Basement Membrane Changes in Breast Cancer Detected by Immunohistochemical Staining for Laminin

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ABSTRACT

The distribution of the basement membrane glycoprotein laminin was studied by the immunoperoxidase technique in benign and malignant human breast tissue and in axillary lymph nodes from patients with breast cancer. An antiserum prepared against rat laminin was used. The specificity of this antiserum against human laminin was studied using the FL cell line of human epithelial-like cells derived from normal amniotic membrane. The antiserum reacted with these cells in immunoperoxidase staining and precipitated metabolically labeled secreted polypeptides which comigrated with polypeptides with molecular weights of 400,000 and 200,000 of rat laminin in sodium dodecyl sulfate:polyacrylamide gel electrophoresis.

The neoplastic cells in malignant breast tissues showed strong cytoplasmic staining for laminin, and a positive reaction was also found in lymph node metastases. In some cases in which only micrometastases were present, these cells also stained strongly for laminin. In nonmalignant breast tissues, the epithelial cells of the ducts were positive for laminin, but the staining was weaker than in the carcinomas. Pretreatment of the fixed tissue sections with trypsin markedly enhanced the staining of basement membranes for laminin. In trypsin-treated sections of normal breast tissue and benign lesions, the laminin staining delineated continuous basement membranes. In carcinomas representing the more differentiated types, basement membranes presumably produced by the tumor cells could be revealed by laminin staining, but they were thinner and discontinuous. The poorly differentiated carcinomas lacked organized basement membranes detectable by laminin staining.

Our studies suggest that staining for laminin may be a useful adjunct test for detection of micrometastases in lymph nodes. The correlation of disintegration of the laminin-containing basement membranes of tumors with increasingly anaplastic appearance supports the notion that basement membranes may play a role in tumor invasion.

INTRODUCTION

Basement membranes separate the epithelium from the mesenchymal tissues. Significant progress has been made recently in studies on the chemistry and biology of the macromolecules that make up basement membranes. Basement membranes are composed mainly of a specialized genetic type of collagen (type IV collagen) which resembles, in some respects, the procollagen forms of the interstitial collagens (2, 14) and of noncollagenous glycoproteins. Among the latter, a noncollagenous glycoprotein component of the basement membrane, termed laminin, has been identified. Laminin has been isolated from basement membranes formed by a mouse tumor (31), from the extracellular matrix of cultured murine endodermal cells (5, 6, 28), and from a rat yolk sac tumor with abundant hyalin (33). Laminin is composed of disulfide-linked polypeptide chains with molecular weights of about 200,000 and 400,000 (6, 23, 28, 31). Laminin is biochemically and immunologically different from fibronectin, another extracellular matrix glycoprotein, and from type IV collagen (6, 28, 31).

Basement membrane proteins may be involved in cellular adhesion to basement membranes and extracellular matrix. Fibronectin mediates the attachment of a variety of cell types (25), whereas type IV collagen(s) and laminin may be primarily involved in the attachment of epithelial cells (3, 15, 30, 32). Malignant cells often display aberrations in their relationship to basement membrane components. For instance, they often lack cell surface fibronectin(s) and, at least in one case, the laminin that their normal counterparts have at the cell surface (12).

Basement membranes are thought to form a protective barrier against the initial infiltration of tissue by malignant cells, and the exceptional ability of metastatic cells to attach to the basement membrane of endothelial cells may play a role in the spread of tumors via circulation (16, 19, 32). These observations emphasize the need for further studies on the relationship of tumor cells to basement membranes. As laminin has been demonstrated recently by immunofluorescence in cultured human breast carcinoma cells (10), we found it of interest to examine the distribution of laminin in malignant and nonmalignant breast tissue with special reference to the basement membranes. We show here that immunocytochemical staining of laminin reveals marked differences in the organization of basement membranes in normal breast tissue and breast cancer.

MATERIALS AND METHODS

Cells. The FL cell line of human epithelial-like cells derived from normal amniotic membrane (9) was obtained from American Type Culture Collection (Grand Island Biological Co., Scotland). The cells were grown in modified Eagle’s minimal essential medium with 10% fetal bovine serum (Grand Island Biological Co.) in tissue culture flasks (A/S Nunc, Roskilde, Denmark) or on coverslips placed in Belco Leighton tubes (Belco Glass, Inc., Vineland, N. J.) in a humidified atmosphere containing 5% CO₂. Cells were routinely subcultured every 4 to 5 days after detachment by 0.01% crystalline trypsin (Novo Industri A/S, Copenhagen, Denmark).

