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Tumorigenic and metastatic properties of "normal" and *ras*-transfected NIH/3T3 cells

(malignancy/organ colonization)

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ABSTRACT **To investigate the role of oncogene activation in the pathogenesis of malignant tumors, we have studied the tumorigenic and metastatic properties of NIH/3T3 secondary transfectants (designated A51) containing an activated c-Ha-*ras-1* gene derived from the human T24 bladder carcinoma cell line and compared them with untransfected NIH/3T3 cells. Whereas subcutaneous implantation of NIH/3T3 cells in the supraclavicular region produced palpable tumors that failed to metastasize, NIH/3T3 cells inoculated in the footpad gave rise to malignant tumors that metastasized to the lung. Under identical conditions and irrespective of the site of implantation, A51 cells formed rapidly growing primary tumors that produced pulmonary metastases. In an assay for experimental metastasis, intravenously injected NIH/3T3 cells gave rise to pulmonary nodules only at high cell inocula and in long-term survivors (90 days after injection). In contrast, A51 cells formed multiple lung tumor colonies detectable 14 days after injection. These results indicate that "normal" untransfected NIH/3T3 cultures contain subpopulations of cells that express malignant properties and that transfection of NIH/3T3 cells with activated c-Ha-*ras-1* accelerates formation of metastases.**

Tumors are classically defined as benign or malignant (1). Benign tumors are noninvasive growths that do not spread to distant organs. Unless located in a functionally vital site (e.g., brain), they pose little threat to the patient and usually can be removed surgically. In contrast, malignant neoplasms are readily invasive, metastasize to organs throughout the body, and eventually kill their host (1). The biochemical events that distinguish malignant from benign neoplasms remain unidentified. Work in several laboratories has implicated oncogene activation in the expression of tumorigenicity but the role of oncogenes in the pathogenesis of malignant versus benign tumors has received little attention. The NIH/3T3 cell line, although immortalized *in vitro*, is reportedly nontumorigenic and is widely used as a recipient cell line for detecting transforming gene sequences isolated from tumorigenic cell lines or tissues (2–6). For example, upon transfection with the activated *ras* gene, NIH/3T3 cells form foci in tissue culture, exhibit anchorage independence, and, in those experiments in which *in vivo* studies have been conducted, produce tumors in nude mice (6–10). However, the behavior of the tumors formed by transfected NIH/3T3 cells has not been rigorously evaluated and it remains unclear whether oncogene activation is an event associated strictly with the pathogenesis of benign neoplasms or whether activation is also an essential feature for expression of metastatic properties. We report that transfection with the activated c-Ha-*ras-1* gene accelerates the tumorigenicity and enhances the metastatic potential of NIH/3T3 cells. Our results also reveal, however, that untransfected NIH/3T3 cells are tumorigenic and metastatic under specific conditions. These findings emphasize the need for greater stringency in the assay of these properties in studies seeking to correlate oncogene activation with the acquisition of specific behavioral properties by malignant tumor cells.

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MATERIALS AND METHODS

Animals. Specific pathogen-free 3- to 4- week-old BALB/c nude mice were obtained from Life Sciences (St. Petersburg, FL). Mice were matched for age, sex, and body weight within each experiment.

Cells. A51 and control NIH/3T3 cultures were obtained from M. Wigler (Cold Spring Harbor, New York) and routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (10). The A51 cell line is a secondary transfectant obtained from transfection of NIH/3T3 cultures with DNA isolated from the T24 bladder carcinoma and contains the activated c-Ha-*ras* gene sequence (11).

Tumorigenicity and Metastasis. Tumorigenicity and spontaneous metastatic potential were assayed by inoculating mice with different cell doses in the footpad (i.m.) or the supraclavicular region (s.c.). Tumor size was monitored at the supraclavicular site every 2–3 days by caliper measurement. For studies on experimental metastasis, different numbers of cells were injected into the tail vein of nude mice. At autopsy the major organs of all animals were examined both grossly and histologically for evidence of metastases. Single sections were prepared from each organ except the lung, in which case multiple sections were examined.

