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Differences in Lipid Composition and Proliferative Activity of Rat Hepatoma-27 Depending on the Target Organ

V. A. Kobliakov[1], O. G. Somova[2], V. F. Kondalenko[1], N. M. Ostashkina[3], A. G. Kandyba[2], T. K. Dubovaya[3], and E. V. Dyatlovitskaya[2]*

[1]Institute of Carcinogenesis, Blokhin Cancer Research Center, Russian Academy of Medical Sciences, Kashirskoe Shosse 24, Moscow, 115478 Russia
[2]Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, Moscow, 117997 Russia; E-mail: dyatl@ibch.ru
[3]Russian State Medical University, ul. Ostrovityanova 1, Moscow, Russia

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Abstract—Proliferative activity and lipid composition (phospholipids and gangliosides) were studied in rat hepatoma-27 transplanted subcutaneously or intrahepatically (as models for primary and metastasizing tumors). The mitotic index of subcutaneously transplanted hepatoma far exceeded that of the intrahepatically transplanted tumor. The overall amounts of both phospholipids and gangliosides increased appreciably in the subcutaneously growing hepatoma (in contrast to the intrahepatically growing tumor) in comparison to the control hepatic tissue. The ganglioside composition in the tumors differs from that in the liver: ganglioside GD3 appears, whereas gangliosides GD1b and GT1b decrease in amount in the intrahepatic tumor compared to the control liver and disappear in the subcutaneously transplanted hepatoma. In both tumor types, the amounts of both phosphatidylethanolamine and sphingomyelin exceed the control values. Comparison of these results with previously reported data concerning the phospholipid and ganglioside composition in the regenerating rat liver indicates that the difference in the lipid composition between the subcutaneously and intrahepatically growing hepatomas-27 is due to their different proliferative status and also their microenvironment.

Key words: proliferation, liver, hepatoma, metastasis, phospholipids, gangliosides

The environment of cells plays an important role in the control of cell functions. The transplantation of tumors into various organs is one approach to *in vivo* study of the role of microenvironment. This method has revealed that various features of tumors, such as morphology, invasiveness, and some biochemical properties, depend on tumor localization. For instance, colon carcinoma transplanted to granular tissue acquires "invasive morphotype", which differs from tumor cells transplanted to the normal tissue [2]. The deoxycytidylate aminohydroxylase activity in the subcutaneously transplanted Novikoff hepatoma cells was 100 times lower than it was in the same hepatoma cells transplanted intraperitoneally [3]. The sulfhydryl group and glutathione contents in the cells of leucosis L1210 were higher after transplantation into liver than after intraperitoneal transplantation [4]. The number of vessels and the level of VEGF mRNA in human rectal adenocarcinoma S174T transplanted subcutaneously into thymus-less mice far exceeded those in the same tumor transplanted intrahepatically [5]. The cytochrome P-450 content was higher in Morris hepatomas after intrahepatic than after intramuscular transplantation [6]. Both constitutive and inducible isoforms of cytochrome P-450 were absent in rat hepatoma-27 cells transplanted intramuscularly [7]. When the same hepatoma cells were transplanted intrahepatically, cytochrome P-450-dependent activities became measurable, and phenobarbital or benz(a)pyrene induced corresponding isoforms of cytochrome P-450 and their specific activities [7].

These data show that microenvironment plays an important role in the control of various cellular functions. However, there is no information on the proliferative potential of tumors growing in ectopic or orthopic organs. Moreover, we know of no studies of the lipid composition of such tumors.

Abbreviations: PL) phospholipids; PE) phosphatidylethanolamine; PSe) phosphatidylserine; PI) phosphatidylinositol; PCh) phosphatidylcholine; SM) sphingomyelin; Sia) sialic acid; Svennerholm's nomenclature is applied to gangliosides [1].

* To whom correspondence should be addressed.

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For this reason, we studied the proliferative potential (determined from the number of mitoses) and lipid composition (phospholipids and gangliosides) of tumors using hepatoma-27 transplanted subcutaneously or intrahepatically as a model.

MATERIALS AND METHODS

The experiments were performed on non-inbred rats weighing 80-120 g. To exclude individual differences, hepatoma-27 was transplanted both subcutaneously and intrahepatically into the same animal. Small bits of tumor tissue were inserted into livers or subcutaneously with a trocar, and the animals were decapitated two weeks after transplantation. The livers of control animals were taken at the same time.

