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Original Article

The Presence of a Type IV Collagen Skeleton Associated with Periductal Elastosis in Breast Cancer

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Using serial sections of frozen and AFA-fixed tissues from 34 breast cancers, we studied the presence of basement membrane material in the areas of elastosis. Various amounts of type IV collagen but not of laminin were demonstrated in areas of periductal elastosis. In some tumors, type IV collagen accumulated beneath the basement membrane. Periductal elastosis in areas of extensive fibrosis showed focal type IV collagen immunoreactivity, indicating remnants of ducts. Interstitial elastosis corresponded with weak type IV collagen reactivity. Each tumor showed type IV collagen immunostaining of the elastotic areas, with various degrees of intensity. Negative crossreactivity of the type IV collagen antibody with elastin was verified in skin biopsies with solar elastosis. Pre-incubation of the antibody with large amounts of elastin demonstrated an identical immunoreactivity. The specificity of the antibody was confirmed by ELISA and by Western blot analysis. To explain the periductal elastosis, we propose the following hypothesis. Excessive production of basement membrane material by the epithelial cells of the ducts leads to formation of a type IV collagen skeleton. This skeleton can act as the matrix for a secondary deposition of elastic material. (*J Histochem Cytochem 38:245-255, 1990*)

KEY WORDS: Basement membrane; Breast cancer; Type IV collagen; Elastosis; ELISA; Immunohistochemistry; Laminin; Western blot.

Introduction

The biological significance of elastosis in breast cancer, defined as the presence of deposits of elastic fibers in abnormal amounts, is a challenging problem (Azzopardi, 1979). Elastic tissue in breast cancer has been mistaken for amyloid because of its affinity for Congo red (Bernath, 1952). Jackson and Orr (1957) first showed unequivocally that the areas of elastosis corresponded to the presence of elastic material.

Elastosis, which is present in 90% of breast cancers (Lundmark, 1972) is usually classified as either periductal, vascular (mostly around veins), or interstitial elastosis. Although part of the interstitial elastosis is probably due to disruption of preexisting structures and hence is a consequence of periductal elastosis, the existence of a primary interstitial elastosis as an etiologically different process remains uncertain. The cell of origin of elastosis and elastic fiber formation is uncertain, and both an epithelial (Kao and Stern, 1986; Shivas and Douglas, 1972) and a mesenchymal origin have been proposed (Nakanishi et al., 1983). Recently, morphological evidence was presented for the existence of "elastic-secreting cells," characterized by a heavy concentration of newly formed elastic fibers of variable maturity along their surfaces (Tamimi and Ahmed, 1987). Apart from breast cancer, elastosis has also been described in benign lesions of the female breast (Tremblay et al., 1977), in gynecomasties (Raju and Lee, 1988), and in a variety of other benign and malignant lesions in salivary glands (David and Buchner, 1980), colon (Smith, 1986), eyelids (Stefanyszyn et al., 1985), skin (Tsuji, 1980), and in several other tissues (Issacson et al., 1985). Whether elastosis precedes or follows the development of breast cancer is not known (Gould and Morales, 1986).

Recent studies showed that the periductal elastotic material was immunostained by antisera to human fetal elastin, lysozyme, and amyloid P component (Mera and Davies, 1987), to lectins, concanavalin A, and wheat germ agglutinin, to some plasma protease inhibitors (Davies and Mera, 1987), and to vitronectin (Loridon-Rosa et al., 1988). Electron microscopy demonstrated elastic fibers with a higher proportion of microfibrils than in the normal mature elastic fibers and an absence of collagen fibers in the central parts of the focal periductal elastotic cuffs (Mera and Davies, 1987; Martinez-Hernandez et al., 1977).

The present work was directed towards study of the basement membranes in the areas of periductal and interstitial elastosis of 34 breast cancers, selected according to the degree of elastosis.

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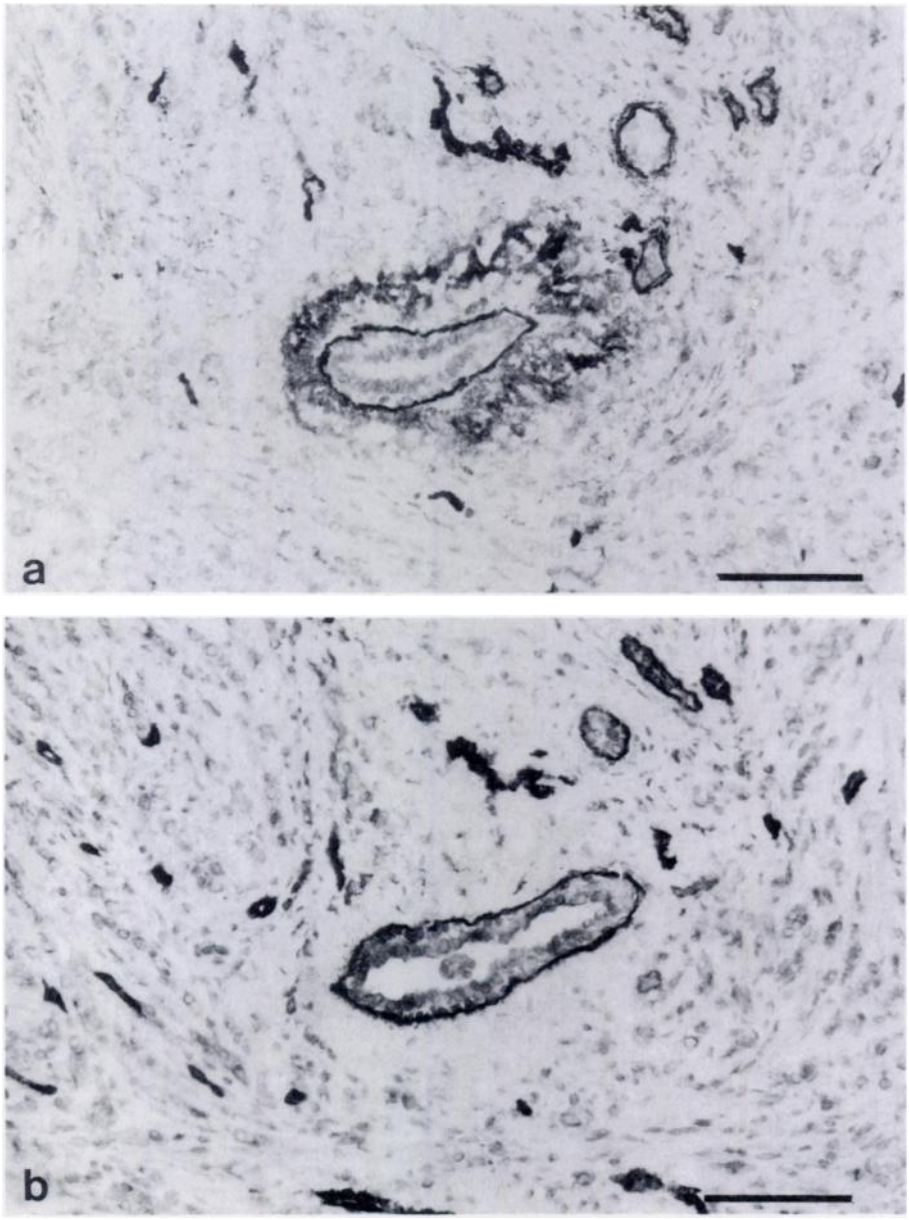

