Interactions of Normal, Dysplastic, and Malignant Mammary Epithelial Cells with Fibronectin in Vivo and in Vitro

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ABSTRACT

Previous studies have demonstrated that normal and malignant mouse mammary cells are indistinguishable in many surface-related properties that often denote transformation of other cell types such as fibroblasts. In the present investigation, the interactions of normal, dysplastic, and malignant mammary epithelial cells with fibronectin in tissues and cultures were examined by indirect immunofluorescence. Cells lining the lumina of ducts and alveoli in normal and dysplastic mouse and human mammary tissues abutted a layer of fibronectin along their basal surfaces that included the region of the basement membrane and the underlying stroma. Moreover, double staining for keratin and fibronectin revealed that myoepithelial cells were surrounded by the matrix protein. In contrast, tumor cells in adenocarcinomas and ductal carcinomas were not directly associated with fibronectin. The accumulation of fibronectin in primary cultures prepared from mouse mammary tissues paralleled the distribution seen in vivo. A matrix of fibronectin formed beneath normal and preneoplastic mammary cells within 4 to 6 days after plating, whereas tumor cells were negative, regardless of the age or density of the culture. This correlation with in vivo results did not extend to cells of established mammary tumor culture lines which readily accumulated pericellular networks of fibronectin. Addition of exogenous fibronectin to primary cultures enhanced formation of a basal matrix by normal cells but had no effect on the negative status of the tumor cells. The results indicate that mammary tumor cells in tissues and in primary cultures have an altered capacity to interact with fibronectin. However, this alteration is not necessarily expressed by established mammary tumor cell lines.

INTRODUCTION

A basic tenet of cancer biology is that changes in cell surface structure and function contribute to the abnormal behavior of tumor cells. However, studies in our laboratory and in others have shown that malignant mouse mammary cells in primary cultures closely resemble normal mammary cells in several surface-related features. The 2 cell types are similar in size and shape (3, 12, 18, 29, 35), surface topography (4, 24), formation of intercellular junctional complexes (29), contact inhibition of cell movement (12, 18, 35), reactivity with lectins (2, 4, 24), expression of mammary-specific surface antigens (9), and organizational and functional aspects of microtubule and microfilament networks (3, 4).

Transformed fibroblasts, in contrast, usually possess numerous alterations in surface properties which distinguish them from their normal counterparts (19, 26). One of the most dramatic changes often accompanying fibroblast transformation is the reduction or loss of fibronectin, a major surface constituent of the normal fibroblast (reviewed in Refs. 19, 34, and 40). The absence or decrease of this extracellular matrix protein has been correlated with expression of many features commonly associated with the transformed phenotype of mesenchymal cells, including alterations in cell morphology, surface topography, lectin-induced agglutinability, disorganization of microfilament bundles, and decreased adhesiveness to substrata. Conversely, the addition of fibronectin to transformed cells has been found to restore many of these features to their normal states (1, 38, 39, 41). However, there is conflicting evidence regarding the expression of fibronectin by transformed fibroblasts in culture and their tumorigenicity in appropriate host animals. While most highly tumorigenic fibroblasts lack fibronectin (10, 16, 19), there are some that display abundant matrix formation by this protein (13, 21, 22).

The minimal surface differences found between normal and neoplastic mouse mammary cells make the relationships and interactions of these cells with fibronectin particularly interesting. Recent investigations on the distribution of the matrix protein in cultures of human and rodent mammary cells have produced disparate results, however. Pearlstein et al. (28), using lactoperoxidase iodination for detection of fibronectin, reported the presence of the protein in explant cultures of normal rat mammary cells but its absence in 2 cell lines derived from mammary carcinomas. Utilizing serially passaged human cell lines and immunofluorescence with antibodies to fibronectin, Smith et al. (32) found that several types of normal epithelial cells and cells from primary carcinomas, including a single breast cell line of each phenotype, readily developed a matrix of fibronectin, whereas cells obtained from metastatic lesions were negative for the surface protein. On this basis, the authors suggested that an absence of fibronectin associated with epithelial tumor cells in culture might be related to an increased aggressiveness and metastatic potential. In contrast, Yang et al. (42), who also used immunofluorescence, were unable to detect fibronectin in primary cultures of either normal or malignant mammary cells from humans and mice.

