Epithelial Component of Host-Tumor Interactions in the Orthotopic Site Preference of a Mouse Mammary Tumor

Fred R. Miller and Donna McInerney

E. Walter Albauchien Department of Immunology, Michigan Cancer Foundation, Detroit, Michigan 48201

ABSTRACT

Mammary tumors grow preferentially upon transplantation into intact mammary glands compared to cleared mammary fat-pads. Both sites provide stroma of the orthotopic site, but the latter lacks epithelial elements. If epithelium from enzymatically dissociated normal mammary glands is added to the tumor cells prior to injection into cleared fat-pads, tumor growth is comparable to that seen in intact mammary fat-pads. The growth-enhancing effects of normal mammary cells are not duplicated by normal kidney or liver cells. These results demonstrate that epithelial-epithelial interactions, as well as stromal-epithelial interactions, are associated with the enhanced growth of mammary tumor cells transplanted into orthotopic sites. The results also suggest that enhancement of tumor growth does not require intact tissue architecture.

INTRODUCTION

Many growth interactions have been described between mouse mammary tissues including those between fetal mammary mesenchyme and fetal mammary epithelium (1–6), between adult mammary fat-pad and fetal mammary epithelium (7), between fetal mammary mesenchyme and adult mammary epithelium (8–9), between normal mammary elements and preneoplastic lesions (10–14), between normal mammary elements and mammary tumor cells (15, 16), and between tumor sub populations from a single mammary tumor (17–21). Tissue interactions in the adult mammal gland are of at least two general types, one involving intraepithelial interactions and one involving fat-pad stromal-epithelial interactions. Evidence for the first is seen in the mutual inhibition exhibited between normal epithelium and preneoplastic lesions (12), the genotypically mosaic tumors described by Slemmer (16), and the preferential growth of tumor cells in the intact fat-pad versus the cleared fat-pad from which glandular tissue has been surgically removed (15). Evidence for the second is seen in the absolute requirement for a fat-pad for transplantation of normal mammary epithelium (13) and preneoplastic mammary lesions (22) and by the relative preferential growth of tumor cells in cleared fat-pads versus the subcutis (15).

Whether these growth interactions are mediated by soluble factors or by a mechanism requiring cell-to-cell contact, tissue architecture may be important for the interplay of regulatory factors in the mammary gland. DeOme et al. (10) and Ethier and Ullrich (11) found that intact glandular architecture is involved in preneoplastic regulation in vivo; the dispersion, prior to transplantation, of apparently normal mammary glands from virgin mice resulted in an increased rate of formation of HANs and subsequent tumor formation. To assess the role of mammary architecture architecture on tumor behavior in vivo, we

determined if reconstitution, in which cells from enzymatically dissociated mammary gland were mixed with tumor cells, reproduced the site effects on the growth of mammary tumors.

MATERIALS AND METHODS

Tumor. Mouse mammary tumor line 66 was derived from a spontaneously arising mammary tumor in a female BALB/cF3H mouse (23). The tumor line is maintained in vitro in DME-10, 2 mM glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and mixed nonessential amino acids (1 mM) at 37°C in a 10% CO2 air atmosphere. Cells for injection into mice were removed from monolayer cultures by treatment with 0.25% trypsin in 0.05% EDTA, washed once, and suspended in HBSS for injection (20 μl injection volume).

Mice. An inbred BALB/c mouse colony was established at the Michigan Cancer Foundation with mice Cesarean derived from BALB/cF3H mice purchased from the Cancer Research Laboratory, University of California, Berkeley, CA. Mice used for experiments described here were supplied by this Michigan Cancer Foundation breeding colony.

Clearing and Fat-Pad Injections. Three-wk-old female mice were anesthetized with 65 mg of pentobarbital per kg of body weight. The left No. 4 mammary fat-pads were cleared; i.e., the epithelial elements were surgically removed (22). The wounds were closed with stainless steel clips which were removed 2 wk later. Fat-pads were injected at 14 wk of age. Mice were anesthetized, and a small incision was made near each No. 4 gland so that cell injections could be verified visually. The wounds were closed with steel clips which were removed 2 wk later. Tumors were measured twice weekly in two perpendicular dimensions, and the tumor volume was calculated by the formula (a x b2 x π/2), where b is the smaller of the two measured diameters.

