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Heterogeneous distribution of actin, myosin, fibronectin and basement membrane antigens in primary and metastatic human breast cancer *

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Summary. The distribution of actin, myosin, fibronectin and basement membrane antigens has been studied by indirect immunofluorescence in benign and malignant human breast lesions. While benign tumors showed only minor differences from normal mammary tissue, tumors of different histological types displayed a heterogeneous distribution of the antigens studied. Heterogeneity was observed within the same tumor, among different neoplasms and between primary tumors and autologous metastases. As a common characteristic, most of the tumors did not stain for actin and myosin, the pattern being similar to that found in myoepithelial cell distribution. In transformed epithelia there was often a lack of detectable actin with a myosin-positive fluorescence. Staining for both proteins was diffused to most of the cell cytoplasm. Staining for fibronectin was seen in only a minority of the cases, with medullary tumors being the most positive. Basement membrane stain was either absent or decreased and fragmented, except in rare ductal, i.e. papillary, carcinomas. Medullary tumors displayed an almost continuous, though fragmented basement membrane in approximately 70% of cases.

Key words: Actin – Myosin – Fibronectin – Basement membrane – Breast cancer

Experimental and clinical data indicate that most animal and human tumors, at least at the time they become clinically apparent, consist of cell populations that are characterized by different phenotypes and biological behaviors (Fisher 1980; Hart et al. 1981). Thus, the study of this heterogeneity has become relevant not only for an understanding of tumor cell biology, but also because it may provide additional criteria for tumor neoclassifications

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and for assessing whether given tumor cell phenotypes could be predictive of the cell response to therapy as well as of their metastatic potential and organ tropism (De Baetselier et al. 1980; Shearman et al. 1980).

In the present study we have analyzed whether proteins which control cell motility, i.e., actin and myosin, as well as components of the pericellular matrix (fibronectin and basement membranes), which regulate cell-substrate interactions and cellular scaffolding, undergo changes in distribution in human breast cancers. The data presented here indicate that mammary tumors express these antigens to a variable degree. This heterogeneity can be detected within individual tumors, in neoplasms of different histological types, and by comparing primary tumors with metastatic foci.

Materials and methods

Tissues

Tissue fragments were obtained immediately after tumor removal. The specimens were trimmed into small cube-shaped samples and snap-frozen in liquid nitrogen. The breast tumors collected included benign lesions: fibrocytic disease (3 cases), fibroadenomas (2 cases), cytosarcoma phyllodes (3 cases); the malignant lesions included: infiltrating ductal carcinomas (10 cases), invasive lobular (2 cases), papillary (1 case), tubular (3 cases), adenoid cystic (1 case), medullary (7 cases) and colloid (1 case) carcinomas. Histological classification of the tumors was in accordance with the recommendations of the World Health Organization (1982).

In five infiltrating ductal carcinomas and one lobular carcinoma, both the primary tumor and one axillary metastasis were obtained. Normal breast tissue was obtained from four different patients undergoing surgery for mammoplasty. All of the specimens were from patients that had not undergone chemo- or radiation therapy.

Antisera

The serum of a patient with chronic active hepatitis as proven both clinically and histopathologically was used as a source of anti-smooth muscle actin antibodies. Serum specificity was assessed by absorption with purified actin; this was kindly performed by Dr. F. Bottazzo (Middlesex Hospital, London). The adsorption removed all the cell-staining patterns characteristic of anti-actin human sera (Bottazzo et al. 1976). The serum did not contain rheumatoid factor, but had low titers of antinuclear antibodies, which were removed by absorption with insoluble nucleoproteins (Tan 1967).

Anti-smooth muscle myosin, a gift from Dr. U. Groschel-Stewart (Technische Hochschule, Darmstadt, Federal Republic of Germany) was produced in rabbits after short-term immunization with purified smooth muscle myosin extracted from chicken gizzard (Groschel-Stewart et al. 1976).

Anti-human plasma fibronectin, which extensively cross-reacts with the cell-associated glycoprotein, was produced in rabbit and characterized as described previously (Natali et al. 1981).

Anti-human whole-soluble-antigen antiserum was produced in rabbits, using an antigen (renal basement membrane) preparation (Marquardt et al. 1973) kindly provided by Dr. Frank J. Dixon (Scripps Clinic, La Jolla, USA). In separate experiments, the antiserum was shown to be devoid of species and organ specificity.

Rabbit anti-laminin antiserum (lot no. 20104) was purchased from Bethesda Research Laboratory (Gaithersburg, Ma., USA). The specificity of the reagent, established by the manufacturer by enzyme-linked immunoassay and immunoprecipitation, was not further characterized.

Prior to their use in indirect immunofluorescence (IIF), antimyosin, anti-bm and laminin antisera were extensively absorbed with AB RH+ red blood cells (rbc) and insolubilized

normal human plasma (Avrameas et al. 1969). Anti-actin and fibronectin were absorbed only with rbc.

Fluorescein isothyocianate (FITC)-labelled antisera to human and rabbit immunoglobulins (Ig) were purchased from Cappel Labor (Cochranville, PA., USA). Antisera were repeatedly absorbed with rbc and insolubilized normal human plasma (anti-rabbit Ig antiserum). Both antisera had a fluorescein/protein ratio of 3 and were employed at the antibody concentration of 1 mg/ml.

Indirect immunofluorescence

The tissue substrates for IIF consisted of 4-μm cryostat sections, which were fixed for 10 min in absolute acetone. In separate experiments, this fixation procedure was shown not to interfere with the immunoreactivity of the different antigens studied. Once fixed in acetone, the sections were either immediately processed for IIF or stored at −35° C until use. These storage conditions proved not to alter the reactivity of tissues with different antisera.