Figure 1. Frozen sections of infiltrating duct carcinoma of female breast. (a) Granular type IV collagen immunoreactivity in an area of periductal elastosis. (b) No laminin immunoreactivity in the same area. Original magnification × 200. Bars = 100 μm.

Materials and Methods

Human Tissues

Samples of 34 cases of breast cancer with different degrees of periductal and interstitial elastosis were selected for the study: five contained no elastin, 12 a small amount, nine a moderate amount, and eight a marked amount of elastin. For control and comparison with non-malignant conditions, the following cases were studied: six benign breast lesions consisting of two cystic mastopathies, two fibroadnomas, and two gynecomasties, two normal breast samples (one adult and one neonate), two skin biopsies of solar elastosis, two skin biopsies of extensive amyloidosis, two biopsies of a massive macrophage infiltration, and two biopsies of elastic arteries. All techniques were performed on tissues obtained during surgical procedures, which had been rapidly frozen in liquid nitrogen within 15 min and stored at −80°C. In addition, the adjacent tissue freshly fixed in AFA (85% alcohol, 10% formalin, 5% acetic acid) for 8 hr was studied. Fifteen serial frozen and paraffin sections, 4 μm thick, were cut from each sample to allow comparison of the different stains. Light microscopy was performed on cryostat and paraffin sections stained with HE, orcein (Unna, 1891), and with a Giemsa–elastin staining consisting of a first staining with orcein followed by the Giemsa method (1904).

Antibodies

The following antibodies were used: (a) a polyclonal antibody directed against purified type IV collagen, collected by pepsin digestion from human placenta; and (b) a polyclonal antibody against laminin purified from the murine EHS tumor.

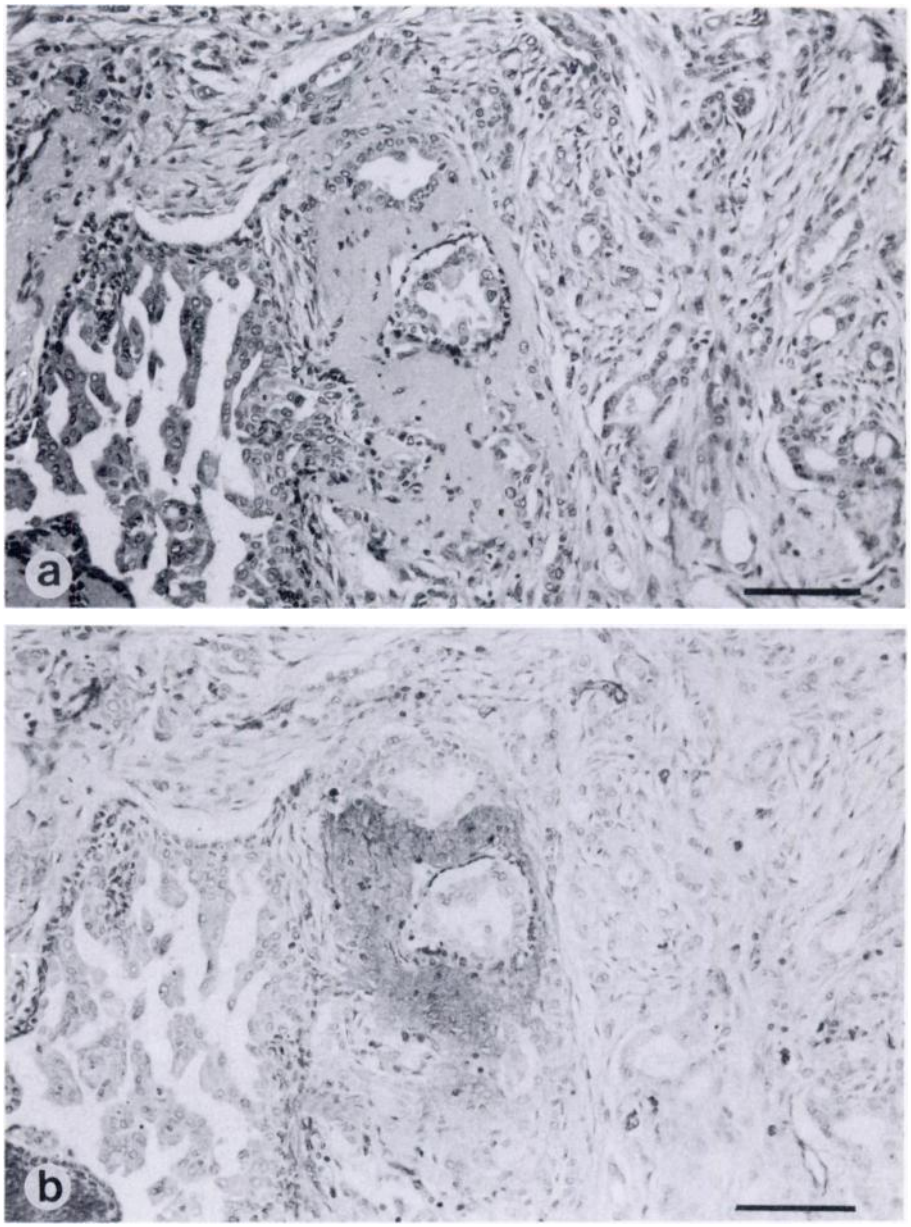

Figure 2. AFA-fixed paraffin sections of infiltrating duct carcinoma of postpartum female breast. (a) Elastosis around a duct bordered by a neoplastic epithelium (HE). (b) Adjacent section showing layered type IV collagen immunoreactivity of the periductal elastosis. Original magnification × 160. Bars = 100 μm.