Detection of Activated c-Ha-*ras* Oncogene and Human *Alu* Sequences. For preparation of DNA (12), cell monolayers established from primary tumors or metastatic foci were dispersed into phosphate-buffered saline (P_i/NaCl), pelleted, rinsed, resuspended in 10 mM Tris·HCl, pH 8.0/0.35 M NaCl/1 mM EDTA, lysed in 0.5% $NaDodSO_4$, and treated for 4–12 hr with Pronase (0.1 mg/ml) at 37°C. DNA was extracted with phenol, ethanol precipitated, and dissolved in 10 mM Tris·HCl, pH 8.0/1 mM EDTA. For transfer to nitrocellulose (13), the gel was soaked twice in 0.2 M HCl, twice in 0.5 M NaOH (each for 15 min), and once (1 hr) in 20× SSPE buffer (0.2 M sodium phosphate, pH 7.4/3.6 M NaCl/0.02 M EDTA) with addition of 1 M Tris/1.5 M HCl as necessary to maintain neutrality and then blotted for 2 hr in the same buffer. Hybridization to the human c-Ha-*ras* sequence was carried out at 72°C in 6× SSPE buffer with pSKT24, a derivative of the activated human c-Ha-*ras* cDNA plasmid pPS22 as probe. For the *Alu* blot, the probe was BLUR-8 and the hybridization was at 65°C. Molecular weight markers were pBR322 digested with either *Eco*RI (4.4 kilobases) or *Hin*cII (3.3 and 1.1 kilobases).

Table 1. Tumorigenicity and spontaneous metastasis of NIH/3T3 and A51 cells

Cell line	Route of inoculation	Cell inoculum $\times 10^{-6}$	Days to maximum tumor burden (range)*	Incidence of pulmonary metastasis
A51	i.f.p.	0.5	19 (18–25)	7/8
A51	s.c.	1.0	18 (16–20)	5/9
3T3	i.f.p.	0.5	106 (84–125)	2/5
3T3	s.c.	1.0	46 (32–50)	0/10

Monolayer cultures of NIH/3T3 or A51 cells were harvested with 0.25% trypsin/0.25% EDTA, washed once with Dulbecco's modified Eagle's medium/10% fetal calf serum and three times with P_i/NaCl, pH 7.2, and then suspended in P_i/NaCl at 10^7 cells per ml. Viability was greater than 90% as determined by staining with trypan blue. Nude mice were inoculated s.c. at the supraclavicular region or in the hind footpad (i.f.p.). Animals bearing supraclavicular primary tumors, 2–3 cm in diameter, were autopsied when moribund. The tumor-bearing limb of mice inoculated in the footpad was amputated when the primary tumors reached 1–1.5 cm in diameter. Mice were autopsied when moribund, usually 40 days after amputation of the limb.

*Mean number of days required for primary tumor to reach a diameter of 1–1.5 cm (i.f.p.) or 2–3 cm (s.c.).

RESULTS

Mice injected in the supraclavicular region with untransfected NIH/3T3 cells developed large primary tumors (approximately 2 cm in diameter) 32–50 days after injection (Table 1). No evidence of macroscopic or microscopic metastatic disease was detected in the organs of these animals. As a more sensitive indicator of metastasis, lungs from tumor-bearing animals were minced and injected i.p. into naive nude mice. No tumor growth was observed in these animals following observation for 65 days, supporting the conclusion that the original recipient animals were free of metastatic disease. In contrast, A51 cells inoculated at the supraclavicular site formed tumors that grew approximately twice as fast as those produced by control NIH/3T3 cells and also gave rise to histologically detectable lung metastases (Table 1). No metastases were detected in other organs.

In contrast to their behavior when implanted s.c. in the supraclavicular region, NIH/3T3 cells injected into the footpad produced slow-growing tumors that metastasized to the lung. To ensure that these metastases did not arise from accidental seeding of tumor cells into the circulation during the footpad injection, a similar number of control mice were inoculated in the footpad with an identical number of tumor cells and the footpad amputated 15 min later. Neither tumor growth nor metastases was observed in these animals after 160 days (data not shown). When A51 cells were inoculated into the footpad they produced rapidly growing tumors that formed macroscopic metastases in the lung (Table 1). No metastases were found in other organs.

In the organ colonization assay, A51 cells at each inoculum tested formed readily visible pulmonary tumor nodules as early as 14 days after injection. In contrast, experimental metastases were not detected in animals injected with NIH/3T3 cells until after 90 days (Table 2). Regardless of their site of growth (supraclavicular region or footpad), the implanted tumors and the pulmonary metastases produced by NIH/3T3 and A51 cells were histologically indistinguishable fibrosarcomas with no indication of a host response (Fig. 1).