In brief, the IIF was performed as follows: the cryostat sections were incubated with antisera for 30 min at room temperature in a moist chamber. They were then washed with phosphate (0.01 M)-buffered saline (0.15 M) (PBS), pH 7.2, and layered with an appropriate dilution of FITC-labelled antiserum for an additional 30 min. After a final wash with PBS, sections were mounted in PBS-buffered glycerol and observed on a Leitz Ortholux II fluorescence microscope equipped with phase-contrast microscopy and epi-illumination. For each biopsy, adjacent cryostat sections stained with 0.5% toluidine blue in PBS provided the histological control of the lesion.

The specificity of the fluorescent patterns observed was assessed by staining sections with normal rabbit and human sera as well as with antisera (anti-fibronectin and bm) absorbed with the homologous antigen.

Results

Immunohistochemical reactivity of normal breast tissue and benign breast lesions

Indirect immunofluorescence of normal mammary gland tissue sections with specific antisera showed that smooth muscle actin and myosin are present in the breast ducts in two distinct cell types. The first is represented by elongated cells, which run along the periphery of the epithelial ducts around the inner aspect of the basement membrane. These cells most likely represent myoepithelial cells. The staining with both antisera is intense and diffused to whole cytoplasm The second cell type is represented by the epithelial cells, in which both contractile proteins appear to be confined mainly to the cell apical portion (Fig. 1 a and b). A specific weak stain for fibronectin was detected along the basement membrane of both the mammary ducts and the vessel walls (Fig. 1 c). No convincing staining of the epithelial cells was observed.

Characteristically, the whole stromal portion of the gland was stained by antisera to actin, myosin and fibronectin, with a fluorescent pattern arranged in strands with no specific orientation. Staining with anti-basement membrane and laminin antibodies outlined a fine, continuous ribbon-like fluorescence surrounding single epithelial ducts (Fig. 1 d). In two breast fibroadenomas and in three cases of fibrocystic disease of the breast, the fluorescent patterns observed with the battery of antisera employed were almost identical with those described in normal breast tissue. In fibroadeno-