Type IV collagen was purified from human placenta as described by Foidart et al. (1981), who assessed its purity by estimating the ratios of 3- to 4-hydroxyproline and hydroxyproline to proline, and by the pattern produced on polyacrylamide gel electophoresis before and after peptide mapping procedures. Laminin was purified from a murine tumor that produces a basement membrane matrix (Timpl et al., 1978, 1979; Orkin et al., 1977). About 200 μg of each protein dissolved in 0.2 ml of PBS was emulsified with an equal volume of Freund's complete adjuvant (Difco; Detroit, MI) and injected into the footpads and posterior nuchal area of New Zealand White rabbits. Immunization was repeated 2 weeks later by injection of the same dose of antigen emulsified in incomplete Freund's adjuvant. Each week for 2 months blood was drawn from the ear of each rabbit, after which the animals were exsanguinated.

Affinity-purified antibodies to type IV collagen or to laminin were prepared from their respective antisera by cross-immunoadsorption (Nowack et al., 1976). The specificity of the antibodies was verified by radioimmunoassay and by blockage of immunofluorescence and competition studies as previously reported (Yaoita et al., 1978).

Immunohistochemical Procedure

Cryostat sections were air-dried and fixed in acetone for 10 min at −20°C. The endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol and nonspecific staining was prevented by the use of 20% normal swine serum. The first antibodies were added at dilutions of 1:200 and incubated overnight at 4°C. After washing in PBS, the sections were incubated for 30 min with swine anti-rabbit immunoglobulin (DAKO; Glostrup, Denmark) diluted 1:50 in PBS with 10% normal swine serum and

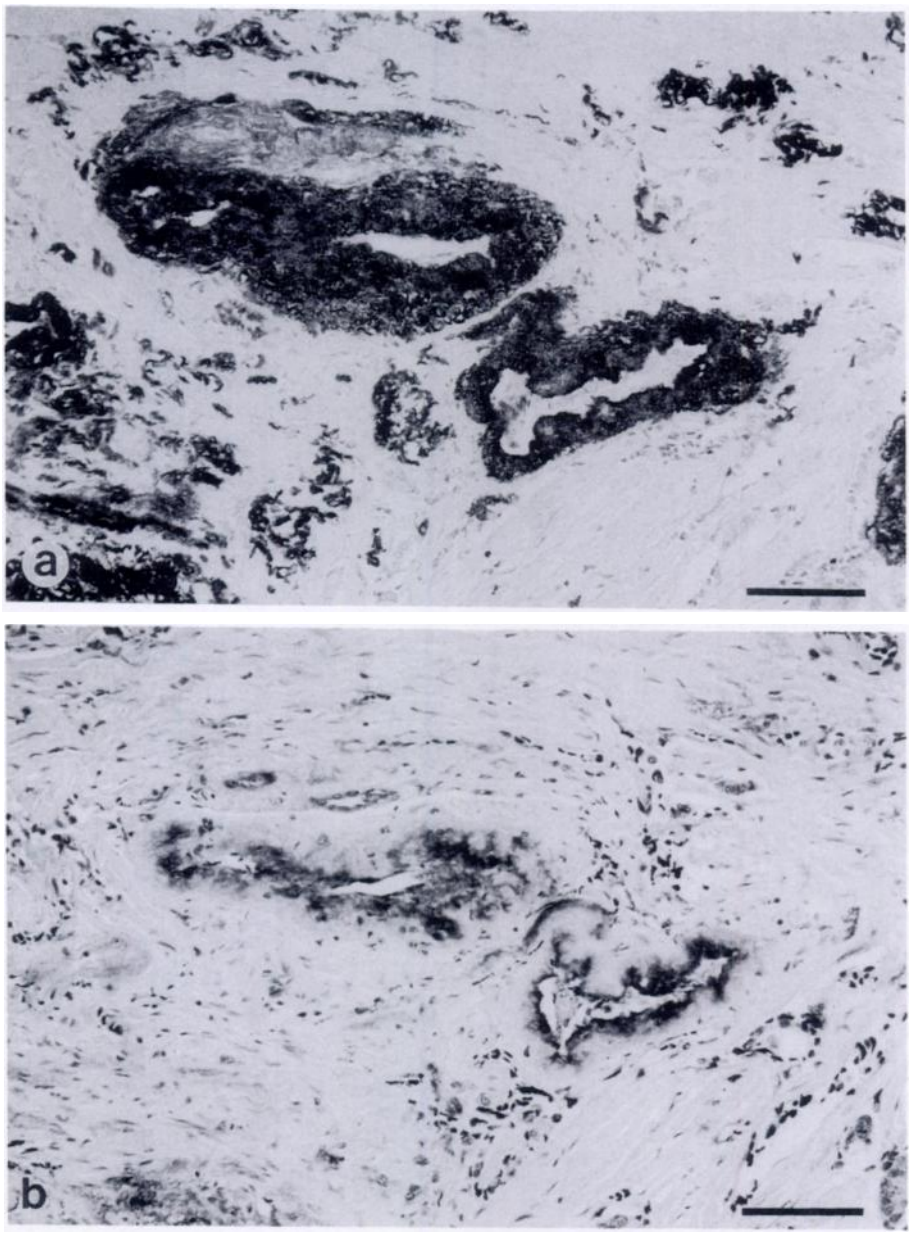

Figure 3. AFA-fixed paraffin sections of infiltrating duct carcinoma of female breast. (a) Elastin stain showing black deposits in the areas of periductal elastosis. (b) Layered type IV collagen immunoreactivity is accentuated beneath the basement membrane in an adjacent section. Original magnification × 160. Bars = 100 μm.

