Cell Proliferation Induced by Triiodothyronine in Rat Liver Is Associated with Nodule Regression and Reduction of Hepatocellular Carcinomas

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ABSTRACT

Previous studies have demonstrated that short-term treatment with peroxisome proliferators decreased the size and number of γ-glutamyl transpeptidase or placental glutathione S-transferase (GSTP)-positive hepatic hyperplastic lesions. In this study, we have examined the effect of the hormone triiodothyronine (T3), which, similarly to peroxisome proliferators, is a strong liver mitogen and a ligand of nuclear receptors, on the growth of GSTP-positive nodules generated by the resistant hepatocyte model and on the development of hepatocellular carcinoma. Hepatic hyperplastic nodules were induced in male Fischer rats by a single dose (150 mg/kg) of diethylnitrosamine, followed by a 2-week exposure of the animals to 2-acetylaminoflourene and partial hepatectomy. Nine weeks after diethylnitrosamine administration, rats were switched to a diet containing 4 mg/kg T3 for 1 week (experiment 1) and sacrificed during T3 feeding or were exposed to seven cycles of T3-supplemented diet (1 week/month per 7 months), and sacrificed 6 months after the last cycle (experiment 2). Results showed that T3 treatment for 1 week caused a 70% reduction in the number of GSTP-positive nodules (14/cm² in T3-fed rats versus 44/cm² of control animals), as well as GSTP-positive area (12% versus 43% of controls). Reduction in the number of GSTP-positive nodules observed 1 week after T3 feeding was associated with a strong increase in the labeling index of enzyme-altered nodules compared with that of controls (labeling index was 64 and 31%, respectively). No significant differences in the apoptotic index were observed between the two groups. Results from experiment 2 did reveal that although rats treated with diethylnitrosamine + 2-acetylaminoflourene developed 100% hepatocellular carcinoma and 33% of them showed lung metastasis, only 50% of rats exposed to repeated cycles of triiodothyronine developed hepatocellular carcinoma with no lung metastasis. This study indicates that cell proliferation per se might not necessarily represent a promoting condition for putative preneoplastic lesions and demonstrates an anticarcinogenic effect of T3.

INTRODUCTION

Liver cell proliferation is considered to play an important role in the several steps of carcinogenic process, initiation, promotion, and progression (1). Although the exact mechanism by which cell proliferation plays a role in initiation is not known, its involvement in events such as fixation of a miscoding lesion in the newly made DNA has been entertained (2–5). A second site at which cell proliferation exerts a critical effect is the promotion of carcinogen-initiated cells. Increased incidence of preneoplastic lesions and tumors has been observed when carcinogen treatment was followed by compensatory regeneration induced by repeated PH or multiple treatment with necrogenic agents (6–8). On the contrary, the effect of agents that cause liver hyperplasia without previous cell loss/death (primary mitogens) on the growth of carcinogen-induced preneoplastic hepatoctyes is far less clear. Indeed, although long-term treatment with PPs3 gives rise to HCC (9–11), a short-term treatment with PPs results in a decreased number and size of preneoplastic lesions induced by genotoxic carcinogens and identified by either adenosine triphosphate, γ-glutamyl transpeptidase, or GST staining (12–14). The latter effect has been attributed to the inhibitory action of these agents on the marker enzyme used to identify preneoplastic lesion (14) and not to a real growth-inhibitory effect. However, Chen et al. (15) have shown that a short-term exposure to the PP ciprofibrate reduced from 40% to <5% the L.I. of preneoplastic nodules generated by the R-H protocol (16), and this effect was associated with a rapid decrease in area and number of the nodules. This raises the important issue as to whether induction of liver cell proliferation is always a promoting condition for preneoplastic lesions or whether the nature of the proliferative stimulus is important in determining the type of response of carcinogen altered cells.

The hormone T3 resembles PPs in that: (a) it is a ligand of a nuclear receptor (TRs) of the same superfamily of steroid hormone nuclear receptors (17); (b) it is an inducer of peroxisome proliferation (18); and (c) it is a potent hepatomitogen (19, 20). However, the effect of T3 on the hepatocarcinogenic process is not known. Recently, evidence was obtained in our laboratory that a short exposure to a mitogenic dose of T3 exerts an inhibitory effect on the number of DENA-induced GSTP-positive hepatocytes.4 To further investigate the effect of hepatocyte proliferation induced by primary mitogens on the growth of carcinogen-induced putative preneoplastic lesions, we have examined the effect of T3 on the progression of hepatic nodules, induced in rats by the R-H model, to HCCs.