Table 2. Experimental metastasis of A51 and NIH/3T3 cells

Cell inoculum	Frequency of detectable lung colonization (days after injection)		
	A51 cells	NIH/3T3	
10^4	4/6 (14)	0/2 (14)	1/4 (90)
10^5	6/6 (14)	0/2 (14)	1/3 (90)
10^6	6/6 (14)	0/2 (14)	2/3 (90)

NIH/3T3 and A51 cells were harvested as described in Table 1 and injected at the indicated cell concentrations into the tail vein of 3- to 4-week-old nude mice. The presence of metastases was assessed macroscopically and microscopically 14 and 90 days after injection.

The presence of the activated c-Ha-*ras-1* oncogene in A51 cells was a stable phenotypic trait both *in vitro* and *in vivo*. c-Ha-*ras-1* and human *Alu* sequences were identified in DNA extracted from stock A51 cells grown *in vitro* and from cultures established from an A51 footpad tumor and pulmonary metastases produced by these cells. Parallel experiments conducted on DNA prepared from cultured NIH/3T3 cells and an NIH/3T3 footpad tumor were negative for these sequences (Fig. 2).

DISCUSSION

The current investigation contains observations that are germane to future experimental efforts to implicate a causal role for oncogenes in the pathogenesis of neoplasia. First, control untransfected NIH/3T3 cultures contain subpopulations of cells that are tumorigenic as judged by their ability to produce tumors at two anatomical locations, to form pulmonary metastases from a primary tumor growing in the footpad, and to generate lung colonies following intravenous injection. These findings contrast with several reports in which control NIH/3T3 cells have been described as nontumorigenic (6–8). The reasons for this discrepancy are not clear but may reflect differences in animal suppliers, site of tumor inoculation, insufficient observation times for the test animals, and the use of different NIH/3T3 cell stocks. Two other groups (7, 10) have reported that NIH/3T3 cells transfected with DNA from nonneoplastic tissue were tumorigenic in nude mice, but similar observations were not recorded for naive NIH/3T3 cultures.

We consider it significant that spontaneous metastasis of control NIH/3T3 cells was detected only when a particularly sensitive assay for tumor cell spread (inoculation in the footpad followed by amputation of the primary tumor) was used and that test animals were allowed to live until 140–160 days after injection. No metastases were observed in nude mice bearing supraclavicular NIH/3T3 tumors. Previous assays of the tumorigenicity of NIH/3T3 cells have involved inoculation of cells into the flank or supraclavicular region and assay of tumor formation after only a few weeks (6–8, 14). Studies using the footpad as a site of injection, although commonly employed for other tumor cell lines (1), have not been reported using NIH/3T3 cells. Differences in the tumorigenicity and metastatic potential of the same tumor cell population inoculated at distinct anatomical sites are not unexpected since there are several reports demonstrating the importance of implantation site in the expression of metastatic properties (15–18). Culture history also appears to influence NIH/3T3 tumorigenicity. In studies not conducted in synchrony with those recorded here, we have observed wide interexperimental variation in the rate at which NIH/3T3 cells form primary tumors, particularly at the

