MULTICELLULAR ORIGIN OF FIBROSARCOMAS
IN MICE INDUCED BY THE CHEMICAL
CARCINOGEN 3-METHYLCHOLANTHRENE*

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Determination of the number of cells from which a neoplasm arises can provide clues to its mode of origin. For example, if a tumor arises as the result of a rare event such as a somatic mutation, unicellular (clonal) origin is anticipated. In contrast, a multicellular origin might be found for a neoplasm that develops from a field of altered cells. This question can be investigated in organisms with two or more genetically distinct types of cells. Most such studies have been done in humans using as a marker immunoglobulin or glucose-6-phosphate dehydrogenase in females with X-chromosome inactivation mosaicism (1). The data suggest that the majority of human neoplasms are clonal in origin at the time of study. Interpretation of these indirect investigations of tumorigenesis in man could be facilitated by direct studies of tumor development in animals. Such studies have been hampered by lack of suitable X-linked markers, but recently Nielsen and Chapman (2) described an electrophoretic variant for the X-linked enzyme phosphoglycerate kinase (PGK-1) in feral mice.

Because only one of the two X-chromosomes is active in XX somatic cells, a female mouse heterozygous at the X-linked PGK locus for the usual \( \text{Pgk-1}^b \) gene and the variant \( \text{Pgk-1}^a \) has two populations of cells. In the cells of one population, \( \text{Pgk-1}^b \) is active, with consequent production of type B isoenzyme, whereas in cells of the other population, \( \text{Pgk-1}^a \) is active and type A isoenzyme is synthesized. Tumors with a clonal origin in \( \text{Pgk-1}^b/\text{Pgk-1}^a \) mice should therefore display B or A-type PGK, whereas those with a multicellular origin may exhibit both B and A isoenzymes. We utilized this isoenzyme system as a marker to study tumors induced by the chemical carcinogen 3-methylcholanthrene (MCA).

Materials and Methods

Animals. Male feral mice with the PGK variant (PGK type 1 A) originally trapped in Denmark (2), were kindly given to us by Dr. Vernon M. Chapman, Roswell Park Memorial Institute, Buffalo, N. Y. We bred these animals with females from inbred strains homozygous for the usual \( \text{Pgk-1}^b \) and used the \( F_1 \) hybrids heterozygous for the wild type and variant PGK genes \( (\text{Pgk-1}^b/\text{Pgk-1}^a) \). The \( F_1 \) mice were maintained on a standard diet and were injected with carcinogen when they weighed \( \approx 20-25 \) g (between 8 and 12 wk of age).

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Abbreviations used in this paper: DEM-Tc, Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum, 100 U of penicillin and streptomycin per liter, and 25 mM Hepes; MCA, 3-methylcholanthrene; PGK, phosphoglycerate kinase.
Induction of Tumors. Mice were shaved and injected subcutaneously with a single dose of MCA (Sigma Chemical Co., St. Louis, Mo.) dissolved completely in 0.2 ml olive oil by heating in boiling water. Four mice were injected with 0.2 mg MCA and five were injected with 2.0 mg. The animals were examined twice weekly for palpable tumors or other manifestations of malignancy. They were killed and underwent complete autopsy after progressively growing tumors were identified.

Dissection of Tumors. Each tumor was dissected free of connective tissue and divided into at least two to five sections depending upon its nodular architecture. In most neoplasms, each nodular section was subdivided into small fragments measuring ≈ 3- to 5-mm diameter. Subsequently, each such fragment was divided into three equal portions. The two outer portions were used for PGK analysis and the center piece was utilized for histologic examination and for the establishment of tissue cultures. Blood, parenchymal organs, and muscle fragments adjacent to the tumors were also tested for PGK.

PGK Assay. PGK phenotypes were determined with a slight modification of the method described by Chen et al. (3). Gels were stained (4) and the activity of the PGK isoenzymes was quantitated visually using known mixtures as standards. We found that a minor population in a mixture of cells can be detected with this method if it contributes 2-5% of the total PGK activity.

Tissue Culture. Tissues were finely minced and placed in Falcon flasks containing Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum, 100 U of penicillin and streptomycin per liter, and 25 mM Hepes (DEM-Tc). Cells in the primary cultures were analyzed for PGK and cells in the second passage, for chromosomes.

