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Evidence for Single-Cell Origin of 3-Methylcholanthrene-induced Fibrosarcomas in Mice with Cellular Mosaicism[1]

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ABSTRACT

Fibrosarcomas produced by s.c. injection of 5 μg of 3-methylcholanthrene in *Pgk-1*^{a}/*Pgk-1*^{b} mice carrying X-chromosome inactivation mosaicism for the phosphoglycerate kinase (*PGK-1*) gene contained only one type of phosphoglycerate kinase in seven of eight cases (88%), as judged from its electrophoretic mobility, indicating the single-cell origin of the tumor.

INTRODUCTION

Fialkow (2) has presented evidence for the monoclonal origin of various human tumors in females heterozygous for the X-chromosome-linked gene for glucose-6-phosphate dehydrogenase and hence consisting of 2 types of enzymatically different somatic cells due to inactivation of one of a pair of X-chromosomes (4). On the contrary, in mice heterozygous for the X-chromosome-linked gene of the enzyme PGK[2] (5), Reddy and Fialkow (6) reported that fibrosarcomas induced with MC contained both PGK phenotypes. This result indicates that in mice the tumor originates from multiple cells. However, Reddy and Fialkow used a very high dose of MC. Moreover, it is difficult to determine the enzyme type of solid tumors from the electrophoretic pattern because the tissue is contaminated with the blood and supporting tissue of the host.

In the present experiments, we used a low dose of carcinogen and solved the technical difficulty of contamination by transplanting the original tumor into 2 types of mice, each having only one type of PGK, and then determining the PGK type of the tumor after regrowth. We report here the presence of tumors with a single phenotype in mice with cellular mosaicism.

MATERIALS AND METHODS

Animals. Mice with the *Pgk-1*^{a} gene producing an electrophoretically variant type of PGK (5) against a C3H/HeHa background were obtained from Dr. Verne M. Chapman, Roswell Park Memorial Institute, Buffalo, N. Y., and raised in our laboratory. C3H/He mice with the usual type of PGK gene, *Pgk-1*^{b}, were obtained commercially (Charles River Japan). Female heterozygous mice of the genotype *Pgk-1*^{a}/*Pgk-1*^{b} were produced in our laboratory by crossing *Pgk-1*^{a} males and *Pgk-1*^{b} females.

Carcinogen Treatment. MC (a product of Fluka) was dissolved at 5 mg/ml in olive oil by heating and diluted to 50 μg/ml with olive oil. A 0.1-ml aliquot of this solution was injected s.c. into the right groin of *Pgk-1*^{a}/*Pgk-1*^{b} mice, 8 weeks old, as described previously (7). The injection site was visible as a swollen area (5 mm in diameter) for more than 1 week. Mice were kept in an isolating rack and were given mouse diet (CE-1; Clea Japan) and water *ad libitum*.

Tumors. Tumor formation in the mice was monitored by palpation at least once a week. When tumors reached 1 cm in diameter, they were excised and cut into 4 parts. Two pieces from each part were transplanted with a transplantation needle into 2 mice having only A-type PGK (A host) and B-type PGK (B host), respectively. The rest of the tumor was used for histological examination. After regrowth in each host, the transplanted tumors were excised for assay of PGK.

Electrophoresis of PGK. The excised pieces of tumor were washed with distilled water and homogenized with a mechanical homogenizer (type PT 10/35 Polytron; Kinematica GmbH) in PGK buffer (3). The supernatant of this homogenate was used for PGK assay. Electrophoresis was carried out on a gel plate (15 x 14 cm) of 12% starch (Connaught Laboratories) for 17 hr at 5 V/cm and 4°. The gel plate was then cut into 2 slices, and PGK was detected as nonfluorescent spots due to conversion of NADH to NAD in the PGK assay system (3). The spot of A-type PGK was usually 5 cm from the origin, and that of B-type PGK was 3 cm from the origin. A detectable spot of PGK was obtained with 2×10^4 tumor cells (2% of the cells usually applied).