1% bovine serum albumin (BSA). After another series of washings, the sections were incubated for 30 min with the rabbit peroxidase–antiperoxidase complex (DAKO) diluted 1:100 in PBS with 10% normal swine serum and 1% BSA. After a further wash with PBS, the antigens were visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St Louis, MO) for 10 min. Hematoxylin was used as counterstain. The same procedure was performed on the AFA-fixed paraffin sections, although these first underwent proteolytic digestion with 0.1% pepsin (Boehringer; Mannheim, FRG) in acetic acid, pH 2.6, at 37°C for 2 hr (Willebrand et al., 1986; Barsky et al., 1984).

Control Procedures

Histology. In general, the specificity was assessed as follows: On one slide the specific antiserum was omitted and was replaced by the diluent buffer PBS–1% BSA; on a second slide the specific antiserum was replaced by a diluted non-immune rabbit serum; and a third slide was incubated with an unrelated specific polyclonal rabbit antiserum directed against human epidermal keratin (Ramaekers et al., 1982).

In particular, the specificity of the type IV collagen antibody was further tested because of the conspicuous immunoreactivity in elastotic areas. An absorption control was performed consisting of a pre-incubation for 3 hr of the anti-human type IV collagen antibody diluted 1:100 with different concentrations of elastin K (100, 200, 400, and 800 μg/ml) before application on the sections. Elastin K was obtained from Sodichimic (Geneva, Switzerland). It is a soluble elastin purified from calf ligamentum nuchae as described by Keeley et al. (1976). This soluble elastin was characterized by amino acid analysis content of hydroxyproline, lysine, hydroxylysine des-

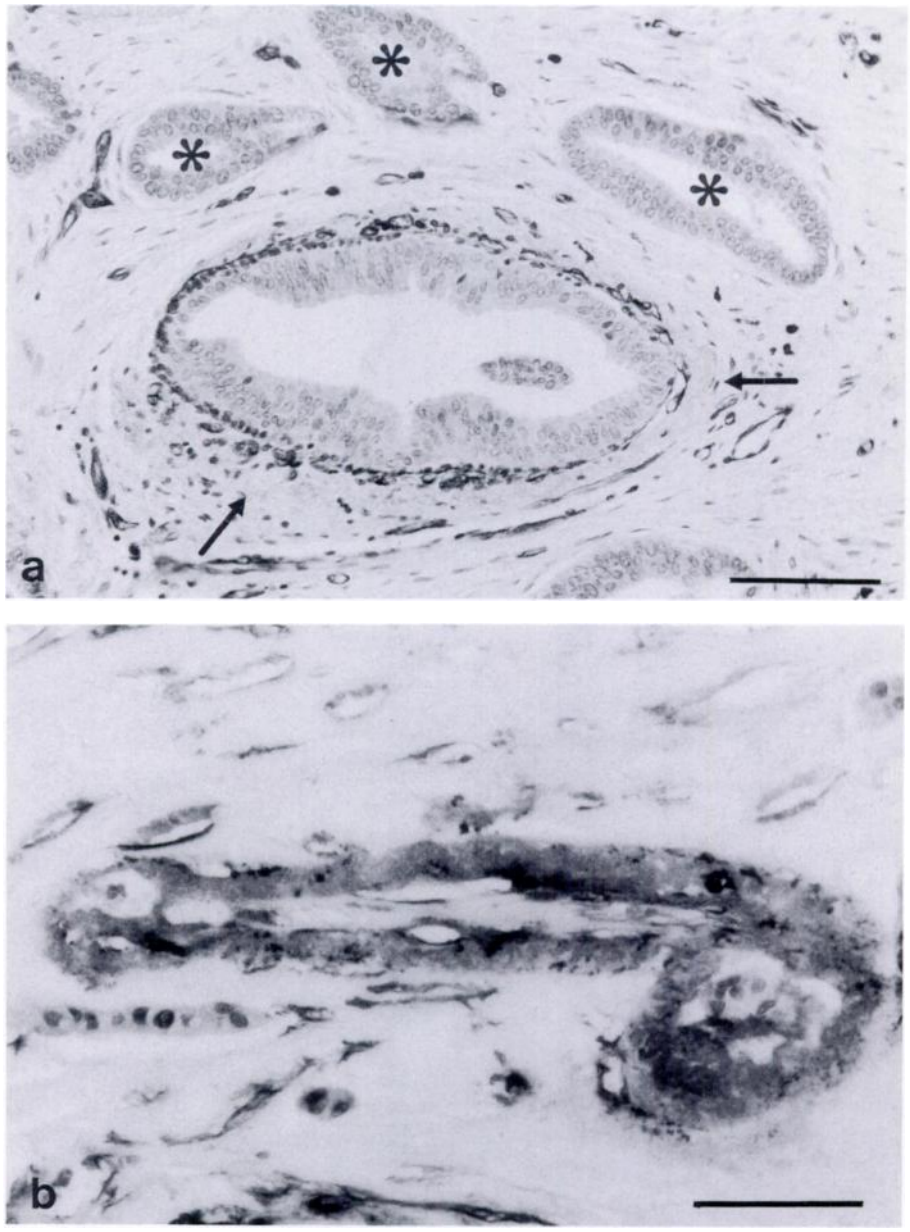

Figure 4. Type IV collagen immunostaining of AFA-fixed paraffin sections of female breast carcinomas. (a) Tubular carcinoma with layered type IV collagen immunoreactivity around the intraductal part of the carcinoma (arrows) but not around the infiltrating tubules (asterisks). (b) Scirrhous carcinoma with the remnants of a duct recognizable by the type IV collagen immunostaining. Original magnifications: a × 200; b × 230. Bars = 100 μm.

mosine and isodesmosine, and by slab gel electrophoresis, which showed a molecular weight ranging from 70,000 to 300,000 daltons.

To exclude crossreactions with elastin, amyloid P or lysozyme (Mera and Davies, 1987) control slides of arteries, solar elastosis, amyloidosis, and macrophage infiltrations were immunostained with the type IV collagen antibody.