Cloning of Tumor Cells in Culture. A single cell suspension of tumor cells was obtained from cell cultures by dissociation with 0.25% trypsin. The cells were washed twice in Hank's balanced salt solution and adjusted to a concentration of 8-10 cells per milliliter in DEM-Tc. 0.1 ml of this suspension was dispensed into each well of Microtest-II plates (Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.). The plates were incubated at 37°C for 12 h in a 5% CO₂, 100% humidity atmosphere. Wells appearing to contain one cell were selected and 0.1 ml more of DEM-Tc was added. After a colony appeared, the cells were transferred to a 25-cm² flask for clonal amplification and then tested for PGK.

Histopathology. Tissues were fixed in 10% formalin in phosphate-buffered salt solution. The sections were stained with hematoxylin and eosin. Estimates were made of the percent of tumor cells and of nontumor cell admixture without knowledge of the isoenzyme results (5).

Chromosomal Studies. 0.02 ml of colchicine was added to the tissue culture and after 2 h the cells were removed from the flask with trypsin and treated with hypotonic KCl (0.05 M) for 15 min at room temperature. After fixation in acetic acid:methanol (1:3), the cells were stained with Giemsa. For each culture, 50 randomly selected metaphases were scored at 1,000 ×.

Results

PGK Phenotypes in Normal Tissues. Each of 140 specimens of solid tissue from six heterozygous mice exhibited both B and A PGK types (Table I). Generally, there were equal amounts of B and A isoenzyme activities.

MCA-induced Tumors. Two of four F₁s (feral mice × BALB/c) injected with 0.2 mg MCA, and three of five F₁s injected with 2.0 mg MCA developed palpable tumors. The neoplasms were noted at the sites of injection after latent periods of 19-25 wk and had gross and histologic features typical of fibrosarcomas with anaplastic spindle-shaped cells and numerous mitoses (Figs. 1 and 2). Large fibroblast-like cells grew in culture from the primary tumor explants, formed colonies within a few days, and, multilayers within 2-3 wk (Figs. 3 and 4). Chromosomes in the cultured cells were examined at the second passage. Almost every cell studied from all five tumor lines was hyperploid. In each case, the modal number was tetraploid or hypotetraploid (range, 75-80 chromosomes).

PGK Phenotypes in MCA-induced Tumors and Tumor Cultures. Both B and A types of
PGK (double-enzyme phenotypes) were found in each of the five tumors (Table II). In general, PGK phenotypes in multiple fragments from the same tumor nodule were similar to each other. However, single enzyme phenotypes were observed in at least one fragment from a single nodule in two tumors (3 and 5). Nodule G from the tumor in animal 3 showed only type A enzyme; two other nodules from this same tumor displayed predominantly type B PGK and tissue culture lines derived from these nodules showed only type B enzyme.

PGK Phenotypes in Clones. The results given in Table III and Fig. 5 show that some of the “clones” grown from cell cultures derived from both tumors tested displayed only A-type PGK and the others showed only B-type enzyme. Both types of clones were hyperploid and displayed multi-layered growth.

Discussion

The finding of single-enzyme PGK phenotypes in some tumor nodules and culture lines confirms the fact that this locus undergoes X-inactivation. Consequently, the occurrence of a double-enzyme phenotype in each MCA-induced tumor suggests that each growth contains multiple neoplastic clones. However, before this conclusion can be accepted it is necessary to exclude the possibilities that the tumor samples were heavily contaminated with normal tissue (e.g., blood vessels) or that the tumor arose from a single cell in which both Pgk-1<sup>b</sup> and Pgk-1<sup>a</sup> were active.

Noteworthy contamination with nonneoplastic cells was rendered unlikely by histologic examination of tumor sections adjacent to the samples assayed for PGK. Less than 10% of the cells were judged to be nonneoplastic in almost all instances. Furthermore, cells cultured from the tumors showed double-enzyme phenotypes and yet they exhibited characteristics typical of transformed cells: hyperploidy and multilayered growth. Many of these cultures had the same proportion of PGK B to A isoenzyme as was observed in the tumors directly. The findings in tumors 3 and 5 of some samples with all or mostly type B PGK and others in the same tumor with predominantly or all type A isoenzyme indicate that at least each of these neoplasms had more than one neoplastic clone.

To further evaluate the possibility that the double-enzyme phenotypes found in the
tumors and cell cultures derived from them reflect the phenotypes of the neoplastic cells themselves, attempts were made to establish clones from two cultures which exhibited equal proportions of A- and B-type PGK. We do not have independent verification that these “clones” indeed arose from single cells. In fact, the finding that as 50% of them had double-enzyme phenotypes suggests that they arose from more
than one cell. Nonetheless, the facts that in each case some “clones” of type A and others of type B were observed, and that cells within these “clones” retained the characteristics typical of transformed cells found in the mass cultures, hyperploidy and multilayered growth, indicate that the tumor cell cultures contained multiple clones (some with PGK type A and others with type B).

