Orotic Acid, Nucleotide-Pool Imbalance, and Liver-Tumor Promotion: A Possible Mechanism for the Mitoinhibitory Effects of Orotic Acid in Isolated Rat Hepatocytes

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Abstract

This study was designed to determine the possible mechanism by which orotic acid exerts its mitoinhibitory effect on rat hepatocytes in primary culture. Orotic acid inhibited, dose-dependently DNA synthesis in hepatocytes induced by epidermal growth factor, transforming growth factor α, hepatocyte growth factor, acidic fibroblast growth factor, or plasma from rats exposed to various liver cell-proliferative stimuli, such as two-thirds partial hepatectomy, lead nitrate, cyproterone acetate, ethylene dibromide, or a diet deficient in choline. Further, orotic acid inhibited DNA synthesis even when added 24 h after the hepatocytes were primed with transforming growth factor α. Taken together, these results suggested that the target site may not be at the level of the growth-factor receptor and receptor-mediated early events. In a preliminary experiment, orotic acid inhibited the expression of the ribonucleoside diphosphate reductase gene. Exposure to orotic acid results in an imbalance in nucleotide pools characterized by an increase in uridine nucleotides and a decrease in adenosine nucleotides. It is hypothesized that this imbalance in nucleotide pools inhibits the expression of the ribonucleoside diphosphate reductase gene and, therefore, is a likely target for the mitoinhibitory effect of orotic acid.

Introduction

Nucleotides, in addition to being precursors of template-directed nucleic acid synthesis, play a key role in major biological processes. For example, after conversion to nucleotide sugars, nucleotides participate in glycosylation reactions of proteins and lipids, including those of the membranes. Further, uridine nucleotides participate in the synthesis of polysaccharides, cytidine nucleotides in the synthesis of lipids, and adenosine and guanosine nucleotides in the synthesis of proteins. Although the ATP-ADP system is involved in the mainstream of energy transfers, 5'-di- and triphosphates of other ribo- and deoxyribonucleosides also participate in cellular energy transfer. As second messengers, nucleotides also influence signal-transduction mechanisms. In addition, nucleotides also regulate the activity of enzymes such as RNR1 (1, 2) and galactosyltransferase (3). Because nucleotides participate in several cellular functions, an imbalance in the levels of nucleotides may have profound effects in the pathogenesis of many disease processes.

One of our approaches to understanding the carcinogenic process is identifying those metabolic events that, when perturbed, influence pathogenesis. Nucleotide pools appear to be an important cellular influence on carcinogenesis. An imbalance in nucleotide pools can disturb the homeostasis of both DNA

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3 The abbreviations used are: RNR, ribonucleoside diphosphate reductase; EGF, epidermal growth factor; HGF, hepatopoietin-A or hepatocyte growth factor; TGF-α, transforming growth factor α; FGF, fibroblast growth factor; 2/3 PH, two-thirds partial hepatectomy; PRPP, 5-phosphoribosyl-1-pyrophosphate; PPP, platelet-poor plasma; OTC, ornithine transcarbamoylase.

The present study reflects our continued efforts to comprehend the mechanism by which orotic acid and the resulting imbalance in the nucleotide pools promote liver carcinogenesis. Operationally, promotion results in focal proliferation of initiated hepatocytes to form foci of enzyme-altered hepatocytes and hepatic nodules (18, 19). Therefore, any mechanism proposed for promotion should be able to explain how focal proliferation is achieved. Accordingly, a promoter can act as a mitogen selectively on initiated hepatocytes or can mitoinhibit the noninitiated hepatocytes differentially while permitting the initiated hepatocytes to respond to growth stimuli and form foci and/or nodules. Orotic acid is not a mitogen to hepatocytes (12), but orotic acid is a mitoinhibitor (20, 21). We provide evidence from in vitro experiments to suggest that one of the target sites at which orotic acid exerts its mitoinhibitory effect is beyond the growth-factor receptor and the growth factor-mediated early events.

