ISOLATION AND CHARACTERIZATION OF A SERIALLY CULTIVATED, NEOPLASTIC, EPITHELIAL CELL LINE FROM THE N-NITROSO METHYLUREA INDUCED RAT MAMMARY ADENOCARCINOMA

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SUMMARY

A new in vitro model for human breast cancer is described. Derived from an N-nitrosomethylurea (NMU) induced rat mammary adenocarcinoma, this serially cultivated cell line has been demonstrated, by a variety of criteria, to be an authentic neoplastic, rat mammary epithelial cell line. The criteria used include morphological and growth characteristics; the presence of specific cell surface antigens; steroid hormone receptors; hormone responsiveness; casein production; karyotype and isoenzyme profile analysis; anchorage independent growth and oncogenicity. Inasmuch as the NMU cell line possesses high concentrations of glucocorticoid and androgen receptors, it may provide a useful model for study of the action of these hormones in human breast cancer. In addition, the NMU line may serve as a valuable in vitro model in which to assess the effects of a variety of endogenous and exogenous agents known to influence mammary tumor growth in vivo, including drugs, nutrients, and growth factors.

Key words: mammary tumor cell culture; validation; characterization.

INTRODUCTION

Since its introduction in 1975, the NMU induced mammary tumor has been regarded as an improvement over the better known dimethylbenz[a]anthracene (DMBA) tumor as a model for human breast cancer (1). The primary reasons are its close histological similarities to human breast cancer and that the NMU tumor has been reported to exhibit metastases to the lung, bone, and spleen coupled with hypercalcemia, syndromes commonly associated with human breast cancer (1). Although the results of several recent studies have cast doubt on the capacity of this chemically induced autochthonous mammary tumor to metastasize (2,3), other studies (4-6) suggest that it may, by virtue of its hormone response patterns, bear a close resemblance to human breast cancer. Specifically, although prolactin is the preeminent hormone governing the growth and development of the DMBA tumor (7,8), this hormone apparently plays a lesser role in the growth of the NMU tumor (4-6) and human breast cancer (9,10).

Because the environment of cultured cells can be controlled to a far greater extent than that of an in situ solid tumor, and because in vitro experiments can be performed without interference from host related factors such as immune reactions, established cultures of neoplastic mammary epithelial cells provide a valuable living model with which to investigate the direct cellular effects of in vivo growth regulating agents such as hormones, nutrients, and drugs. However, before such studies can be initiated, the authenticity of any putative mammary cell line must first be established. Accepted criteria for validation of a neoplastic mammary epithelial cell line are as follows: (a) accurate histopathological classification of the tumor of origin, (b) proof of interspecies specificity (isozyme profiles and karyotype), (c) evidence of epithelial nature (morphology/antigenic determinants), (d) evidence of organ specificity (synthesis of differentiated product/presence of hormone receptors), and (e) oncogenicity (11).

In a previous report (12) the isolation and characterization of normal and neoplastic cell lines
derived from virgin rat mammary fat pads and DMBA induced rat mammary adenocarcinoma were described. Accordingly, to provide an appropriate and reliable in vitro mammary tumor model system, the present report describes the biochemical and morphological properties of a new epithelial cell line derived from an NMU induced rat mammary adenocarcinoma.

**MATERIALS AND METHODS**

*Induction of mammary tumors.* Mammary tumors were induced in female Sprague-Dawley rats (Camm Res., Wayne, N J) by a modification of the procedure of Gullino et al. (1). N-Nitrosomethylurea obtained from Ash Williams Inc., Detroit, MI, was wet in 3% acetic acid and dissolved in distilled H2O at 10 mg/ml. The NMU solution was then injected once at 25 mg/kg rat into etherized animals on Day 50 of age. Injections were via the dorsal tail vein. Induction of tumors was assessed by palpation.

Approximately 5 months after NMU injection animals were killed by CO2 euthanasia. Mammary tumors were excised surgically under aseptic conditions, cleared of surrounding connective tissue, and cut into 2 mm³ segments for cell culture preparation. The remainder of the tumor was fixed in buffered formalin, sectioned, and stained with hemotoxylin and eosin (H&E) for histopathological examination. Animals were housed three to a cage in a temperature 74 ± 2 °C, light (14 h cycle), and humidity (50%) controlled room. The animals were fed the recommended NIH-07 diet (13). Both food and tap water were provided ad libitum.

*Tissue dissociation and isolation of neoplastic epithelial cells.* Cell isolation, consisting of both enzymatic and mechanical techniques, was essentially as described in (12). Tumor fragments were minced in a drop of Eagle’s minimal essential medium (MEM) with Earle’s salts and 2 mM L-glutamine (GIBCO, Grand Island, NY) using a sterile iris scissors. The finely minced tissue brei, containing both parenchymal and stromal elements, was then placed in a small Erlenmeyer flask containing 5 ml MEM, 0.1% crystalline collagenase (Worthington Biochemical Corp., Freehold, NJ), 4,000 U penicillin and 2,000 µg streptomycin, and incubated at 37°C in a gyratory water bath for 1 h. The cell suspension was then pipetted vigorously to further disperse cells and filtered through two layers of sterile gauze.

The filtrate was centrifuged at 100 xg to remove cell debris, and the resulting pellet was resuspended in 20 µg/ml each MEM supplemented with 20% fetal bovine serum (FBS) (GIBCO), and a hormone combination consisting of insulin (bovine pancreas, crystalline) (Sigma, St. Louis, MO), prolactin (NIH-P-S-12, ovine) obtained gratis from the National Institute of Arthritis, Metabolism and Digestive Disease, and hydrocortisone sodium succinate (Upjohn, Kalamazoo, MI). The dispersed cells and the cell clusters were plated out differentially. This is based on the idea that fibroblasts attach to the dish more readily than epithelial cells (14). Initially, the cells and cell clusters were plated into 16-mm tissue culture dishes (Falcon Plastics, Oxnard, CA) and incubated in a humidified 5% CO2 incubator at 37°C. At about 4 to 8 h intervals, the suspended cells in each dish were removed from the incubator and decanted to another dish. Fresh growth medium was added to the initial dishes. The process was repeated about 10 times. The initial dishes usually contain fibroblasts predominantly, but the cells plated out in the subsequent dishes are mostly epithelial cells. One week later the attached cells were subjected to differential trypsinization. The dishes were rinsed repeatedly with calcium-magnesium free trypsin solution (GIBCO) at 40°C. The cells remaining attached to the dishes were refed with fresh growth medium and allowed to grow. When confluency was attached, the cells were subjected again to differential plating as mentioned above, except that the supernatant solution was decanted to another dish after 1 h. This was repeated three times, and 24 to 48 h later spindle shaped cells, if present, were removed by mechanical means, leaving only the polygonal shaped epithelial-like cells. This was achieved by either piercing with a glass pipette or scraping with a rubber policeman. A pure epithelial culture was established in about 4 wk.

*Routine culture techniques.* Once growth was established the concentration of serum was lowered from 20 to 10%, the serum was changed from fetal bovine to calf (North American Biological, Miami, FL), and hormone supplementation was discontinued. To subculture, growth medium was aspirated and the cells washed once with Dulbecco’s phosphate buffered saline (PBS) (Ca**, Mg**-free) and dissolved with 0.05% trypsin and EDTA 0.02% (GIBCO). Treatment with trypsin was for 3 to 5 min at 37°C. Trypsin was then removed and the cells reincubated for 2 to 3 min. When 95% of the cells had detached...