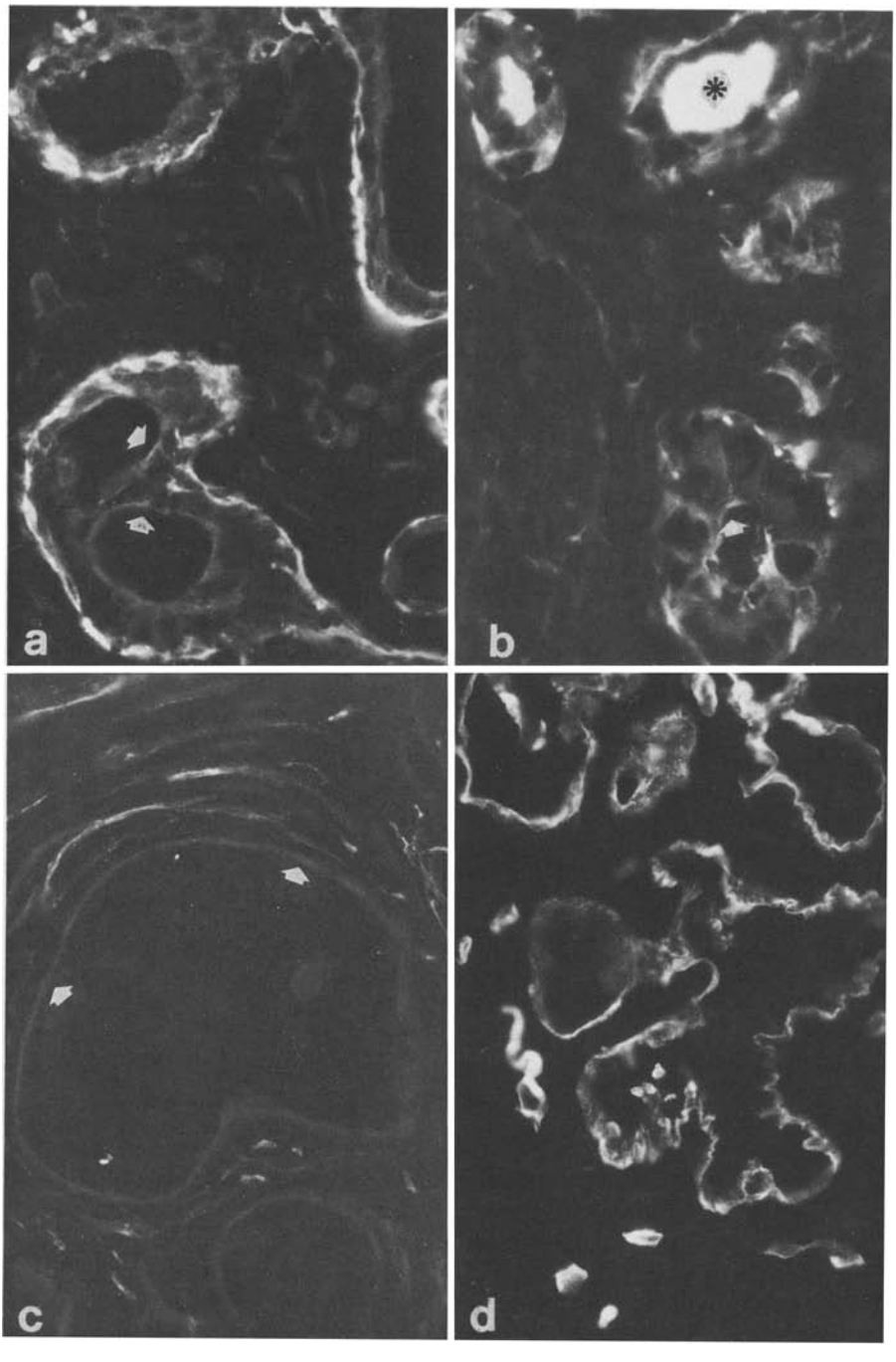

Fig. 1 a–d. Distribution of actin (**a**), myosin (**b**), fibronectin (**c**) and laminin (**d**) on cryostat sections of normal breast tissue detected by IIF. Staining with anti-actin and myosin antisera is localized in the cytoplasm of basal myoid cells and at the apical portion of the epithelial cells (*arrows*). A faint continuous stain for fibronectin is present along the basement membrane (*arrows*), which is outlined by anti-laminin antibodies, with a linear fluorescent pattern. The *black asterisk* marks the autofluorescence of epithelial duct lumen. **a:** × 400; **b, c, d:** × 640

mas, however, and varying from normal breast tissue (not shown here), the myosin stain was increased in myoepithelial cells, which looked like irregularly branched cells. Basement membrane was increased in thickness, often with apparent splitting. In cystic disease, epithelial cells lining the wall of the cysts displayed a normal distribution of actin and myosin, although staining of the cell glycocalyceal region resulted only with anti-myosin antibodies (Fig. 2a and b). Myoepithelial cells were intensely stained by the two antisera, had a globoid shape and somehow appeared hypertrophic (Fig. 2a and b). Staining for fibronectin was clearly increased on the cyst wall (Fig. 2c). Several capillaries were detected running along the basement membrane of the cysts, which was intact in its distribution (Fig. 2d) but was frequently found to be split and interdigitating myoepithelial cells (Fig. 2e).

Immunohistochemical reactivity of primary breast carcinomas of different histological types

Table 1 presents the way in which breast tumors of different histological types reacted with a panel of antibodies. The following common characteristics were found. In most of the tumors no staining of myoepithelial periductal cells could be observed with antisera to actin and myosin, except in one lobular carcinoma. The positivity of the tumor cells with the two antisera, when present, displayed a pattern which was completely different from that of normal breast tissue. In fact, the staining was either diffused cytoplasmic, more pronounced around the nucleus and underneath the plasma membrane, or entirely absent (Fig. 3a–d). Cytoplasmic staining for both contractile proteins was also seen in tumor cells infiltrating the stroma and, in some cases, clearly outlined clusters of tumor cells. The staining for both contractile proteins in most instances showed a complete dichotomy,

Table 1. Expression of actin (Act), myosin (Myo), fibronectin (Fbr), basement membrane (Bm) antigens and laminin (Lm) in human breast tumors

Histological tumor type	Act[b]	Myo[b]	Fbr	Bm/Lm[b]
Fibroadenoma	2/2[a]	2/2	2/2	2/2
Cystic hyperplasia	3/3	3/3	3/3	3/3
Cystosarcoma phylloides	2/3	2/3	1/3	3/3
Infiltrating ductal carcinoma	4/10	8/10	2/10	5/10[c]
Lobular carcinoma	1/2	2/2	0/2	0/2
Tubular carcinoma	1/3	3/3	1/3	0/3
Papillary carcinoma	1/1	1/1	0/1	1/1
Adenoid cystic carcinoma	0/1	0/1	0/1	0/1
Medullary carcinoma	2/7	5/7	4/7	5/7
Colloid carcinoma	1/1	1/1	0/1	1/1

[a] No. positive/no. tested by IIF

[b] The stain was highly variable in intensity and distribution within each histological tumor type

