# Promoting Effect of *o*-Aminoazotoluene on Hepatocarcinogenesis is Accompanied by the Increase in Inflammatory and Proliferative Processes in Liver Tissue and Decrease in the Concentration of Free Thyroxin in the Blood

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*o*-Aminoazotoluene in noncarcinogenic doses promoted the development of liver tumors in female ICR mice induced by administration of diethylnitrosamine during early ontogeny. Severe inflammatory infiltration and proliferation of oval cells were found in liver tissue of these animals. The concentration of free thyroxin decreased in the blood. Our results supplement published data that promoters of hepatocarcinogenesis inhibit thyroid function.

**Key Words:** *hepatocarcinogenesis; diethylnitrosamine; o-aminoazotoluene; promotion; thyroid hormones*

Carcinogenesis is a complex multistep process, which proceeds in 3 stages: initiation (heritable change in the cell genetic program), promotion (primary division of initiated cells), and progress (progressive increase in malignancy and instability of the genome). Stage 2 is reversible and, therefore, serves as a target for the treatments that reduce the risk for tumor development. There are a lot of factors, which can induce the inhibitory or stimulatory effect on tumor development at this stage (starvation, diets, plant and chemical preparations, *etc*.) [6,9,13]. The agents stimulating tumor development usually cause oxidative stress, inflammation, activation of cell proliferation, and inhibition of apoptosis in target tissues [7,8,13]. However, direct mitogen-induced proliferation of hepatocytes does not affect the number and size of pre-tumor structures [3]. Moreover, administration of thyroid hormones suppresses hepatocarcinogenesis (despite the increase in proliferative processes) [10]. The promoting effect of phenobarbital on hepatocarcinogenesis in mice is partly mediated by a decrease in the concentration of thyroid hormones in the blood [1,2]. We hypothesized that the decrease in the concentration of these hormones can result from treatment with another promoter of hepatocarcinogenesis, *o*-aminoazotoluene (OAT). The increase in inflammation and proliferation of oval cells after administration of OAT probably serves as a factor for the promoting effect of this agent.

The severity of proliferative and inflammatory processes in liver tissue and concentration of thy-

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roid hormones in the blood of mice were studied after the induction of hepatocarcinogenesis by single administration of diethylnitrosamine (DENA) during early ontogeny and promotion of tumor development with OAT.

## MATERIALS AND METHODS

Previous studies showed that after administration of DENA to suckling mouse pups, the stage of initiation of hepatocarcinogenesis in females proceeds as strongly as in males. However, tumor development in female animals was suppressed due to deficiency of promoting factors [1]. Hence, the promoting effect of OAT was studied on female mice. Our study was conducted in accordance to the Directive of the Council of the European Economic Society (86/609/EEC). Parent ICR mice were maintained under natural light/dark conditions and had free access to PK 120-1 mixed feed (Laboratorsnab) and water (3 females and 1 male per cage). Female pups (12-14 days of life) received intraperitoneal injection of 0.5% aqueous solution of DENA (Sigma) in a single dose of 0.01 mg/g. The animals were separated from mothers on day 30 of life. Fifty percent of 2-month-old animals received intraperitoneal injections of OAT (ICN Biomedicals Inc; 0.1 ml 22.5% oil solution) 1 time per 2 weeks for 20 weeks. Each animal received 10 injections of OAT. The remaining mice served as the control. The mice receiving the same dose of OAT in the same period of life served as positive control. The mice were decapitated at the age of 10 months. The blood was sampled. Body weight and weight of the liver were measured. Free thyroxin concentration in blood plasma was measured by the radioimmune method using FT4 reagents (Immunotech). The total number and size of tumors and pre-tumor nodules in the liver were estimated under a binocular magnifier with an ocular micrometer (magnification ×8). For morphological study, tissue samples were fixed with 10% formalin and embedded into paraffin. Sections were stained with hematoxylin and eosin. The relative volume of inflammatory infiltrates and oval cells was measured by the point method (magnification ×20). We used a grid (8×8 $mm^2$) consisting of 256 points. Sections were photographed under an Axioscop 2 plus microscope (Karl Zeiss) using AxioVision software.

The significance of intergroup differences was estimated by Student's *t* test.