The interpretation of these conflicting results therefore raises important questions. Are the differences between the studies due to the types of cultures used (cell lines versus primary cultures)? Which cell types in mammary tissues are associated with fibronectin and in what manner? Which in vitro system most closely reflects that situation? Does the ability (or inability) of a cell line to interact with fibronectin represent the selection of a minority population from the original tumor cell population or an adaptation of the cells to cultivation in an artificial environment? In order to investigate these points, we have exam-
ined the association of mammary cells with fibronectin in various normal and pathological tissues and in primary and long-term cultures.

MATERIALS AND METHODS

Cell Lines. The established mouse mammary tumor cell lines ESD/BALB-CL3, MTV-L/BALB-CL2, and DMBA/BALB-CL2 were a gift from Dr. Janet Butel, Baylor College of Medicine. These are clonal lines derived from mammary adenocarcinomas developing in BALB/c mice treated with different agents [17β-estradiol, mouse mammary tumor virus, and 7,12-dimethylbenz(a)anthracene, respectively] (8). The growth characteristics of these lines in vitro and in vivo have been reported (8). The ductal and NMuMG cell lines were provided by Dr. Daniel Medina, Baylor College of Medicine. The ductal line was established from a primary ductal carcinoma arising in a BALB/c mouse treated with 7,12-dimethylbenz(a)anthracene (14). The NMuMG line originated from the normal mammary gland of a NAMRU mouse but produces benign cystic adenomas when tested in nude mice (27).3 The Nil 8 hamster cell line was obtained from Dr. Richard Hynes, Massachusetts Institute of Technology. All lines were propagated by serial subculturesevery 3 to 4 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 5 μg insulin per ml (Sigma Chemical Co., St. Louis, Mo.), 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and 50 μg gentamicin per ml. These cultures consisted primarily of epithelial cells, with about 5% or less of the population being fibroblasts. Verification of the type of cells growing in the cultures by transplantation studies in syngeneic mice has been reported elsewhere (3).

Antisera. Rabbit antiserum against hamster cell surface fibronectin was a gift from Dr. Richard Hynes. Its preparation and characterization have been published (23). Guinea pig antiserum against total human keratin proteins of the stratum corneum was prepared by the procedure described in Ref. 33 and was generously provided by Dr. Tung-Tien Sun, Johns Hopkins Medical School. In double-immunodiffusion tests, this antiserum reacts strongly with the keratin proteins with molecular weights of 65,000, 63,000, and 55,000, but not with the polypeptide with a molecular weight of 46,000.5 Rhodamine-and fluorescein-conjugated goat anti-rabbit immunoglobulin antisera and fluorescein-tagged goat anti-guinea pig immunoglobulin antisera were purchased from Cappel Laboratories, Inc., Cochranville, Pa.

Indirect Immunofluorescence. Sections of mouse and human mammary tissues were cut at a thickness of 4 to 6 μm on an IEC cryostat (IEC, Boston, Mass.) and collected on glass coverslips. Two or more sections of each specimen were stained with toluidine blue and eosin and examined for histological classification. Other sections were air dried for 30 to 60 min at 37°, fixed for 10 to 15 min in absolute acetone at —20°, air dried again, and hydrated in HBSS. They were then incubated at 37° with anti-fibronectin antiserum (diluted 1:30) for 45 min, followed by gentle washing in several changes of HBSS for a total of 30 min at room temperature. The sections were next incubated for 45 min at 37° with fluorescein-conjugated secondary antiserum, washed as before in HBSS, and finally mounted in glycerol:HBSS (9:1, pH 9.0) on glass slides. Cultured cells attached to glass coverslips were washed twice in HBSS, fixed for 30 min in 3% formaldehyde at room temperature, rinsed in HBSS, and in some cases treated with absolute acetone for 7 min at —20°. Incubation with the primary and secondary antisera was then conducted as described for the tissue sections. Controls were treated in the same manner either with rabbit preimmune serum or with HBSS substituted for the primary antiserum, and they always gave negative results. For double staining to detect fibronectin and filaments of keratin-like proteins simultaneously, cultured cells were fixed for 5 min in absolute methanol at —20°, followed by 5 min in acetone. Tissue sections were fixed as before in acetone. They were then incubated with a mixture of the rabbit antiserum to fibronectin and the guinea pig antiserum to keratin proteins at final dilutions of 1:30 each. After washing, the cells or sections were treated with a mixture of rhodamine-conjugated goat antibodies to rabbit immunoglobulins and fluorescein-conju-