Activity of both X chromosomes has never been observed in a diploid somatic cell,
## Table II

**PGK Phenotypes in Normal Tissues and in MCA-induced Tumors in Feral × BALB/c F₁ Mice Heterozygous for PGK**

| Animal No. | Normal tissues | Tumors |
|------------|----------------|--------|
|            | Percent A enzyme | MCA Time interval* | Size | Specimen‡ | Percent A enzyme |
|            | Blood cells | Muscle | mg | wk | mm | Direct | Cultured cells |
| 1          | 65 | 50 | 0.2 | 19 | 26 × 18 | A | 30 | ND§ |
|            | B | C | D | 40 | 50 | 5 | 50 | 45 |
|            | E | 40 | 50 | 50 | 20 | 20 | 50 |
| 2          | 50 | 50 | 0.2 | 21 | 5 × 5 | A | 20 | 50 |
|            | B | C | D | 20 | 0 | 0 | 60 |
|            | E | 30 | 30 | 50 |
| 3          | 50 | 55 | 2.0 | 19 | 20 × 16 | A | 20 | 50 |
|            | B | C | D | 20 | 0 | 0 | 60 |
|            | E | 30 | 30 | 50 |
| 4          | 50 | 40 | 2.0 | 25 | 29 × 12 | A | 50 | 50 |
|            | B | C | D | 35 | 0 | 0 | 65 |
|            | E | 35 | 50 |
| 5          | 50 | 50 | 2.0 | 20 | 25 × 20 | A | 50 | 50 |
|            | B | C | D | 35 | 0 | 0 | 65 |
|            | E | 35 | 50 |
|            | F | G | 30 | 50 |

* Time elapsed between injection of MCA and autopsy.
‡ The tumor was first divided into sections (A-G) according to its nodular architecture. Sections were subdivided into fragments (1-7) each measuring 3-5 mm in diameter.
§ ND, not done.
TABLE III

PGK Phenotypes in “Clones” Derived from Tumor Cell Cultures

| Animal No. | Tumor specimen | No. studied | PGK phenotypes |
|------------|----------------|-------------|----------------|
|            |                |             | A No. | B No. | A/B No. |
| 1          | D              | 21          | 5     | 7     | 9       |
| 3          | F              | 20          | 5     | 5     | 11      |

* All clones from each tumor cell culture were derived from a single flask.

Fig. 5. Starch gel electrophoresis of clones separated from fragment F of the tumor from animal 3. Tissue homogenates in slots 2 and 8 exhibit 100% A-type PGK. Slots 1, 6, and 9 show predominantly A-type PGK. Slots 3 and 10 exhibit 100% B-type PGK. Slots 4, 5, and 7 show A- and B-type PGK.

normal or neoplastic (1). However, the cells grown in culture from the MCA-induced tumors were hypotetraploid. We do not known the significance of this finding, but if the neoplastic cells were tetraploid or hypotetraploid before culture, it is probable that two X-chromosomes were active in each cell. Nonetheless, it is very unlikely that the double-enzyme phenotypes are explained by origin from single cells with both an active \( \text{Pgk-1}^b \) and an active \( \text{Pgk-1}^a \) rather than origin from multiple cells. This conclusion is supported most strongly by the findings in tumors 3 and 5. For example, in tumor 3 some small samples displayed only B-type PGK whereas others showed only A-type isoenzyme, indicating the presence of at least two cell lines. A similar cell composition is likely for tumors 1, 2, and 4 even though all the samples from these neoplasms showed both B and A-types PGK. The tetraploidy in the cells in these neoplasms (if indeed they were tetraploid before culturing) must have arisen either by endoreduplication (chromosome replication without cell division) or by cell fusion. A tetraploid cell that arises as a result of endoreduplication presumably would have two active \( \text{Pgk-1}^b \) genes or two active \( \text{Pgk-1}^a \) genes (depending upon which PGK gene was active in the diploid cell before endoreduplication), but not one active \( \text{Pgk-1}^b \) and one active \( \text{Pgk-1}^a \). Thus, more than one endoreduplicated tumor cell progenitor for each tumor would be required to explain the double-enzyme phenotypes. The possibility that the tumors arose from a single tetraploid cell which was the result of fusion between two diploid cells with an active \( \text{Pgk-1}^b \) and \( \text{Pgk-1}^a \), respectively, was rendered very unlikely by the demonstration of both PGK type A and type B clones derived from tumors with double-enzyme phenotypes.

