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Tumorigenicity of morphologically distinct transformed foci induced by 3-methylcholanthrene in BALB/c-3T3 cells

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Abstract

Studies have been performed in our laboratory to determine the preneoplastic and neoplastic potential of cells from morphologically different types of transformed foci. Transformed foci induced by 3-methylcholanthrene in BALB/c-3T3 cells were classified into five morphologically distinct types based on size (2–3, 3–4 and >4 mm in diameter), invasiveness (smooth vs. invading margins) and other properties (piling vs. spread). In our previous report, we showed that cells from all five types grew in soft agar, transformed normal NIH 3T3 cells and formed foci on normal layer of BALB/c-3T3 cells. In this study, the neoplastic/tumorigenic potential of cells from the five different types of transformed foci was investigated in nude mice. About two million cells from each transformed focus were injected into 4-week-old nude mice. Non-transformed BALB/c-3T3 cells were used as control. The results of this study indicate that all the 45 athymic mice injected with different transformants developed tumors between 2 and 4 weeks after injection. Tumors were not observed in eight mice injected with non-transformed BALB/c-3T3 cells. All tumors were histopathologically confirmed fibrosarcomas. These findings indicate that all five morphologically different foci show tumorigenicity and that any foci of size ≥ 2 mm regardless of invasiveness and piling could be scored as positive during the cell transformation assay.

Keywords: Cell transformation; Nude mice; Tumorigenicity; 3-Methylcholanthrene; BALB/c-3T3 cells

1. Introduction

Several cell culture systems have been developed for the in vitro transformation assay and have been used to study the mechanism of chemical carcinogenesis as well as to evaluate the carcinogenic potential of environmental chemicals [1]. Of these, the BALB/c-3T3 cell [2,3] and C3H 10T1/2 assay systems [4,5] were designed to allow the expression of transformed foci of high cell density and aberrant cell morphology on a confluent monolayer and contact-inhibited non-transformed cells. The BALB/c-3T3 system has been demonstrated to be sensitive to the transforming activity in a variety of carcinogens [1–3,6,7]. Reznikoff et al. [4,5] identified three different types of morphologically altered foci in C3H/10T1/2 clones induced by different chemicals of which only two were scored as transformants. Similar classification and scoring was used by Di-

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Paolo et al. [8] in the BALB/c-3T3 system. Smith et al. [9] have shown that tumorigenicity varies among transformed clonal populations that are induced by different carcinogenic agents or are of spontaneous origin.

Although several attempts have been made to classify foci by different authors, the problem still remains as to which types of foci are potentially tumorigenic and capable of inducing tumors when injected into isogenic host animals. It is generally accepted that only types 2 and 3 were scored as transformants. However, the question of whether other types are potentially tumorigenic and should also be scored as transformants remains unanswered. In our laboratory, BALB/c-3T3-transformed foci induced by 3-methylcholanthrene have been classified into five most common types that appear in cell culture and each focus characterized based on several properties such as size, invasiveness and piling properties. From the earlier study, we reported that all five types of foci were anchorage-independent, capable of focal reconstruction, forming colonies when normal NIH 3T3 cells were transfected with transformed DNA and activated certain proto-oncogenes. The aim of this study is to investigate the neoplastic/tumorigenic potential of all five types of transformed foci using the nude mice model.

2. Materials and methods

2.1. Cell culture and chemicals

BALB/c-3T3 clone A32-1-13 (a generous gift from E.A. Matthews, Hazelton Laboratories America, Kensington, MD) was used in this study. The culture was confirmed to be mycoplasma-free and the cells were grown in 75 cm^2 tissue culture flasks (Corning, NY) containing 15 ml of minimal essential medium (MEM; Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS, Sigma). Cells at passage 5 were used for the transformation studies. Exponentially growing cells were seeded onto a 25 cm^2 flask at a density of 3×10^4 cells. 3-Methylcholanthrene (MCA, Sigma) stock solution was prepared in dimethyl sulfoxide (DMSO) at 2 mg/ml which was further diluted with complete medium to a final concentration of 4 μg/ml.

2.2. Cell transformation assay

The cells were exposed to MCA (4 μg/ml) for 72 h and then rinsed with 5 ml phosphate-buffered saline solution (PBS, pH 7.2) three times. Thereafter, the cultures were split and maintained with 5 ml medium containing 7.5% FBS. Individual flasks were re-fed twice a week with 5 ml of maintenance medium (7.5% FBS) for a period of 4 weeks in humidified 5% CO_2 at 37°C. The foci formed were isolated, classified into five different types based on size, invasiveness and piling characteristics as shown in Table 1 and Fig. 1.