The results demonstrate that T3 administration for 1 week, despite its powerful mitogenic capacity, resulted in a 70% reduction of the number of GSTP-positive lesions with no increase in the size of the remaining nodules. In addition, repeated exposures of nodule-bearing rats to T3 caused a 50% reduction in the incidence of HCCs and 100% inhibition of lung metastasis. Our data add further support to the notion that the growth response of preneoplastic lesions is dependent upon the nature of the proliferative stimulus and indicate that T3 possesses anticarcinogenic effect in the liver.

MATERIALS AND METHODS

Animals. Male Fischer F-344 (100–125 g) purchased from Charles River (Milano, Italy) were maintained on a laboratory diet (Ditta Mucedola, Milano, Italy). The animals were given food and water ad libitum with a 12-h light/dark daily cycle and were acclimatized for 1 week before the start of the experiment.

Guidelines for the Care and Use of Laboratory Animals were followed during the investigation. DENA, T3, and 2-AAF were purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental Protocol I. Rats were injected i.p. with a single dose of diethylnitrosamine (DENA), dissolved in saline, at the dose of 150 mg/Kg body weight (Fig. 1). After a 2-week recovery period, rats were placed on a

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3 The abbreviations used are: PP, peroxisome proliferator; T3, 3,5,3'-triiodothyronine; GSTP, glutathione S-transferase; DENA, diethylnitrosamine; 2-AAF, 2-acetylaminofluorene; R-H, resistant hepatocyte; BrdUrd, bromodeoxyuridine; L.I., labeling index; A.I., apoptotic index; PH, partial hepatectomy; HCC, hepatocellular carcinoma.

4 Unpublished data.

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with 2 N HCl for 1 h and incubated with trypsin 0.1% for 20 min and then with normal goat serum for 20 min at room temperature. The sections were then incubated for 2 h with an anti-BrdUrd monoclonal antibody (1:200) and for 30 min with peroxidase goat antirabbit IgG. The sites of peroxidase binding were detected with diaminobenzidine. A segment of duodenum, an organ with a high rate of cell proliferation, was included from each rat to confirm delivery of BrdUrd into hepatocytes. The sections were then incubated with 2 N HCl for 1 h and incubated with trypsin 0.1% for 20 min and then with normal goat serum for 20 min at room temperature. The sections were then incubated for 2 h with an anti-BrdUrd monoclonal antibody (1:200) and for 30 min with peroxidase goat antirabbit IgG. The sites of peroxidase binding were detected with diaminobenzidine. A segment of duodenum, an organ with a high rate of cell proliferation, was included from each rat to confirm delivery of the DNA precursor. The location of GSTP in the liver was determined by using an antirat GSTP polyclonal antibody (Biotrin, Dublin, Ireland) and Dako Envision alkaline phosphatase goat antirabbit (K4018; Dako Corp.). The sites of phosphatase binding were detected with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate system (K598, Dako Corp.).

**Measurement of GSTP-positive Nodules.** GSTP-positive foci were measured with a computer-assisted image processor, programmed for the threedimensional calculation by Campbell et al. (21). Only foci >76 μm in diameter were measured.

**Determination of Labeling Index.** Random microscopic fields were scored for BrdUrd-positive hepatocytes within GSTP-positive lesions. The L.I. was calculated as BrdUrd-positive hepatocyte nuclei/100 hepatocyte nuclei. At least 2500 hepatocytes/rat were scored. The same procedure was used to obtain the L.I. for surrounding GSTP-negative hepatocytes.

**Determination of Apoptotic Index.** The incidence of apoptotic bodies within randomly selected nodules was determined in H&E-stained sections by scoring at least 3500 hepatocytes/rat. Only apoptotic bodies containing nuclear fragments were recorded. The A.I. was calculated as number of apoptotic bodies/100 hepatocytes.

**Classification of Liver Tumors.** Histological typing of liver tumors was performed according to the classification proposed in “Histological Typing of Liver Tumors of the Rat” (22).

**Statistical Analysis.** Comparison between treated and control groups was performed by Student’s f test.

**RESULTS**

Previous studies have described a mitogenic activity of a single dose of T3 in rat liver (19). Preliminary experiments performed with various T3 concentrations revealed that feeding a diet supplemented with 4 mg/kg of T3 for 1 week induced a 10-fold increase in synthetic activity of hepatic DNA (data not shown). Subsequent immunohistochemical studies showed that hepatocyte L.I., following 1-week exposure to this concentration of T3, was approximately 31% versus 4% of controls (Fig. 3). No evidence of liver cell damage could be observed throughout the experimental period. The concentration of 4 mg/kg of T3 was selected for all future experiments.