Biochemistry. For biochemical control of the specificity of the type IV collagen antibody, direct and indirect ELISA was performed. The direct technique was done by coating a polyvinyl chloride (PVC) tube with 10 μg/ml type IV collagen overnight at 4°C in Tris-saline, pH 7.6. Afterwards, serial dilutions of the antibody against human type IV collagen ranging from 1:20 to 1:5120 were incubated in the tube. The same procedure was done after pre-incubating the antibody with 10 μg/ml elastin in Tris-saline. Indirect ELISA was done by coating a PVC tube with 10 μg/ml elastin overnight at 4°C in Tris-saline, pH 7.6. Afterwards, as described before, different dilutions of the antibody against human type IV collagen were incubated in the tube. After extensive washings (five times for 5 min each in PBS), the rabbit antibody to human type IV collagen bound to the walls of the tubes was revealed by incubation for 30 min with peroxidase-conjugated swine anti-rabbit immunoglobulin, diluted 1:20 in PBS. A further control consisted of immunostaining the Western blot of the elastin and human collagen type IV antigen with the antibody against human type IV collagen.

Results

On paraffin-embedded and HE-stained sections, the periductal

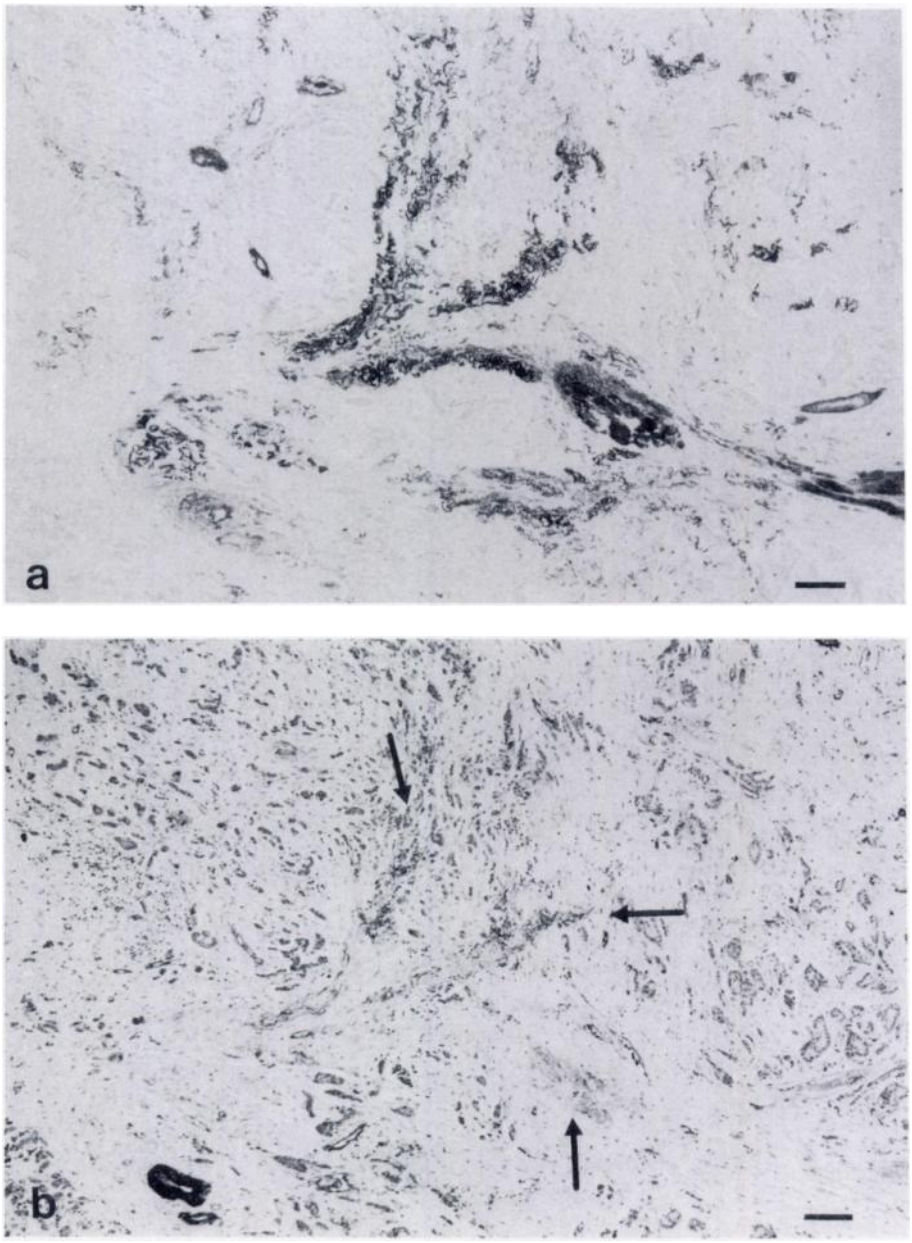

Figure 5. AFA-fixed paraffin sections of a scirrhous carcinoma of female breast. (a) Elastin stain showing the interstitial elastosis. (b) Adjacent section illustrating distinct immunoreactivity for type IV collagen (arrows). Original magnification × 65. Bars = 100 μm.

elastosis present in most of the cases of breast cancer consisted of a broad band of granular eosinophilic material surrounding main ducts lined by a neoplastic or non-neoplastic epithelium. With the elastin staining this elastosis appeared as black deposits. The same elastotic depositions were observed around some veins, and sporadically around an artery. Tumors with extensive collagenous stroma showed rounded or linear slit-like lumina bordered by atrophic epithelium or endothelium surrounded by similar elastotic material. Occasionally no remnant of a lumen could be observed. Half of the tumors showed interstitial elastosis present as elastotic material dispersed in the collagenous stroma, without any relation to ductal structures.

In serial frozen sections, a fine granular type IV collagen cuff but no laminin immunoreactivity was seen in areas of pronounced periductal elastosis (Figure 1). In areas of interstitial elastosis, weak granular type IV collagen immunoreactivity but no laminin staining was observed. Both type IV collagen and laminin were distinctly stained in the basement membranes of all ducts and vessels.