Origin of each tumor from more than one cell could come about in at least two ways: (a) a relatively large number of cells could be affected (transformed) simultaneously by the carcinogen; (b) the MCA initially altered only a single cell and this
alteration subsequently led to recruitment to the tumor of hitherto normal neighbor cells (e.g., through release, or activation, or both of an oncogenic factor).

The results obtained in these animals with X-linked mosaicism contrast with those obtained by Iannaccone et al. (6) in chimeric mice made by the amalgamation of two embryos, each producing a different electrophoretic variant of the enzyme glucose phosphate isomerase. The smallest samples of normal tissues that could be analyzed almost invariably contained both isoenzymes, but almost all chemical carcinogen-induced tumors had single-enzyme phenotypes. Those workers concluded that the epidermal tumors produced could not have arisen from more than eight cells and in all likelihood were clonal in origin. There are several possibilities to explain our failure to observe similar results. First, we studied fibrosarcomas, whereas Iannaccone et al. (6) studied predominantly epidermal tumors. Second, in our experiments the MCA was completely dissolved in the solvent. Third, we used X-linked mosaic mice, whereas Iannaccone et al. (6) used chimeras. The patch size is probably larger in chimeras (7, 8); consequently, the probability that a tumor with multicellular origin will have a single-enzyme phenotype is greater.

Because MCA-induced tumors have unique tumor specific transplantation antigens (9), it had been assumed by some that all such neoplasms are clonal in origin. However, Prehn and co-workers (10) sampled opposite poles of nine tumors and found evidence for at least two tumor-specific transplantation antigens in one neoplasm. If more extensive sampling had been done, it is conceivable that similar findings would have been made in other tumors. Another possibility is that the Fs between these feral mice and BALB/c are particularly susceptible to tumor transforming events. Thus, such animals may have tumors of multicellular origin in contrast to inbred strains of mice with MCA-induced tumors.

These results also suggest an explanation for the difference in immunogenicity noted between spontaneously occurring tumors and chemically induced neoplasms. The former are not at all or only weakly immunogenic (11, 12, 13), whereas chemically-induced tumors are highly immunogenic (14, 15). Furthermore, immunogenicity increases with increasing carcinogen doses even if the latency period for tumor development does not change (15). It is possible that tumors induced with high doses of carcinogen are strongly immunogenic because they are multicellular in origin and, therefore, have diverse tumor-associated transplantation antigens. On the other hand, spontaneously occurring neoplasms or those induced by low doses of carcinogen are more likely to be clonal in origin, have only one type of tumor-associated antigen and, therefore, be weakly immunogenic. Recently it was reported that immunogenicity of AKR leukemia cells is markedly augmented if several clones, each with a distinct antigen(s), are used as the immunogen (16).

Although every MCA-induced neoplasm we studied contained multiple neoplastic clones, we do not know if the neoplasms had a multicellular origin or if there were multiple primaries which fused to simulate one tumor. Perhaps this distinction is essentially semantic. In either event, the results suggest that at the doses and in the animals we used the tumorigenic event induced by MCA may not be as rare as had been previously supposed. A critical question will be to determine whether tumors induced with low doses of MCA under circumstances more likely to simulate spontaneous neoplasms in man, will have clonal origin. And finally, the results of these experiments indicate that it should not be assumed in investigations using MCA-induced tumors that the neoplasms necessarily have a clonal origin.
Summary

The cellular origin of tumors induced by the chemical carcinogen 3-methylcholanthrene (MCA) was studied in mice with X-chromosome inactivation mosaicism. Because only one of the two X-chromosomes is active in XX somatic cells, a female heterozygous at the X-linked phosphoglycerate kinase (PGK-1) locus for the usual $Pgk^{-1}$ gene and the variant $Pgk^{-1b}$ has two populations of cells, in the cells of one population, $Pgk^{-1b}$ is active and B-type enzyme is synthesized, whereas in cells of the other population, A-type enzyme is produced. Both enzyme types are found in normal tissues from these mosaic mice. A tumor developing from a single cell exhibits only one of the two PGK enzyme types, whereas a tumor with a multicellular origin expresses both enzymes (i.e., it has a double-enzyme phenotype).

Five fibrosarcomas developing at the site of injection of 0.2 or 2.0 mg of MCA were analyzed. 36 of 38 fragments from the five tumors had double-enzyme PGK phenotypes. One piece from each of two tumors showed a single-enzyme phenotype. Histological, cell culture, and cloning studies indicate that the double-enzyme phenotypes reflect the presence of both types of malignant cells and not admixture of normal with neoplastic elements in the specimens tested for PGK. The results suggest strongly that these fibrosarcomas have a multicellular origin.

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