Detection of Basement Membrane Components and Basal Cell Keratin 14 in Noninvasive and Invasive Carcinomas of the Breast

Ria H. W. Wetzels, Roland Holland, Urbain J. G. M. van Haastel, E. Birgitte Lane,* Irene M. Leigh;† and Frans C. S. Ramaekers
From the Department of Pathology, University Hospital Nijmegen, Nijmegen, The Netherlands, the Imperial Cancer Research Fund, Clare Hall Laboratories, Potters Bar, Hertfordshire,* and the Experimental Dermatology Laboratory, London Hospital, London,† United Kingdom

Using immunohistochemistry, the distribution patterns of basement membrane components type VII collagen (monoclonal antibody LH7.2), type IV collagen, and laminin were investigated in normal and malignant human breast tissue and compared with that of keratin 14 (monoclonal antibody LL002), which is expressed only by the basal (myoepithelial) cells in the secretory epithelia of the mammary gland. In normal breast tissue as well as in intraductal carcinomas, a more or less continuous basement membrane was observed at the epithelial stromal interface. Unlike laminin and type IV collagen, type VII collagen was not detected in the basement membrane of blood vessels. The keratin 14 antibody stained the basal cell layer of normal ducts and ducts with in situ cancer. In 85% of the invasive carcinomas no basement membrane or basal cells were detected. In 13 cases, however, laminin, type IV collagen, and/or type VII collagen were detected around tumor nests and individual tumor cells. Five of these tumors also showed a positive reaction with the keratin 14 antibody. In five cases keratin 14 expression was found without detectable basement membrane components. It is concluded that 18 of 103 invasive ductal breast carcinomas examined in this study exhibit a basal cell phenotype as determined from the expression of keratin and the deposition of basement membrane components. (Am J Pathol 1989, 134: 571–579)

Basement membranes are complex extracellular matrix structures that separate epithelial and endothelial cells from underlying connective tissue. Ultrastructural studies have shown that most basement membranes consist of an electron-dense layer (lamina densa) and an electron lucent layer (lamina rara or lamina lucida) adjacent to the cell membrane. Constituents of the basement membrane are divided into two groups: 1) intrinsic components, such as type IV collagen, laminin, heparan sulphate proteoglycan, and 2) extrinsic components, such as fibronectin and type V collagen. Recently, a new type of collagen, type VII collagen, has been described. This type of collagen has been demonstrated to be the major protein in the anchoring fibrils projecting from the lamina densa into the subjacent connective tissue. Type VII collagen has been shown to occur in a more or less tissue-specific manner. Thus far, only the basal lamina of stratified epithelia, such as epidermis, oral, esophageal, and cervical epithelium, and urothelium of the bladder have been shown to contain type VII collagen. Those epithelia that are composed of different cell types, sweat gland epithelium or breast epithelium, for example, containing myoepithelial cells next to glandular cells, possess a type VII collagen containing basement membrane.

Basement membranes play an important role in tumor progression. The occurrence of basement membrane in neoplastic breast lesions has been studied extensively using antibodies to laminin and type IV collagen. In normal breast tissue, in benign breast lesions, and in situ malignancies the basement membrane always surrounds ducts and tubules, whereas in invasive breast carcinomas it is often absent. In intraductal mammary carcinomas, fragmentation and absence of basement membrane was reported in areas of microinvasion. Various conflicting views exist in the literature with respect to the presence or absence of basement membrane components in infiltrating mammary cancers. According to some investigators, invasion of breast carcinoma into stroma is associated with complete loss of extracellular laminin or type IV collagen.
collagen staining, whereas invasive cells may gain intracytoplasmic staining for laminin. Others were able to demonstrate extracellular laminin staining in invasive breast carcinomas, but did not find intracytoplasmic reactivity. Charpin et al. have suggested that these discrepancies may be caused by differences in the specificities of the antibodies used in the various studies.