[c] Positive cases showed weak and fragmented staining

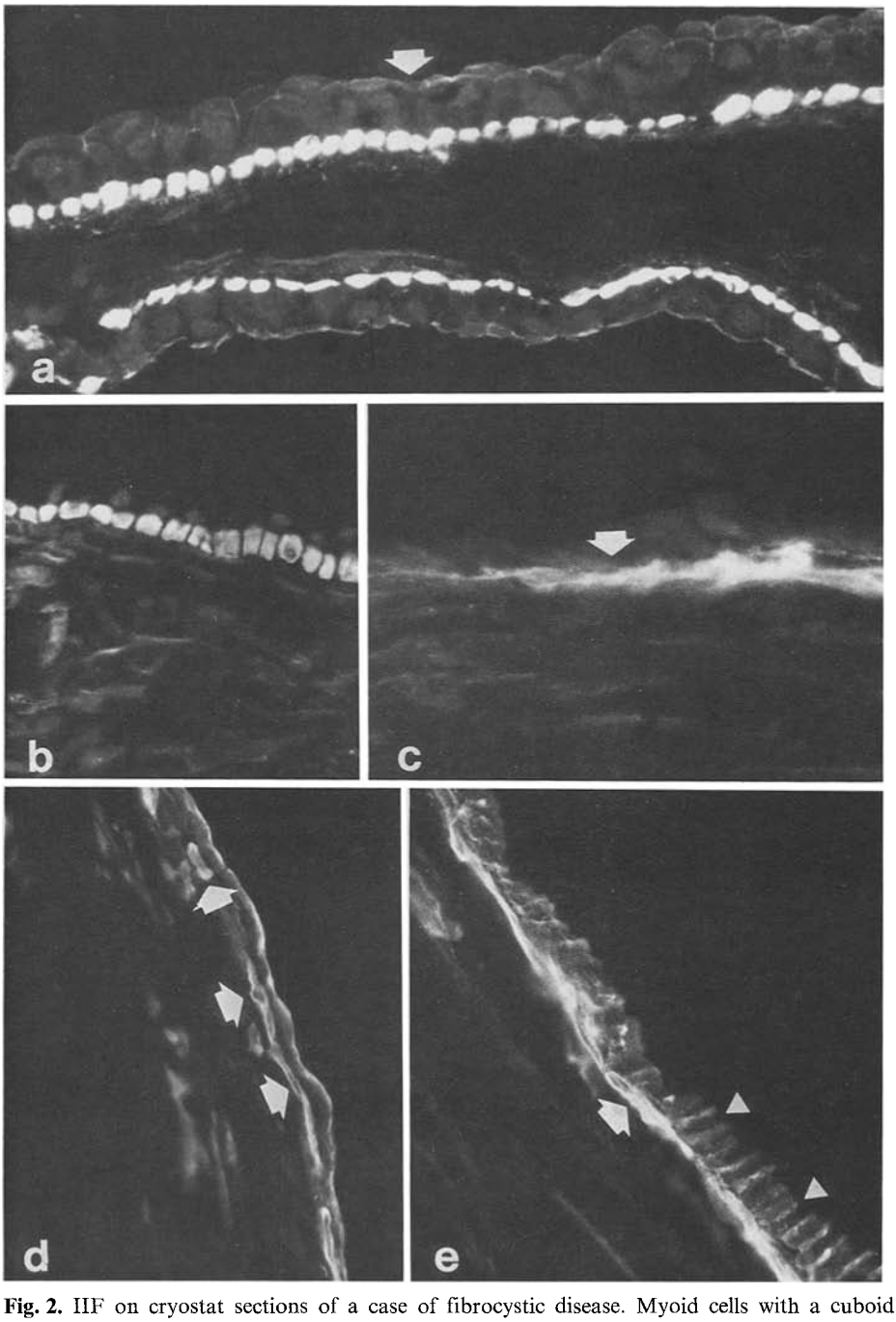

Fig. 2. IIF on cryostat sections of a case of fibrocystic disease. Myoid cells with a cuboid shape in a cyst wall are intensely stained by myosin (**a**) and actin (**b**) antibodies. Specific stain for myosin is also present along the luminal side of the epithelial cells (**a**, *arrow*). Stain for fibronectin is stronger than in normal breast tissue and has a split distribution (**c,** *arrow*). Numerous vessels are present along the basement membrane (**d,** *arrows*) of the cysts, which sometimes appear split (**e,** *arrow*) and in some section planes with a sowteeth distribution (**e**, *arrow-head*). **a, b:** ×400; **c:** ×640; **d, e:** ×400