Enzymatic Tissue Dissociation. Liver, kidney, and normal mouse mammary glands were isolated from midpregnant mice by treatment of minced tissues with 1 mg/ml of collagenase type III (Worthington Biomedial Corp., Freehold, NJ) and 1 mg/ml of hyaluronidase type I (Sigma Chemical Co., St. Louis, MO) in Waymouth's medium (20 ml/g of tissue) for 90 min at 37°C in a shaking water bath followed by a 15-min treatment with 12.5 mg/ml of protease type IX (Sigma) (10 μg/ml of tissue) at 37°C in a shaking water bath. Cells were washed 3 times by centrifugation in HBSS and counted with a hemacytometer.

These cells were then mixed with tumor cells for injection. In one experiment we used an alternative procedure to prepare mammary cells enriched for epithelial cells. Minced mammary tissues were incubated for 45 min at 37°C in a HBSS solution (12.5 μg/ml of tissue) with 40% fetal calf serum, 1.77 mg/ml of collagenase type III, and 0.04 mg/ml of DNase type IV (Sigma). The tissue was then washed 3 times by centrifugation in HBSS and allowed to sediment at 1 x g at 4°C for 15 min in HBSS. The supernatant was discarded, 1 x g sedimentation repeated, and the supernatant again discarded. Cell aggregates in the sediment were layered over a 5-ml fetal calf serum cushion and centrifuged at 20 x g for 1 min. The pellet was suspended in DME-10 further supplemented with 10% NCTC 109 medium (M. A. Bioproducts, Walkersville, MD), 20 mM glucose, bovine crystallin insulin (8 μg/ml), and 1 mM oxaloacetic acid and plated in tissue culture dishes for 1 h at 37°C in 10% CO2. Nonadherent cell aggregates were cultured in T-25 flasks at a concentration of approximately 5 x 106 cells/ml in this medium for 7 days. These cells were removed with 0.25% trypsin in 0.05% EDTA, washed in HBSS, and mixed with tumor cells for injection. Cleared fat-pads in 4 mice were injected with 5 x 106 mammary cells alone to determine the capacity of these cultured cells to reconstruct the normal ductal structure in the mammary fat-pad.
Whole Mount Preparation. Twelve wk after injection, fat-pads previously cleared and injected with cultured mammary epithelial cells were removed, fixed, and stained as follows. Skin with attached mammary gland was fixed in 15% formalin for 24 h before the gland was peeled from the skin. Fat was removed in four changes of acetone followed by three changes in 95% ethyl alcohol. The glands were stained with hematoxylin overnight, washed in running water, dehydrated with ethyl alcohol and toluene, and stored in methyl salicylate.

RESULTS

Fig. 1 illustrates the growth of tumors after injection of $1 \times 10^3$ line 66 cells into contralateral cleared and intact No. 4 mammary fat-pads of individual mice. In this experiment, enhanced tumor growth in intact fat-pads clearly occurred in seven of the ten mice, whereas a tumor arose first in the cleared fat-pad in only one mouse. In one animal a tumor never appeared in the cleared fat-pad.

The preferential growth of tumor 66 in intact mammary glands was masked by the addition of normal mammary epithelial cells. Tumors arose in cleared glands just as rapidly as in intact glands if normal mammary epithelial cells were mixed with the tumor cells prior to injection (Fig. 2). The median times for tumors to reach a size of 100 mm$^3$ were nearly identical for those initiated by $1 \times 10^3$ line 66 cells alone injected into intact glands (24.8 days) and for those initiated by $1 \times 10^3$ line 66 cells mixed with $6.25 \times 10^3$ dissociated normal gland cells injected into either intact glands (25.1 days) or cleared fat-pads (24.8 days). Tumors initiated by $1 \times 10^3$ line 66 cells alone injected into cleared fat-pads took significantly longer to reach a size of 100 mm$^3$ (median, 35.4 days). The ability of enzymatically dissociated normal mammary epithelial cells to reconstitute cleared fat-pads was dose dependent (Fig. 3).

