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Dose Response of Monoclonal Tumor Induction with 3-Methylcholanthrene in Mosaic Mice[1]

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ABSTRACT

Inductions of monoclonal tumors, as judged by their single phenotype of phosphoglycerate kinase, in mice with X-chromosome inactivation mosaicism, were observed after s.c. injection of doses of 3-methylcholanthrene of 0.005 to 2 mg/mouse. The tumors induced (79% fibrosarcomas) exhibited the single-enzyme phenotype with 93% frequency at the lowest dose and with 57% frequency at the highest dose, indicating the dependence of monoclonal tumor induction on the dose of carcinogen.

INTRODUCTION

The methods available for demonstrating the single somatic cell origin of tumors are: observation of the specific type of immunoglobulin in tumors such as myelomas; the karyotype of tumor cells, especially the presence of a common marker chromosome, in a tumor; the isoenzyme phenotype of tumors produced in a female host with cellular mosaicism due to X-chromosome inactivation (4); and, more recently, the uniformity of a mutated oncogene in a tumor.

The isoenzyme method has been widely applied to various types of tumors. Fialkow (1) reported in his review that, in humans with X-inactivation mosaicism, tumors exhibited the single-enzyme phenotype, with respect to glucose-6-phosphate dehydrogenase, strongly suggesting their single-cell origin. However, Reddy and Fialkow (8) reported that, in mice with cellular mosaicism with regard to PGK-1[3] (5), fibrosarcomas induced by high doses of MCA had multiple-PGK phenotypes. On the contrary, later observations showed the induction of tumors with a single-PGK phenotype in mosaic mice under different conditions (9, 11, 14).

These discrepant results suggest that the dose of carcinogen may affect the results. It is important to examine this possibility, because humans are usually exposed to relatively low doses of environmental carcinogens, whereas experimental animals are treated with high doses, and thus extrapolation of data in animals to humans may not be valid.

To examine the clonal nature of tumors with respect to the dose of carcinogen applied, we measured the yields of tumors with a single-PGK phenotype induced by doses of MCA from the lowest to the highest dose possible. In the present report, we show that the monoclonal character of tumors is dependent on the dose of carcinogen applied.

MATERIALS AND METHODS

The materials and methods used in this experiment were essentially as described previously (11), except that a wide range of doses of carcinogen was used.

Mosaic Mice. Mice with mutation *Pgk-1*^{a} of the *Pgk-1* locus linked to the X-chromosome, coding for the variant PGK (A-type), against a C3H/HeHa background were originally obtained from Dr. Verne M. Chapman, Roswell Park Memorial Institute, Buffalo, NY, and were mated with commercially available C3H/He mice (Charles River Japan) with the usual PGK gene (B-type), *Pgk-1*^{b}, as described previously (11). Female heterozygous offspring of the genotype *Pgk-1*^{a}/*Pgk-1*^{b} were used in this experiment. Mice were maintained in an isolated rack with free access to mouse diet (CE-1; Clea Japan) and water.

Carcinogen Treatment. MCA (Fluka, Buchs, Switzerland) was dissolved in olive oil at concentrations of 0.05 to 20 mg/ml, and volumes of 0.1 ml of these solutions were injected s.c. into the right groin of mice of about 8 weeks old, as described previously (11). The treated area was a 5-mm-diameter circle.

Tumors. The time course of tumor formation and the growth rates of tumors were followed as described previously (12). When tumors reached about 1 cm in diameter, about 90% of the tumor mass was excised surgically. The host was then maintained for experiments on radiation treatment of regrown tumors, which will be described elsewhere. Each tumor was cut into 4 sections, and pieces (about 1 cu mm) from each section were transplanted into a mouse with A-type PGK and a mouse with B-type PGK, respectively, to avoid an AB-type PGK background of the host (11). The overall transplantation efficiency was 87.3%. When these 4 pairs of transplants had grown to 1 cm in diameter, they were used for PGK assay. Sections of tumors were also stained with hematoxylin-eosin and examined histologically.

PGK Assay. The type of PGK was determined as described previously (11). Pieces of tumor were homogenized and centrifuged, and the supernatants were subjected to electrophoresis on 12% starch gel for 17 hr at 5 V/cm and 4°. A-type and B-type PGKs were located as distinct nonfluorescent spots after reaction with an NADH-NAD$^+$ system. A detectable PGK spot could be seen with extract from 2×10^4 tumor cells, which was about 2% of the number of cells usually used. The presence of the 2 types of PGK was confirmed in the blood and homogenates of the liver, kidney, heart, and skin of mosaic mice, and the blood PGK of each mosaic mouse was checked. The PGK type of the original tumor was determined from at least 2 and usually 4 pairs of PGK patterns, as shown in Fig. 1.

