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Immunohistochemical localization of collagens and fibronectin in human breast neoplasms

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Summary. Forty four specimens from neoplastic, hyperplastic and normal human breast tissues were studied for localization of collagens and fibronectin. Affinity purified antihuman type I, III and IV collagens and antifibronectins were utilized by the indirect immunoperoxidase technique on fixed and paraffin-embedded sections. 86% of the cell cytoplasm of infiltrating ductal and 83% of the lobular cancers were positively stained for collagen type I and III. Collagen type IV, however, was detected in 100% of infiltrating ductal and 83% of lobular carcinomas. Focal cytoplasmic staining is a predominant feature for all antigens in the intraduct carcinoma while a diffuse pattern is encountered in the infiltrating types. Intact basement membranes in various lesions always stained for type IV collagen and showed variable staining for type III collagen and fibronectin. Epithelia of normal, benign, hyperplastic breast and most medullary carcinoma were negative for the three collagen types.

Our results are in favour of the view that infiltrating breast carcinoma cells produce inappropriately the majority of collagens and inconsistently other proteins such as fibronectin.

Key words: Breast neoplasms - Collagens - Fibronectin and immunoperoxidase

Introduction

Several carcinomas exhibit a hard texture, hence they have been given the designation «scirrhous». The hard consistence of a tumour is proportional mainly to the degree of stromal proliferation versus the cancer cell mass. The origin of the stroma especially the collagenous part however, is still a matter of controversy. The traditional belief (based on morphological studies) that it represents a host reaction against the cancer cells (Willis, 1952) has been challenged by the morphological sparsity of stromal collagen producing cells (Douglas and Shivas, 1974; Shivas and Mackenzie, 1974).

Detection of the enzyme prolyl hydroxylase which catalyses the conversion of proline to hydroxyproline (a major imino acid in various collagen types), has been used as a marker for collagen synthesis in many cell types (Gondelberg and Green, 1969). Several reports have shown that many fibroblastic and non-fibroblastic cell lines are capable of collagen production (Langness and Udenfriend, 1974; Guy et al., 1976: Berman and Foidart, 1980; Limsemmayer et al., 1977). Malignant cells such as parietal yolk sac carcinoma produces both type IV and V basement membrane collagens (Van Ness and Simpkins, 1979). In a previous study we have shown that human breast duct carcinoma cells express both prolyl hydroxylase and collagen antigen (Al Adnani et al.,1975). This was demonstrated immunohistochemically by the indirect immunoperoxidase procedure on fresh frozen tissue (Al Adnani et al., 1975). The antibody which we used was raised in rabbit against tyrosylated rat tendon collagen. This antibody was not characterised in respect of the molecular types of collagen with which it reacts (Kirrane and Robertson, 1968).

The present study therefore, was undertaken to investigate the localization of interstitial collagens type I and III as well as basement membrane collagen type IV in human breast (benign and malignant) epithelial lesions. In this investigation affinity purified antibodies to human collagens type I, III and IV were used on formalin-fixed, paraffin-embedded tissues of various benign and malignant epithelial lesions by the indirect immunoperoxidase procedure. Since fibronectin often codistributes with collagen (D'Ardenne and McGee, 1984), and is produced by a variety of normal and neoplastic cells (Yamada and Olden, 1978; Alitalo and Vaheri, 1982), fibronectin was concomitantly examined with collagens in these human breast lesions.

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Materials and methods

Preparation of collagens and anticollagen antibodies:

Interstitial collagen type I and III were purified from human placenta by limited pepsin digestion, salt fractionation and chromatography, and monospecific polyclonal antibodies were prepared as described elsewhere (Al Adnani, 1985).