**Effect of T3 on the Number and Size of GSTP-positive Nodules.** In agreement with previous data (23), livers from rats exposed to DENA + AAF + PH and sacrificed 6 weeks after release of 2-AAF
showed the presence of several macroscopically evident, white nodules merging from the surface. Immunohistochemically, two types of nodules could be easily identified: those characterized by a uniform GSTP staining and therefore classified as persistent nodules; and those showing a progressive loss of GSTP staining (remodeling nodules, 22 per cm²). The total number of GSTP-positive nodules was 41 per cm² (Table 1). Feeding T3 for 1 week caused a dramatic change in the macroscopic appearance of the liver. Indeed, most of T3-fed rats exhibited a liver characterized by a smooth surface, with only a few protruding nodules. Accordingly, histological observation of liver sections stained with H&E did reveal the presence of very few nodules in T3-fed rats, in contrast with the large number found in DENA + 2-AAF + PH group. Quantification of the number of GSTP-positive nodules in T3-treated rats showed a 3-fold reduction in their number (from 41 to 14; Table 1). Reduction of the number of GSTP-positive nodules was accompanied by a decrease in the percentage of area occupied by GSTP-positive hepatocytes (Fig. 4 and Table 1). Interestingly, the reduction in the number of GSTP-positive nodules by T3 was associated with an increased proliferative activity of hepatocytes both in the residual GSTP-positive nodules (L.I., 64% versus 42% of controls) as well as in surrounding liver (L.I., 31% versus 7% of controls). Size distribution of the nodules in the two groups (Fig. 5) showed that no selective growth of some subpopulations of putative preneoplastic nodules had occurred in T3-treated rats.

Effect of T3 on L.I. of GSTP-positive Nodules. The above results clearly show that T3 feeding for 1 week, despite its mitogenic potency, exerted an inhibitory effect on the number of GSTP-positive nodules. To determine whether the disappearance of the vast majority of the nodules observed in T3-fed rats could have occurred as a consequence of an initial inhibition of hepatocyte proliferation within the nodules, rats treated as described in experimental protocol 1 were given BrdUrd in drinking water (24) and sacrificed 2, 4, and 7 days after starting of T3 diet. As shown in Table 2, although T3 feeding for 1 week exerted a strong inhibitory effect on the number of GSTP-positive nodules, confirming the results presented in Table 1, treatment with T3 for only 2 or 4 days did not cause any significant change in number and/or size of GSTP-positive nodules. L.I. of hepatocytes was determined by analyzing the number of BrdUrd-positive hepatocyte nuclei within the GSTP-positive nodules, using a double immunohistochemical technique. As shown in Fig. 6 and Table 3, feeding of T3 for 2 days caused a striking increase in L.I. of GSTP-positive nodules compared with that of nodules from rats maintained on a basal diet (51 and 12%, respectively); the range of L.I. was 47–57% in nodules from T3-treated rats versus 10–24% of controls. An increased L.I. in T3-liver nodules was also observed at 4 days (55% versus 21% of controls). As mentioned above, despite the increased L.I., no difference in the percentage of GSTP-positive area, either in the mean area of GSTP-positive nodules between T3-fed rats or the control group, was observed at 2 and 4 days. Moreover, when distribution analysis of nodule size was performed, no indication of a shift of T3 nodules to higher classes was observed (Fig. 7).

Effect of T3 on A.I. of GSTP-positive Nodules. On the basis of the findings that liver nodules generated by several promoting proto-

![Fig. 4. Photomicrographs of liver sections from rats treated with DENA + 2-AAF (A) and DENA + 2-AAF + T3 (B), as described in experimental protocol 1. Sections were stained for GSTP and counterstained with hematoxylin. ×15. GSTP nodules undergoing remodeling (arrowheads) are present together with GSTP-persistent nodules.](image-url)
Rats were sacrificed 2, 4, and 7 days after starting the T3 diet. DENA 1 animals/group. Total number of persistent nodules scored was 235 in rats treated with 1 although three of the five tumors seen in the DENA 1 all tumors in this group were classical trabecular HCCs (Fig. 8 8A), not shown). All rats exposed to the complete R-H protocol in untreated rats or in animals treated with DENA alone (data months after initiation. Macroscopically, no tumors could be observed in these animals. No livers weighing up to 50 g were observed except for one in T3-exposed animals, a single clearly defined tumoral mass was observed. In contrast to the T3-unexposed group, a single clearly defined tumoral mass was observed in these animals. No livers weighing >50 g were observed in tumor-bearing animals treated with T3. Histological observation confirmed the presence of tumors in all rats exposed to DENA + AA + PH in the absence of T3 (Table 4) and revealed that all tumors in this group were classical trabecular HCCs (Fig. 8A), although three of the five tumors seen in the DENA + AAF + PH + T3 group were adenocarcinomas with frequent cystic or papillary cystic patterns (Fig. 8, C and D). Moreover, in the DENA + AAF + PH group, metastases to the lung were found in 5 of 15 animals (33%; see Table 4 and Fig. 8B); no metastases were found in T3-exposed animals.