RESULTS

Results for the PGK tumor types induced with various doses of MCA in *Pgk-1*^{a}/*Pgk-1*^{b} mosaic mice are shown in Table 1. All but one tumor induced in this and previous (11) experiments with the lowest dose of MCA (0.005 mg/mouse) were of the single-PGK phenotype. The percentage induction of tumors with the single-PGK phenotype decreased to 57% with an increase in the dose of MCA to 2 mg/mouse. This dose dependence is shown more clearly in Chart 1, where the percentage of tumors with a single-PGK phenotype is plotted as a function of the dose of MCA.

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[3] The abbreviations used are: PGK, 3-phosphoglycerate kinase (EC 2.7.2.3); MCA, 3-methylcholanthrene; DMBA, 7,12-dimethylbenz(*a*)anthracene.

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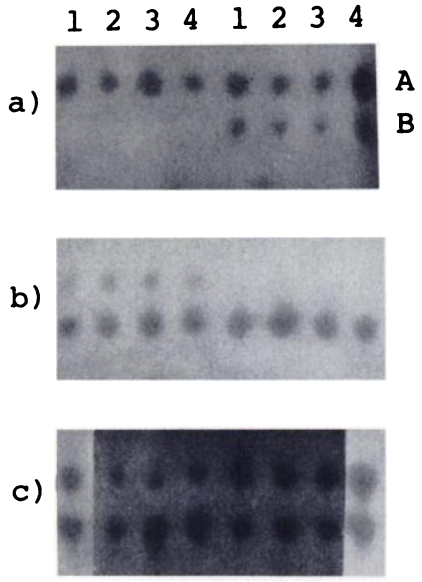

Fig. 1. Electrophoretic PGK patterns of tumors induced by MCA. A tumor was cut into 4 sections, and 2 pieces from each section were transplanted into mice with A-type (*1* to *4*, *left*) and B-type (*1* to *4*, *right*) PGK, respectively. The PGK types of the regrown tumors were determined by electrophoresis (*bottom* to *top*). *a*, A-type tumor (fibrosarcoma induced with 0.25 mg MCA); *b*, B-type tumor (squamous cell carcinoma induced with 0.05 mg MCA); *c*, AB-type tumor (fibrosarcoma induced with 2 mg MCA).

Table 1

PGK types of tumors induced by various doses of MCA in mosaic mice

Dose of MCA (mg/mouse)	No. of tumors of each PGK type					
	Fibrosarcoma		Other types of sarcoma[a]	Squamous cell carcinoma	Total	
	(A, B, AB)	%[b]	(A, B, AB)	(A, B, AB)	(A, B, AB)	%[c]
0.005	(11, 2, 1)	93	(0, 1, 0)	(0, 0, 0)	(11, 3, 1)	93
0.025	(9, 2, 2)	85	(1, 0, 0)	(0, 0, 0)	(10, 2, 2)	86
0.05	(11, 3, 1)	94	(0, 0, 1)	(0, 1, 1)	(11, 4, 3)	84
0.25	(8, 1, 4)	69	(1, 1, 0)	(0, 1, 1)	(9, 3, 5)	71
0.5	(10, 0, 2)	83	(0, 1, 2)	(2, 0, 1)	(12, 1, 5)	72
2.0	(2, 2, 5)	44	(1, 0, 1)	(1, 2, 0)	(4, 4, 6)	57

[a] Myogenic sarcomas, liposarcomas, and pleiomorphic sarcomas.
[b] Percentages of fibrosarcomas with a single-PGK phenotype at each dose of MCA. Previous data with 0.005 mg of MCA are included.
[c] Percentages of tumors of all histological types with a single-PGK phenotype. These values are plotted in Chart 1.

The cumulative incidences for all tumors in mosaic mice after 300 days are also plotted in Chart 1. Only one tumor was seen at each injection site. The efficiency of tumor induction in mosaic mice did not differ from that in commercial female C3H/He mice or mice with A-type PGK (data not shown). The time course of appearance of tumors is reported elsewhere (12). Histologically, the tumors were fibrosarcomas (79%), other types of sarcomas (10%; see Table 1, Footnote *a*), and squamous cell carcinomas (10%).

Among the fibrosarcomas with a single-PGK phenotype, tumors with A-type PGK (A-type tumors) predominated over those with B-type PGK, except at the highest dose of MCA. The average volume-doubling times (12) of A-, B-, and AB-type tumors in mosaic mice were 2.5, 2.7, and 2.6 days, respectively.

DISCUSSION

Several problems in interpretation of results on mice with X-inactivation mosaicism were mentioned in a previous report (11).