Tissues. Samples of human female breast tissue sent to the laboratory for histological evaluation were investigated. In cases of cancer,
mastectomy was performed, and the axillary fat was removed to be examined for lymph node metastases. A total of 22 malignant tumors [20 infiltrating ductal carcinomas, not otherwise specified (8), one colloid carcinoma, one medullary carcinoma] and 10 normal mammary glands (7 cases of fibrocystic disease, 2 fibroadenomas, one granulomatous mastitis) were examined.

**Antisera and Immunoprecipitation.** An anti-rat laminin serum prepared by immunizing a rabbit against laminin isolated from rat yolk sac tumor (33) was used. Briefly, the tumor was homogenized in 4 M NaCl, and laminin was extracted from the insoluble residue with 0.5 M NaCl in 0.05 M Tris buffer, pH 7.0, and the extract was fractionated on a column of Sepharose 6B. Laminin eluted in the void volume was further purified on heparin-Sepharose (27). The resulting laminin gave 2 bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis at molecular weights of 200,000 and 400,000 (Fig. 2). Affinity-purified antibodies were isolated from anti-rat laminin serum by adsorption to and elution from purified rat laminin coupled to Sepharose. The antisera stained basement membranes in rat tissues by immunocytochemical technique (33) and precipitated the laminin polypeptides from culture media of rat yolk sac tumor cells (33) and normal and transformed rat kidney cells.

An anti-mouse laminin serum prepared by immunizing a rabbit with laminin isolated from the extracellular matrix of endodermal cells was also used. The preparation and characterization of such a serum have been described (27, 28). This antisera was used in some cases for additional verification of the staining results.

Subconfluent cultures of FL cells were labeled with [35S]methionine (Amersham, England), by adding 5 μCi/ml to the culture medium for 24 hr (Eagle's minimal essential medium without methionine supplemented with 1% fetal bovine serum). Immunoprecipitation and gel electrophoresis were carried out as described before (26, 33).

**Immunoperoxidase Staining.** For immunoperoxidase investigation, the cultured cells were fixed for 20 min at room temperature, and the tissue blocks were fixed overnight at 4°C. The fixation was done in 95% ethanol/glacial acetic acid (99:1, v/v) (26). The tissue samples were dehydrated in ethanol and xylene, processed for paraffin embedding at 58°C, and serially sectioned. The sections were deparaffinized, washed with PB, and depleted of endogenous peroxidase by incubating for 30 min with an 0.5% solution of 30% hydrogen peroxide in methanol at room temperature. Some sections were pretreated with crystalline trypsin (Novo Industri, A/S, Copenhagen, Denmark) at various concentrations (0.05 to 100 μg/ml in PB) for 5 to 15 min at 37°C. The sections were then washed with PB for 10 min and preincubated with a 1:10 dilution of normal swine serum (Dako, Copenhagen, Denmark) for 15 min at room temperature. The immunoperoxidase staining was processed by using either the indirect double-layer technique or the unlabeled antibody peroxidase-antiperoxidase method as described by Sternberger (29). The antisera were diluted 1:50 or 1:1000, respectively, and incubated for 60 min at room temperature. The bound peroxidase was localized with 3-amin-9-ethylcarbazole (Sigma Chemical Co., St. Louis, Mo.) or 3,3′-diaminobenzidine tetrahydrochloride (Sigma) as chromogen and, in some cases, followed by 1 min of hematoxylin counter-staining.

Normal rabbit serum instead of the antisera was used as a control. Anti-rat laminin serum from which antilaminin had been removed by absorption to rat laminin coupled to Sepharose served as an additional negative control. For histological examination, paraffin sections were stained with hematoxylin and eosin. In some cases, tissue sections processed after formalin fixation in the routine surgical pathology laboratory were also stained for laminin. As the background staining in these cases was often high, only results obtained with sections fixed in ethanol/glacial acid are presented.