In the serial AFA-fixed paraffin sections the localization and organization of type IV collagen were crisp, allowing a detailed description. Similar to the frozen sections, only type IV collagen immunoreactivity was present as a broad cuff in the elastosis surrounding the small and large ducts. This cuff consisted of type IV collagen layers composed of fine intertwined fibers and membranes,

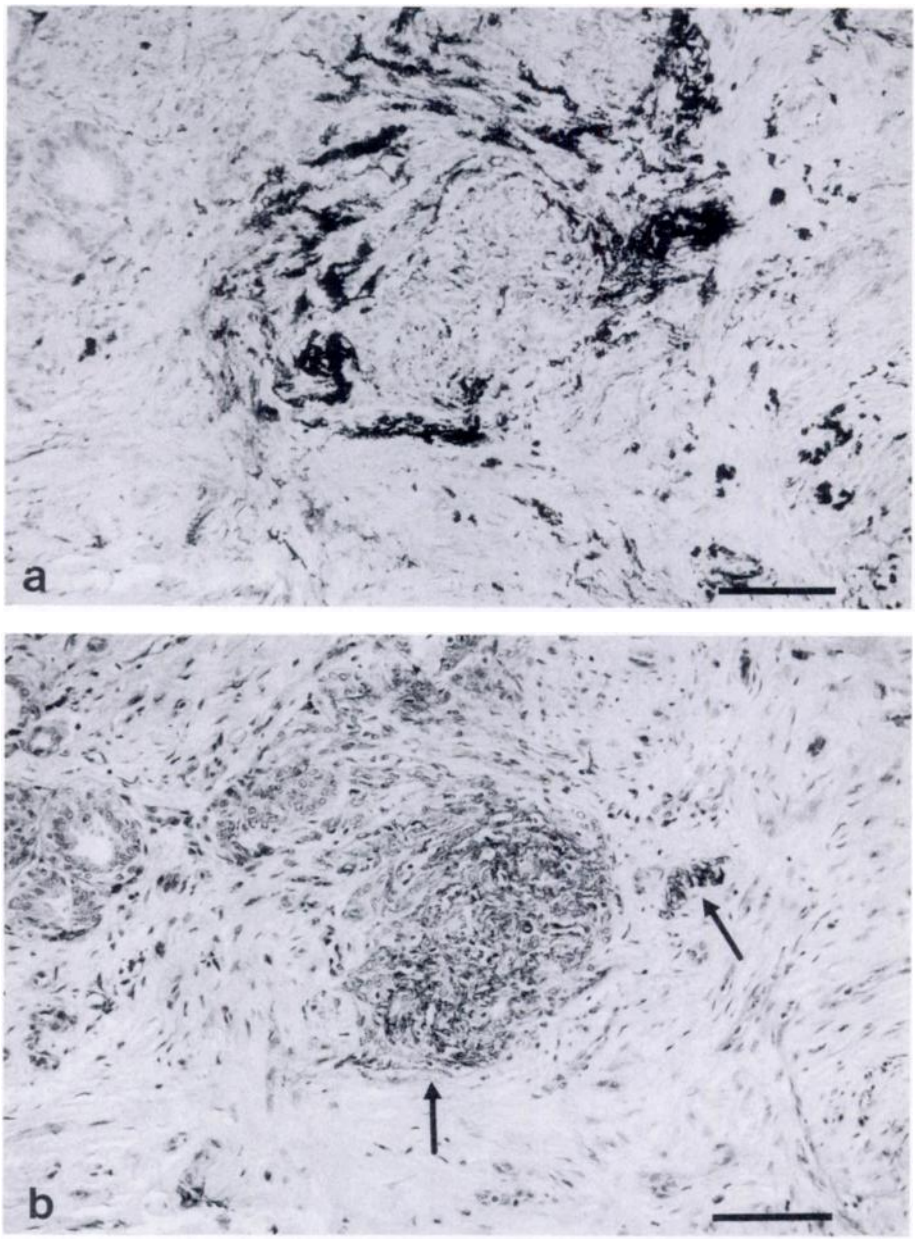

Figure 6. AFA-fixed paraffin sections of cystic mastopathy of female breast. (a) Elastin stain showing the presence of elastin in an area of sclerosing adenosis. (b) Adjacent section with distinct immunoreactivity for type IV collagen in the areas of elastosis (arrows). Original magnification × 160. Bars = 100 μm.

most conspicuous in infiltrating carcinomas with an extensive intraductal component, in tubular carcinomas with a radial scar, and in the postpartum breast (Figures 2 and 4a). Occasionally the type IV collagen staining was accentuated just beneath the basement membrane, diminishing progressively to the outer side of the elastotic cuff (Figure 3). Elastosis around slit-like lumina showed a weaker type IV collagen staining. The periductal elastosis of all tumors corresponded to a type IV collagen immunoreactivity ranging from strong to weak. In scirrhous carcinoma the remnants of ducts could be identified by their type IV collagen cuff (Figure 4b). In the areas of interstitial elastosis, locally weak but distinct fibrous immunostaining for type IV collagen was observed (Figure 5). In the areas of vascular elastosis, distinct type IV collagen immunoreactivity was present but did not reveal a conspicuous increase of basement membrane material, probably owing to the normal presence of basement membranes around the smooth muscle cells of these vessels.

In general, the normal and benign breast tissue showed a distinct continuous basement membrane around the ducts and the lobules with type IV collagen and laminin, without interstitial staining in the stroma. However, in the areas of sclerosing adenosis in a cystic mastopathy, and around some ducts filled with a hyperplastic epithelium, areas of weak to moderate immunoreactivity for type IV collagen within elastotic material were observed (Fig-