Basal cells and myoepithelial cells are thought to be involved in basement membrane production. In former studies actin and myosin antibodies were used to identify myoepithelial cells in breast cancers, but such antibodies do not exclusively stain myoepithelial cells. In the last few years antibodies to keratins have been shown to distinguish between different epithelia. The exact keratin content of myoepithelial cells has, however, not been determined yet. Several studies indicate the presence of keratin 5 and 14 in the myoepithelial cell layer.

In this study we examined the pattern of basement membrane deposition in normal breast and in a series of breast carcinomas using a monoclonal antibody to type VII collagen together with antibodies to laminin and type IV collagen, and compared the distribution patterns of these three basement membrane components during invasion of tumor cells. A positive correlation was observed between the expression of type VII collagen and the presence of keratin 14 expressing (basal) cells. Type VII collagen was detected only in the presence of keratin 14.

Materials and Methods

Tissues

This study used 131 breast biopsies and mastectomy specimens. Table 1 gives an overview of the types of human breast lesions used. The samples were frozen and stored in liquid nitrogen. Cryostat sections (5–7 μ) were air-dried for 24 hours at room temperature and stored at −20°C until use.

Antisera

The following antibodies were used:

1) The mouse monoclonal antibody 4E10 (IgG1), directed against human laminin, was used as ascitic fluid from Balb/c mice, absorbed on human plasma proteins coupled to sepharose, and diluted 1:1000 for the indirect immunoperoxidase technique.

2) The mouse monoclonal antibody LH 7:2 (IgG3), directed against type VII collagen, was used as culture supernatant and diluted 1:10 for the indirect immunoperoxidase and immunofluorescence technique.

3) The mouse monoclonal antibody to type IV collagen was raised in human kidney glomeruli as described previously. The specificity of the monoclonal antibody was determined by Gusterson et al. and it was diluted 1:5000 for the indirect immunoperoxidase and immunofluorescence technique.

4) The polyclonal rabbit antiserum 57E/2, directed against rat laminin, was absorbed on rat plasma proteins coupled to sepharose and diluted 1:50 for the indirect immunofluorescence technique.

5) Antiserum 57E/2 absorbed on rat laminin coupled to sepharose was used as a control.

6) The polyclonal rabbit antiserum Z 810, directed against human laminin, was absorbed on human plasma proteins coupled to sepharose and diluted 1:50 for the indirect immunofluorescence technique.

7) The mouse monoclonal antibody RCK 102, directed against keratin 5 and 8 and staining several epithelial tissue types, was used as undiluted culture supernatant in the indirect immunoperoxidase technique.

8) The mouse monoclonal antibody LL002, directed against keratin 14, was used as a culture supernatant and diluted 1:20 for the indirect immunoperoxidase and immunofluorescence technique.

Antibodies 4E10, 57E/2, 57E/2 control, and Z 810 were a gift from Dr. U. Wewer, and the antibody to type IV collagen was obtained from Prof. Dr. W. W. Hancock and Prof. Dr. B. Gusterson.

Immunocytochemistry

Indirect Immunoperoxidase Technique

Cryostat sections were fixed in acetone (−20°C) for 20 seconds and washed in phosphate-buffered saline (PBS, pH 7.4) for about 10 minutes. After preincubation for 15 to 30 minutes with 10% normal goat serum in PBS to reduce background staining, the sections were incubated with
the appropriate primary antibody for 30 minutes at room temperature at the dilutions indicated above. After three washes with PBS for 10 minutes each, the peroxidase-conjugated rabbit-anti-mouse IgG (Dakopatts, Glostrup, Denmark, diluted 1:25 in PBS with 5% human AB serum) was applied to the sections for 30 minutes at room temperature. After a second series of washing steps with PBS the peroxidase was detected with 3-amino-9-ethylcarbazole (AEC; Aldrich Chemical Co., Brussels, Belgium). For this purpose 40 mg of AEC was dissolved in 10 ml N,N-dimethylformamide (Merck, Darmstadt, FRG) and added to 190 ml of sodium acetate buffer (0.05 M, pH 4.95). Hydrogen peroxide was added to a final concentration of 0.01% (vol/vol). Sections were incubated for 10 minutes with this mixture, rinsed with tap water, counterstained with hematoxylin, and mounted with Kaisers glycerin-gelatine (Boom B. V., Meppel, The Netherlands).