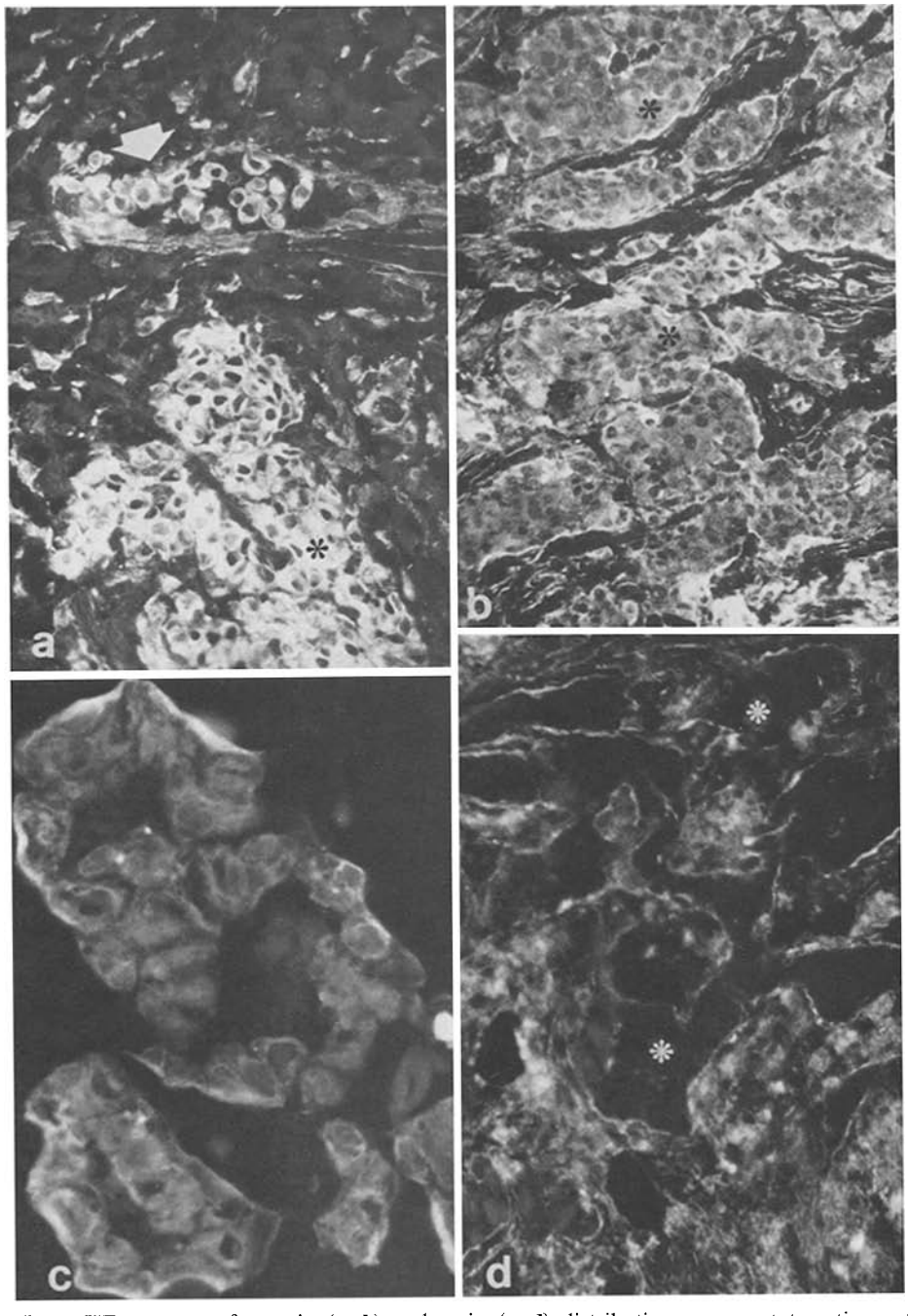

Fig. 3. IIF patterns of myosin (**a, b**) and actin (**c, d**) distribution on cryostat sections of breast tumors. Myosin is homogeneously present in the cytoplasm of tumor cells of two intraductal carcinomas (*black asterisks*). In panel **a**, tumor cells inside the vessel lumen (*white arrow*) display myosin-specific stain. Actin has a diffused cytoplasmic distribution in cells from a mucoid carcinoma (**c**), while it is present only with a strand-like pattern in the stroma of the same intraductal carcinoma, strongly positive for myosin in panel **a** (*white asterisks* mark tumor cell nests). **a, b:** $\times 160$; **c:** $\times 400$; **d:** $\times 160$

Table 2. Expression of actin (Act), myosin (Myo), fibronectin (Fbr), basement membrane (bm) antigens and laminin (Lm) in primary breast carcinomas and autologous axillary metastases

Patient	Histological tumor type	Primary tumor				Metastasis			
		Act	Myo	Fbr	Bm/Lm	Act	Myo	Fbr	Bm/Lm
Co	Tubular carcinoma	−	++	−	−	−	++	−	−
Df	Lobular carcinoma	−	−	−	±	±	+	−	−
Le	Infiltrating ductal carcinoma	−	+	−	±	−	++	−	−
Et	Infiltrating ductal carcinoma	−	+	−	±	−	+	−	−
Po	Infiltrating ductal carcinoma	−	±	−	±	±	+	−	±
Ri	Infiltrating ductal carcinoma	+	±	−	±	+	++	±	±

± Isolated cell nest positive or homogeneously weakly positive bm fragmented and thin; + Variable stain among tumor cells; ++ Homogeneous stain

especially in cases of infiltrating ductal carcinoma, the most frequent pattern being a loss of actin stain with a positive, often increased myosin fluorescence.

A specific tumor cell fluorescence for fibronectin was detected in only two cases of infiltrating ductal carcinoma, in one case of sarcoma phylloides, and in four of seven medullary-type tumors. The fluorescence stain observed on the cell membrane displayed a spotty distribution (Fig. 4a) and was highly heterogeneous. In medullary-type tumors, a network of fluorescent stain could also be observed within the tumor cell nests (Fig. 4b). In all other tumors studied, fibronectin was present at the periphery of the tumor cell nests with a pattern paralleling that of basement membrane.

Staining with anti-basement membrane and laminin antibodies was either absent, barely detectable or highly heterogeneous in most infiltrating ductal, tubular, lobular, cystic adenoid and colloid carcinomas. A reduced or fragmented pattern of staining was seen in the remaining histological types of tumor (Fig. 5a–d). Papillary carcinomas displayed an almost normal distribution.

Comparison of primary tumor and autologous metastasis as regards immunohistochemical staining

An attempt was made to evaluate the expression of the different antigens studied with regard to the primary tumor and its metastatic foci. To this end, tumor tissues from four infiltrating ductal carcinomas, one tubular, and one lobular carcinoma were assayed, together with the autologous axillary lymph node metastasis involved using indirect immunofluorescence.

As summarized in Table 2, the expression of all the antigens showed some degree of heterogeneity between the two tumor sites, except for fibro-

