Distribution of Myoepithelial Cells and Basement Membrane Proteins in
the Normal Breast and in Benign and Malignant Breast Diseases

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ABSTRACT

An immunocytochemical method for fixed and paraffin-embedded human breast biopsies is reported for the detection of myoepithelial and epithelial cells using antibodies to myosin and keratin, respectively, and of basement membranes using antibodies to laminin and type IV collagen. Using these markers, myoepithelial cells can be clearly distinguished in the normal breast and in the benign breast diseases sclerosing adenosis, epitheliosis, and fibroadenoma. In sclerosing adenosis, myoepithelial cells form a major cellular component. A stromally derived spindle cell is identified which stains with myosin but not with keratin antibodies (myofibroblast). These cells are seen in one-fifth of the fibroadenomas and in one-half (9 of 18) of the infiltrating carcinomas. Although cells staining with myosin antibodies are seen in the infiltrating component of all 18 carcinomas examined, elongated cells staining with both myosin and keratin antibodies (myoepithelial-like) are seen in only one infiltrating carcinoma where they are interposed at the stromal-epithelial junction of the infiltrating tumor cells. In contrast to the situation in benign breast diseases, mature myoepithelial cells form a very minor component of the majority of infiltrating ductal carcinomas. Basement membrane proteins, laminin, and type IV collagen are present in normal breast, benign breast disease, and grade I infiltrating ductal carcinomas but are absent in carcinomas of grades II and III.

INTRODUCTION

The relative contribution made by myoepithelial cells to both benign and malignant breast disease is still debated (1, 3, 8, 17, 23, 27, 31). Numerous histological methods have been used to identify myoepithelial cells, but the techniques used have relied mainly upon poorly characterized enzymatic histochemical or nonspecific staining methods (25, 32, 33, 35). Characteristic properties of myoepithelial cells, at least in rodents, are the presence of actin and myosin-containing myofilaments and keratin-containing intermediate filaments and the production of basement membrane proteins, fibronectin, laminin, and type IV collagen (45). Immunofluorescence studies using antibodies to actin (12–14, 17, 28) and myosin (17, 26) have demonstrated that these proteins occur in certain cells in frozen sections of both benign (26, 28) and malignant (12–14, 16, 35) human breast diseases, and keratin immunoreactive materials have been found in both the normal and malignant human (5, 18, 37, 40) and rodent breasts (6, 44). None of these proteins is unique to myoepithelial cells. Thus, the keratins occur in epithelial cells, myosin myofilaments occur in smooth muscle cells, fibronectin is produced by stromal cells, and laminin and type IV collagen are produced by endothelial cells. This paper describes the demonstration of keratin, myosin, laminin, and type IV collagen using immunocytochemical localization techniques with specific antibodies in fixed and paraffin-embedded specimens from a range of normal, benign, and malignant breast biopsies. This technique yields a superior cytology to that obtained previously using immunofluorescent localization of single antibodies on frozen breast sections and delineates the myoepithelial cell in benign breast diseases from other reactive stromal cells, e.g., myofibroblasts. These results are discussed in relation to the normal developmental program of the myoepithelial cell in the breast.

MATERIALS AND METHODS

Tissue Processing. Biopsy specimens were used from 35 unselected cases of human breast disease including 18 infiltrating ductal carcinomas. At least 2 pieces of tissue from each specimen were cut into slices approximately 3-mm thick and fixed in 60% methanol:30% chloroform:10% acetic acid overnight at 4°C. Tissues were processed through 5 changes of absolute alcohol, one change of absolute alcohol and 1.1.1. trichlorethane (1:1 v/v), 2 changes of 1.1.1. trichlorethane, and 2 changes of wax. After a further hr in wax under vacuum, the slices were finally embedded in fresh wax, the wax blocks cooled at 4°C, and 3-μm-thick sections were cut on a sledge microtome fitted with disposable blades (Sukura Fine Technical Co., Japan). Serial sections were mounted on albumin-coated slides and dried overnight at 45°C. Sections were dewaxed by immersion in fresh xylene for three 5-min periods and then by immersion in fresh absolute alcohol for a further three 5-min periods. The sections were rehydrated firstly in water and then finally in Ca²⁺-, Mg²⁺-free PBS.²