Indirect Immunofluorescence

In the indirect immunofluorescence technique fixation and preincubation were performed as described above for the indirect immunoperoxidase technique.

The sections were incubated with a PBS solution containing an appropriate monoclonal antibody or polyclonal antiserum at the dilutions indicated above for 45 to 60 minutes at room temperature. After repeated washings in PBS the FITC-conjugated goat-anti-rabbit IgG (Nordic, Tilburg, The Netherlands; dilution 1:25 in PBS), the FITC-conjugated rabbit-anti-mouse IgG (Nordic, dilution 1:25 in PBS) or Texas Red conjugated sheep F(ab')-anti-mouse Ig (New England Nuclear, Dreieich, FRG; dilution 1:40 in PBS) were applied to the sections for 45 to 60 minutes at room temperature. After three washes with PBS for 10 minutes each, nuclei were stained by incubating the cells for 15 minutes with Hoechst 33258 (0.1 μg/ml in 22 mM citric acid, 56 mM disodium hydrogen phosphate). Sections were washed again with PBS and mounted in Gelvatol (Monsanto, St. Louis, MO). Slides were viewed with a Leitz Dialux 20 EB microscope equipped with epifluorescent illumination. Pictures were taken using 400 ASA Tri-X film (Kodak, Rochester, NY) with an automatic camera.

Results

The reactivity patterns of the different basement membrane antibodies and the keratin antibodies on normal and malignant breast tissues are summarized in Table 2 and depicted in Figures 1 to 4.

Distribution Pattern of Basement Membrane Markers

Normal Human Breast

In normal human breast, laminin and type IV collagen appeared as a relatively thick layer in basement mem-

branes surrounding ducts and lobules (Figure 1A–C). In all cases staining was continuous. Laminin and type IV collagen staining also was observed in basement membrane around blood vessels. The staining pattern of type VII collagen differed from that of laminin and type IV collagen in that the basement membrane surrounding blood vessels was not stained. Furthermore, the staining reaction of the type VII collagen antibody was more punctate than that of the other two basement membrane markers (Figure 1D).

Malignant Breast Tissues

Noninvasive, intraductal carcinomas of the breast were surrounded by a more or less continuous basement membrane as detected with all three basement membrane antibodies (Figure 2A–C). The interpretation of the laminin and type IV collagen staining patterns was sometimes problematic in that it was not always clear whether basement membrane staining was of ductal or of blood vessel origin. Ducts often were surrounded by many blood vessels. In those cases type VII collagen staining was useful, because blood-vascular basement membrane was negative and only basement membrane that surrounded tumors was found to be positive.

In invasive carcinomas basement membrane staining usually was absent around epithelial cells infiltrating the stroma (Figure 2D–F). In 13 of 116 cases, however, an irregular staining reaction around the clusters of the infiltrating epithelial cells was observed when the antibodies to laminin (Figure 2G) and type IV collagen (Figure 2H) were used. In three of these cases type VII collagen also was visible (Figure 2I, J), but staining was always weaker. In the other ten cases, type VII collagen staining was totally absent in the invasive tumor areas. The intraductal component of invasive carcinomas showed a more or less continuous basement membrane with all three basement membrane antigens. The normal ducts, seen in the same specimen, also were positive, except for one case where several normal ducts stained with the antibodies to laminin and type IV collagen, but not with the type VII collagen antibody. In our samples no intracytoplasmic staining was observed with any of the basement membrane antigens.