2.3. Animals

Athymic BALB/c (4 weeks old) female nude mice (nu/nu) were obtained from Harlan Sprague–Dawley. Mice were housed in a centralized animal facility accredited by the American Association for Accreditation of Laboratory Animal Care and USDA and maintained according to the recommendations established in the NIOSH Guide for the Care and Use of Laboratory Animals. The animals were housed in a glove box under sterile conditions and provided with sterile feed and water ad libitum. Animals were divided into six groups ($n = 9$/group), one group received about 2 million non-transformed BALB/c-3T3 cells/site by subcutaneous injection into axil-

Table 1
Classification of transformed foci into five distinct types based on size, invasiveness and piling properties

Type I	2–3 mm in diameter, parallel alignment of cells, non-invasive, not piled up
Type II	2–3 mm in diameter, invasive, piled up, similar to standard type II foci
Type III	~4 mm, invasive, piled up, crisscrossed, similar to standard type III foci
Type IV	Large size, > 4 mm, non-invasive, parallel alignment, smooth margin, piled up
Type V	Large size, > 4 mm, diffuse, two to three layers of piling, invasive

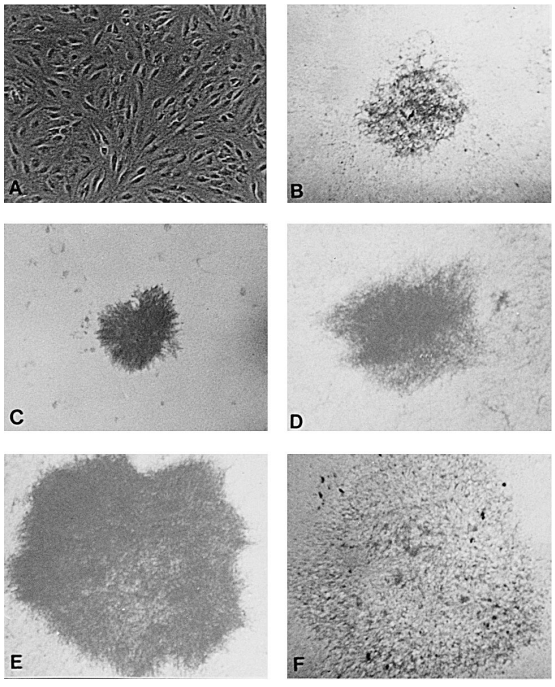

Fig. 1. Morphologically different types of foci. (A) Non-transformed BALB/c-3T3 cells showing contact inhibition and growth in monolayer. (B) Type I foci showing parallel alignment of cells and non-invasive. (C) Type II foci showing invasive and piling up properties. (D) Type III foci having crisscrossed margins, piling up properties and invasive. (E) Type IV foci with large size, non-invasive showing parallel alignment and smooth margins. (F) Type V large foci, diffused and invasive.

lary region. Similarly, other groups received either type I, II, III, IV or V focal cells. Mice were observed for tumor formation by palpating at weekly intervals. When the tumors were 1.5–2.0 cm in size, the tumors were removed ascetically. After determining the tumor weight and size, the tissues were used for histological examination and in establishing a primary cell line. Lungs were perfused with 10% buffered formalin and also processed for histology.

2.4. Statistical analysis

Pairwise multiple comparisons were done using the Student–Newman–Keul's method. One-way analysis of variance and normality was also performed. Fisher exact test and χ^2-test were performed to define if the contingency is significantly different than that expected from random occurrence.

3. Results

3.1. Cell transformation

The cell transformation experiment using BALB/c-3T3 cells treated with MCA resulted in the formation of many foci. Among them, the most common five morphologically different types of foci were analyzed (Table 1, Fig. 1). As described elsewhere, the criteria for selection of foci were size, invasiveness, piling-up and other properties. Three isolates of each type of foci were used for characterization and further analysis.

3.2. Characterization of morphologically transformed cells

Morphologically transformed clonal population, which was determined based on cell density, grew