Basement membrane type IV collagen was prepared from pepsin digests of human placenta according to the method described by Glaville et al. (1979) and its modification by Kuhn et al. (1981). Briefly human placenta, after extensive washing in cold water was homogenized in 10 volumes of 0.5% formic acid and stirred gently at 4°C overnight. The digested material was warmed to 12°C and 50 mg/litre solid pepsin was added, then digestion continued for 4-6h at 12°C. After centrifugation for 1h at 10,000g, solid NaCl was added to the supernatant to a final concentration of 0.7M. The precipitate was collected by centrifugation at 20,000g for 30 min at 4°C, dissolved in 30m Tris-HCl, ph 7.4 containing 0.2M NaCl. Undissolved particles were removed by centrifugation and solid NaCl was added to a final concentration of 1.8M, left overnight at 4°C and the precipitate collected by centrifugation at 20,000g. The precipitate was dissolved in 30mm Tris HCl, ph 7.6, containing lM NaCl Collagen type IV (so called spiders) was recovered from the solution by addition of solid NaCl to a final concentration of 1.8M. The precipitate was redissolved in 30mm Tris HCl, lM NaCl. On polyacrylamide gel electrophoresis, analysis of the latter gave a single band very close to the stacking gel. The purified material was stored at -20°C and use as antigen. Antibody to collagen type IV was raised in an albino rabbit as described for anti type I and type III (Al Adnani, 1985) and the specificity and titre were checked by the Elisa method (see Figure 1).

Immunohistology:

4μm thick consecutive sections of formalin-fixed, paraffin-embedded blocks from various neoplastic, hyperplastic and normal breast tissue were cut. The indirect immunoperoxidase procedure was used as previously described (Al Adnani et al., 1975) except that sections were pretreated with 0.1% trypsin in PBS at 37°C for 30 min. Antihuman, collagen type I, III and IV were diluted 1:5 and antihuman fibronectin (Dakopatt, Denmark) 1:20 in PBS. Parallel control sections were treated with normal rabbit serum, antisera absorbed with their parent antigens or an inappropriate antibody namely antihuman α_1-antitrypsin (Dakopatt, Denmark). Swine-antirabbit IgG - peroxidase conjugate was also obtained from Dakopatt, Denmark.

Results

Immune Reactivity and specificity of anticollagen type I, III and IV:

Figure 1 shows the titration curves of the anticollagens indicating its specificity.

Immunohistology:

Table 1 shows the distribution of the various antigens in the breast lesions examined while the number and percent positive for collagen types and other proteins is shown in Table 2.

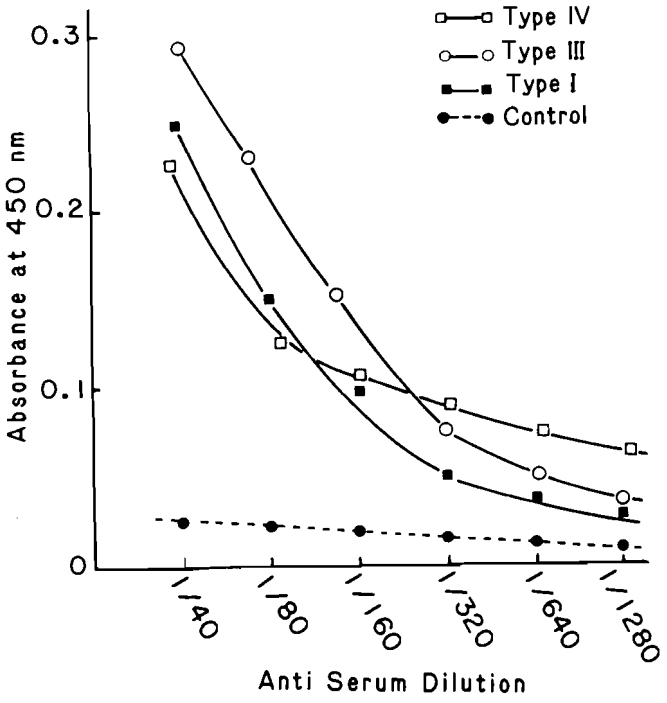

Fig. 1. *ELISA titration curves*
Wells of polystyrene microtitre plates were coated with 5 micrograms of either collagen type I, III, or IV treated with double dilution of the respective antibody or control and the proper IgG-peroxidase conjugate diluted 1:20. The peroxidase reaction was developed by using diaminobenzidine-H_2O_2 substrate and read at 450 nm in a titer-Tec multiscanner. To both washing buffer and diluant, 0.05% Tween-20 was added to prevent nonspecific absorption of proteins. The control is the mean results of using either normal rabbit serum or antisera absorbed with its parent antigen.