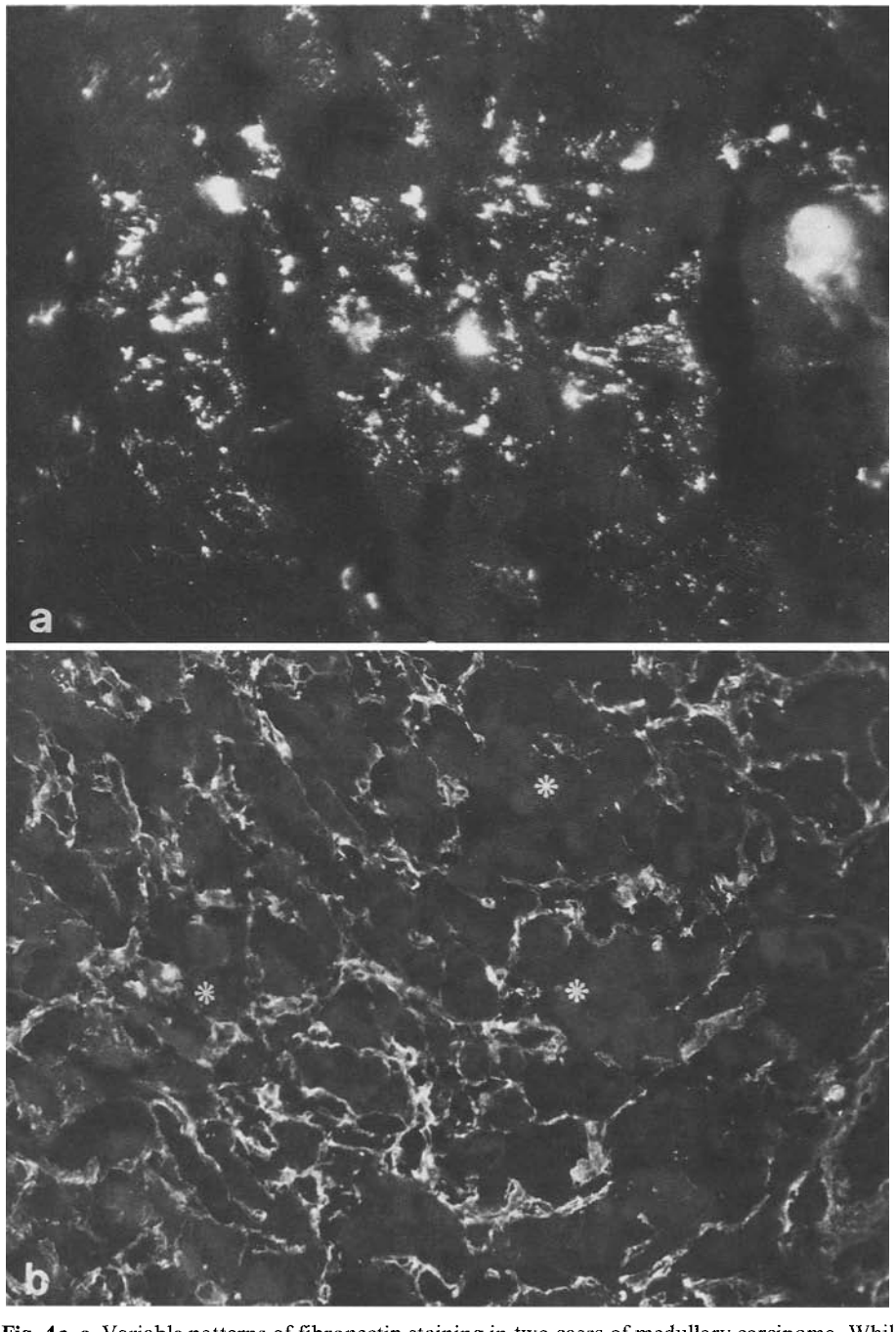

Fig. 4a–c. Variable patterns of fibronectin staining in two cases of medullary carcinoma. While in panel **a** the stain outlines single tumor cell boundaries in a punctate pattern, in panel **b**, a network of fibronectin stain surrounds the tumor cell nests (*asterisks*) of variable sizes. **a:** ×1000; **c:** ×160