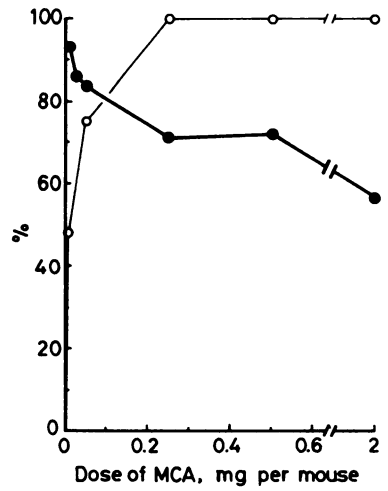

Chart 1. Percentages of tumors with a single-PGK phenotype (●) and 300-day cumulative tumor incidences (○) in *Pgk-1*^{a}/*Pgk-1*^{b} C3H/He mosaic mice, plotted as functions of the dose of MCA.

These include fineness of mosaicism, the stability of X-inactivation during tumorigenesis, and the possibility of formation of tumors with a single-PGK type from multiple origins.

The patch size and the fineness of mosaicism are essential problems, which are difficult to examine when an isoenzyme is used as a marker. Recently, we used heterozygous mosaic mice with Cattanach translocation on one of a pair of X-chromosomes, so that inactivation occurring on one of the 2 distinguishable X-chromosomes could be visualized in each individual cell. The results obtained showed the fine mixture of the 2 types of cells in 1 cu mm of the fibrous tissue and the presence of only one type of cells in tumors.[4] Furthermore, reversal of X-inactivation during tumorigenesis is unlikely, judging from current information on X-inactivation (3).

Tumors with multiple-PGK phenotypes were interpreted as representing the tumors of multiple cellular origins. These tumors might be formed by coalescence of independent monoclonal tumors. We interpret the present results as showing that tumors originate from a single cell if a sufficiently low dose of carcinogen is applied but that they tend to originate from multiple cells at higher doses of carcinogen. However, the incidence of multiple-PGK-phenotype tumors rose to a steady level of only 43%, even with the highest dose of MCA, where the number of original tumor cells can be estimated as 2 from Chart 1. This was possibly because the dose of carcinogen was not high enough, but we consider it as an indication of the existence of systemic resistance of the host to tumors since tumors of multiple origin should have been formed, judging from the fact that after MCA treatment the mutation or transformation frequencies of cells *in vitro* reach 10^{-4} or more. Of these multiple transformants, only one may escape suppression by the host and develop from the latent state into a tumor. It is interesting that, in PGK mosaic mice, preneoplastic nodules induced in the liver by 2-acetylaminofluorene p.o. were reported to have a single-PGK phenotype (7). This finding indicates that, after carcinogen treatment, clones of mutated or precancerous cells that have not yet become malignant are present at a considerably high frequency. The clonal evolution of tumor cells (6), by which a tumor acquires biological variety (2), is thought to occur at a later stage of malignant

[4] N. Takagi and H. Tanooka, manuscript in preparation.

growth.

Recently, Reddy and Fialkow (10) reported that most papillomas (82%) produced in the skin of PGK mosaic mice by treatment with DMBA followed by 12-*O*-tetradecanoylphorbol-13-acetate, an initiation-promotion regimen, had a single-PGK phenotype, whereas on repeated application of DMBA alone the proportion was only 54%. However, Taguchi *et al.* (15) found that, with lower doses of DMBA alone, most (93%) of the papillomas that developed exhibited a single-PGK phenotype and that, on continued application of DMBA, these papillomas progressed to malignant squamous cell carcinomas, maintaining the same single-PGK phenotype as in the original papilloma. Here again, there seems to have been a dose-response problem.

In the present study, A-type tumors predominated over B-type tumors. Since mice with A-type PGK were as susceptible to carcinogen as were mice with B-type PGK, and no selection of A-type or B-type tumor cells was observed during growth after transplantation of a mixture of the 2 types of tumor cells (11), this predominance probably reflects an imbalance in the cellular composition of normal tissue ($A/B = 3/2$) arising from imbalanced X-inactivation controlled by an X-chromosome-controlling element (13) and provides further evidence for the single-cell origin of tumors. However, this imbalance was not seen in AB-type tumors (Fig. 1*c*), possibly because most AB-type tumors originated from 2 cells (one A and one B). The possibility of the 2-cell origin at the highest dose of MCA is supported by the fact that the incidence of the single-PGK phenotype tumors was 57% at this dose.

Tumors with a single-PGK phenotype produced in mosaic mice can also be used to distinguish true recurrence from formation of new second tumors after experimental therapy of the primary tumor, as will be reported elsewhere.

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