The cell cytoplasm of infiltrating carcinomas of duct (86%) or lobular 83% origin were positive for interstitial collagen type I and III. Whereas type IV collagen was detected in 100% of infiltrating ductal carcinomas and 83% of the lobular type. Only 1/3 of metastatic carcinoma cells in lymph nodes were positive for all collagen types. The cells of 1/3 of medullary carcinomas examined were positive for type IV collagen types. In contrast all epithelia of normal, hyperplastic lesions and fibroadenomas were negative for the three collagen types (Figs. 2, 3). Staining for fibronectin was variable both in neoplastic, hyperplastic and normal breast epithelia (Table 2, Figs. 4, 5). The detection of the inappropriate protein, α_1- antitrypsin was also variable; the highest positive rate was in medullary carcinoma and lactating breast (2/3) (Table 2). Treatment with non-immune serum, or sera absorbed with

its parent antigens, showed negative staining (Table 1).

The pattern of staining of cancer cells for all types of collagen (as well as with other proteins) was mainly focal. This was particularly so in the pre-invasive intraductal lesions with antibody to type IV collagen (Fig. 6). In the scirrhous component, diffuse staining of columns of cells was the predominant pattern (Fig. 7) for all collagen types.

Anti type III characteristically stained blood vessels especially capillaries both in neoplastic and other lesions (Fig. 8). Anti type IV collagen strongly stained the basement membrane of benign, hyperplastic, normal breast lobules and intraduct carcinomas (Figs. 2, 3 and 6). No intact basement membranes could be detected around invasive cancer cells. In infiltrating carcinomas, positive staining of stroma for collagen type I, III, IV, and fibronectin was observed and even for α_1-antitrypsin in a few cases (Table 2).

Fig. 2. Lactating breast showing strong positive staining of basement membrane and negatively stained epithelial cells. Anti type IV collagen. × 250