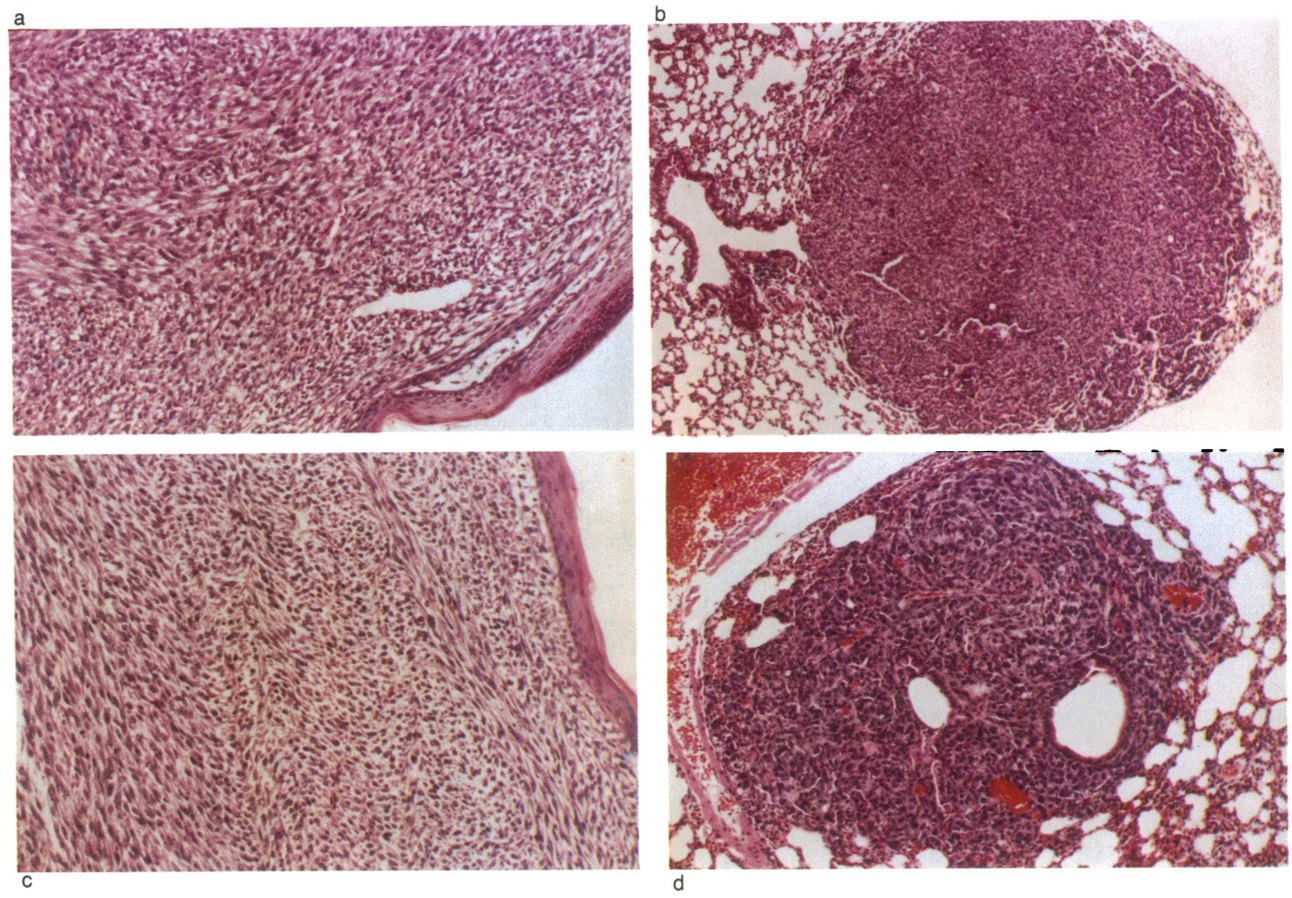

FIG. 1. Histology of primary tumors and pulmonary metastases produced by NIH/3T3 and A51 cells. (*a*) NIH/3T3 primary tumor (i.f.p.), 106 days after injection. (*b*) NIH/3T3 pulmonary metastasis, 50 days after amputation of primary tumor (i.f.p.). (*c*) A51 primary tumor (i.f.p.), 19 days after injection. (*d*) A51 pulmonary metastasis, 21 days after amputation of primary tumor (i.f.p.).