Antibodies. Keratins were purified from human callus and analyzed on sodium dodecylsulfate-polyacrylamide gels as described by Sun and Green (41). The molecular weights of the keratins were 43,000, 45,000, 47,000, 47,000 and 69,000. Antisera were raised by dissolving 1 mg of keratins in 0.5 ml of 1 M urea and 0.14 M β-mercaptoethanol in PBS; this solution was emulsified with 1 ml of Freund's complete adjuvant, and the resultant mixture was injected s.c. into the nuchal region of rabbits at monthly intervals. The resultant antisera gave a single immunoprecipitin band in Ouchterlony diffusion tests with the purified keratins. These antisera are henceforth called anti-keratin. Rabbit antisera to pig uterine smooth muscle myosin (30, 43) and the mouse type IV collagen (24) and laminin (42) have been described previously. The last 2 antisera specifically precipitated collagen IV and laminin, respectively, as analyzed on sodium dodecyl sulfate-polyacrylamide gels (45). IgG fractions of all antisera were obtained by precipitation of the original sera with 25% ammonium sulfate. The precipitates were collected by centrifugation, dissolved in 0.025 M NaCl:0.05 M Tris-HCl (pH 7.5), and then chromatographed on DEAE-

² The abbreviation used is: PBS, phosphate-buffered saline.
cellulose columns. All fractionated antisera were diluted between 1/100 and 1/500 with 0.5% bovine serum albumin (w/v) in PBS before use. Second antibodies were obtained by conjugating affinity-purified sheep anti-rabbit γ-globulin to alkaline phosphatase with glutaraldehyde (7).

Immunocytochemical Staining. The binding of specific rabbit antisera to histological sections was visualized by an indirect procedure using alkaline phosphatase-conjugated anti-rabbit second antibodies. When sections were partially digested with proteases before incubation with antisera, to enhance the resultant staining (laminin, type IV collagen, and keratin) they were incubated first with PBS and then with trypsin (0.05 mg/ml; type III; Sigma Chemical Co.) in PBS, both at 37°C for 15 min, and rinsed in running water. Staining with antisera was carried out on sections without prior protease treatment unless specified to the contrary in the text. All sections were then treated with 15% acetic acid (v/v) for 5 min at room temperature to inhibit endogenous alkaline phosphatase, subsequently washed in running tap water, and then rinsed with PBS. Sections were incubated with 100 μl of the first antiserum at room temperature for 1.5 hr and then washed with several changes of PBS containing 0.01% Tween 80 followed by several rinses in PBS. They were incubated with 1/100 alkaline phosphatase-conjugated sheep anti-rabbit IgG at room temperature for 1.5 hr. The sections were again washed with 0.01% Tween 80 in PBS, followed by a rinse in PBS. The sections were then washed in running tap water, rinsed in distilled water followed by incubation with 0.5 mg naphthol AS:B1 phosphoric acid (sodium salt) per ml and 0.5 mg Fast Red TR per ml in veronal acetate buffer, pH 9.2, at room temperature for 1 hr. The sections were again washed in running water, counterstained with Mayer’s hemalum, and mounted in buffered glycerin jelly, pH 7.4. The specificity of staining was confirmed by absorption of each of the antisera with 1 mg of the appropriate antigen per ml at room temperature for 1.5 hr. Incubation of histological sections with these absorbed antisera resulted in a complete abolition of the alkaline phosphatase stain in both protease-treated and untreated material.

RESULTS

Previous experiments with different fixatives indicated that the staining of rodent tissues with all the antisera used here was best accomplished by fixation in 60% methanol:30% chloroform:10% acetic acid in preference to the more conventional fixatives (44). Immunostaining was enhanced by pretreatment of the histological sections with proteases (44). Similar results were found with human breast tissues. Unlike the rodent system, prior trypsinization was not required with the keratin antiserum but prior trypsinization of the sections was required to give adequate staining with antisera to type IV collagen and laminin. With the exception of the breast, the binding of the keratin antiserum to different tissues fixed in 60% methanol:30% chloroform:10% acetic acid was similar to that reported for the same tissues fixed in formol 0.9% NaCl solution (37). However, the degree of staining was much stronger using the former method of fixation.

Normal Human Breast. Antibodies to myosin clearly delineated the myoepithelial cells in the resting (Fig. 1) and the lactating mammary gland. Pericytes, smooth muscle cells of the nipple, and blood vessels were stained, but all other stromal elements were consistently negative. Without prior trypsinization, keratin antibodies stained the epithelial cells strongly (Fig. 2) and the myoepithelial cells weakly. This staining pattern was altered by prior trypsinization of the sections whereupon the keratin antibodies stained both the epithelial and myoepithelial cells (Fig. 3). In the lactating breast, the epithelial cells in the nonsecretory areas were strongly stained but the secretory cells were only very weakly stained.

