodular liver (Fig. 4). The pattern of results is very similar to that obtained with hepatocytes from age- and sex-matched control rats that were not exposed to dimethylhydrazine or orotic acid diet (Fig. 4). However, hepatocytes from nodules did not exhibit any mito-

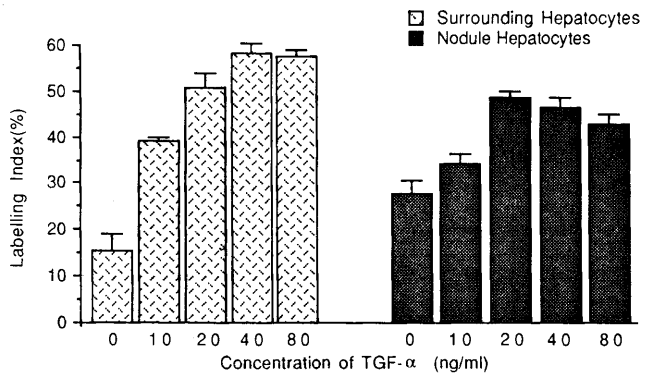

Figure 3. Effect of different concentrations of TGF-α on DNA synthesis in hepatocytes isolated from hepatic nodules and nonnodular surrounding liver tissue. Details concerning the generation of hepatic nodules, isolation of hepatocytes, and measurement of their proliferative response to TGF-α are given in the text. In most of the experiments, hepatocytes from a single nodule of 1 cm or larger were used. The values are the mean ± SD of three dishes. The experiment was repeated two or three times with similar patterns of results.

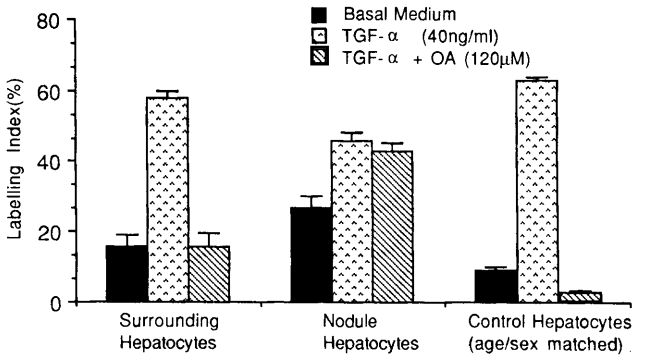

Figure 4. Effect of orotic acid on DNA synthesis induced by TGF-α in hepatocytes from nocules, nonnodular surrounding hepatocytes, and from age- and sex-matched control rats. For experimental details, see legend to Figure 3. Additional details are given in the text. The experiment was repeated three times with similar patterns of results.

inhibition in response to orotic acid. Essentially similar pattern of results were obtained when epidermal growth factor was used instead of TGF-α. Thus, orotic acid at a concentration that inhibited DNA synthesis in hepatocytes isolated from normal rat liver or from surrounding nonnodular liver tissue did not inhibit the DNA synthesis in hepatocytes from hepatic nodules. These results clearly demonstrate that orotic acid is a differential mitoinhibitor in normal hepatocytes and that the hepatocytes from nodules some how escape the mitoinhibitory effects of orotic acid. This observation is consistent with the postulate that orotic acid may promote liver carcinogenesis by a differential mitoinhibitory mode. However, it needs to be demonstrated that the differential mitoinhibitory effects of orotic acid in fact contribute to its promoting effects.

The concept of differential mitoinhibition as a mechanism to selectively amplify the resistant (initiated) hepatocytes to form foci/nodules of enzyme-altered hepatocytes was utilized in the resistant-hepatocyte model of liver tumor promotion (22, 23). 2-Acetylaminofluorene, used in the resistant hepatocyte model, exhibits differential mitoinhibition on normal hepatocytes, whereas the initiated ones are relatively resistant to its mitoinhibitory effects. In this model, 2-acetylaminofluorene coupled with a compensatory liver cell-proliferative stimulus selectively amplifies the carcinogen-induced resistant hepatocytes. Phenobarbital, another liver tumor promoter, also inhibits DNA synthesis in hepatocytes *in vitro* (24, 25) and *in vivo* upon chronic exposure (26; E. Laconi and E. Farber, unpublished observations). Interestingly, phenobarbital inhibits the DNA synthesis also in hepatocytes of the hepatic nodules *in vitro* in response to TGF-α (data to be published elsewhere). Thus, differential mitoinhibition, if it exists at all, is minimal, and one wonders whether it contributes to a significant extent to the promoting effects of phenobarbital (Table I). It is also conceivable that the lack of a strong differential may be one reason

Table I. Comparison of Orotic Acid, Phenobarbital, and the Resistant Hepatocyte Models in Terms of Their Ability to Promote by a Differential Mitoinhibitory Mode[a]

Parameter considered	Models of rat liver tumor promotion		
	2-AAF (RH model)	OA	PB
Mitoinhibition	+	+	+
Differential mitoinhibition	+	+	±
Selection by differential mitoinhibition	+	?	?