**RESULTS**

**Reactivity of Anti-Rat Laminin and Human Laminin.** To study the cross-reactivity of our anti-rat laminin serum with human laminin, we chose the FL cell line of human epithelial-like cells derived from normal amniotic membrane, aware of the fact that epithelial cells from human postpartum amniotic membrane in primary culture have been shown to produce laminin (1). Cultured FL cells showed a strong immunoperoxidase staining reaction with the anti-rat laminin serum (Fig. 1). Immunoprecipitation of culture media of [35S]methionine-labeled cultures with the anti-rat laminin serum followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed 2 polypeptides which comigrated with the polypeptides of rat laminin at molecular weights of 400,000 and 200,000 (Fig. 2).

**Laminin in Breast Cancer Tissue.** Malignant epithelial cells in fixed tissues stained without trypsin pretreatment showed a diffuse or granular cytoplasmic staining for laminin (Fig. 3; Table 1). The staining was found in areas with malignant intraductal elements as well as in areas with invasive growth. The tumors also showed areas negative for laminin staining. The degree of intracellular staining for laminin could not be significantly correlated to the degree of tumor differentiation. However, intense cytoplasmic laminin staining seemed to be more frequently present in invasive tumor cells, especially at the peripheral invading edges of the tumors. Around the malignant intraductal elements at the site of the basement membranes, a weak and inconsistent basement membrane-like linear staining reaction was seen, whereas no such staining could be demonstrated around the infiltrating tumor cells. No other laminin-positive extracellular deposits were observed in the connective tissue or in the lumina of the tumor tubules and ducts.

Basement membranes could be visualized more distinctly by applying the laminin staining to fixed tissue sections treated with trypsin. With trypsin pretreatment, the tumor cells lost most of their cytoplasmic staining for laminin and appeared partly digested. Basement membranes, when present, were visualized around malignant intraductal elements by staining for laminin. In carcinomas that showed a high to moderate degree of differentiation, the invasively growing tumor elements were usually surrounded by a thin but often discontinuous basement membrane (Fig. 4; Table 1). In carcinomas with a

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<td><strong>Immunoperoxidase staining of laminin in 22 primary breast carcinomas</strong></td>
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<td>Infiltrating ductal carcinomas with moderate or low degree of differentiation</td>
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* Staining obtained without trypsin pretreatment.
* Staining obtained with trypsin treatment.
* Numbers in parentheses, cases with the indicated staining reaction.
moderate to poor degree of differentiation, the invasively growing elements in most cases showed no staining reaction consistent with a basement membrane (Fig. 6).

In 7 of the 18 cases of breast cancer in which lymph nodes were found in the axillary fat, samples of the lymph nodes were available to us. These cases represented a random sample of the patients. In 2 of these cases, the nodes were heavily infiltrated with tumor. Approximately one-fourth of the tumor cells were stained for laminin. In 3 cases with micrometastases, tumor cells, predominantly located in the marginal sinus, showed cytoplasmic staining for laminin (Fig. 5). Pretreatment with trypsin abolished the intracytoplasmic staining in the metastases, but no laminin-positive basement membranes were revealed. In 2 cases, the nodes were without metastases, and no laminin staining of the cells was found.

Laminin in Normal Breast Tissue and in Benign Lesions. Without trypsin pretreatment, a weak diffuse cytoplasmic staining for laminin was seen in the luminal epithelial cells of the ducts in normal areas as well as in benign lesions (Fig. 7). The myoepithelial cells showed even weaker staining. The ductal basement membranes could only be identified by a weak staining at the junction of the epithelium and the connective tissue (Fig. 7). After treatment with trypsin, continuous basement membranes with strong staining for laminin were seen in all cases examined (Figs. 8 to 10). The basement membranes of the vessels and nerves showed a weak positive staining for laminin. This was also accentuated by trypsin pretreatment. The connective tissue elements and the inflammatory cells were negative, except for a few histiocytes which appeared positive.

The anti-rat and anti-mouse laminin sera as well as the purified antibodies gave similar staining results. Control reactions with normal rabbit serum and antilaminin serum depleted of antibodies were negative in every case.

DISCUSSION

The present study shows that malignant and nonmalignant breast tissue contains a component reactive with antisera to rat and mouse laminin. Our results indicate strongly that this reactivity is due to the presence of human laminin. The antisera we used have been shown to precipitate the laminin polypeptides from culture media of mouse and rat cell lines (26, 33); in the present work, they were shown to precipitate similar polypeptides from the media of a human cell line FL. Furthermore, treatment of the anti-rat laminin serum with purified rat laminin insolubilized on Sepharose abolished its reactivity with human tissues. Based on these results, we conclude that we are detecting the human equivalent of the rodent laminin in our immunoperoxidase staining.