In order to distinguish a specific effect of mammary epithelium on tumor growth in a fat-pad from a nonspecific effect, we compared mammary epithelium to freshly dissociated kidney and liver cells. As illustrated by Fig. 4, primary cultures from pregnant mouse mammary glands were far more stimu-
to interact and influence organogenesis is typically a well-defined period during fetal development. Stromal-epithelial interactions in the mammary gland are demonstrable from the time that mammary anlagen is identifiable in the 11-day mouse embryo, and progressive changes in epithelial-mesenchymal interactions occur rapidly in the embryo (1-9). The embryonic mesenchyme both induces organogenic differentiation in the fetus and, in the male, mediates the regression of the epithelium in the presence of testosterone (2-4, 6). A reciprocal regulation of differentiation occurs such that mammary epithelium induces the expression of androgen receptors in the mesenchyme in both males and females, and then the mesenchyme, in response to testosterone, induces the regression of the mammary epithelium in the male (24, 25). The adult fat-pad retains mesenchymal inductive activity, capable of inducing both embryonic salivary and mammary epithelium to undergo their respective characteristic branching patterns in vivo (7). Adult mammary epithelium retains competence (i.e., ability to respond to induction), responding to both embryonic salivary and mammary mesenchyme (8). At Day 14 in utero, two mesenchymal condensations are identifiable, the mammary mesenchyme surrounding the mammary epithelium and the underlying fat-pad mesenchyme. The two types of fetal mammary bud mesenchyme, mammary and fat-pad, behave differently when transplanted into adult female fat-pads. The mammary mesenchyme induces ductal hyperplasia and itself becomes "fibroblastic" connective tissue, whereas the fat-pad mesenchyme induces normal gland architecture and becomes fat cells (9). The retention of inductive mesenchyme and competent epithelium in the adult mammary gland may be associated with the ability of the mammary gland to undergo repeated, hormonal-dependent proliferation and involution of the terminal ductal lobuloalveolar units.

Alterations in tissue interactions may be important in the development of preneoplastic hyperplasia and the subsequent progression of such lesions to a malignant state. That mesenchymal-epithelial interactions could be important in mammary tumorigenesis is shown by the reports of DeCosse et al. (26) and Sakakura et al. (27). DeCosse et al. (26) found that a mouse mammary tumor line could be induced to differentiate when cultured transferrin from mouse mammary mesenchyme in vitro. Sakakura et al. (27) found that, when transplanted into cleared fat-pads of mammary tumor virus-positive C3H mice, the ductal-alveolus nodules formed by the implantation of mouse mammary mesenchyme into intact fat-pads were 12 times more likely to form tumors than were normal multiparous mammary gland tissues.

Intraepithelial interactions clearly occur between normal mammary epithelium and both preneoplastic and neoplastic mammary epithelium. Prenaoplastic HANs are inhibited by mammary epithelium in the normal gland (12, 13). The essential role of intact glandular architecture in the homeostatic control of preneoplastic mammary epithelium is demonstrated by reports that the dispersion and transplantation of apparently normal mammary glands result in an increased rate of HAN and tumor formation (10, 11). In addition, enzymatically dispersed line D1 HAN cells were more tumorigenic than D1 HAN tissues transplanted as architecturally intact pieces by trocar (28). Medina et al. (28) found that the addition of dissociated normal mammary cells to the dissociated D1 HAN cells abrogated the affect of dispersion; i.e., D1 HAN cells mixed with normal mammary cells were no more tumorigenic than undispersed D1 HAN pieces and were less tumorigenic than dispersed D1 HAN cells alone. However, in the latter experiments the dissociated normal mammary epithelium reformed the typical ductular architecture of the mammary gland within 10 to 16 wk, whereas the median time for the appearance of tumors was 6 to 8 mo. Therefore, it is difficult to assess the
role of tissue organization since architecture was lacking initially but was reestablished by the time tumors began to form. In our experiments, primary cultures of normal mammary epithelium sporadically formed normal ductal trees within 12 wk of implantation, whereas mixtures of tumor cells and normal mammary cells produced tumors within 2 to 4 wk. Furthermore, lethally irradiated mammary epithelial cells are nearly as active as proliferating cells in enhancing tumor growth (data not shown). Histology sections of tumors growing in intact mammary glands reveal the presence of apparently normal ductal structures. No such structures were detected in sections of tumor line 66, which grows as a "poorly differentiated neoplasm (23), transplanted into cleared fat-pads whether or not tumor cells had been mixed with normal mammary epithelium.

Despite this apparent role of tissue architecture in surveillance in the mammary gland, our data demonstrate the mammary tumor growth in the mammary gland is responsive to the presence of normal mammary epithelium irrespective of the level of tissue organization.

REFERENCES


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