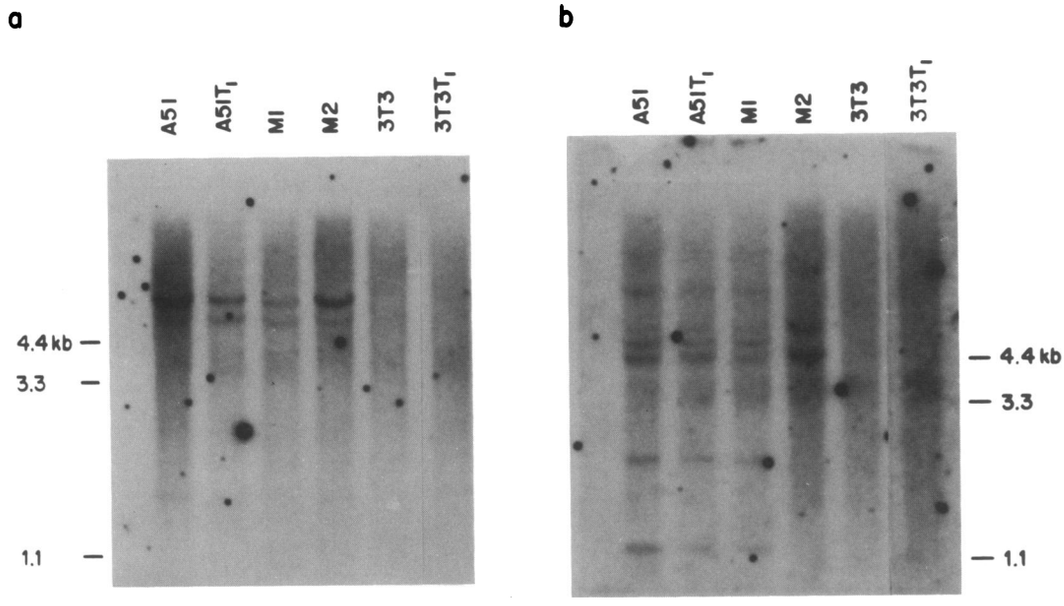

FIG. 2. Detection of the activated c-Ha-*ras-1* oncogene and human *Alu* repeat sequences in a footpad primary tumor $A51T_1$ produced by A51 cells and in pulmonary metastases M1 and M2 arising from the same lesion. 3T3, cultured NIH/3T3 cells; $3T3T_1$, NIH/3T3 footpad tumor. Primary tumors and pulmonary metastases were minced with scissors and treated with trypsin (0.25%) for 5 min at 37°C. Undissociated material was removed by gentle centrifugation (500 × *g*, 2 min) in Dulbecco's modified Eagle's medium/10% fetal calf serum. Single cells were harvested by further centrifugation (2000 × *g*, 5 min), washed three times with P_i/NaCl, suspended in Dulbecco's modified Eagle's medium/10% fetal calf serum and plated into T-75 tissue culture flasks. The cell lines were expanded *in vitro* for approximately one month to generate sufficient cells for analysis. DNA was extracted as described in *Materials and Methods*, and 20 μg was digested with *Eco*RI, electrophoresed through an 0.8% agarose gel, blotted, and hybridized with ^{32}P-labeled *ras* (*a*) or *Alu*-repeat (*b*) plasmids.

supraclavicular site (data not shown). Consequently, in this investigation, animals were not autopsied at a preselected time but instead were necropsied when moribund as a result of advanced neoplastic disease.

In two respects the finding that NIH/3T3 cells are malignant is not surprising. First, in transfection experiments, there is a frequently reported low background rate of foci formation in control NIH/3T3 cultures, usually attributed to "spontaneous transformation" (19–21). Perhaps it is this minority subpopulation of NIH/3T3 cells that is responsible for the formation by NIH/3T3 cells of malignant tumors *in vivo*. Second, BALB/c 3T3 cells have been referred to as tumorigenic (22, 23), but their metastatic properties have not been assayed.

A second noteworthy feature is that transfection of NIH/3T3 cells with the activated c-Ha-*ras* oncogene accelerates significantly the appearance of primary tumors and the development of metastases. When implanted at both the supraclavicular site and in the footpad, A51 cells produced tumors that grew much more rapidly than those formed by NIH/3T3 cells and the time required for the detection of A51 pulmonary metastases was also dramatically reduced. Analogous observations have been made by Land *et al.* (24) in describing the augmented tumorigenicity of rat embryo fibroblasts transfected with two oncogenes (*ras* and simian virus 40 large T) compared with cells transfected with *ras* alone. The mechanism is unknown. The proliferative capacities of NIH/3T3 and A51 cells were indistinguishable *in vitro* (data not shown) but this probably bears little relationship to their growth kinetics *in vivo*.

Finally, because untransfected NIH/3T3 cells express both tumorigenic and metastatic properties, the importance of the present observations in revealing the participation of oncogene activation in the pathogenesis of benign versus malignant tumors cannot be evaluated; however, the data do serve to emphasize that the NIH/3T3 cell line cannot be considered normal and suggest caution in the interpretation of transfection experiments in which NIH/3T3 is used as a recipient cell line.

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