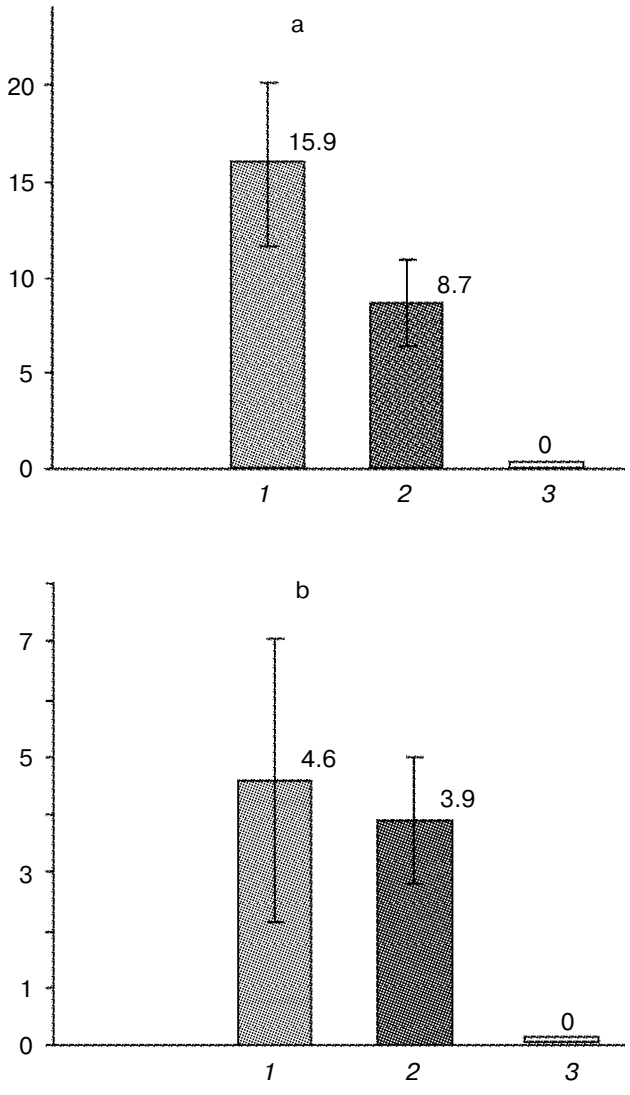
Proliferation was determined from the mitotic index count on paraffin-embedded sections stained with hematoxylin–eosin dye. Apoptotic bodies were counted on the same sections. Results were obtained from seven subcutaneously and five intrahepatically transplanted tumors. Subcutaneously and intrahepatically transplanted tumors were indistinguishable in histological texture as judged by microscopy. The extracted tissues were frozen in liquid nitrogen and stored at –70°C.

Lipids were extracted from tissues by repeated treatment with $CHCl_3$–CH_3OH mixture (2 : 1 and then 1 : 2 v/v) up to their complete extraction. Small aliquots were sampled for qualitative and quantitative analyses of phospholipids, and the main part was repeatedly washed with water by the method of Folch et al. [8]. The overall phospholipid content and relative contents of the individual phospholipids after TLC on HPTLC-glass silica gel plates (10 × 10 cm) (Merck, Germany) in the solvent system $CHCl_3$–CH_3OH–HCOOH (65 : 25 : 4) were determined as described earlier [9].

The aqueous layers from the washing of lipid extracts of each tissue type containing gangliosides and some fraction of the polar and neutral glycosphingolipids were vacuum-evaporated, and the overall amounts of lipid-bound sialic acids were determined in the residues [10]. Gangliosides were separated and identified of by TLC on the plates mentioned above using the solvent system $CHCl_3$–CH_3OH–H_2O (60 : 40 : 9 v/v) containing 0.02% $CaCl_2$ in the presence of brain gangliosides as markers. Gangliosides were cleaved with neuraminidase from *Vibrio cholerae* (Serva, Germany) for 24 h at 37°C as described previously [11]. The lytic products were analyzed on HPTLC plates in the above-mentioned solvent system for gangliosides and also on HPTLC plates impregnated with NaH_2PO_4 [12] in the system *n*-C_3H_7OH–H_2O–28% NH_4OH (6 : 2 : 1). Resorcinol [13] and anthrone [14] reagents were used for the detection of gangliosides, sialic acids, and asialic derivatives. After TLC and staining with resorcinol reagent, the relative amounts of gangliosides were determined by densitometry using CS-920 scanner (Japan) at 580 nm. Protein concentration was determined by the method of Lowry et al. [15].