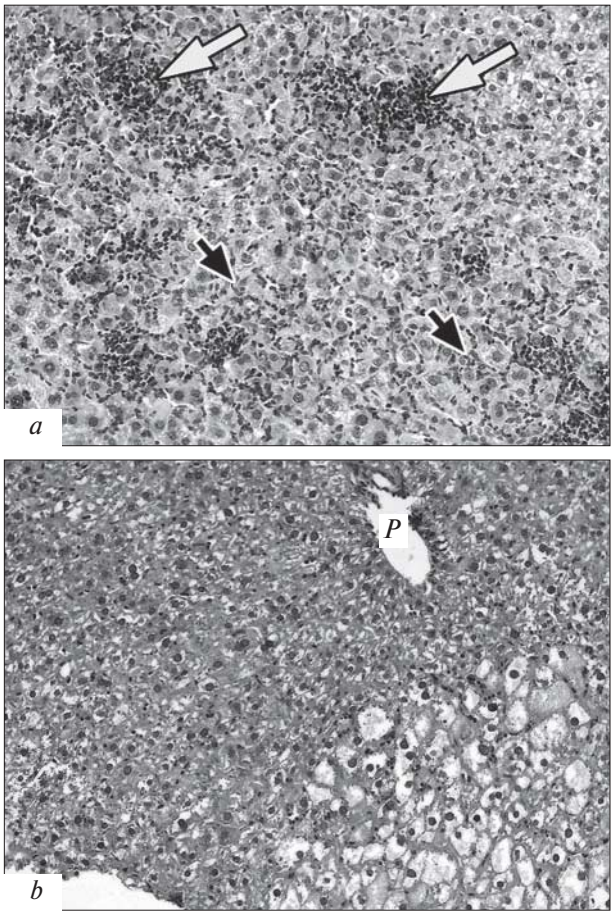
## RESULTS

Course treatment with OAT in the specified dose had no effect on the general physiological state of animals. This conclusion was derived from the absence of differences in body weight of OAT-treated and control mice (Table 1). However, the weight of the liver in OAT-treated mice was 20-30% higher compared to the control. The liver of treated mice was more dense and light and had uneven surface. The mean number of nodules on the liver surface in each animal was 0.5. The diameter of nodules was less than 2 mm. However, the tumor nature of these nodules was not verified histologically. Morphological study of liver sections from these animals revealed hydropic and fatty degeneration of hepatocytes, bridging necrosis of hepatocytes, massive proliferation of oval cells, and local and diffuse lymphocytic infiltration. These changes reflect the development of toxic liver damage with OAT and progression of inflammatory and regenerative processes (Table 1, Fig. 1, *a*). Severe toxic damage

**TABLE 1.** Effect of Chemical Hepatocarcinogens on Physiological and Biochemical Parameters in Female ICR Mice

| Parameter | Group | | |
|---|---|---|---|
| | DENA | OAT | DENA+OAT |
| Number of animals | 13 | 8 | 17 |
| Body weight, g | 36.4±1.0 | 36.3±0.3 | 36.9±1.22 |
| Relative weight of the liver, % | 5.8±0.6 | 6.8±0.2 | 7.7±0.3** |
| Number of tumor and pre-tumor nodules (>2 mm) in the liver per 1 animal | 2.0±1.1 | 0* | 20.1±5.2** |
| Relative volume of inflammatory infiltrates in liver tissue, % | 1.40±0.33 | 7.80±10.55** | 3.70±0.45* |
| Relative volume of oval cells in liver tissue, % | 0.60±0.16 | 5.6±2.0* | 2.9±0.4** |
| Concentration of free thyroxin in blood plasma, pmol/liter | 25.7±1.9 | 19.2±2.0* | 17.1±2.4** |