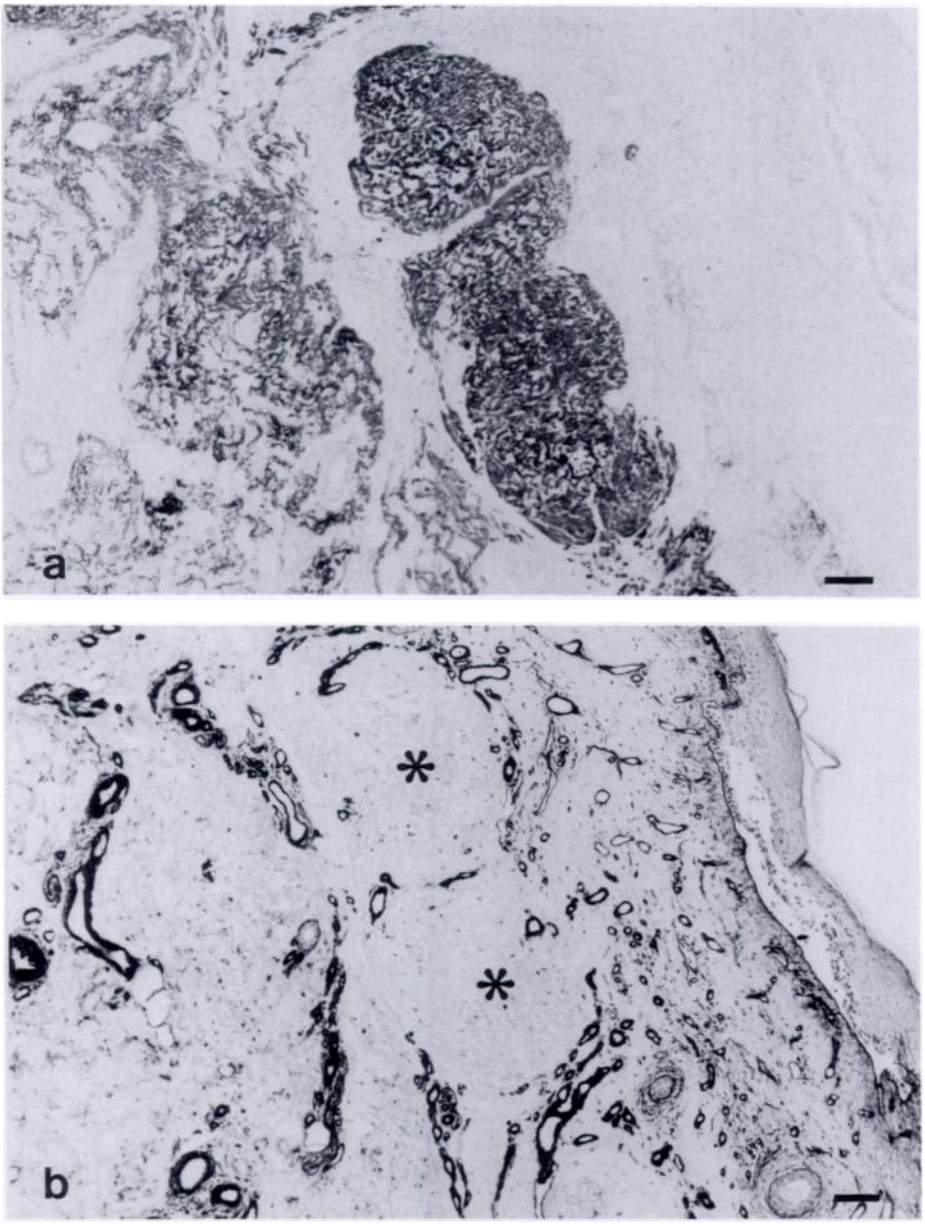

Figure 7. AFA-fixed paraffin sections of solar elastosis of the skin. (a) Elastin stain demonstrating extensive presence of elastin. (b) Type IV collagen immunoreactivity around the vessels but not in the areas of elastosis (asterisks). Original magnification × 65. Bars = 100 μm.

ure 6). The skin sections with solar elastosis (Figure 7), with extensive amyloidosis, and with massive macrophage infiltration, as well as the elastic membranes of the arteries, showed no staining in the abnormal tissue components.

Incubation with the diluent buffer and the non-immune serum gave no staining. Incubation with the polyclonal rabbit antiserum against keratin stained only cells of epithelial origin but no connective tissue or areas of elastosis. After pre-incubation of the type IV collagen antibody with different amounts of elastin, only a very slight attenuation of the staining was noted by pre-incubation with 800 μg elastin/ml. The biochemical control of the specificity of the type IV collagen antibody with direct and indirect ELISA (Figure 8) and Western blot (Figure 9) confirmed the absence of significant crossreactivity between elastin and the type IV collagen antibody used in our study.

Discussion

This study demonstrates the presence of different amounts of type IV collagen in the areas of periductal elastosis using frozen and AFA-fixed material from breast cancers. Possible crossreactivity of the type IV collagen antibody with elastin could be excluded for the following reasons:

1. The human placenta, which is the source of the type IV collagen antigen, is devoid of elastin.
2. No type IV collagen immunoreactivity was observed in skin

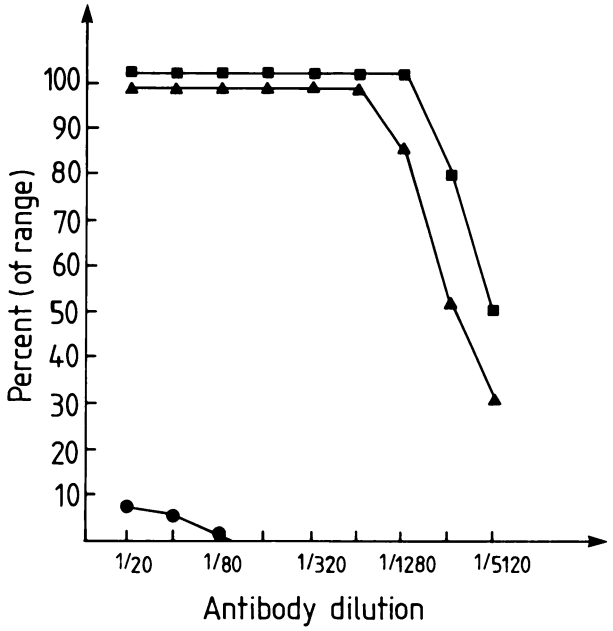

Figure 8. Specificity of anti-type IV collagen antibody by ELISA. Titration of type IV collagen antibodies against elastin-coated PVC tube (indirect ELISA; ●——●) and against type IV collagen-coated PVC tube (direct ELISA), with (▲——▲) and without (■——■) pre-incubation with elastin. Binding of antibody to antigen was measured with peroxidase-conjugated swine anti-rabbit immunoglobulin. Enzyme activity was quantitated optically.