Materials and Methods

Experiments using Defined Growth Factors. Hepatocytes were isolated, by a collagenase-perfusion technique (22), from 180–200-g male Fischer 344 rats (Charles River Breeding Laboratories, St. Constant, Quebec, Canada) maintained on a semisynthetic basal diet (diet 101 from Dyets Inc., Bethlehem, PA). Low centrifugal speeds (50 × g) were used during cell washing to minimize the contamination of hepatocytes with smaller nonparenchymal cells. Cell viability, estimated by the trypsin blue exclusion method, was ≥ 90%. Viable hepatocytes (2 × 10⁸) were cultured on 35-mm dishes coated with collagen (Vitrogen, 60 μg/dish), in modified William’s E medium containing fetal bovine serum (10%, v/v), insulin (20 units/liter), L-glutamine (2 mM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). After an attachment period of 3 h at 37°C in air-CO₂ (95:5), the medium and nonattached cells were removed. The medium was changed to serum-free, modified William’s E medium supplemented with l-proline (2 mM) and sodium pyruvate (10 mM). Dishes contained different growth factors, TGF-α (40 ng/ml), HGF (100 ng/ml), or acidic FGF (20 ng/ml). Control dishes contained only the basal medium. Orotic acid was added as orotic acid methyl ester (Sigma Chemical Co., St. Louis, MO). All dishes also contained [³H]thymidine (5 μCi/dish). The dishes were

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incubated for 48 h. At the end of the experiment, cells were processed for measuring DNA synthesis either by determining labeling index or by determining acid-precipitable radioactivity. For determining labeling index, the cells were washed in cold phosphate-buffered saline, fixed in 10% buffered formalin, and processed for autoradiography (20, 21). For determining the acid-precipitable radioactivity, the cells were washed in cold phosphate-buffered saline and subsequently dissolved in 1.5 ml of 0.33 N NaOH. A sample was taken for DNA precipitation with 1 ml of an ice-cold solution of 40% trichloroacetic acid and 1.2 N HCl. The precipitate was then filtered onto 0.45-Mm Millipore filters and washed with ice-cold 5% trichloroacetic acid. The air-dried filters were dissolved in 1 ml of Cellosolve (Fisher Scientific, Canada) and counted with 10 ml of Aquasol (NEN, Canada) in a scintillation counter. The data are expressed as mean cpm/dish ± SD of three dishes. In experiments with HGF, the conditions of the experiment and media were slightly different from those described above and were detailed earlier (23, 24).

Experiments using Platelet-poor Plasma. PPP was prepared (25) from control sham-hepatectomized rats or rats subjected to 2/3 PH, treated with lead nitrate (10 umol/100 g i.v.), ethylene dibromide (10 mg/kg intragastrically), or cyproterone acetate (10 mg/100 g intragastrically) 24 h before being killed, or from rats exposed to a choline-deficient diet for 5 days. After the hepatocytes were allowed to attach for 3 h, PPP was added to the medium (20% final concentration). Some dishes also contained orotic acid. The effect of orotic acid on DNA synthesis in hepatocytes stimulated by PPP was monitored by using the methodology described above.

Addition of Orotic Acid 24 h after Priming the Hepatocytes with Growth Factors. In some experiments, after the 3 h for attachment the medium was changed to a serum-free medium and the growth factor was added to the medium in appropriate dishes. Twenty-four h later, the medium was washed off, fresh serum-free medium containing different concentrations of orotic acid and [3H]thymidine was added, and the dishes were incubated for another 24 h. At the end of the experiment, cells were processed for measuring DNA synthesis as described above. Routinely, the effect of EGF (20 ng/ml) on the induction of DNA synthesis and the effect of orotic acid (120 μM) on EGF-induced DNA synthesis in hepatocytes were determined as positive controls. All experiments were repeated at least two or three times and gave similar patterns of results.

Results and Discussion

The first series of experiments was designed to determine whether orotic acid would inhibit the growth response of hepatocytes, in primary culture, to several growth factors that presumably use different receptors. The rationale was that, if this occurs, it is likely that at least one site of action is not at the level of growth-factor receptor. The results presented in Figs. 1 and 2 indicate that orotic acid dose dependently inhibited the growth response of hepatocytes to both TGF-α and HGF. A comment should be made on the differences in dose response of orotic acid towards TGF-α and HGF. Experiments dealing with HGF were done by Pediaditakis and Michalopoulos (23, 24) at Duke University, with a slightly different medium. Currently we are exploring the possibility of whether the supersensitivity of hepatocytes to orotic acid when HGF was used was because of the differences in the composition of the basal medium.