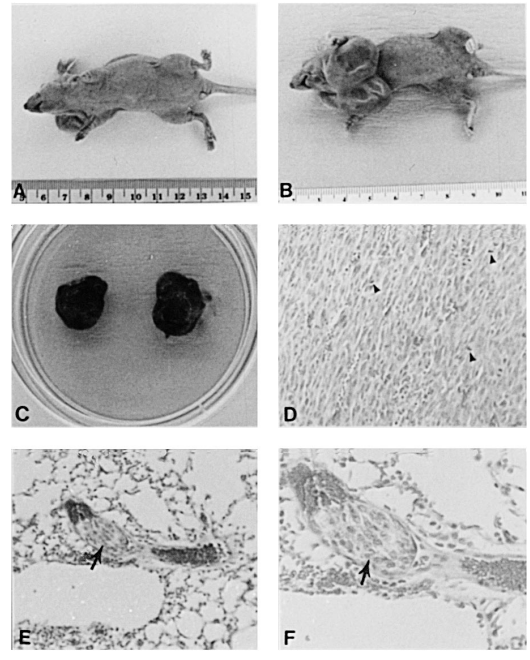

Fig. 2. Tumorigenicity of transformed cells when injected into nude mice. (A) Mouse injected with non-transformed BALB/c-3T3 cells. (B) Animal showing tumor at the sites of injection with type III foci. (C) Tumor removed ascetically for culturing of cells. (D) Histopathological section of a tumor — arrows indicate rapidly dividing mitotic cells. (E) Transformed fibroblastic cells metastasized and localized in the lungs (25×). (F) Higher magnification of E (100×).

faster than the non-transformed BALB/c-3T3 cells. They retained their original characteristic even after repopulating at a lower density to obtain true to origin clones and also to eliminate any non-transformed BALB/c-3T3 cells. Non-transformed BALB/c-3T3 cells maintained the characteristics of growing in monolayer, showed contact inhibition when cells came in close proximity and showed decreased growth rate after 30–40 passages. Contact inhibition was determined qualitatively: if there was a monolayer of cells even after the cells were confluent for over 3 days, then they were considered contact-inhibited. On the contrary, all five types of transformed focal cells showed aggressive growth characteristics once confluent or even before they attained confluency. Extensive crisscrossing was also seen in some types of foci, whereas others had parallel alignment of cells.

3.3. Tumorigenicity

Tumorigenicity of non-transformed and morphologically transformed foci was assessed by injecting cells into 4-week-old athymic nude mice. Three clones were obtained from each type and passage was kept constant (three to four passages). Three mice were used per isolate and, therefore, nine mice with two sites each received one type of focal cells. Tumors were formed in animals injected with morphological transformants (Fig. 2B, C) between 14 and 23 days after inoculation. The animals were sacrificed when the tumor was about 1.5–2.0 cm in length and width. Table 2 shows the summary of average size of tumors, days until tumor appearance and histopathological changes when cells were injected into nude mice. As shown, it was in the second week that the tumors appeared and the animals were sacrificed any time between 14 and 23 days except the control. The control animals were observed for 90 days to ascertain if any spontaneous tumor would appear. No tumors were seen in all eight mice injected with normal BALB/c-3T3 cells (Fig. 2A). Types I and IV foci showed tumor as early as 8 days and the animals were euthanized on the 14th day when the tumor were at a size of 1.5–2.0 cm.

Histopathological results indicated that all tumors sectioned and stained with hemotoxylin and eosin were fibrosarcomas (Fig. 2D). Histological sections of tumors showed a high incidence of mitotic cells,

Table 2
Gross tumor size, and histopathology of tumor and lungs

Types of foci	Days until tumor appearance	Average tumor size (cm)		Tumor type	Histopathology (Lungs)
		Right	Left		
Ia	14	1.5 × 2.0	1.0 × 1.8	FS	Focal, moderate pulmonary hemorrhage
Ib	16	1.0 × 1.8	1.8 × 1.8	FS	No significant lesions
Ic	22	1.6 × 1.8	1.7 × 1.9	FS	No significant lesions
IIa	17	1.5 × 1.8	1.6 × 1.8	FS	No significant lesions
IIb	17	1.5 × 1.7	1.5 × 1.8	FS	No significant lesions
IIc	17	1.5 × 1.8	1.2 × 1.7	FS	No significant lesions
IIIa	16	1.4 × 1.9	1.3 × 1.8	FS	No significant lesions
IIIb	20	1.5 × 1.8	1.5 × 1.5	FS	Multifocal, mild pulmonary hemorrhage
IIIc	17	1.8 × 1.2	1.8 × 1.8	FS	No significant lesions
IVa	14	1.5 × 2.0	1.5 × 1.8	FS	No significant lesions
IVb	23	1.6 × 2.0	1.6 × 1.9	FS	No significant lesions
IVc	18	1.5 × 2.3	1.6 × 2.0	FS	Intravascular tumor, thrombus*
Va	16	1.5 × 1.8	1.5 × 2.2	FS	No significant lesions
Vb	14	1.8 × 2.0	1.8 × 2.0	FS	No significant lesions
Vc	16	1.4 × 2.1	1.6 × 2.2	FS	No significant lesions
Control**	–			Normal skin	No lesions

*Animals showing metastasis to lungs.
**Animals sacrificed at 90 days.
FS = fibrosarcoma.