Antibodies to laminin and type IV collagen delineated the basement membrane (Fig. 4) as a single line around both the lobules and ducts. No individual cells were stained with these antibodies. No breaches were seen in the basement membrane in either the lactating or resting gland.

Benign Breast Disease. Because of the confusing nomenclature in benign breast disease, it is necessary to define some of the conditions described in the text. These definitions are taken from Azzopardi (8). He also provides an excellent account of the histological appearances of the subtypes of adenosis.

Adenosis. This is a nonneoplastic glandular hyperplasia. Distinct varieties can be recognized, namely blunt duct adenosis, nodular adenosis, and sclerosing adenosis.

Epitheliosis. This is the name for the solid and quasisolid benign epithelial proliferation which is found predominantly in small ducts, ductules and lobules.

In the 4 cases of adenosis, the myoepithelial cells were stained with the antimyosin serum, and the epithelial cells were identified by the strong staining with the anti-keratin serum. In blunt duct adenosis, the myoepithelial cells of the terminal ducts were very prominent with a ragged appearance to their outer surface. There was a spectrum of changes from blunt through nodular to sclerosing adenosis with a progressive loss of epithelial cells. The reduction in number and distortion of the acini is demonstrated by the keratin staining (Fig. 5). At the same time, there was a proliferation of myoepithelial cells which took on a spindle cell morphology (Fig. 6) and which appeared to have migrated into the intralobular stroma. In these regions, there was a diffuse staining with antibodies to laminin and type IV collagen in the intralobular stroma around the myoepithelial cells (Fig. 7). The myoepithelial cells in regions of sclerosing adenosis and nodular adenosis were weakly positive with the anti-keratin serum before trypsinization and strongly positive after trypsinization of the histological sections. In the normal glands and benign breast diseases, neither the epithelial nor the myoepithelial cells showed cytoplasmic staining with antibodies to laminin or type IV collagen.

In the 4 cases of epitheliosis examined, the majority of the intraluminal cells were strongly keratin positive, and a minor population was myosin positive. In one case, cells similar to those identified morphologically and by their keratin content as epithelial-like, contained myosin-immunoreactive materials. The basement membrane and normal external myoepithelial cell layer were still intact in areas of epitheliosis. Areas of apocrine metaplasia showed an accentuation of the myoepithelial cells and weak keratin positively of the epithelial component.

In fibroadenomas, the staining patterns with antibodies to myosin, keratin, and the basement membrane proteins were similar to those found in the normal breast. In the stroma of one fibroadenoma, spindle-shaped cells stained very strongly with myosin antibodies. Morphologically similar cells in an adjacent section failed to stain with keratin antiserum even after trypsinization, a technique which enhanced the myoepithelial cell staining in the adjacent ducts.

Intraduct and Infiltrating Carcinoma. Tumors were graded in accordance with Bloom and Richardson (11). In the majority of the intraduct lesions, the myoepithelial cells were prominent...
The basement membrane was delineated with antibodies to laminin. Infiltrating carcinomas were graded according to Bloom and Richardson (11), and each tumor biopsy was from a different patient. In one tumor, the infiltrating myosin-positive cells were randomly mixed in the tumor. In one tumor, however, not all of the 18 tumors examined, the infiltrating myosin-positive cells failed to stain with antibodies to keratin even after trypsinization of sections. The presence of keratin-positive cells, the infiltrating islands of tumor was too large to be explained by attenuation of the myoepithelial cells (Fig. 9). There was also loss of definition of the basement membrane proteins around some areas of intraduct carcinoma.

The infiltrating tumor cells were heterogeneously stained with both anti-keratin and anti-myosin sera. In the 18 infiltrating carcinomas studied, all contained keratin-positive cells, the proportion varying from approximately 25% to greater than 75% (Fig. 10). All of the tumors contained some myosin-positive cells with some individual cells strongly stained. There was no correlation between cytological appearance and antibody staining with the anti-myosin and pre-keratin serum. In 17 out of the 18 tumors examined, the infiltrating myosin-positive cells were randomly mixed in the tumor. In one tumor, however, there was a minor population of strongly myosin-positive malignant cells which were spindle shaped and interposed at the stromal-epithelial junction of the infiltrating islands of tumor cells (Fig. 11) suggestive of myoepithelial cell differentiation.