**DISCUSSION**

Cell proliferation can influence carcinogenesis by a number of mechanisms (29). This has led to the hypothesis (30, 31), although controversial (32–35), that cell proliferation per se may be carcinogenic and carcinogens that increase cell proliferation may be operating exclusively by this mechanism. As far as the liver is concerned, one of the best examples of nongenotoxic carcinogens is represented by the class of PPs. These compounds, indeed, despite their lack of measurable mutagenic activity, are very potent in their capacity to induce HCC in rodents (9–11). However, it is also evident from the literature that in response to most PPs, the liver becomes refractory to the mitogenic activity in a short time period (36–39). Therefore, it is difficult to causally relate a very late event such as cancer occurrence with the transient proliferative response evoked by these agents in the target organ. Moreover, it has been shown that hepatocyte proliferation induced by single or repeated treatment with hyperplastic agents, unlike liver regeneration after two-thirds PH or necrogenic compounds, does not support initiation of chemical hepatocarcinogenesis (35, 40). Furthermore, short-term treatment with PPs often results in a decrease in the number and size of preneoplastic lesions identified through phenotypic markers such as γ-glutamyl transpeptidase or GSTP (12–15).

To know more about the relationship between mitogenesis and preneoplastic growth, in the present study, we have examined the effect of a single or repeated short-term treatment with the hormone T3 on the growth and progression of GSTP-positive nodules generated by the R-H protocol. Two findings were the most significant in this study: (a) a short-term treatment (1 week) with T3 was able to dramatically reduce the number of GSTP-positive nodules; and (b) repeated cycles of T3 to nodule-bearing rats caused a 50% decrease in HCC development and a complete inhibition of lung metastasis.

Loss of GSTP-positive nodules occurred despite a striking increase in hepatocyte proliferation, both within the nodules as well as surrounding liver. Therefore, the mechanism responsible for the disappearance of the nodules by T3 appears to be different from that proposed for ciprofibrate by Chen et al. (15), who observed an inhibitory effect by this agent on nodule growth.

Another factor influencing growth of preneoplastic lesions is the rate of cell death. Indeed, promoting agents, such as phenobarbital (25) and dioxin (41), are thought to act, at least in part, by inhibiting apoptosis; on the other hand, dietary restriction and 3-adenosyl-
methionine, potent inhibitors of carcinogenesis, appear to exert their anticarcinogenic effect by inducing an increased apoptotic incidence in preneoplastic lesions (42–44). Our results, however, did not sup-
port a significant role for apoptosis in the regression of GSTP-positive nodules induced by T3. Indeed, although it is conceivable that the higher A.I. observed 2 days after T3 might in part explain the lack of increase in nodule size at that particular time point (compared with controls), no difference in A.I. between T3-fed and control rats was observed at later time points.

Although, at the present, it is difficult to draw any conclusion, at least two possibilities can be envisaged to explain the dramatic reduction in the number of nodules observed in T3-fed rats, despite the concomitant increase in hepatocyte proliferation: (a) the loss of GSTP-positive foci is attributable to inhibition of GSTP expression by T3, similarly to what was proposed for PPs (14), and not a to a real

Table 3 Effect of T3 on L.I. and A.I. of GSTP-positive nodules after T3 administration

Five weeks after 2-AAF release, rats were fed T3 for 1 week and given BrdUrd (1 mg/ml) in drinking water. Rats were sacrificed 2, 4, and 7 days after starting the T3 diet. For determination of L.I. and A.I., at least 2500 and 3500 nodule hepatocytes/rat, respectively, were counted.