Separation of Tumor Cells. The excised tumor tissue was minced and treated with 0.5% trypsin in phosphate-buffered saline (16 g/liter NaCl, 0.4 g/liter KCl, 2.3 g/liter $NaHPO_4$, 0.4 g/liter KH_2PO_4) at room temperature. Separation of intact tumor cells was observed by phase-contrast microscopy. The action of trypsin was terminated at a suitable time by adding fetal calf serum, and then the tumor cells were washed by centrifugation and suspended in Eagle's medium.

RESULTS

Tumors developed in 8 of 16 mice (50%) 200 days after MC injection, and they were identified histologically as fibrosarcomas. Fig. 1 shows the electrophoretic patterns of PGK of one of the tumors assayed under various conditions. The blood of this *Pgk-1*^{a}/*Pgk-1*^{b} mouse contained 2 types of PGK, as did homogenates of its kidney, liver, and lung (data not shown). The original tumor appeared to contain 2 types of PGK. However, extensive washing of the original tumor with distilled water to remove RBC or further treatment with trypsin and collagenase decreased the intensity of the spot of B-type PGK appreciably, although not completely (Fig. 1). After transplantation into an A host, the tumor tissue gave only the spot corresponding to A-type PGK, with none corresponding to B-type PGK. After transplantation into a B host, the tumor tissue gave both spots (Fig. 1). Moreover, tumor transplanted into an A host after passage through a B host contained A-type PGK only (data not shown).

Thus, the B-type PGK in this tumor is thought to be due to contamination of blood and supporting connective tissue of the host, and the tumor shown in Fig. 1 was concluded to contain type A PGK only. Comparable results were obtained with a tumor containing B-type PGK. The tumor showed only the B-type pattern after passage through a B host and both types after passage through an A host. A tumor with both PGK types exhibited both PGK patterns after passage through either an A

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[2] The abbreviations used are: PGK, phosphoglycerate kinase; MC, 3-methylcholanthrene.

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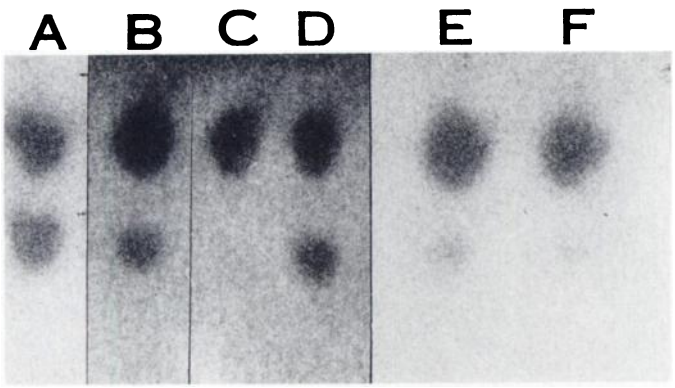

Fig. 1. Electrophoretic patterns of PGK of a tumor assayed under various conditions. Direction of electrophoresis was *bottom* to *top*. *Upper* and *lower* spots are those of A-type and B-type PGK, respectively (distance from center to center, 2 cm. *A*, blood of *Pgk-1*^{a}/*Pgk-1*^{b} mouse; *B*, original tumor; *C*, tumor transplanted into an A host; *D*, tumor transplanted into a B host; *E*, original tumor, minced and washed with distilled water; *F*, original tumor further treated with 0.1% trypsin and 0.1% collagenase in phosphate-buffered saline for 30 min at 20°. The PGK type of this tumor was deduced to be A.

or a B host (data not shown). Although the PGK type could be determined with the original tumor tissue, we used the transplantation method in this study, since it gave clearer results.

Results on the PGK types of tumors induced with 5 μg MC are summarized in Table 1. PGK types were judged from 4 pairs of PGK patterns. Of 8 tumors examined, 5 exhibited A-type, 2 showed B-type, and 1 exhibited both types. Accordingly, the frequency of single-phenotype tumors was 88%.