Because several authors have described the usefulness of basement membrane staining to detect invasion of tumor cells, we carefully examined this phenomenon. From the data obtained in this study we conclude that artifacts introduced during cryosectioning may be falsely interpreted to be disruptions of the basement membrane by invading tumor cells. In some instances, however, a positive staining reaction of basement membrane antibodies can support the diagnosis of a noninvasive carcinoma. For example, Figures 3A and B show a branching duct with a bud filled with tumor cells, whereas small tu-
Table 2. Staining Reactions of Normal Human Breast Tissues and Breast Carcinomas with Antibodies to Basement Membrane Components and to Keratin 14

<table>
<thead>
<tr>
<th>Histology</th>
<th>Number</th>
<th>Laminin</th>
<th>Coll. IV</th>
<th>Coll. VII</th>
<th>Keratin 14</th>
</tr>
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<tbody>
<tr>
<td>Normal mammary gland</td>
<td>2*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Basement membrane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal cells</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal carcinoma in situ</td>
<td>15</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Basement membrane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal cells</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>85</td>
<td>-</td>
<td>-</td>
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<tr>
<td>2</td>
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</table>

* Next to these two cases of normal breast (obtained from autopsy) normal mammary gland tissues were observed in virtually all tumor cases examined.

Tumor nests are lying in close proximity of a bigger duct. With conventional staining methods, the cells in the bud and the tumor nests could be interpreted as infiltrating tumor cells. Staining with the antibodies to laminin, type IV collagen, and type VII collagen, however, shows an intact basement membrane around the ducts, indicating that the cells of the intraductal carcinoma in this case do not infiltrate the underlying stroma.

Distribution of Keratin 14 in Normal and Malignant Breast Tissues

In normal breast tissue the monoclonal antibody LL002 specific for keratin 14 stained the basal cells and myoepithelial cells but not the secretory columnar epithelial cells of ducts and lobules (Figure 4A–C). A similar staining pattern was seen in ductal carcinomas in situ (Figure 4D–

Figure 1. Indirect immunofluorescence (A) and immunoperoxidase (B–D) staining patterns of normal breast tissue incubated with anti-laminin (57H/2; A, X30; 4610; B, X280), anti-Type IV collagen (C, X280) and anti-Type VII collagen (LI7.2; D, X280).
Invasive carcinomas, however, were almost always negative with the antibody to keratin 14 (Figure 4G). Only 10 of 116 cases of invasive carcinomas showed a positive reaction, either homogeneously (Figure 4H) or more focally (Figure 4I). For comparison, the keratin antibody RCK 102 stained all tumor cells in such cases (Figure 4J). Surprisingly, five of ten keratin-14-positive tumor cases also showed a positive reaction with the basement membrane markers laminin and type IV collagen. The three cases that were positive for all three basement membrane markers (including type VII collagen) also stained with the antibody to keratin 14. In the five other keratin-14-positive tumors no basement membrane reactivity was found.
Discussion

This study demonstrates the distribution of type VII collagen in normal breast tissues as well as invasive and noninvasive breast carcinomas, in comparison with two well characterized basement membrane constituents, laminin and type IV collagen. It also is correlated with the distribution of keratin 14 containing basal cells.

The study of the presence and distribution of basement membrane components in invasive breast carcinomas has led to several contradictory reports in the literature. In support of the literature, we were able to demonstrate laminin and type IV collagen in normal breast around ducts and surrounding in situ ductal carcinomas. Type VII collagen shows a similar reaction pattern in these benign tissues and noninvasive lesions. In invasive breast tumors, most data in the literature suggest the absence of basement membrane around the tumor cells, although some authors have found basement membrane components in infiltrating breast carcinomas, rectal carcinomas, and colonic carcinomas.