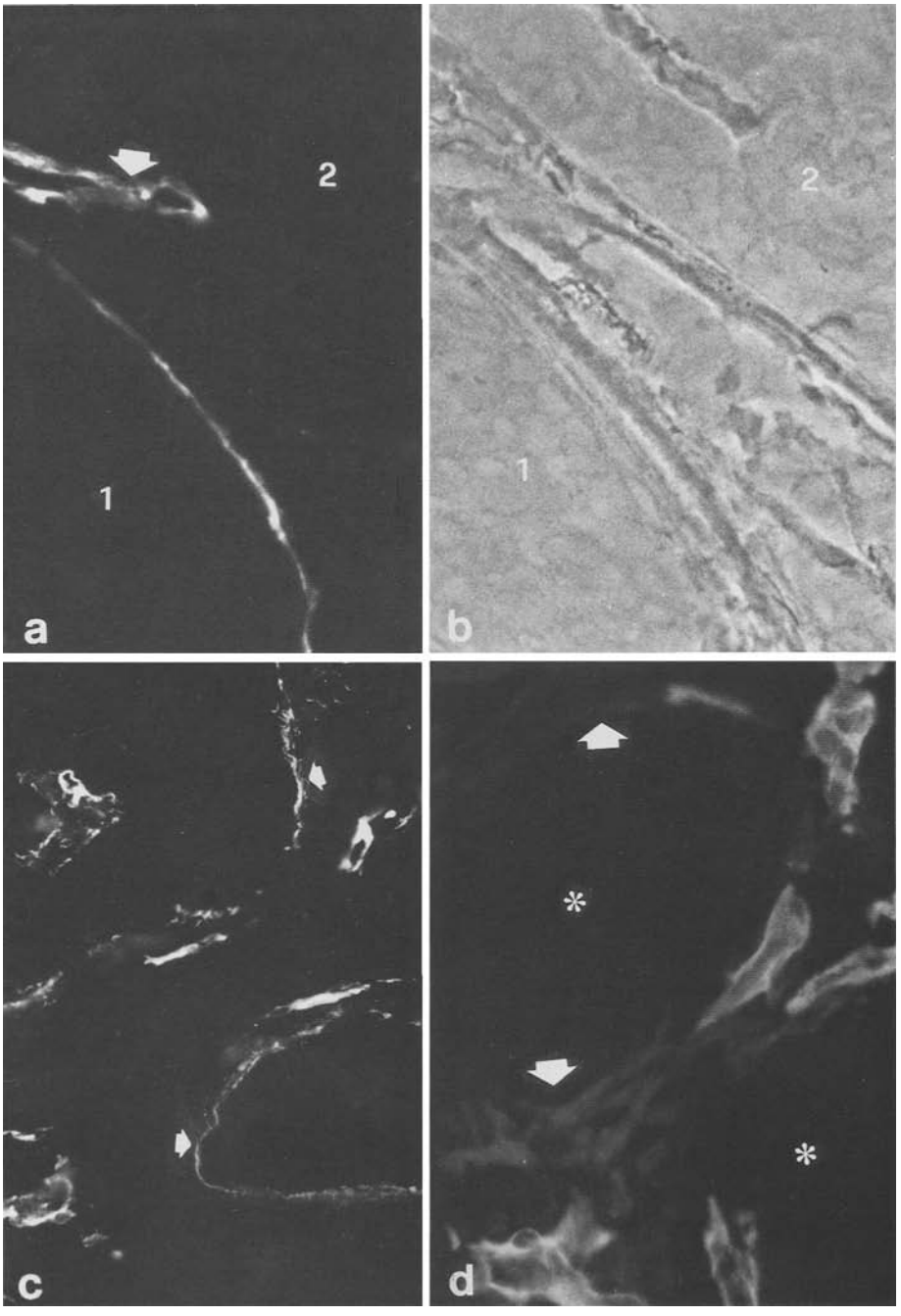

Fig. 5a–d. Variable distribution of basement membrane antigens in breast carcinomas. Cryostat sections of a case of infiltrating ductal carcinoma observed on IIF (**a**) and phase-contrast microscopy (**b**) show that specific basement membrane stain surrounds the tumor area (1), while in the tumor area (2), it is absent. The *arrow* in **a** marks a vessel wall. In cases of medullary carcinomas (**c**), the neoplastic cell nests appear to be homogeneously outlined by basement membrane (*arrows*). In a case of adenoid cystic carcinoma (**d**) no basement membrane stain (*arrows*) surrounds the tumor cell nests (*asterisks*). **a, b, d:** × 640; **c:** × 400