3 D. Medina, personal communication.
4 The abbreviations used are: HAN, hyperplastic alveolar nodule; HBSS, Hanks' balanced salt solution.
5 T-T. Sun, personal communication.
gated goat antibodies to guinea pig immunoglobulins. Controls in these experiments included incubating the tissue sections and cell cultures with each primary antiserum individually in conjunction with the secondary antiserum for the other primary antiserum. Under these conditions, no staining was observed. The stained tissue sections and cultured cells were examined in a Zeiss Photomicroscope Ii equipped with epifluorescence optics, and photographs were taken on Kodak Tri-X film.

**Incubation of the Mammary Cells in Culture Fluid from Nil 8 Cells.** Medium of Nil 8 cultures that had just reached confluency in 25-sq cm flasks (4 days postsubculturing) was decanted and replaced with 4 ml of fresh Dulbecco’s medium containing 5% fetal calf serum. After the cultures had incubated for 48 hr at 37°C, this medium was collected, centrifuged at 2000 × g, followed by filtration through an 0.22-μm filter (Millipore Corp., Bedford, Mass.) to remove any cells, and finally mixed with an equal volume of fresh medium containing 5% fetal calf serum. The diluted Nil 8 medium was then added to 2-day-old primary cultures of mouse mammary cells growing on glass coverslips that had been washed 3 times in serum-free Dulbecco’s medium. Samples were processed for indirect immunofluorescence after 24, 48, and 120 hr of treatment. The medium of cultures to be tested at 120 hr was replaced with freshly prepared Nil 8 medium at the end of the first 48 hr.

**RESULTS**

**Fibronectin in Mouse and Human Mammary Tissues.** Several different types of human and mouse mammary tissues were surveyed by immunofluorescence to determine the association of the epithelial cells with fibronectin (Table 1), and the same results were obtained with both species. The histology of normal mouse mammary gland is shown in Fig. 1 as a reference for the structures and cells mentioned in the dark-field photomicrographs. Control sections treated with rabbit preimmune serum were always negative (Fig. 2). In normal mammary gland of virgin, pregnant, and lactating females stained by immunofluorescence with the rabbit anti-fibronectin antiserum, no fluorescence was associated in fibronectin.

In primary malignant mammary neoplasms, fibronectin was confined to stromal areas. No staining was detected within the clusters of tumor cells. This was true in mice for primary adenocarcinomas developing from the HAN (Fig. 8a) and in human primary ductal carcinomas (Fig. 8b). The same picture was seen in human metastatic lesions of breast cancer (Fig. 8c) and in the murine MXT 3590 transplanted ductal carcinoma (Fig. 8d).

**Deposition of Fibronectin in Mammary Cell Cultures.** The studies on the tissue sections suggested that malignant mammary tumor cells differed from normal mammary cells and from cells of benign mammary lesions in that the former cell type usually had no direct association or contact with fibronectin. To determine if this difference extended to cells cultured in vitro, normal, preneoplastic, and malignant mouse mammary cells grown in primary cultures were tested for the presence of fibronectin and were compared with each other and with cells of 5 established mammary tumor cell lines. Only mouse tissues were used in these investigations because of the ease of growing them in culture and because the in vivo growth potential of each type of culture has been confirmed in transplantation experiments (3).