The presence of myoepithelial cells in infiltrating carcinomas was not related to the degree of overt histological differentiation of the tumor when assessed by the criteria of Bloom and Richardson (11). In one tumor which was a very well-differentiated adenocarcinoma, no peripheral tumor cells stained with anti-myosin serum, but the islands of well-differentiated acini were still surrounded by a clearly defined basement membrane. The relationship of basement membrane to the degree of differentiation is shown in Table 1. In 9 out of 18 infiltrating carcinomas, there were prominent myosin-positive benign proliferating spindle cells (Fig. 12). As with fibroadenomas, these cells failed to stain with antibodies against keratin (Fig. 13) even after prior trypsinization, suggesting that they were of stromal origin, probably myofibroblasts.

### DISCUSSION

An immunocytochemical technique is described for the demonstration of keratin, myosin, laminin, and type IV collagen in paraffin-embedded biopsies of human breast. Using these antisera, it appears to be possible to distinguish epithelial, myoepithelial, and stromal cells and to delineate basement membrane.

Myoepithelial cells constitute a large proportion of benign breast lesions, while in the majority of infiltrating carcinomas examined there are very few identifiable myoepithelial cells. These results are in agreement with earlier morphological studies (1, 8, 23). However, morphological criteria are no longer entirely satisfactory for the recognition of myoepithelial cells since a similar spindle cell, the myofibroblast (20, 29), has recently been described in certain human breast diseases (2, 3, 8, 36) including infiltrating carcinomas (1, 29, 38). In our studies, myofibroblasts are observed in one fibroadenoma and one-half of the infiltrating carcinomas. These cells stain strongly with antibodies to myosin but not with antibodies to keratin even under conditions shown to enhance the staining of myoepithelial cells. This strongly supports the view that myofibroblasts originate from the stromal rather than the epithelial component of the breast (29, 38). Positive stromal markers have not been used to identify these cells, and in these studies it is not possible to exclude the possibility that trace amounts of keratins are present but that the concentrations are too small to be detected immunocytochemically or that the epitope recognized by the antibody is masked. The presence of large amounts of polymerized myosin in myofibroblasts does not necessarily suggest that they represent a new cell type since similar activated fibroblasts occur in other situations, e.g., in Dupuytren's contracture (19) and in granulation tissue (36).

There is disagreement concerning the distribution of keratins in the breast. This has arisen because laboratories have used various sources of keratins for immunization purposes (5, 16, 18) and have tested the keratin antibodies on different species, and tissues have been fixed and processed in different ways. In our studies, anti-human keratin serum stains myoepithelial cells in the rat and lining epithelial cells in human breast tissue. The enhancement of staining for myoepithelial cells in human breast reported by Schlegel et al. (37) was seen in our studies only after prior treatment of the sections with trypsin. Recently, Altmannsberger et al. (5) have reported that fixation of breast tissues in alcohol gives satisfactory staining results which is contrary to our experience (44). Finally, changes in cellular staining patterns can be produced with antibodies raised in different species against the same immunogen (6). Reliable comparisons can be made between results obtained in different laboratories if antibodies are clearly defined in terms of their normal tissue distribution, the method of tissue fixation, and the tissue-processing schedule used.

In epitheliosis, both ultrastructural (3, 8, 26) and histochemical (8) techniques have suggested elements of both myoepithelial and epithelial differentiation with the conclusion that this condition may be due to a proliferation of "indeterminate" cells (31). In our cases, myosin-positive cells formed a minor component in the intraduct proliferation of epitheliosis, although in one case the majority of the cells in an area of solid epitheliosis were positively stained, supporting the potential role of an indeterminate cell. In sclerosing adenosis, it has been suggested that the lesion appears initially as an increase in numbers of acini and then develops through a series of phases in which the myoepithelial cells play an ever increasing role until the epithelial elements become compressed and the myoepithelial cells take on a spindle cell morphology (8). Our results with anti-myosin staining are consistent with this interpretation.

The generation of cellular heterogeneity within breast lesions initially depends on the underlying developmental program of the normal breast. Our data would suggest that in infiltrating...
carcinomas phenotypic heterogeneity is marked but that differentiation is predominantly towards a cell with epithelial as opposed to myoepithelial characteristics. This could arise either by transformation of a normal epithelial cell or by transformation of a stem cell with selection for a reduced capacity to differentiate into myoepithelial cells. Analogies can perhaps be drawn with carcinogen-induced rat mammary tumors where stem cells capable of both replicating and yielding myoepithelial cells have been described (10). The apparent phenotypic heterogeneity of the carcinoma cells could then be generated either by clonal and/or environmental variation in the control of gene expression or by retention of the last vestiges of the capability to differentiate to myoepithelial cells. These 2 explanations may not be mutually exclusive since clonal and/or environmentally induced variation could still occur in the vestigial stem cell as it does in certain rat cell lines (34). Similar considerations can also be applied when considering the histogenesis of human lung tumors (22).