[a] Abbreviations used in tables: 2-AAF, 2-acetylaminofluorene; RH, resistant hepatocyte; OA, orotic acid; PB, phenobarbital.

why phenobarbital takes several months to achieve tumor promotion.

Recently, orotic acid was also shown to promote duodenal carcinogenesis induced by azoxymethane (27) and mammary carcinogenesis induced by dimethylbenzo(*a*)anthracene (28) and to enhance *N*-nitroso-(2-hydroxypropyl)(2-oxypropyl)amine-induced renal mesenchymal tumors and lung adenomas (29). It is not known whether orotic acid exerts a differential mitoinhibition in these organs similar to that seen in the liver.

The discussion so far has been focused on orotic acid as a promoter. One question that needs to be explored is whether orotic acid also has the potential to be a progressor. In this context, progression is defined as a process whereby the nodules undergo cellular evolution to malignant neoplasms (14). This question stems from two observations: (i) It takes 10–15 weeks of exposure for diets containing 1% orotic acid to induce foci of enzyme-altered hepatocytes in rats initiated with diethylnitrosamine or 1,2-dimethylhdrazine (10). The foci formed are fewer and smaller compared to those seen with the resistant hepatocyte model of liver tumor promotion. (ii) However, by the end of 1 year, nearly 100% of the initiated rats exposed to orotic acid develop hepatocellular carcinomas, with about 50% of them metastasizing to the lungs (35–70% depending on the initiating protocol) (13). In many of the existing models of rat liver tumor promotion, it is difficult to draw a clear cut demarcation between where the tumor promotion ends and tumor progression starts. Thus, there is an urgency to develop models with good synchrony of the lesions and with clearly identifiable boundaries between tumor promotion and progression, and to determine biological, biochemical, or molecular markers that have functional significance to tumor progression. In recent years, the need to develop such models to study tumor progression has been increasingly recognized.

Thus, in summary, the results obtained so far indicate that orotic acid is not a carcinogen or an initiator to the liver, but a promoter of carcinogenesis in the liver. It exerts a differential mitoinhibition in hepatocytes from normal and nonnodular surrounding liver tissue, while the hepatocytes from nodules are resistant to the mitoinhibitory effects of orotic acid. Whether this differential plays any role in orotic acid-induced liver tumor promotion needs to be established.

This study was supported by funds from NIH CA 37077. We thank Lori Cutler for her secretarial assistance.