Several aspects of our findings on human laminin are of interest. The presence of cytoplasmic staining for laminin in ductal epithelial cells in areas with normal breast morphology suggests that laminin is continuously synthesized by the normal epithelium. The biological significance of this synthesis is not clear, but it may be that basement membranes are in a state of continual turnover (18), and that this synthesis is needed to replenish laminin that has been broken down. Since malignant cells stained even more strongly for laminin than did the normal epithelial cells, it seems reasonable to conclude that carcinoma cells are also able to synthesize laminin in vivo. The significance of this synthesis is not known, but recent results on the cell adhesion-promoting properties of laminin (3, 30, 32) suggest that laminin could be important for the attachment and growth of such tumor cells.

We have noted previously that antilaminin stains poorly the hyalin material of a rat yolk sac tumor and Reichert’s membrane in the rat embryo (33). This is in spite of the fact that these structures are known to contain abundant amounts of laminin (6, 13). In the present study, we also observed weak and inconsistent staining for laminin in basement membranes of malignant and nonmalignant human breast tissues. The visualization of basement membranes by laminin staining could be greatly improved by pretreatment of the tissue sections with trypsin. The weak staining of the basement membranes without trypsin treatment could be due to masking of the antigenic determinants by other macromolecules or lack of penetration of the antibodies. The fact that increased staining was observed after treatment with trypsin may be taken as an argument for either possibility. The improved method for laminin staining allowed us to study the organization of basement membranes in malignant and nonmalignant breast tissues in some detail.

Previous ultrastructural and histochemical works have suggested that malignant breast tumors only occasionally possess basement membranes (7, 11, 20). Quite recently, Pitelka et al. (21) reported that well-differentiated malignant mouse mammary tumor lines had intact basement membranes demonstrable by electron microscopy, whereas a poorly differentiated tumor line lacked them. Our results are in agreement with these observations and also with earlier investigations of basement membrane changes in human cervix carcinoma (24). As visualized by laminin staining, a progressive lack of basement membranes seemed to parallel an increasing degree of tumor dedifferentiation. The reason for this lack of basement membranes may be an insufficient synthesis of basement membrane components other than laminin and/or a mechanism for their increased destruction. In any event, the absence of a basement membrane barrier may facilitate tumor spread.

Finally, we would like to discuss the possible utility of laminin staining in the detection of micrometastases in regional lymph nodes. The normal cell population and the extracellular material of lymph nodes, with the exception of the vascular basement membranes, did not stain for laminin. This made it possible to detect single malignant cells by laminin staining. Micrometastases of individual tumor cells in axillary lymph nodes can be difficult to identify in routinely stained sections. Recently, antibodies to type IV collagen (17) and to milk fat globule membrane (4) have been proposed as possible tools for immunohistochemical detection of metastatic breast carcinoma cells. The demonstration of laminin by immunoperoxidase staining may be a valuable adjunct to early detection of micrometastases. These have been proposed to be derived from subclones of tumor cells selected by the metastatic process to have a special potential for metastasis (22). Such subclones often lack characteristics of the original tumor, especially those associated with the differentiated state. The accentuated cytoplasmic staining for laminin that we invariably found in the metastatic cells suggests that the presence of this marker is not dependent on the differentiated state of the cells and is therefore perhaps less likely to be lost. It will be important to evaluate in long-term clinical studies whether the laminin-pos-
Malignant nature. Lymphocytes are marked

Immunoperoxidase technique with hemotoxylin counterstaining, x 420.

tumor cells (7) is seen, but no basement membranes (arrows) delineate the tumor islets from the stroma. Basement membranes of the vessels are positive for laminin.

Acknowledgments

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REFERENCES


Fig. 7. An area with normal breast morphology from a case with fibrocystic disease. Cells (secretory type) lining the ducts are stained for laminin. Basement membranes of the ducts (arrow) are faintly stained. Immunoperoxidase technique, × 225.

Figs. 8 to 10. Basement membranes visualized by immunoperoxidase staining for laminin after pretreatment with trypsin.
Fig. 8. Ductal proliferation and dilations in fibrocystic disease. × 325.
Fig. 9. Apocrine metaplasia in fibrocystic disease. Counterstained with hematoxylin, × 400.
Fig. 10. Sclerotic adenosis with waved and ragged basement membranes. Counterstained with hematoxylin, × 300.
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