The cells in primary cultures were examined for fibronectin at various times postseeding up to 1 week. In living cultures or in cultures fixed only with formaldehyde prior to staining with the anti-fibronectin antiserum, no fluorescence was associated with the upper surfaces of the epithelial cells in any of the cultures (Fig. 9). The lack of staining was not altered by the length of time the cells were in culture or by the density of the cultures. The few stromal fibroblasts in the cultures began to
display a fibrillar matrix of fibronectin which is characteristic of that cell type (19) within 2 days. This pericellular accumulation continued to increase in density around the cells with time (Fig. 9, a and c), and thus provided a useful positive control for the technical accuracy of the staining method. In contrast to the cells in the primary cultures, cells of 4 malignant mammary tumor lines all had fibrils of fibronectin visible within 2 days after subculturing. These rapidly increased in number and by 4 days had formed a moderate to heavy 3-dimensional scaffolding around most of the cells in the cultures (Fig. 10, a to d). Cells of the NMuMG line, however, which produces benign cystic adenomas in vivo (27), were negative until 3 to 4 days postplating. At this time, small speckles of fluorescence began appearing along cell-cell borders (Fig. 10e). Although these speckles elongated into short fibrils and became more numerous during the next 2 to 3 days, they remained confined to areas adjacent to cell-cell contacts and never developed into the elaborate networks seen with the malignant cell lines (Fig. 10f).

When the primary cultures were treated with acetone after formaldehyde fixation to facilitate penetration of antibodies into and beneath the cells (38), no fibronectin was seen around any of the epithelial cells in the primary cultures for the first 3 days postseeding. However, by 4 days, intensely fluorescent strands of material appeared underneath the epithelial colonies in the region between the cells and the substratum (Fig. 11a). By 6 to 7 days, an elaborate fibrillar matrix had developed and spread beneath most of the epithelial colonies (Fig. 11, b and c), although interestingly, most of the cells in the epithelial islands were devoid of intracellular staining. The basal accumulation of fibronectin occurred in both the normal and preneoplastic cultures but was not detected in the tumor cell cultures established either from the primary HAN-derived adenocarcinomas or from the MXT3590 transplanted ductal mammary tumor (Fig. 11, d and e). Many fibroblastic cells in all the primary cultures contained brightly fluorescent intracellular granules as well as pericellular fibrils. The same was true for cells of the 4 malignant lines. Cells of the NMuMG line, however, did not contain cytoplasmic granules or basal fibrils.

Localization of Cytoplasmic Fibronectin and Keratin-like Filaments in Myoepithelial Cells. As no evidence of intracellular fibronectin was seen within the normal and preneoplastic epithelial cells, the source of the fibronectin that was deposited beneath these cells was unclear. On the basis of the recent report by Hayman and Ruoslahti (17), at least part of this material probably was derived from serum. Moreover, since intracellular granules were present in many fibroblastic cells, fibronectin produced by these cells also may have contributed to the matrix formation beneath the epithelial cells. Because myoepithelial cells in the tissue sections appeared to be enveloped by fibronectin, we attempted to determine if this cell type might provide a third source of the glycoprotein. When primary cultures derived from mammary tissues are stained with the guinea pig antisera to human keratin proteins, approximately 15% of the cells, presumably myoepithelial cells, are positive for the filaments. We therefore conducted a double staining procedure using the techniques described earlier for the tissue sections to determine if granules of intracellular fibronectin and the keratin-like filaments were localized in the same cells. As shown in Fig. 12, several cells displaying both cytoplasmic components were observed in cultures of normal mammary cells. Neither structure was trypsin sensitive. There were also cells, presumably fibroblasts, which contained only perinuclear granules of fibronectin but no filaments that reacted with the antisera to keratin protein, as well as cells which possessed the keratin filaments but did not have detectable intracellular fibronectin.

Attempts to Reconstitute Surface Fibronectin of Mammary Tumor Cells. Several explanations might account for the lack of basal deposition of fibronectin in the primary cultures of tumor cells. One possibility is that the tumor cells have decreased numbers of or structurally altered receptors for interacting with fibronectin. Alternatively, the receptors on the cells might be adequate, but the levels of fibronectin in the cultures were insufficient to permit accumulation of the glycoprotein by the cells. We attempted to test the latter possibility by providing the cultures with an abundant exogenous supply of fibronectin. Confluent cultures of the Nil 8 hamster cell line produce and release large quantities of biologically active fibronectin into the culture medium (23). Primary cultures of normal and neoplastic mammary cells 2 days postseeding therefore were incubated for various time periods with culture medium from Nil 8 cells, as described in “Materials and Methods.” In cultures of normal mammary cells treated with the Nil 8 culture fluid, increased fibril deposition and meshwork formation was evident within 24 hr, both beneath the epithelial cells and around the fibroblasts. Control cultures fed with regular medium were just beginning to exhibit thin wisps of fluorescent material at this time. By 48 hr, the basal matrices associated with the normal epithelial cells in the treated cultures were dense enough to obscure most cell outlines (Fig. 13a). The control cultures did not achieve this level of matrix accumulation until 2 to 3 days later (compare Fig. 13a with Fig. 11, a and b). In contrast, the tumor cells incubated in Nil 8 fluid remained negative during the entire 120-hr period of treatment, although fibroblasts in these cultures rapidly formed their typical fibril networks (Fig. 13b). Thus, these experiments suggest that the tumor cells may have a reduced capacity to interact with fibronectin, even when it is plentiful.