RESULTS

The figure (panel (a)) shows that the mitotic activities in hepatoma cells transplanted subcutaneously and intrahepatically differ: the intrahepatic tumor contains fewer mitotic cells than the subcutaneously growing tumor. It should be noted that a negligible number of



Mitotic activity and apoptotic body count in cells of hepatoma-27 depending on the target organ: a) mitotic activity; ordinate, number of mitoses per 1000 cells; the difference between *1* and *2* is significant ($p < 0.05$); b) apoptotic body count; ordinate, number of apoptotic bodies per 100 cells; *1*) subcutaneously transplanted hepatoma; *2*) intrahepatically transplanted hepatoma; *3*) control liver.

mitoses is found in normal liver without any stimulation. Thus, the intrahepatically transplanted tumor is more similar to the liver tissue in proliferative potential than the subcutaneously transplanted tumor.

The apoptotic level was determined by the count of apoptotic bodies. As seen from the figure (panel (b)), the subcutaneously transplanted and intrahepatically growing tumors do not vary in this parameter. Apoptotic bodies were not found in the control liver.

Certain differences in phospholipid composition were noted between the rat liver and hepatoma-27 transplanted subcutaneously or intrahepatically. The difference in the total phospholipid content is noteworthy (Table 1). The phospholipid level is virtually the same in the liver and in the hepatoma-27 transplanted to the liver, but it is increased sharply in the subcutaneously growing hepatoma when compared to the control. As for the relative contents of the individual phospholipids, differences between the liver and the hepatomas exist for some of them. For instance, both hepatoma types have significantly increased level of PE and slightly increased level of SM. Yet it should be mentioned that the overall amount of PSe and PI in intrahepatic hepatoma-27 is intermediate between those in the liver and in the subcutaneous hepatoma. Regarding the main phospholipid component of cell membranes, PCh, its level in the hepatomas in comparison to the liver remains virtually unchanged.

Interestingly, these data on phospholipids are unlike our results published in 1970 [16]. In comparison with the previous data, the PCh level is increased and PSe + PI level is decreased in subcutaneously transplanted hepatoma-27. This indicates that the phospholipid composition of the tumor changed with multiple passages.

Some differences between the liver and the tumors, as well as between both types of hepatoma-27, were also disclosed in studies of gangliosides. As can be seen from Table 2, the overall amount of gangliosides (lipid-bound sialic acids) is equal in the liver and in the intrahepatically growing hepatoma, but is sharply increased in the subcutaneously transplanted hepatoma. The gangliosides GM3, GM1, GD1a, GD1b, and GT1b, which contain N-acetylneuraminic and/or N-glycolylneu-

Table 1. Phospholipid composition of rat hepatoma-27 cells transplanted subcutaneously and intrahepatically (mean value of three measurements)

Tissue	P, μmoles/mg protein	PL composition, %					
		cardiolipin	PE	PSe + PI	PCh	SM	lyso-PCh
Liver (4)	0.183	3.9 ± 0.1	22.4 ± 0.2	8.4 ± 0.4	52.8 ± 1.3	8.8 ± 0.1	3.7 ± 0.1
Intrahepatically transplanted hepatoma (9)	0.199	5.5 ± 0.5	27.1 ± 1.1	6.0 ± 0.2	49.0 ± 1.1	10.8 ± 0.1	1.6 ± 0.1
Subcutaneously transplanted hepatoma (7)	0.262	5.4 ± 0.1	28.1 ± 0.6	3.3 ± 0.1	50.6 ± 2.0	10.5 ± 0.2	2.1 ± 0.2

Note: The number of animals is given in parentheses.

Table 2. Ganglioside composition of liver and the rat hepatoma-27 cells transplanted subcutaneously and intrahepatically (mean value of three measurements)

Tissue	Sia, nmoles/mg protein	Gangliosides, %					
		GM3	GM1	GD3	GD1a	GD1b	GT1b
Liver (4)	0.8	29.4 ± 1.9	20.8 ± 0.3	—	9.7 ± 1.1	28.5 ± 1.2	11.6 ± 1.3
Intrahepatically transplanted hepatoma (9)	0.8	24.2 ± 0.2	10.3 ± 0.1	26.0 ± 0.5	29.9 ± 0.5	5.4 ± 0.1	4.2 ± 0.1
Subcutaneously transplanted hepatoma (7)	4.7	61.0 ± 3.3	7.4 ± 0.2	31.5 ± 3.1	traces	—	—

Note: The number of animals is given in parentheses.

raminic acid, were identified in the liver by TLC as the main components. (Both sialic acids were also identified among the hepatoma gangliosides.) The ganglioside GD3 that is common for many tumor types (see review [17] and the original papers reviewed there) was discovered in both hepatoma types. On the other hand, the gangliosides GD1b and GT1b are drastically decreased in amount in the intrahepatically transplanted hepatoma compared to the liver and disappear completely in the subcutaneously transplanted tumor. We suggested earlier that the ganglioside GT1b is absent in subcutaneously transplanted hepatoma-27 [18]. It is conceivable that some gangliosides of the *c*-series may be present in all of the studied tissues [19], but we were unable to identify them because we lacked the needed specific monoclonal antibodies.