1. Rajalakshmi S, Sarma DSR, Sarma PS. Studies on orotic acid fatty liver. Biochem J **180**:375–378, 1961.
2. Von Euler LH, Rubin RJ, Handschumacher RE. Fatty liver induced by orotic acid. II. Changes in nucleotide metabolism. J Biol Chem **238**:2464–2469, 1963.
3. Marchetti M, Paddu P, Caldarera CM. Metabolic aspects of orotic acid fatty liver: Nucleotide control mechanisms of lipid metabolism. Biochem J **92**:46–51, 1964.
4. Rao PM, Nagamine Y, Roomi MW, Rajalakshmi S, Sarma DSR. Orotic acid, a new promoter for experimental liver carcinogenesis. Toxicol Pathol **12**:173–178, 1984.
5. Rogers S. Inhibitory influence of normally occurring pyrimidine precursor upon methylcholanthrene carcinogenesis. Proc Soc Exp Biol Med **96**:464–465, 1957.
6. Lin J-K, Tung T-C. Effect of orotic acid on DAB-hepatocarcinogenesis in rats. J Formosan Med Assoc **64**:501–511, 1965.
7. Sidransky H, Verney E. Influence of orotic acid on liver tumorigenesis in rats ingesting ethionine, N-2-fluorenylacetamide and 3′-methyl-dimethylamino-azobenzene. JNCI **44**:1201–1215, 1970.
8. Columbano A, Ledda GM, Rao PM, Rajalakshmi S, Sarma DSR. Dietary orotic acid, a new selective growth stimulus for carcinogen altered hepatocytes in rat. Cancer Lett **16**:191–196, 1982.
9. Rao PM, Nagamine K, Ho R-K, Roomi MW, Laurier C, Rajalakshmi S, Sarma DSR. Dietary orotic acid enhances the incidence of γ-glutamyltransferase positive foci in rat liver induced by chemical carcinogens. Carcinogenesis **4**:1541–1545, 1983.
10. Laurier C, Tatematsu M, Rao PM, Rajalakshmi S, Sarma DSR. Promotion by orotic acid of liver carcinogenesis in rats initiated by 1,2-dimethylhydrazine. Cancer Res **44**:2186–2191, 1984.
11. Laconi E, Vasudevan S, Rao PM, Rajalakshmi S, Sarma DSR. Orotic acid, a novel tumor promoter: Studies on the mechanism of its action. In: Banbury Reports. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, Vol 25, pp85–90, 1987.
12. Elliot TS, Robinson JL, Visek WJ. Influence of dietary orotic acid on hepatocarcinogenesis in Fischer 344 and Sprague-Dawley rats. FASEB J **46**:746, 1987.
13. Laconi E. Studies on rat liver tumor promotion by orotic acid. Doctoral thesis, University of Toronto, Toronto, Canada, 1988.
14. Farber E, Sarma DSR. Biology of disease: Hepatocarcinogenesis—A dynamic cellular perspective. Lab Invest **56**:4–22, 1987.
15. Sarma DSR, Rao PM, Rajalakshmi S. Liver tumor promotion by chemicals: Models and mechanisms. Cancer Surv **5**:782–798, 1986.
16. Sheikh A, Yusuf A, Laconi E, Manjeshwar S, Rao PM, Rajalakshmi S, Sarma DSR. Orotic acid inhibits DNA synthesis in rat liver following partial hepatectomy. Proc Am Assoc Cancer Res **32**:154, 1991.
17. Laconi E, Li F, Semple E, Rao PM, Rajalakshmi S, Sarma DSR. Inhibition of DNA synthesis in primary cultures of hepatocytes by orotic acid. Carcinogenesis **9**:675–677, 1988.
18. Pichiri-Coni G, Coni P, Laconi E, Schwarze P, Seglen PO, Rao PM, Rajalakshmi S, Sarma DSR. Studies on the mito-inhibitory effect of orotic acid on hepatocytes in primary culture. Carcinogenesis **11**:981–984, 1990.
19. Lea M, Luke A, Assad A, Ayyala S. Inhibitory effects of orotate on precursor incorporation into nucleic acids. Chem Biol Interact **75**:49–59, 1990.
20. Cesarone CF, Scarabelli L, Giannoni P, Gallo G, Orunesu M. Relationship between poly(ADP-ribose) polymerase activity and DNA synthesis in cultured hepatocytes. Biochem Biophys Res Commun **171**:1037–1043, 1990.
21. Manjeshwar S, Sheikh A, Pichiri-Coni G, Coni P, Rao PM, Rajalakshmi S, Pediaditakis P, Michalopoulos G, Sarma DSR. Orotic acid, nucleotide pool imbalance, and liver tumor promotion: A possible mechanism for the mito-inhibitory effects of orotic acid in isolated rat hepatocytes. Cancer Res **52**:2078s–2081s, 1992.
22. Solt DB, Farber E. New principle for the analysis of chemical carcinogenesis. Nature **263**:701–703, 1976.

23. Solt DB, Cayama E, Tsuda H, Enomoto K, Lee G, Farber E. Promotion of liver cancer development by brief exposure to dietary 2-acetylaminofluorene plus partial hepatectomy or carbon tetrachloride. Cancer Res **43**:188–191, 1983.
24. Eckl PM, Meyer SA, Whitcombe WR, Jirtle RL. Phenobarbital reduces EGF receptors and the ability of physiological concentrations of calcium to suppress hepatocyte proliferation. Carcinogenesis **9**:479–483, 1988.
25. Manjeshwar S, Sheikh A, Rao PM, Rajalakshmi S, Michalopoulos G, Pediaditakis P, Sarma DSR. Inhibition of DNA synthesis by orotic acid and phenobarbital in hepatocytes in vitro. Proc Am Assoc Cancer Res **32**:157, 1991.
26. Barbason H, Rassenfosse C, Betz EH. Promotion mechanism of phenobarbital and partial hepatectomy of DENA hepatocarcinogenesis cell kinetics effect. Br J Cancer **47**:517–525, 1983.
27. Rao P, Laconi E, Rajalakshmi S, Sarma DSR. Orotic acid, a liver tumor promoter, also promotes carcinogenesis of the intestine. Proc Am Assoc Cancer Res **27**:147, 1986.
28. Elliot TS, Visek WJ. Effects of orotic acid feeding on carcinogenesis. FASEB J **3**:A474, 1989.
29. Kokkinakis DM, Scarpelli DG, Oyasu R. Enhancement of N-nitroso(2-hydroxypropyl)(2-oxypropyl)amine-induced tumorigenesis in Sprague-Dawley rats by orotic acid. Carcinogenesis **12**:181–186, 1991.