The effect of orotic acid was also examined by using acidic FGF as the inducer of DNA synthesis. Orotic acid again inhibited the response of hepatocytes to acidic FGF. However, the stimulation of DNA synthesis by acidic FGF, compared with the controls, was not consistently statistically significant between different experiments. Because of these variations the data are not presented. In addition to these defined growth factors, we also examined the effect of orotic acid on the growth response of hepatocytes stimulated by plasma from rats subjected to various liver cell-proliferative stimuli. These include 2/3 PH, liver mitogens such as lead nitrate, cyproterone acetate, and ethylene dibromide (26), and a choline-deficient diet (27). The results presented in Table 1 indicate that orotic acid inhibited DNA synthesis in hepatocytes stimulated by plasma from rats subjected to the different types of liver cell-proliferative stimuli. These data, together with our earlier observation that orotic acid inhibited DNA synthesis in hepatocytes induced by EGF (20, 21), suggest that one of the target sites for the mitoinhibitory effect of orotic acid may be beyond events related to growth factor receptors. This statement may be valid, because EGF and TGF-α, HGF, and acidic FGF appear to have different

Table 1 Effect of orotic acid (OA) on DNA synthesis in hepatocytes induced by plasma from rats subjected to several liver-cell-proliferative stimuli

<table>
<thead>
<tr>
<th>Proliferative stimuli used to prepare 20% PPP</th>
<th>Labeling index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without OA</td>
<td>With OA</td>
</tr>
<tr>
<td>Control</td>
<td>20 ± 2.0</td>
</tr>
<tr>
<td>2/3 PH</td>
<td>39 ± 2.5</td>
</tr>
<tr>
<td>Lead nitrate</td>
<td>37 ± 3.1</td>
</tr>
<tr>
<td>Ethylene dibromide</td>
<td>41 ± 3.4</td>
</tr>
<tr>
<td>Cyproterone acetate</td>
<td>40 ± 2.9</td>
</tr>
<tr>
<td>Choline-deficient diet</td>
<td>65 ± 3.8</td>
</tr>
</tbody>
</table>

Fig. 1. Inhibition of TGF-α-induced DNA synthesis by orotic acid (OA) in hepatocytes in primary culture. Isolated hepatocytes were incubated with basal medium (BM) or basal medium containing TGF-α and different concentrations of orotic acid. All dishes contained [3H]thymidine. After 48 h of incubation, the cells were processed for measurement of acid-precipitable radioactivity. Other details are given in the text. Values are the mean ± SD of three dishes. The experiment was repeated 3 times with a similar pattern of results.

Fig. 2. Inhibition of HGF-induced DNA synthesis by orotic acid (OA) in hepatocytes in primary culture. Experimental conditions were identical to those given in the legend to Fig. 1. The basal medium (BM) used in these experiments was different from that used in the experiments in Fig. 1, and its composition was described earlier (23, 24). Values are the mean ± SD of three dishes. The experiment was repeated 2 or 3 times.
receptors (28, 29). Even though we do not know the nature of the plasma growth factors induced by liver-cell-proliferative stimuli, the in vivo response of the liver to 2/3 PH and different liver mitogens, in terms of induced expression of c-fos and c-myc, is different (30, 31).

The next series of experiments was designed to determine whether orotic acid inhibits DNA synthesis by interfering with the growth factor-mediated early events. The protocol in these experiments was to prime the hepatocytes with a growth factor for 24 h, wash the cells free of the growth factor, and study the effect of orotic acid. The rationale was that, if orotic acid still inhibits DNA synthesis, then the target site may not be growth factor-mediated early events.

The results indicated that orotic acid can inhibit TGF-α-induced DNA synthesis in hepatocytes irrespective of whether orotic acid is added at the beginning of the experiment (i.e., together with the growth factor) (Fig. 1) or 24 h after the hepatocytes are primed with the growth factor (Fig. 3). Similar patterns of results were obtained when EGF (21) and acidic FGF (data not shown) were used instead of TGF-α.

Taken together, these results suggest that one of the target sites for the inhibition by orotic acid of DNA synthesis in hepatocytes is at some point beyond growth factor receptor- and growth factor-mediated early events. We, therefore, hypothesized that the target site could be some step related to the cell cycle.

