TRANSPLACENTAL AND DIRECT ACTION OF ORTHOAMINOAZOTOLUENE OF ORGAN CULTURES OF EMBRYONIC MOUSE LIVER

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UDC 612.35:612.646/.014.46:
615.277.4.032.013.85/-85.23

The transplacental and direct action of orthoaminoazotoluene (OAAT) were studied in organ cultures of embryonic liver of CBA mice, with a high incidence of hepatoma. By its direct action in vitro OAAT induced a marked decrease in survival of organ cultures of embryonic mouse liver compared with the control. When OAAT acted transplacentally, its toxic effect was replaced in the last stages of cultivation by a growth-stimulating effect and the number of living experimental cultures was 30% higher than in the control.

KEY WORDS: orthoaminozotoluene; organ cultures of liver; transplacental action; carcinogenic effect.

The possibility of a transplacental carcinogenic effect on the progency of experimental animals has been demonstrated for various classes of carcinogens [9]. However, comparatively few studies have been made of the transplacental action of the hepatotropic carcinogen orthoaminoazotoluene (OAAT) [3,6,7]. In previous investigations to study transplacental carcinogenesis the writers used organ cultures of the kidneys and lungs of animal embryos subjected to transplacental carcinogenic action in utero [4,8,9]. Similar experiments were carried out on organ cultures of the liver, which were studied by E. A. Luria in relation to various aspects of their hematopoietic function [5]. The main subject of interest was whether organotypical growth of the hepatic epithelium is possible and its sensitivity to carcinogenic agents.

This paper describes the results of a comparative study of the survival of organ cultures of the embryonic liver of intact mice and cultures after exposure to the transplacental and direct action of OAAT.

EXPERIMENTAL METHOD

To study the transplacental action of OAAT it was given to the female CBA mice by mouth in 0.2 ml sunflower oil two or three times in a total dose of 24 mg, starting from the 16th day of pregnancy. The animals were killed on the 19th-20th day of pregnancy and the liver of the embryos was explanted in organ culture.

To study the direct action of OAAT it was added to the nutrient medium at the time of explantation in a concentration of 0.001 mg/ml. After 3 or 4 days, when the nutrient medium was first changed, cultivation continued without the carcinogen. In both series of experiments organ cultures of embryonic liver of intact CBA mice at the same period of embryogenesis served as the control. The method of multiple cultures developed by Luria [5] was used for cultivation in the modification adopted in the writers' laboratory [4]. The supporting substrate consisted of AUFS Millipore filters with a pore diameter of 0.6–0.9 μ, on which 10 to 15 pieces of embryonic liver, measuring 0.5-1 mm, were explanted. The filters were supported on tantalum grids, held in deep watch glasses containing the nutrient medium. The medium consisted of 80% medium No. 199 and 20% bovine serum. To 100 ml of this medium 400 mg glucose, 7 mg ascorbic acid, and 20 mg glutamine were added. Immediately before use, 10% chick embryonic extract prepared from 11-day embryos in Hanks's solution in the ratio of 1:1 was added to the medium. The watchglasses with the explants were placed in Petri dishes and incubated at 37°C in an atmosphere consisting of 95% air and 5% CO_2 with a relative humid-

Department for the Study of Carcinogenic Agents, Oncologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR L. M. Shabad.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 82, No. 9, pp. 1117-1119, September, 1976. Original article submitted February 18, 1976.

ity of 100%. Cultivation continued for 24 h. The explants were investigated at different times of the experiment in total preparations made after fixation of the cultures in 70-80° alcohol and staining with hematoxylin.

EXPERIMENTAL RESULTS

On the 2nd-3rd day of cultivation small monolayer areas of epithelium consisting of large, polygonal cells, surrounded by migrating blood cells and macrophages, appeared around most of the explants of embryonic liver from the intact mice.

By the 4th day the tissue of the explant was visibly flattened: From the stratified center, where degenerative changes were observed, it gradually changed into a monolayer epithelial membrane which, as a rule, appeared around most of the viable explants. At the periphery, inside the fragment the original structure of the embryonic liver was preserved and intensive hematopoiesis occupied extensive areas as well as small separate foci. On the undersurfaces of the filters connective-tissue cells with small, dark, irregular nuclei could be seen.

On the 5th-6th day in some cases small irregular ingrowths arising from the peripheral part of the fragment and consisting of small basophilic cells were observed in the monolayer epithelial zone of growth. These peripheral projections as a rule were separated from the surrounding epithelial cells by a single layer of connective-tissue cells (Fig. 1).