Since A-type tumors were more frequent than B-type tumors, the possibility of their selection during tumor growth was examined. This possibility also applied to PGK type of tumors transplanted into A- and B-hosts. A suspension of a 1:1 mixture of the 2 types of tumor cells (1.5×10^3 cells each) in 0.1 ml was injected s.c. into A- and B-hosts, respectively. After regrowth, the tumors were assayed for PGK. Both types of PGK were found in tumors grown in either host, indicating the absence of selection (data not shown).

DISCUSSION

Before drawing any conclusions from the present results, several problems should be considered. (*a*) The area of mouse skin treated with a carcinogen should cover a large number of patches of A- and B-type cells. The cellular mosaicism produced by X-chromosome inactivation provides a fine intermixture of 2 types of cells in mice. With heterozygous mice carrying X-chromosome-linked normal pigment and albino alleles, Deol and Whitten (1) showed that the patch size is less than 90 μm in sections of the retina and less than 0.02 sq mm in diameter in sections of the inner ear. In the skin, this patch size is considered to be much smaller. In our experiments, the carcinogen-treated area was 5 mm in diameter (20 sq mm), as measured from the size of the swollen skin after injection of carcinogen solution. Consequently, the treated area should be large enough to cover a large number of A- and B-type cell patches. (*b*) It should be assumed that X-chromosome inactivation is not disturbed during cellular tumorigenesis. (*c*) No selection of one type of cells should occur during tumor growth. This selection seems unlikely from results on transplantation of a mixture of the 2 types of cells, which resulted in equal growth of both types of cells, although initial number of cells at transplantation was large (3×10^3 cells).

On the basis of the above considerations, our finding that most tumors produced in mosaic mice had a single PGK phenotype provides evidence for the single-cell origin of tumors.

It is unlikely that a single PGK phenotype was produced by multiple cellular events, since the probability that, in a population of cells consisting of equal numbers of A- and B-type cells, n independent cellular events occur in A-type cells only or in B-type cells only is $(½^n) \times 2$, *i.e.*, $½^{n-1}$; consequently, 88% incidence of single-phenotype tumors cannot be explained in the case of $n \geq 2$. In this work, A-type tumors were produced at higher frequency than were B-type tumors, but this imbalance needs confirmation.

The C3H/He mice used in the present work are more sensitive to MC than were ICR/JCL (7) mice. The dose of MC used in the present work, 5 μg/mouse, is in the middle of the linear dose response range for tumor induction. The sensitivity to MC of the F_1 hybrids of feral and BALB/c mice used by Reddy and Fialkow (5) is unknown. However, the doses of MC that they used, 0.2 and 2 mg/mouse, seem very high, and the chance for multiple cellular events would probably have been higher in their work. This difference in the dose of carcinogen applied may explain the apparent discrepancy between their results

Table 1

PGK types of 8 fibrosarcomas produced with 5 μg MC assayed after transplantation into A- and B-hosts

Tumor	Transplantation no.	PGK type of tumor after transplanted into		Deduced PGK type of original tumor
		A host	B host	
1	1	A	AB	A
	2	A	AB	
	3	A	AB	
	4	A	AB	
2	1	A	AB	A
	2	A	AB	
	3	A	AB	
	4	A	AB	
3	1	A	AB	A
	2	A	AB	
	3	A	AB	
	4	A	AB	
4	1	A	AB	A
	2	A	AB	
	3	A	AB	
	4	A	AB	
5	1	A	AB	A
	2	A	AB	
	3	A	AB	
	4	[a]	AB	
6	1	AB	B	B
	2	AB	B	
	3	AB	B	
	4	AB	B	
7	1	AB	B	B
	2	[a]	B	
	3	AB	B	
	4	[a]	B	
8	1	AB	AB	AB
	2	AB	AB	
	3	AB	AB	
	4	AB	AB	

[a] Transplantation failed.

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and ours, because the ratio of the frequency of induction of a single PGK phenotype to that of multiple PGK phenotypes should vary depending on the dose of MC.

The above considerations also explain the predominance of monoclonal type tumors in the human population (2), since most human tumors are thought to be produced by exposure to a relatively low dose of environmental carcinogens. Detailed studies are required on the dose dependence of formation of the single PGK phenotype tumors.

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