Earlier work suggested that for orotic acid to induce its biological effects it needs to create an imbalance in nucleotide pools, characterized by an increase in uridine nucleotides and a decrease in adenosine nucleotides (5–8). Consistent with this postulation are the observations that adenine, which inhibits the metabolism of orotic acid by competing for PRPP, also inhibits the efficacy of orotic acid to induce fatty liver (5–8), liver-tumor promotion (16), and inhibition of EGF-induced DNA synthesis (21). This argument favors the suggestion that the target sites for mitoinhibitory effects mediated by orotic acid could be enzymes whose expression and/or activity are regulated by the levels of nucleotide pools. It is tempting to speculate that one of the target sites for orotic acid to induce its mitoinhibition could be RNR. RNR is a key rate-limiting enzyme in DNA synthesis, and its activity is regulated by nucleotides. ATP is one of the positive effector molecules for RNR (1, 2). In rat liver exposed to orotic acid, there is not only a decrease in the levels of ATP but also an increase in uridine nucleotides (5–8).

In a preliminary study, we observed that orotic acid inhibited EGF-induced expression of RNR in hepatocytes and that such an inhibition was counteracted by adenine (32). It is very likely that an imbalance in nucleotide pools inhibits DNA synthesis by regulating the expression of the gene for RNR and perhaps other genes sensitive to the levels of nucleotides and deoxynucleotides.

One of the significant features of the orotic acid model of tumor promotion is that orotic acid, being a normal cellular constituent and a precursor of pyrimidine nucleotides, may have the potential to be a multiorgan tumor promoter. Interestingly, orotic acid appears to promote azoxymethane-induced carcinogenesis in rat duodenum (33) and dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats (34).

Another interesting feature of the orotic acid model of tumor promotion is that there are metabolic and genetic disorders that are associated with increased levels of orotic acid. These disorders may pose increased risk of tumor promotion. For example, feeding a diet deficient in arginine, a urea cycle amino acid, results in increased levels of orotic acid (35–37) and an imbalance in hepatic nucleotide pools similar to that caused by feeding orotic acid (37). In addition, an arginine-deficient diet also promotes liver carcinogenesis in both rats and mice (37).

Certain urea cycle disorders, such as deficiency in OTC (38, 39), arginosuccinate synthetase (40), arginosuccinate lyase (41), or arginase (42) or a decreased transport of ornithine to mitochondria (43), are associated with increased levels of orotic acid. If the excess orotic acid is utilized for the synthesis of uridine nucleotides, this could result in an imbalance in nucleotide pools similar to that found in rat liver after administration of orotic acid. Interestingly, livers of OTC-deficient patients exhibited a higher ratio of uridine to adenosine nucleotides, compared with that in the non-OTC-deficient patients (44).

Although no extensive epidemiological data are available about these disorders and cancer incidence, there have been a few reports about hepatocellular carcinoma in patients with orotic aciduria (45, 46).

Orotic aciduria is also seen under conditions of hepatic insufficiency resulting from partial hepatectomy (47), CCl4 toxicity (47), portacaval shunt (48), hepatic cirrhosis, and alcoholic hepatitis in rats (49) and humans (47). Interestingly, these altered conditions are associated with an increased risk of liver-cancer development. However, implicating orotic acid as the risk factor under these conditions needs further study.

There are also genetic and metabolic disorders that result in increased levels of uridine nucleotides without a concomitant increase in orotic acid. Folic acid deficiency results in up to a 10-fold increase in dUMP because of inadequate levels of folic acid needed in the conversion of dUMP to TMP (50). Interestingly, folic acid deficiency is known to enhance cell transformation (50). In addition, Lesch-Nyhan syndrome is attributed to a deficiency of hypoxanthine guanine phosphoribosyl transferase, the enzyme that catalyzes the conversion of hypoxanthine and guanine to inosinic acid and guanylic acid in the presence of PRPP. Deficiency of this enzyme makes the PRPP available for orotic acid to form orotidylic acid and results in a 6-fold increase in uridine nucleotides (51). One wonders whether such an imbalance in nucleotide pools has any role in the pathogenesis of this disease process.

In view of the important roles nucleotides play in various cellular functions, an imbalance in their levels not only may
disturb the homeostasis of the cell but also may play an important role in the pathogenesis of several disease processes. It is very likely that imbalances in different nucleotides may have different pathogenic effects. Perhaps of greater significance is the prospect that, by correcting the imbalance, one may interrupt or slow down the pathogenesis of the process.

Note Added in Proof


References


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