Some investigators found laminin staining in well-differentiated areas, whereas laminin staining was absent completely in poorly differentiated areas. In most cases of invasive breast carcinomas we found no basement membrane staining around the tumor cell nests. However, in 13 tumors we could show extracellular basement membrane staining, either in the whole specimen that was examined or in part of it, by using antibodies to laminin and type IV collagen. In some cases a positive reaction was found with the type IV collagen antibody only (see Table 2). The antibody to type VII collagen displayed a slightly different distribution pattern. Those carcinomas that were negative for laminin or type IV collagen also were negative for type VII collagen. In 3 of 13 cases, the invasive carcinomas positive for laminin and type IV collagen also were positive for type VII collagen. In these tumors the type VII collagen staining was always less intense than that of the other basement membrane constituents, indicating that the amount of type VII collagen in these regions is low compared with that of the other components. In the other ten carcinomas type VII collagen staining was absent completely.

In 10 of the 116 infiltrating carcinomas a strong and homogeneous reaction was observed with the keratin 14 antibody, recognizing basal (myoepithelial) cells in alveoli and ducts of normal human breast. In the larger ducts, however, luminal cells also were stained. Surprisingly, half of the invasive tumors positive for keratin 14 also were positive for laminin, type IV collagen, and in part for type VII collagen (see Table 2). Some authors, using antibodies to other keratins (eg, keratin 5) staining myoepithelium, suggested that the homogeneously reactive tumors represent malignant cells of myoepithelial origin, whereas those that are unreactive represent malignancies of luminal epithelial origin. Because the myoepithelial cell is regarded by several authors as the source of (breast) basement membrane, the presence of basement membrane material is in accordance with the appearance of keratin 14 positive (myoepithelial) tumor cells. The other half of the keratin 14 positive cases were negative for all basement membrane markers, however. This is in accord with earlier findings of Gusterson et al, who showed that, in a minor proportion of infiltrating ductal carcinomas in which myoepithelial cells (indicating myoepithelium) were found, no stainable quantities of basement membrane material could be detected.

Extension of ductal carcinoma in situ into small ducts within the confines of lobules, a phenomenon called cancerization, can be erroneously regarded as evidence of invasion in some cases. Staining by the antibody to keratin 14, however, indicating the presence of an intact basal cell layer, together with labeling of laminin, type IV collagen, and type VII collagen, strongly emphasizes the intraductal nature of such tumor cells. We also observed some branching ducts with ductal extensions into the stroma. In those cases a continuous basal layer was al-
ways found around the buds, indicating that no invasion was taking place.

Type VII collagen has been found to underly both stratified and pseudostratified epithelial tissues, as well as combined epithelia.\textsuperscript{23,24} In contrast to laminin and type IV collagen, type VII collagen thus far has not been found in basement membrane of nonepithelial tissues such as blood vessels, muscle, or nerve tissue. This property
makes this antibody useful in tumor pathology for specifically recognizing basement membrane around ducts and lobules in intraductal carcinomas. These lesions are often surrounded by several blood vessels, the basement membrane of which reacted with the laminin and type IV collagen antibodies. In several cases it was not clear whether the stained basement membrane belonged to the blood vessels or whether the basement membrane surrounded the malignant ducts. Using the type VII collagen antibody, a clear distinction could be made between these two types of basement membrane.

In summary, we can conclude that in normal human breast tissue and in non-invasive carcinomas laminin, type IV collagen and type VII collagen were detected in basement membranes surrounding ducts and lobules, while the antibody specific for keratin 14 stained the basal and myoepithelial cells. In most invasive carcinomas no basement membrane constituents could be detected. In 11% of the invasive tumors, however, basement membrane proteins could be demonstrated around the tumor nests. In part of these basement membrane-positive invasive carcinomas a correlation between basement membrane deposition and a basal cell nature (as concluded on basis of keratin 14 expression) of the tumor cells might exist. Although keratin 14 could be expressed in the absence of type VII collagen, type VII collagen was never detected in the absence of keratin 14. This suggests that type VII collagen is synthesized only in the presence of basal cells. Type IV collagen and keratin 14 did not appear to be independent. Unlike laminin and type IV collagen, type VII collagen is not present in basement membranes around blood vessels. This property makes the antibody to type VII collagen most useful in recognizing basement membrane around in situ ductal and lobular malignancies.

References

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