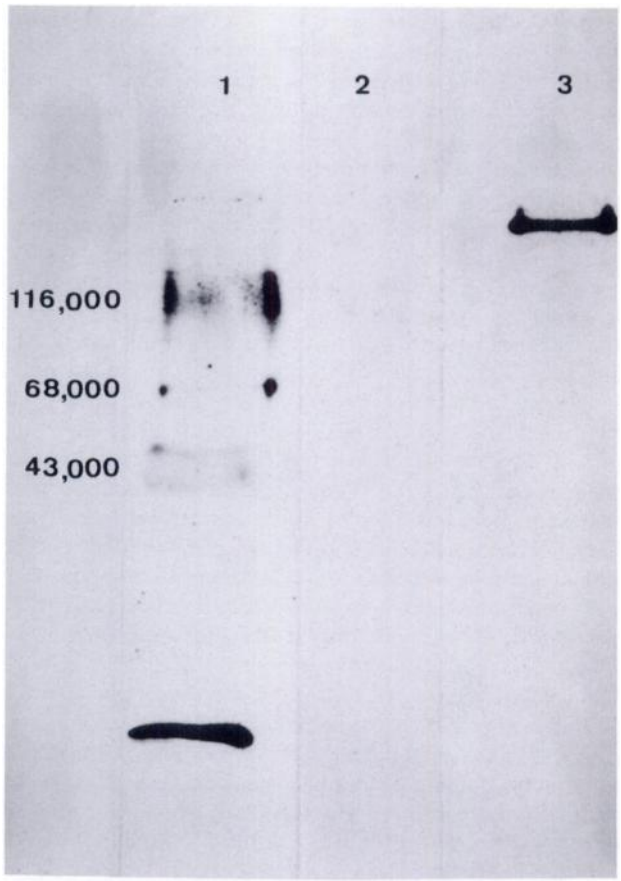

Figure 9. Immunostaining with the type IV collagen antibody (50 μg/ml in PBS) of the Western blot of elastin (50 μg; **lane 2**) and human type IV collagen (25 μg; **lane 3**) showing a specific reaction only with type IV collagen. **Lane 1** shows the reference proteins stained with Coomassie blue: 116,000 daltons (β-galactosidase), 68,000 daltons (bovine serum albumin), and 43,000 daltons (ovalbumin).

sections with extensive solar elastosis or in the membrana elastica of arteries.

3. Pre-incubating the type IV collagen antibody with large amounts of elastin showed no altered immunostaining of the sections.
4. The ELISA technique showed no significant reaction of the type IV collagen antibody with elastin.
5. Western blot analysis showed specific staining of the type IV collagen antibody with type IV collagen but not with elastin.

Until now, an association between basal membrane-like material and elastin was noted only in embryonic aorta (Kadar, 1974), in cultured smooth muscle cells of immature guinea pig aorta (Ross, 1971), and in pleomorphic adenoma of the salivary gland (David and Buchner, 1980).

A strong positive correlation was found between the degree of elastosis in breast tumors, the amount of estrogen receptors (Giri et al., 1987; Rasmussen et al., 1985), and the severity of epithelial hyperplasia (Parfrey and Doyle, 1985). The different amounts of type IV collagen can be due to variations in type IV collagen production dependent on a variety of factors, such as hormonal stimulation and intraductal epithelial proliferation. Under experimental conditions, type IV collagen production by acini and ducts isolated from virgin rat mammary glands was dependent on the presence of such supportive hormones as insulin, progesterone, and estradiol (Liotta et al., 1979).

Parfrey and Doyle (1985) showed that periductal and interstitial elastosis was present in cystic mastopathies and increased progressively with the severity of the epithelial hyperplasia, increasing further in periductal and infiltrating duct carcinoma. Egger and associates (1982) stated that narrow ducts with a conspicuous periductal elastosis mark the site of active growth and the zone "at risk" for carcinogenesis. We found slight amounts of type IV collagen in the areas of elastosis around some hyperplastic ducts and in sclerosing adenosis, demonstrating also in benign breast lesions a relation between the accumulation of type IV collagen and the presence of elastin.

The origin and significance of interstitial elastosis are a matter of debate. Interstitial elastosis probably arises in a variety of ways. Jackson and Orr (1957) suggested a collapse of the ducts encircled by elastotic material and a transition to interstitial elastosis. We found remnants of ducts recognizable by a type IV collagen cuff and small but distinct amounts of type IV collagen present in the areas of interstitial elastosis. This pattern is consistent with a transition from periductal to interstitial elastosis.

The absence of type IV collagen in solar elastosis indicates that the formation of elastosis is not always associated with deposition

of type IV collagen. Chen et al. (1986) also noted the absence of basement membrane material in solar elastosis. Kligman (1986) reported an increased production of elastic fibers, with increased breakdown of dermal connective tissue, following sun damage. Roach (1983) proposed that differences in stress and strain may stimulate elastin production in the aorta of mammals. The deposition of elastin is probably only a secondary event after the accumulation of type IV collagen, damage to the dermal connective tissue, or a stress factor.

To explain the periductal elastosis in breast cancer, the following hypothesis is proposed. There exists a cyclic production and degradation of basement membrane material by benign and malignant epithelial cells. Many authors have already demonstrated the biosynthesis and turnover of basement membrane material by tumor cells (Martinez-Hernandez and Amenta, 1983; Pierce and Spiro, 1977). This production of type IV collagen forms a skeleton. The exaggerated presence of type IV collagen beneath the basement membrane suggests, in particular, a process originating from epithelial cells. The absence of laminin may be owing to the higher sensitivity to proteolytic enzymes or to lack of laminin formation by the transformed cells. It is known that different basement membranes have different ratios of type IV collagen and laminin. For instance, although lens capsule contains some laminin, at least 90% of its dry weight is type IV collagen. It is therefore plausible that some proteases could degrade the small amounts of laminin, with persistence of some immunoreactive type IV collagen. Liotta et al. (1977) found that tumor cells could degrade collagenous and noncollagenous components of the basement membrane. Tumor cells are believed to secrete hydrolytic enzymes or to induce host cells to secrete enzymes that can degrade the matrix in localized regions (Liotta, 1986). Recently, protease treatment of cryostat sections revealed that laminin could be removed more easily from all tissues than could type IV collagen (Leu and Damjanov, 1988). The discrepancy between laminin and type IV collagen staining may also depend on the presence of some proteases inhibiting selectively type IV collagenase and contributing to the accumulation of type IV collagen (Davies and Mera, 1987; Wooley, 1982). However, degradation of laminin was not demonstrated in this study, and the problem of the low levels of identifiable laminin remains unsettled. In a second stage, the type IV collagen skeleton could form the matrix for secondary deposition of elastotic material, probably secreted by stromal cells (Tamimi and Ahmed, 1987; Nakanishi et al., 1983).

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