DISCUSSION

Studies of the same tumor type growing in different organs are of both theoretical and practical importance. Such a model allows on one hand to elucidate what cell functions depend on the tumor microenvironment. On the other hand, the tumor growing in the organ of origin may serve as a prototype of a primary tumor, whereas the tumor proliferating in other organ may be considered as a model for the metastasis. The possibility for the creation of reliable models for primary tumor and metastasis development is important for chemotherapy.

Most studies devoted to the role of cell environment in the control of cell function have been done with cell cultures. Using cell matrixes and cultures of various cell types, it was shown that the mitotic index and various intracellular functions depend on the cell environment. For instance, hepatocytes grown on Vitrogen have higher proliferative potential than the same cells grown on Matrigel [20]. Hepatocytes grown in collagen gel showed less expression of the *c-myc* gene than the same cells grown on plastic [21]. Glycoproteins isolated from the membranes of cells in contact inhibition were able to inhibit the growth of fibroblasts, whereas glycoproteins extracted from cells lacking contact inhibition had no such ability [22]. The differentiation indexes also varied depending on the microenvironment in cell cultures. A culture of hepatocytes grown on glass or plastic for 2-3 days loses the ability to express cytochrome P-450, but when grown on Matrigel, the level of cytochrome P-450 remains close to that detected in the liver for a long time [23]. Hepatocytes grown in three-dimensional collagen gel keep their liver-specific functions for a long time [24]. The differentiation status of parenchymal liver cells determined from their testosterone-metabolizing activity was stabilized in comparison with monoculture when the cells were co-cultivated with non-parenchymal epithelial liver cell [25].

Model studies using tumor transplantation into various organs are relatively few in number. To summarize these, it can be concluded that tumor cells subjected to transplantation "perceive" the changes in their microenvironment between the initial and remote organ, as well as between different remote organs. Hepatomas used as the transplanted tumors, when growing intrahepatically, were found to be demonstrating more distinctive differentiation indexes than after transplantation into remote organ [6, 7]. All known studies have been devoted to the functioning of various enzymatic systems; the studies of the proliferative properties of tumor cells depending on the target organ have not been reported previously. We have shown in the present study that the cell microenvironment influences the proliferative status.

The data also suggest that the intrahepatically growing hepatoma-27 (a model for primary tumor) is intermediate in lipid composition between the normal rat liver and subcutaneously transplanted hepatoma (a model for metastasis). The results suggest that ganglioside biosynthesis is modified in both tumors (synthesis of the complex components of the ganglio-series is inhibited), which results from the tumor development process. However, this modification depends on the environment of the tumor. In the case of the subcutaneously transplanted hepatoma, this deterioration is more pronounced, which leads to the "simplification" of ganglioside composition up to the complete loss of some gangliosides, such as GD1b and GT1b. The drastic increase in both phospholipid and ganglioside contents in the subcutaneously transplanted (rather than in the intrahepatically transplanted) tumor compared to the normal homologous tissue has also engaged our attention and appears to be associated with the tumor growth. The increase in the relative levels of PE and SM is notable among the similar changes in the lipid compositions of both hepatomas (in comparison with the control).

It is interesting to correlate the data on the lipid composition with the differences in the proliferative status of the tumor. There was no similarity between these results and known data on the changes in ganglioside composition of regenerating rat liver during the course of proliferation (0-96 h) [26], whereas the level of lipid-bound sialic acids increases drastically in the subcutaneously transplanted hepatoma-27 possessing high proliferative status in comparison with the normal tissue (the present study) and decreases in the regenerating liver [26]. As for phospholipid composition, no significant difference was observed between the regenerating rat liver and control liver tissue [27], whereas the data of the present study shows distinctions in phospholipid contents between the subcutaneously and intrahepatically transplanted hepatomas, which have dissimilar proliferative statuses. These data indicate that the distinctions in lipid

compositions of hepatomas transplanted to diverse organs may be derived not only from different mitotic activities, but also from the different microenvironment of the tumor.

Thus, the present study shows effects of the microenvironment on growth and lipid composition of the same tumor type.

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