DISCUSSION

Results of the present study provide evidence that mammary carcinoma cells differ from normal and dysplastic mammary epithelial cells in their interactions with fibronectin both in vivo and in vitro. The distribution of fibronectin was essentially identical in mouse and human mammary tissues. The similarities between the murine HAN and the human samples of fibrocystic disease are especially notable. The mouse D2 HAN line has been in serial transplantation for over 10 years (25), whereas all of the lesions in the human breasts were, of course, primary occurrences. In addition, an excellent correlation was found between the association of this glycoprotein with epithelial cells in the mammary gland and its association with these cells in primary cultures. The surface polarity exhibited by normal ductal and alveolar cells in mammary tissues was maintained by these cells in culture in that only their basal surface was involved in interacting with fibronectin. The same situation was found with cells of the murine HAN neoplasia both in

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vivo and in vitro. This implies that the receptors for the glycoprotein on these cells are confined to the region of the cell surface which participates in cell-substratum attachment. In contrast to the normal and dysplastic cells, mammary tumor cells, whether from a primary or transplanted neoplasm, had no detectable association with fibronectin in either environmental setting. The basis for this lack of interaction has not been determined.

The correlation between in vivo and in vitro results did not include malignant mammary tumor cell lines which developed extensive matrices, although they were originally derived from the same type of mammary tumors as were the tumor cells in the primary cultures. This ability of the tumor cell lines to interact with fibronectin therefore may reflect an adaptation of the cells to long-term culture or to the selection of a fibronectin-positive subpopulation from the initial tumor mass. A similar inconsistency between the distribution of fibronectin in tissues and its expression by a cell line was reported by Quaroni et al. (31). They demonstrated that epithelial cells in the rat small intestine were in contact with the matrix protein only along their basal surface, whereas fibronectin was limited to regions of cell-cell contact in cultures of an established intestinal epithelial cell line.

The picture obtained from sections of normal tissues implied that myoepithelial cells were enveloped by and contained fibronectin. Synthesis and maintenance of the basement membrane in epithelia are considered to be primarily functions of the epithelial cells rather than of the stroma (5, 7, 30). Production of fibronectin by myoepithelial cells would support this concept, and the location of these cells between the basement membrane and the cells facing the lumen make them ideally situated for such a role. It is thus tempting to speculate that one of the specialized functions of this cell type might be to produce and maintain a scaffold of fibronectin which helps anchor them and the ductal and alveolar cells to the basement membrane. To our knowledge, this is the first example of fibronectin occurring on the epithelial side of the basement membrane. The limits of resolution of light microscopy, however, necessitate a more detailed study of the mammary tissues by immunoelectron microscopy in order to establish the exact distribution of fibronectin. Results of the double staining experiments with cultures of normal cells lend further credibility to the idea that myoepithelial cells, in addition to stromal fibroblasts, may synthesize the glycoprotein. At the same time, despite the eventual accumulation of fibronectin in untreated cultures of normal and preneoplastic cells, it is probably not the main mediator of adhesion for any of the mammary cells in culture, regardless of their neoplastic state. Normal, preneoplastic, and neoplastic mammary cells all attach very firmly to glass or plastic substrata long before any fibronectin is detectable (data not shown). A more definitive determination of the roles of the various mammary cells in the synthesis and utilization of fibronectin will require separating populations of each cell type for radiolabeling studies.