**Note.** *$p<0.05$ and **$p<0.01$ compared to DENA-treated animals.



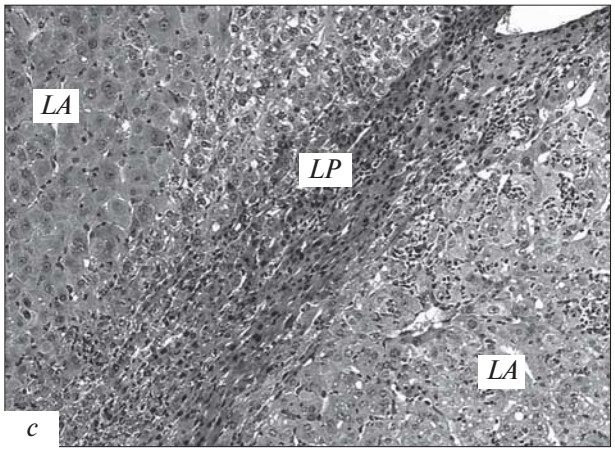




**Fig. 1.** Morphological signs of liver tissue in female ICR mice after administration of hepatocarcinogens. (*a*) Mouse liver after chronic administration of OAT. Light arrow, massive lymphocytic infiltration; dark arrow, proliferation of oval cells. (*b*) Absence of lymphocytic infiltration in mice after single administration of DENA during early ontogeny. Lower right corner: focus of light hepatocytes. P, portal tract. (*c*) Large cell and small cell liver adenoma (LA) after combined administration of DENA and OAT. LP, regions of the liver parenchyma. Staining with hematoxylin and eosin. ×20.

to the liver and inflammatory infiltration of liver tissue were not revealed in DENA-treated mice (Table 1, Fig. 1, *b*). Tumors and pre-tumor nodules with a diameter of 1-6 mm (mean diameter 2.5±0.7 mm) were found on the liver surface of 50% animals. A tumor >1 cm was found in only 1 mouse. It was histologically verified as moderately differentiated hepatocarcinoma. Other structures appeared as hepatocellular adenomas.

The number of tumors and pre-tumor nodules in the liver of mice receiving OAT after DENA was one order of magnitude higher than in animals of the DENA group (Table 1). The size of these structures was much greater. The diameter of structures was more than 1 cm in 23.4% mice (mean diameter 6.4±0.9 mm). Liver adenomas prevailed in these animals. Vascular tumors were identified in several mice. Examination of liver sections from animals of this group also revealed hydropic and fatty degeneration of hepatocytes, strong proliferation of oval cells, and lymphocytic infiltration. As differentiated from OAT-treated mice, these changes were accompanied by the presence of numerous tumor and pre-tumor nodes and focuses of hepatocytes (Table 1, Fig. 1, *c*).

Our results indicate that the rate of formation and number of preneoplastic structures and liver tumors are much higher after single injection of DENA during early ontogeny and subsequent chronic treatment with OAT (as compared to individual administration of each compound). Therefore, OAT has a promoting effect on DENA-induced hepatocarcinogenesis.

The increase in inflammatory and proliferative processes in liver tissue after administration of OAT probably contributes to the promoting effect of this compound. At the same time, these processes can result in a decrease in the concentration of thyroid hormones in the blood. Previous studies showed that the increase in the concentration of proinflammatory cytokines suppresses functional activity of thyroid cells and decreases blood content of free thyroxin and triiodothyronine [5,11]. Free thyroxin concentration in OAT-treated animals of both groups was much lower compared to DENA-treated control animals (Table 1). However, administration of DENA to 12-14-day-old animals had no effect on free thyroxine concentration in adult mice. By the end of study, the concentration of this hormone in the blood of DENA-treated animals did not differ from that in intact mice (23.5±1.0 pmol/liter). An-

other possible mechanism for OAT-induced decrease in blood thyroxin concentration is the increase in the rates of metabolism and elimination of thyroid hormones due to activation of microsomal enzymes in the liver. This mechanism was described for the substances that promote the development of liver tumors, including phenobarbital, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (synthetic agonist of the constitutive androstane receptor) [4], and several peroxisome proliferators [8]. These compounds induce activity of microsomal enzymes in the liver (cytochromes P-450 2B) that metabolize thyroid hormones and decrease their concentration in the blood [4,12]. OAT also induces activity of cytochromes P-450 [14]. It remains unclear whether these changes accelerate metabolic conversion of thyroid hormones.

Thyroid hormones *in vivo* and *in vitro* modulate neoplastic transformation of cells. The increase in blood concentration of thyroid hormones can be followed by inhibition of liver tumor development [1,10]. The promoting effect of phenobarbital on hepatocarcinogenesis correlates with its ability to decrease the concentration of thyroid hormones [2]. The influence of hepatocarcinogenesis promoter OAT is accompanied by a decrease in the concentration of free thyroxin in the blood. Probably, the ability to decrease the concentration of thyroid hormones is a general property of various promoters of hepatocarcinogenesis.

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