On the following days a further flattening of the explant and an increase in the severity of the degenerative changes in the center were observed, with the formation of primary central foci of necrosis and a simultaneous increases in size of the monolayer epithelial membrane around the fragments.

By the 12th day of cultivation the tissue of most explants was greatly flattened and consisted of a sheet of hepatic epithelium formed by large polygonal cells with clear boundaries (Fig. 2). The connective-tissue cells continued to grow into the pores of the filter and were arranged on its under surface as a network of small branching cells, forming a "bed" for the epithelial layer. Basophilic hepatocytes with the characteristic polygonal shape and with the clear outlines of regularly arranged cells, separated from the surrounding tissue by a single layer of connective-tissue cells, were seen at the boundary between the monolayer epithelial membrane and the tissue of the explant. In some explants the central foci of necrosis at this time were replaced by epithelial tissue. Hematopoiesis continued in the cultures.

By the 16th day the central foci of necrosis were absent in most explants. Starting from this time there was a gradual diminution of hematopoiesis and replacement of the hepatic epithelium by connective tissue.

On the 23rd-24th day extensive secondary foci of necrosis appeared in the cultures and occupied the peripheral part of the explant itself and the zone of growth around it. Hematopoiesis had ceased. However, areas of tissue formed by the small basophilic hepatocytes described above still remined viable.

During the direct action of OAAT on organ cultures of embryonic liver of intact mice, the formation of the epithelial zone of growth around the explant was inhibited. For instance, from the 7th through the 17th day of cultivation it was observed less frequently in the experimental series than in the corresponding control (Table 1). In the experimental cultures regeneration of epithelial tissue in the zone of the central foci of necrosis was delayed and degenerative changes were observed more frequently at the periphery of the explants. The intensity of hematopoiesis was less than in the control.

In liver explants from mouse embryos subjected to the transplacental action of OAAT in utero, inhibition

TABLE 1. Transplacental and Direct Action of OAAT on Organ Cultures of Embryonic Mouse Liver

Duration of cultivation, days	Control			Transplacental action of OAAT, 24 mg per mouse				Direct action of OAAT, 0.001 mg/ml			
	number of explants studied	number of living explants		number of explants studied	number of living explants		P	number of explants studied	number of living explants		P
		absolute	%		absolute	%			absolute	%	
4—6	70	29	41,4	116	19	16,4	<0,001	7	2	28,5	>0,1
8—9	53	39	73,4	40	16	40,0	<0,001	39	18	46,6	<0.001
12—13	46	29	63,0	29	20	69,0	>0,1	30	9	30,0	<0,01
15—17	112	66	58,0	115	76	69,1	<0,001	40	16	40,0	<0,05
20	20	9	45,0					21	11	52,3	>0,1
23—24	74	8	10,8	35	15	43,0	<0,001	7	2	28,5	>0,1

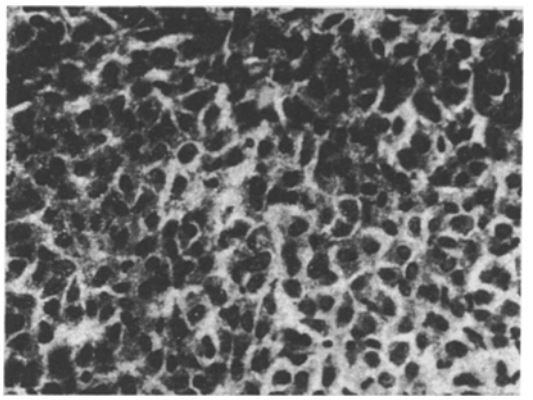

Fig. 1. Embryonic liver tissue from intact CBA mouse. Tissue of explant with monolayer epithelial zone of growth, 300 ×.