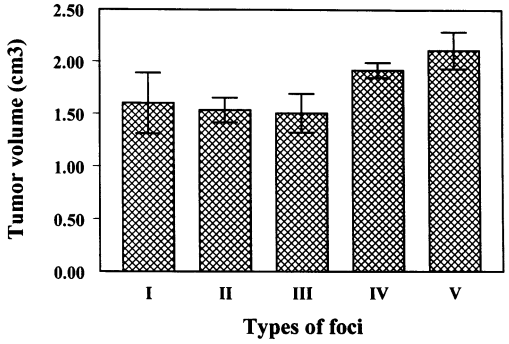

Fig. 3. Tumor volume of all five types of foci at the end of the experimental term. The growth rate of tumors was monitored by measuring the diameters of the eliptical tumors in two perpendicular dimension, and the tumor volume was calculated according to the formula: $4/3\pi r_1^2 r_2$ (r_1 and r_2 are the short and the long tumor radii, respectively).

increased cytoplasmic to nuclear ratio, and invasion of neighboring tissues. There was no significant lesions in the animals injected with types II and V foci; mild focal pulmonary hemorrhage was observed in one of the isolates of types I and III which was presumed to be terminal. The left lung was perfused to study metastatic potential of tumor. It was observed that in type IV, one of the transformed foci had metastasized to the lung and had localized in the alveolar region (Fig. 2E,F). The growth rate of tumors was monitored and the diameters of the elliptical tumors in two perpendicular dimension were measured, and the tumor volume was calculated according to the formula: $4/3\pi r_1^2 r_2$ (r_1 and r_2 are the short and the long tumor radii, respectively). Fig. 3 shows the tumor volume of all five types of foci. These results indicate that all the five types of foci were tumorigenic and one of the types had metastasized into the lungs.

4. Discussion

Cell transformation of BALB/c-3T3 cells was first reported by Kakunaga [2] and his methodology has remained essentially unchanged [10]. Many different chemicals have been tested using this assay. Recent modifications have increased the sensitivity of the BALB/c-3T3 transformation assay protocol [11,12]. Using the recommended assay evaluation criteria, the BALB/c-3T3 transformation assay has been shown to have a high specificity and sensitivity. However, the main disadvantage of BALB/c-3T3 cell transformation assay is its subjectivity in the scoring of foci. Since several morphological types of foci can be formed, researchers face uncertainty as to the type of foci which are actually capable of inducing tumors when injected into immunodeficient host animals.

In the present study, foci induced by MCA in the BALB/c-3T3 cell systems were classified into five morphological types based on their size, invasiveness and piling properties. Two types (Types II and III) classified in this study correspond to the currently used types for scoring the end points of cell transformation [10]; however, three other types of foci were classified that do not currently exist in scoring transformation assay but are most commonly found in the chemically induced cell transformation. Lu et al. [13] have modified the classification of foci into five different types; however, their criterion for modification was simply the progressively transformed state of cells compared with the initial ''normal'' BALB/c-3T3 cells. Smets [14] hypothesized that an affected cell acquires the ability to produce tumors when a critical number of independent phenotypic (and underlying genotypic) changes occur simultaneously. According to Smets' hypothesis, such individual phenotypic/genotypic alterations may be insufficient to render an affected cell tumorigenic. Also, transformation in vitro occurs by degrees or steps through a spectrum of qualitatively distinct and independent phenotypic changes, leading to a variety of stable preneoplastic phenotypes and tumorigenesis.

Malignancy of cultured cells has been convincingly demonstrated by the tumorigenicity of cells when transplanted into isogenic/immunologically deficient. Nude mice offer an excellent animal model to test the tumorigenicity of morphologically different phenotypes. Four-week-old animals were injected with five different types of transformants and observed for tumor formation and growth. The results showed that all the five types of transformants produced tumors in nude mouse within 2–4 weeks after injection. None of the mice injected with non-transformed BALB/c-3T3 cells showed tumors, thus indicating the tumorigenicity of the morphologically

different transformants irrespective of their size and invasiveness.

Lungs were chosen as a target organ to detect the metastatic potential of the different transformants. It was observed that one of the cell lines had metastasized into lungs and localized in the alveolar region. No fibroblast cells were found in mice injected with other types of transformed cells. The reason why the other cell lines showed no metastasis could be the fact that the tumors appeared and grew so fast (2–4 weeks) that the animals had to be sacrificed because they started to suffer due to the size of the tumor. It is possible that there was not enough time for the tumors to disseminate to longer distances within a short period of time. In conclusion, our study indicates that these morphologically different transformed foci are capable of producing tumors. Therefore, all five types of foci should be scored as positive for the cell transformation assay.

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