Previous in vitro (25, 35) and in vivo (44) studies with antibodies to collagen IV and laminin have shown that the bulk of the mammary basement membrane is laid down by the myoepithelial cells in the young and adult rat. The thickening of the basement membrane in association with scarring, observed here by immunolocalization techniques and by others using ultrastructural techniques (3, 8), suggests that the myoepithelial cell is also the source of human breast basement membrane. The presence of basement membrane proteins in Grade I carcinoma (Table 1) appears to contradict this hypothesis since fully mature myoepithelial cells were not seen in these tumors. However, epithelial cells in the mammary glands of pre- and neonatal rats appear to be associated with the synthesis of the basement membrane prior to the appearance of mature myoepithelial cells.3 Thus, either the better-differentiated carcinomas consist of a class of developmentally early breast epithelial cells or they retain some primitive myoepithelial-like cells with the capacity for making basement membrane proteins.

The use of antibodies to identify basement membrane proteins and myoepithelial cells has also been useful in studies of early tumor invasion. Earlier investigators using immunofluorescent antibodies to actin, noted defects in the myoepithelial cell layer in some in situ carcinomas (12, 14), and ultrastructural studies have suggested that these may be associated with breaches in the basement membrane (21). More recent studies using antibodies to laminin on frozen (39) and paraffin-embedded (4, 9) sections have demonstrated that defects in the basement membrane occur in association with local microinvasion. It has also been suggested that the presence of basal lamina when identified using the periodic acid-Schiff technique is a useful differentiation feature in distinguishing scaring adenosis and infiltrating carcinoma (15). Thus, the loss of myoepithelial-like cell characteristics and of basement membrane proteins in the spectrum of disease states from the normal breast to the highly malignant carcinoma may reflect the same underlying change in the cellular composition of the diseased lesions.

REFERENCES


Myoepithelial Cells and Basement Membrane Proteins


Fig. 1. Photomicrograph of a lactiferous sinus stained for myosin. The peripheral myoepithelial cells are clearly delineated. × 250.

Fig. 2. Photomicrograph of a segmental duct stained for keratins indicating the strong positivity of the lining epithelial cells and weaker staining of the myoepithelial cells. × 500.

Fig. 3. Photomicrograph of the same duct as shown in Fig. 2 but at a deeper level stained for keratins after trypsinization. Both the lining epithelial cells and the myoepithelial cells are strongly positive. × 530.

Fig. 4. Photomicrograph of a portion of a lobule stained for laminin, demonstrating the basement membrane adjacent to the myoepithelial cells and the darkly stained stromal blood vessels. × 350.

NOVEMBER 1982
Figs. 5 and 6. Photomicrographs of an area of sclerosing adenosis. Sections were cut through the same area but at a slightly different level and stained for keratins (Fig. 5) and myosin (Fig. 6). There is strong cytoplasmic staining of the few residual distorted acini and weak staining of the intervening spindle cells with the anti-keratin antisera. These spindle cells are strongly stained with the anti-myosin sera (Fig. 6). × 250.

Fig. 7. Photomicrograph of an area of sclerosing adenosis stained for laminin. The laminin is very diffuse and strongly positive, masking the myoepithelial cells and outlining residual distorted acinar structures. × 530.

Fig. 8. Photomicrograph of an area of intraduct carcinoma stained for myosin. The myoepithelial cells are very prominent and give a "saw-toothed" appearance. × 280.
Myoepithelial Cells and Basement Membrane Proteins

Fig. 9. Photomicrograph of an area of intraduct carcinoma stained for myosin demonstrating loss of myoepithelial cells in some areas. × 125.

Fig. 10. Photomicrograph of an infiltrating ductal carcinoma stained for keratin. The majority of the tumor cells are stained, and a few cells are very strongly positive. × 280.

Fig. 11. Photomicrograph of an infiltrating ductal carcinoma stained for myosin. Strongly positive malignant spindle cells are identifiable at the stromal-tumor interface suggesting areas of myoepithelial differentiation. × 280.
Figs. 12 and 13. Photomicrographs of an infiltrating ductal carcinoma stained for myosin (Fig. 12) and keratin (Fig. 13). The tumor cells are weakly positive with myosin and the stromal spindle cells are strongly positive. The same region stained for keratins fails to stain the stromal cells, but focal tumor cells are strongly positive. × 280.
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