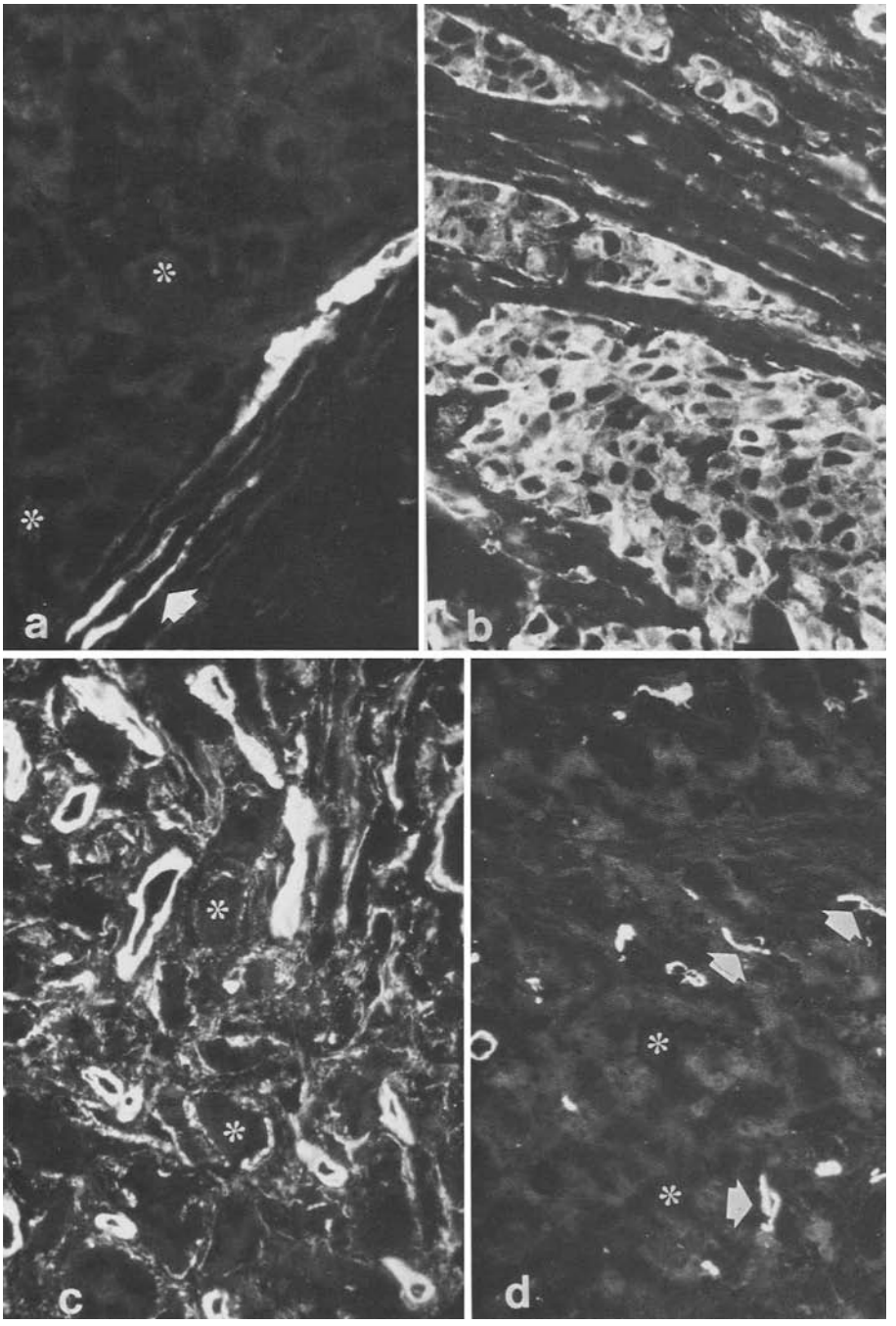

Fig. 6a–d. Heterogeneous expression of myosin (**a, b**) and basement membrane (**c, d**) antigens in primary tumors and in autologous axillary metastases. Myosin stain is barely detectable in primary ductal carcinoma cells (**a,** *asterisks*), where only the vessel media react with the antiserum (*arrow*), while it is homogeneously strong in the axillary metastasis (**b**). Staining for basement membrane is present in primary ductal carcinoma with an interrupted pattern around the tumor cell nests (**c,** *asterisk*) and continuously around the vessel walls. In the autologous metastasis (**d,** *asterisks*) only the capillary walls (*arrows*) are stained by the bm antiserum. **a:** ×400; **b:** ×640; **c, d:** ×160

nectin, which displayed a rather homogeneous behavior. Furthermore, four of six metastases lost all reactivity with anti-basement membrane and laminin antisera (Fig. 6a–d).

Discussion

As described for other plasma membranes, as well as for cystoplasmic breast tumor-associated antigens and histocompatability antigens (Natali et al. 1983; Weiss et al. 1981; Horan-Hand et al. 1983), the present study has shown that actin, myosin, fibronectin and basement membranes also have a heterogeneous expression in these neoplasias. This heterogeneity, which is present within individual tumors, as well as in tumors of different histological types, and both primary tumors and autologous metastatic tissue, was shown to have a variable effect on the antigens studied.

Differences in expression of cellular contractile proteins have been reported in different human tumors, including breast cancer (Gabbiani et al. 1976). In accordance with previous studies (Gabbiani et al. 1976), we have found that the two proteins do undergo a cellular redistribution in each transformed breast epithelium. At present, it is still unclear whether this is due to an actual increase in concentration or to a change in molecular organization, as described by Low et al. (1981) in skin tumor-associated actin. Contrary to the report of Gabbiani et al. (1976), who also employed active chronic hepatitis antibodies in indirect immunofluorescence, we did not find a high percentage of actin-positive breast tumors. In most of the neoplasias a decrease or absence of actin was paralleled by normal or increased staining for myosin.

Although the biochemical basis of this pattern of expression of the two contractile proteins is unknown, it does not appear to be confined to transformed cells, having been also observed in myoid cells of the seminiferous tubules following experimental cryptorchidism (Bellocci et al. 1980).

The functional importance of this phenomenon in breast tumors and the extent of its occurrence in other neoplasias have not yet been established, but may be relevant in altering cell surface motility and the turnover of plasma membrane-associated antigens. In this context it should be remembered, in fact, that integral components of plasma membrane, such as immunoglobulins (Flanagan et al. 1978) and histocompatibility antigens (Koch et al. 1978), have been shown to be cross-linked to actin upon cell surface polymerization. Modulation (De Baetselier et al. 1980) and degree of expression (Eisenbach et al. 1983) of histocompatibility antigens, at least in experimental tumors, have been demonstrated to influence their metastatic properties.

Fibronectin, the cell surface glycoprotein of high molecular weight, which regulates cell adhesion and positioning (Hynes et al. 1982), was restricted in its expression to breast tumor cells in only one case of sarcoma phyllodes, in two cases of infiltrating ductal carcinoma, in one of six axillary metastases, and in four of seven medullary-type carcinomas. These results are in partial accord with previous reports that describe either a complete

lack of fibronectin in breast tumors (Yang et al. 1980; Labat-Robert et al. 1980; Stenman and Vaheri 1981; Hynes et al. 1982) or the presence of this glycoprotein in a significant number of infiltrating breast adenocarcinomas (Stampfer et al. 1981).

The reason for this discrepancy, if not technical in nature, may reflect differences in the stage of differentiation of the tumors analyzed. No significant correlation could be found between the distribution pattern of actin and fibronectin (Hynes et al. 1978), thus suggesting that no functional relationship exists between the expression of the two antigens in breast carcinoma cells. The overall pattern of fibronectin distribution found in breast tumors does not indicate that this glycoprotein has a relevant role in the biology of these neoplasias in vivo. On the other hand, it should be stressed that medullary-type carcinomas, which are less aggressive than the ordinary invasive ductal type and have a low metastatic potential (Bloom et al. 1970; Fisher et al. 1975), were found to express a higher percentage of fibronectin, either associated with single tumor cells or surrounding tumur cell nests.

As shown in several ultrastructural and immunohistochemical studies reviewed by Ozzello (1979), the present findings indicate that the presence of basement membrane antigens in breast cancer is highly heterogeneous. Further investigations will be necessary to establish whether this heterogeneity reflects the absence of synthesis, or an alteration thereof, increased degradation, or masking by unknown factors of basement membrane components. In agreement with other reports (Foidart et al. 1980; Burtin et al. 1982; Barsky et al. 1983), we were never able to detect cytoplasmic laminin, either in normal or in transformed breast epithelium (Albrechtsen et al. 1981). Although the lower sensitivity of the IIF test employed in this investigation could account for this fact, the heterogeneous expression of basement membrane antigens in primary tumors and between neoplasms and homologous metastases should make us more cautions when employing antisera to basement membrane-associated antigens for localizing breast tumor metastatic foci (Liotta et al. 1979).

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