Myoepithelial cells and fibroblasts together account for a maximum of 20 to 30% of the total cell population in culture. Assuming that these 2 cell types produce and release fibronectin, their combined output along with the small amounts present in serum still may result in a relatively low level of the glycoprotein in the medium. This in turn might explain the slow accumulation of fibronectin on the surface of the epithelial cells in untreated normal and preneoplastic cultures. Supplying an exogenous source of fibronectin accelerated the matrix formation by these 2 cell types but had no effect on the lack of accumulation by the tumor cells in primary cultures. The present experiments do not eliminate the possibility that the mammary tumor cells have adequate receptors but that fibronectin was being destroyed by protease activity. However, this seems unlikely, because fibroblasts juxtapositional to the tumor cells in the cultures displayed an increased rate of meshwork development in the presence of added fibronectin equivalent to that which occurred in the normal cultures. Although the existence of a membrane-bound protease in the tumor cells is possible, further experiments will be needed to determine the actual basis for the decreased surface deposition of fibronectin by the mammary tumor cells.

Several features about the lack of association of fibronectin with the mammary tumor cells are different from those seen with fibroblastic transformants. For instance, the fibronectin matrix of the latter cell type can be reconstituted by cultivation in the presence of large amounts of the protein (1, 39, 41). In addition, the loss of fibronectin by transformed fibroblasts is usually accompanied by an altered morphology and a decrease or disorganization of microfilament bundles (20, 37). We have previously shown that mammary tumor cells in primary cultures possess bundles of microfilaments as well as microtubule complexes that are comparable in quantity, distribution, and function to those of normal and preneoplastic mammary cells (3, 4). Moreover, these 3 types of mammary cells are indistinguishable on the basis of several surface properties, including morphology (3, 12, 18, 29, 35), surface topography visualized by scanning electron microscopy (4, 24), ability to form intercellular junctional complexes (29), and interaction with lectins such as concanavalin A and wheat germ agglutinin (2, 4, 24). At the same time, cells of the established mammary tumor lines are distinctly different from cells in the primary cultures in all of these features except the cytoskeleton (2, 3, 24). The failure of the tumor cells to accumulate fibronectin is thus one of the few surface differences that have been identified among the 3 types of mammary cells in primary cultures.

Our findings on the mammary cells in culture show some similarities with the results of Smith et al. (32) and Yang et al. (42), but they also differ from both in certain important respects. Some of the differences in the 3 studies may stem from the different types of cultures used. Smith et al. (32), using serially passaged human breast cell lines, found that cells derived from normal tissue and a primary neoplasm formed a fibronectin matrix, while cells from a metastatic lesion did not. Yang et al. (42) reported that normal and malignant human breast cells in primary cultures were both negative for fibronectin. Although we surveyed mouse and human tissues and found the same distribution of the glycoprotein in the mammary gland of the 2 species, we limited our in vitro studies to mouse mammary cells for several reasons. Due to the poor yield of epithelial cells from digests of normal human breast tissues, a common source of "normal" mammary epithelial cells from humans is material isolated from milk samples, as in the work of Smith et al. (32) and Yang et al. (42). In contrast to cultures of normal mouse mammary cells, which can be shown to produce normal mammary outgrowths when transplanted into the mammary fat pad of syngeneic hosts (4, 11, 35), the exact identity of the human cells is uncertain. Moreover, even if the...
human cells were indeed actual alveolar or ductal cells, their "normalcy" is still questionable, since they were obtained from material sloughed into the milk rather than from intact functioning tissue. Such cells may not be representative in their behavior or functions of adherent cells present in breast tissues, especially in their interactions with basal matrix components, since they were detached either actively or passively from their in vivo substratum. A more difficult problem to explain is the decreased expression of cell surface LETS protein and tumorigenicity in human cell hybrids. Cell, 13: 1241–1251, 1978.


Interactions of Mammary Epithelial Cells with Fibronectin


Fig. 1. Section of paraffin-embedded mammary gland from a mouse in late pregnancy. de, ductal epithelial cells; ae, alveolar epithelial cells; me, myoepithelial cells; fs, fatty stroma; fs, fibrous stroma; bl, region of the basal lamina. The diameter of the basal lamina itself is below the limits of resolution of the light microscope. H & E, x 345.

Fig. 2. Frozen sections of normal mouse mammary gland stained by immunofluorescence with rabbit preimmune serum (d, ductal lumen; a, alveolar lumen). Note lack of staining. × 560.