<table>
<thead>
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<th>Group</th>
<th>L.I. %</th>
<th>A.I. %</th>
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<tr>
<td>DENA + AAF + PH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days</td>
<td>12 ± 4a</td>
<td>2.38 ± 0.28</td>
</tr>
<tr>
<td>4 days</td>
<td>21 ± 2</td>
<td>2.04 ± 0.19</td>
</tr>
<tr>
<td>7 days</td>
<td>31 ± 5</td>
<td>3.18 ± 0.85</td>
</tr>
<tr>
<td>DENA + AAF + PH + T3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days</td>
<td>51 ± 5b</td>
<td>3.30 ± 0.30</td>
</tr>
<tr>
<td>4 days</td>
<td>55 ± 3b</td>
<td>2.05 ± 0.22</td>
</tr>
<tr>
<td>7 days</td>
<td>68 ± 4b</td>
<td>1.48 ± 0.29</td>
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a Mean ± SE of four animals/group.
b Significantly different from DENA + AAF + PH for P < 0.001.

methionine, potent inhibitors of carcinogenesis, appear to exert their anticarcinogenic effect by inducing an increased apoptotic incidence in preneoplastic lesions (42–44). Our results, however, did not support a significant role for apoptosis in the regression of GSTP-positive nodules induced by T3. Indeed, although it is conceivable that the higher A.I. observed 2 days after T3 might in part explain the lack of increase in nodule size at that particular time point (compared with controls), no difference in A.I. between T3-fed and control rats was observed at later time points.

Although, at the present, it is difficult to draw any conclusion, at least two possibilities can be envisaged to explain the dramatic re-

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>HCC %</th>
<th>Lung metastasis %</th>
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<tbody>
<tr>
<td>DENA + AAF + PH</td>
<td>15</td>
<td>15</td>
<td>100</td>
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<tr>
<td>DENA + AAF + PH + T3</td>
<td>10</td>
<td>5</td>
<td>50</td>
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reduction in nodule number; (b) T3, in addition to its mitogenic capacity, might also interfere with the differentiation program by inducing retrodifferentiation of the nodular hepatocytes to normal-appearing cells via loss of their “resistant” phenotype, a rearrangement to single-cell plates, and an integration into the organizational pattern of the surrounding liver (23). The former possibility (direct inhibition by T3 on GSTP gene expression) appears very unlikely, in virtue of the following: (a) reduction in nodules number was confirmed by analysis of sections stained with H&E; (b) macroscopic observation revealed that livers from T3-fed rats were characterized by a smooth surface, with only a few protruding nodules; and (c) short-term T3 administration did not exert any inhibitory effect on GSTP mRNA or protein levels induced in rat liver by the known GSTP-inducer lead nitrate.

Thus, remodeling (or regression) induced through a redifferentiation program appears to be the most likely explanation for the loss of hepatic nodules caused by T3. T3 is known to possess a nuclear receptor (TRs) of the same superfamily of receptors of PPs (peroxisome proliferator activated receptors), and retinoic acids (retinoic acid receptors and retinoid X receptors), and these receptors have been shown to exert a profound effect on cellular differentiation (45–47).

The observation that the histological appearance of HCCs seen in rats treated with T3 was different from that of rats without T3 also suggests that this hormone, via interaction with and activation of its receptor, may have effects on differentiation of putative preneoplastic hepatocytes.

A second and even more important result achieved by this study was the reduction of HCC development and inhibition of metastases to the lung in rats exposed to repeated cycles of T3. To our knowledge, it has been extremely difficult thus far to reduce the progression of hepatic nodules generated by genotoxic agents to HCC. The fact that the anticarcinogenic effect played by T3 is associated with several cycles of hepatocyte proliferation further supports the notion that cell proliferation per se may not necessarily represent a carcinogenic and/or promoting condition. On the other hand, our data suggest that certain proliferative stimuli may play an anticarcinogenic effect, probably by causing a redifferentiation of preneoplastic cells.

REFERENCES


Fig. 8. Micrographs of sections from rats treated with DENA + 2-AAF (A and B) or DENA + 2-AAF + T3 (C and D) treated according to experimental protocol 2 and sacrificed 16 months after DENA administration. A, neoplastic cells are arranged in the liver in a broad trabecular pattern with slightly dilated sinusoids; H&E, ×100. B, histology of a metastatic tumor in the lung from a rat treated with DENA + 2-AAF. The tumor illustrates trabecular patterns of HCC; H&E, ×100. C, histological appearance of a hepatic tumor from a rat subjected to the treatment of DENA + 2-AAF + T3. The tumor shows distinct adenomatous patterns with dilated lumen and frequent papillary projections; H&E, ×40. D, histological appearance of a hepatic tumor from a rat subjected to the same treatment as the rat shown in C. The tumor shows irregularly arranged acinar and tubular patterns; H&E, ×100.


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