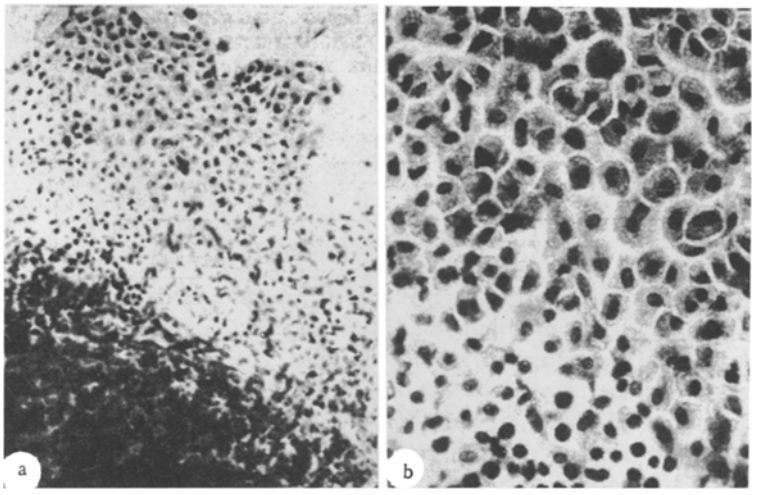

Fig. 2. Embryonic liver tissue of intact CBA mouse, 12th day of cultivation: a) tissue of explant with monolayer epithelial zone of growth (125 ×); b) zone of growth (300 ×).

of formation of the epithelial zone of growth around the fragments also was observed in the first 9 days of cultivation. Degenerative changes were found in their peripheral part more often than in the control. For instance, on the 5th-6th day there were significantly fewer viable explants in the experimental series (16.4%) than in the control (41.4%). On the 7th-9th day the number of such explants in the control increased to 73.4% but in the experimental group only to 40%. During further cultivation an increase in the survival rate was observed in the experimental cultures, but a decrease in the control. This difference was particularly clear on the 23rd-24th day of cultivation, when the number of viable experimental explants was 43% compared with only 10.8% in the control (Table 1). The intensity and duration of hematopoiesis did not differ significantly, however, from the control.

In the dose used and by its direct action in vitro, OAAT thus caused a marked decrease in the rate of survival of the organ cultures of the embryonic mouse liver compared with the intact control. When acting transplacentally, the toxic effect of OAAT in the cultures was replaced by a growth-stimulating effect, which increased with the duration of the experiment.

In previous experiments the writers also observed a marked growth-stimulating effect in cultures of embryonic lung and kidney tissues from animals exposed to the transplacental action of various carcinogens [4, 8, 9]. However, unlike in the experimental cultures of embryonic liver, the growth-stimulating action led to the development of hyperplastic pretumor proliferation and of actual tumors, namely adenomas of the lungs and cystadenomas of the kidneys. The increase in survival rate of the experimental liver cultures from mouse

embryos exposed to the transplacental action of OAAT may perhaps be the result of regeneration of the hepatic epithelium. Such phenomena are observed, for example, in liver cultures from adult animals (mice, rabbits): after preliminary administration of CCl_4 to the animals their liver survived much better in culture than did the liver of intact animals [1,2].

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EFFECT OF HEPARIN ON INCORPORATION OF THYMIDINE-^{3}H INTO A-1 CELLS IN CONTINUOUS CULTURE

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UDC 615.273.53.015.44:/612.014.3:612.6

The effect of heparin on incorporation of thymidine-^{3}H into A-1 cells in continuous culture was studied. Incubation of the cells in medium No. 199 containing heparin (1-200 units/ml) did not lead to any decrease in the percentage of labeled nuclei. The duration of the G_2-and S-periods likewise was unchanged compared with the control. However, the intensity of incorporation of thymidine-^{3}H (mean number of grains above one labeled nucleus) was significantly reduced. The results indicate that heparin affects the permeability of the plasmalemma but has no effect on DNA synthesis. The antimitotic action of heparin is probably connected with its effect on the cell surface.

KEY WORDS: heparin; autoradiography; cell cultures.

The writers previously [4] described quantitative and qualitative changes in the mitotic regime of monolayer continuous cultures of A-1 cells under the influence of heparin. Heparin reduced mitotic activity and caused a phase shift such that the relative proportion of telophases among the dividing cells rose considerably. High doses of heparin (500 units/ml) blocked the transition from telophase to the subsequent interphase and many of the cells blocked in telophase underwent pycnosis and died without completing cytotomy.

To study the extent to which the changes observed in mitosis are linked with a disturbance of DNA synthesis in cells in the S period, an autoradiographic study (using thymidine-^{3}H) was made of continuous cultures of A-1 cells incubated in the presence of heparin.

EXPERIMENTAL METHOD

A suspension of a continuous culture of A-1 cells ($1 \cdot 10^5$ cells in 1 ml medium No. 199 with 10% bovine

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(Presented by Academician of the Academy of Medical Sciences of the USSR A. P. Avtsyn.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 82, No. 9, pp. 1119-1121, September, 1976. Original article submitted March 10, 1976.