Fig. 3. Distribution of fibronectin in normal mammary gland. Frozen sections were stained by immunofluorescence with antibodies to fibronectin. a, longitudinal section through a large duct (d, ductal lumen) of a virgin mouse. Fibronectin is localized in the connective tissue, in the region of the basement membrane, and along the basal surface of epithelial cells lining the lumen. Note the lack of intracellular and apical and lateral surface staining of the epithelial cells. b, cross-section through an alveolar region (a, alveolar lumen) of a lactating human. c, longitudinal section through a duct of a lactating human. The lumens (arrows) is only partially visible. Note similarity of staining patterns in a, b, and c. × 595.

Fig. 4. Double-staining immunofluorescence of a frozen section from the normal mammary gland of a pregnant mouse. a, anti-keratin; b, anti-fibronectin. The positions occupied by myoepithelial cells in a, which outline the cross-section of a duct, are covered by fibronectin in b. Note coincident staining in areas indicated by arrows. The fibronectin also extends into the connective tissue that surrounds the duct. × 630.

Figs. 5 to 7. Localization of fibronectin by immunofluorescence in mammary dysplasias. Note that the patterns resemble those seen in normal tissue (Figs. 3 and 4b).

Fig. 5. Cryostat section through a murine alveolar hyperplastic nodule. The lumina of the alveoli are indicated by arrows. × 482.

Fig. 6. Cryostat section of human breast tissue with fibrocystic disease (arrows indicate lumina) × 450.

Fig. 7. Frozen section of a human fibroadenoma (arrows, lumina). × 450.

Fig. 8. Immunofluorescence demonstrating the distribution of fibronectin in mammary cancers. Frozen sections of primary murine adenocarcinoma that developed from a HAN (a), primary human infiltrating ductal carcinoma (b), human lymph node with a metastatic lesion of ductal carcinoma (c), and the transplanted murine MXT 3590 papillary ductal carcinoma (d). Note that only tumor cells juxtapositional to connective tissue have any contact with fibronectin. × 336.
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Fig. 9. Primary cultures of mouse mammary cells fixed in 3% formaldehyde 6 days after plating and stained by immunofluorescence to detect fibronectin. a, normal mammary cells from an animal in late pregnancy; b, HAN cells; c, cells from a primary adenocarcinoma; d, cells from the transplanted MXT 3590 tumor. Arrows in a and c indicate matrix formation around fibroblasts at the edge of large epithelial cell colonies which are unstained. x 320.

Fig. 10. Deposition of fibronectin in cultures of established mouse mammary tumor cell lines. The cells were fixed in 3% formaldehyde prior to immunofluorescence staining. a to e, 4 days after subculturing; f, 6 days after subculturing. a, ESD/BALB-CL3; b, DMBA/BALB-CL2; c, MTV-L/BALB-CL2; d, ductal 7,12-dimethylbenzanthracene treated; e and f, NMuMG. x 332. Compare with staining in Fig. 9.
Fig. 11. Primary cultures of mouse mammary cells fixed in 3% formaldehyde and then absolute acetone to allow penetration of antibodies into and beneath the cells. Immunofluorescence staining with anti-fibronectin. a, normal mammary cells 4 days postseeding; b, normal mammary cells 6 days postseeding; c, HAN cells 6 days postseeding; d, cells from a primary adenocarcinoma 6 days postseeding; arrows, staining of fibroblasts at edge of colony; e, cells of the MXT 3590 transplanted tumor 6 days postseeding. × 360.

Fig. 12. Double staining of normal mouse mammary cells in primary culture 3 days postplating. Fixation was in absolute methanol and acetone. a, filament system in a cell (presumably myoepithelial) revealed by staining with anti-keratin; note lack of such filaments in the neighboring cell (presumably a fibroblast) on the left; b, granular perinuclear fluorescent pattern found in both cells with anti-fibronectin. × 375.

Fig. 13. Formation of an extracellular matrix of fibronectin in primary cultures of mouse mammary cells after cultivation in medium conditioned by Nil 8 cells. a, normal cells after 48 hr in Nil 8 medium (4 days postseeding); compare with Fig. 11, a and b; b, adenocarcinoma cells after 5 days in Nil 8 medium (7 days postseeding). Arrow, matrix formation around fibroblasts at the edge of the epithelial cell colony. × 375.
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