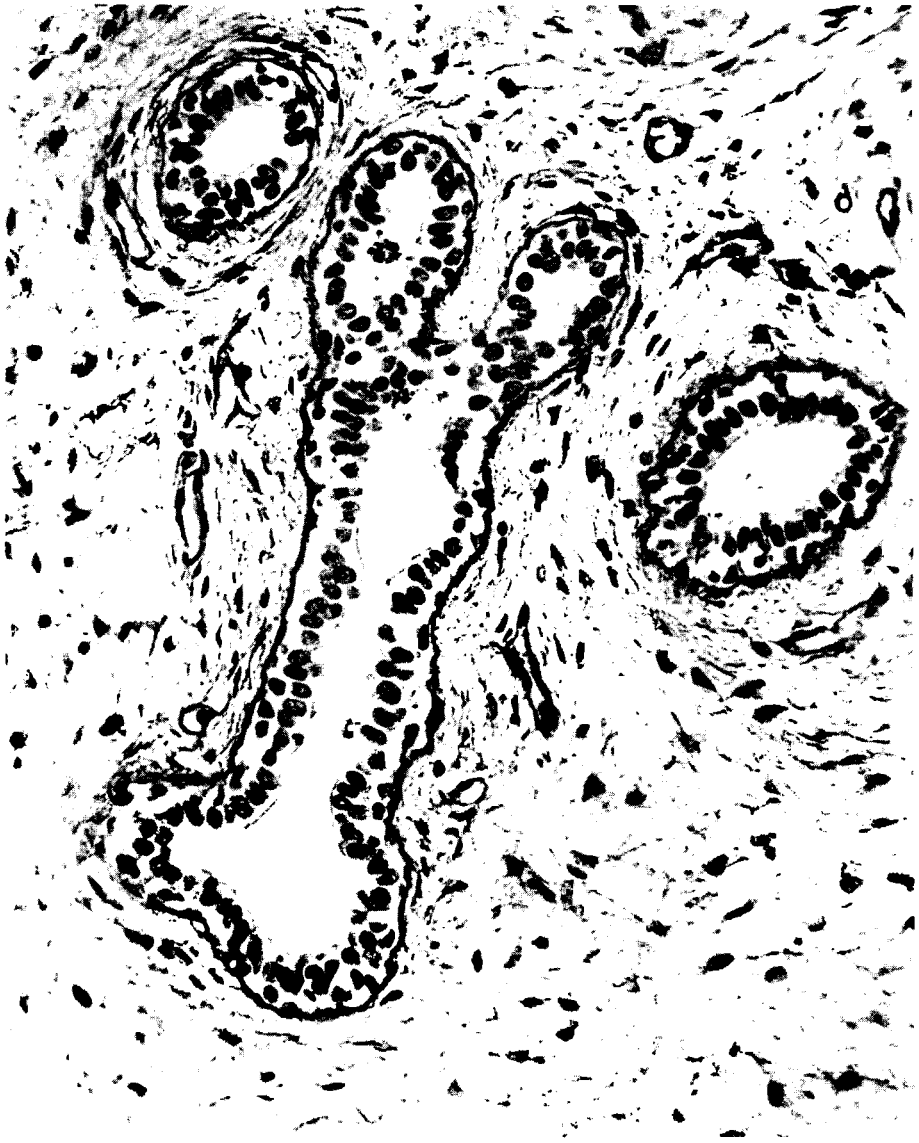

Fig. 3. Fibroadenoma treated with anti type IV collagen serum. Note the intense staining of the basement membrane (arrows) while the epithelium is negative. Anti type IV collagen × 250

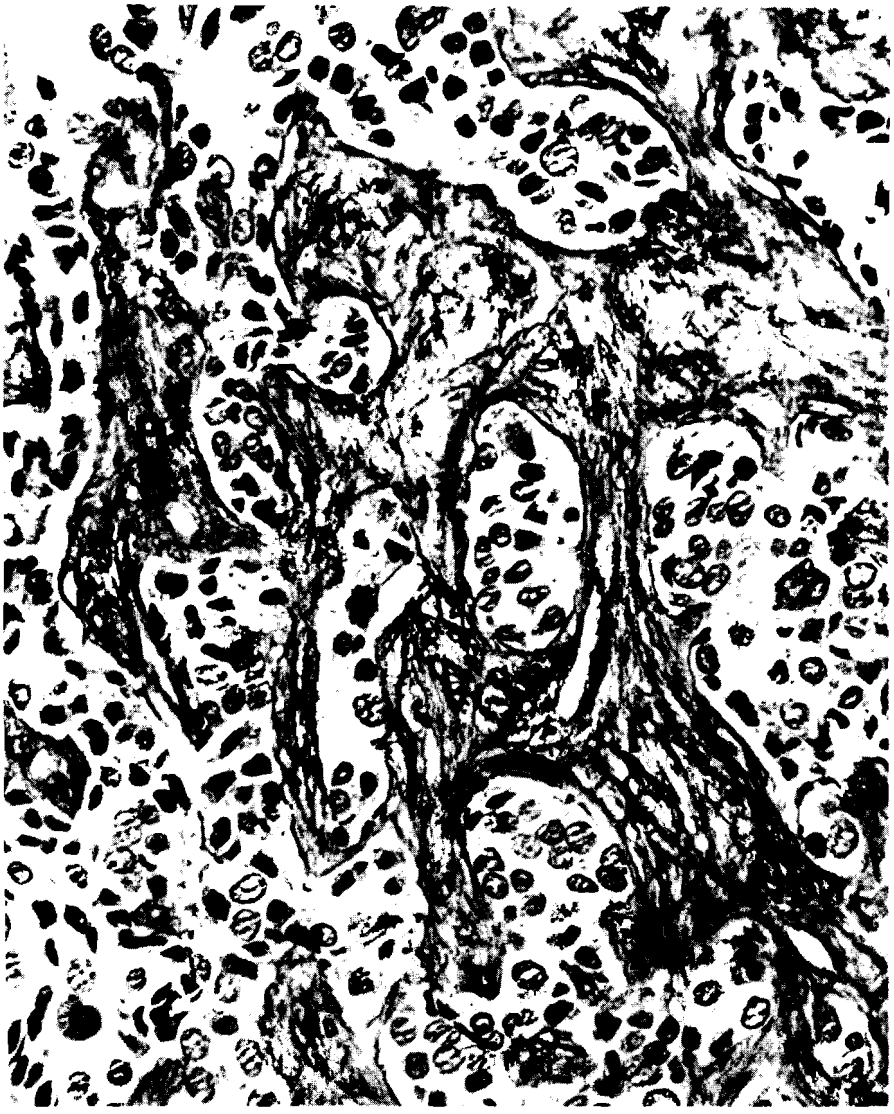

Fig. 4. Infiltrating carcinoma, fibronectin (F) is only detected in the stroma

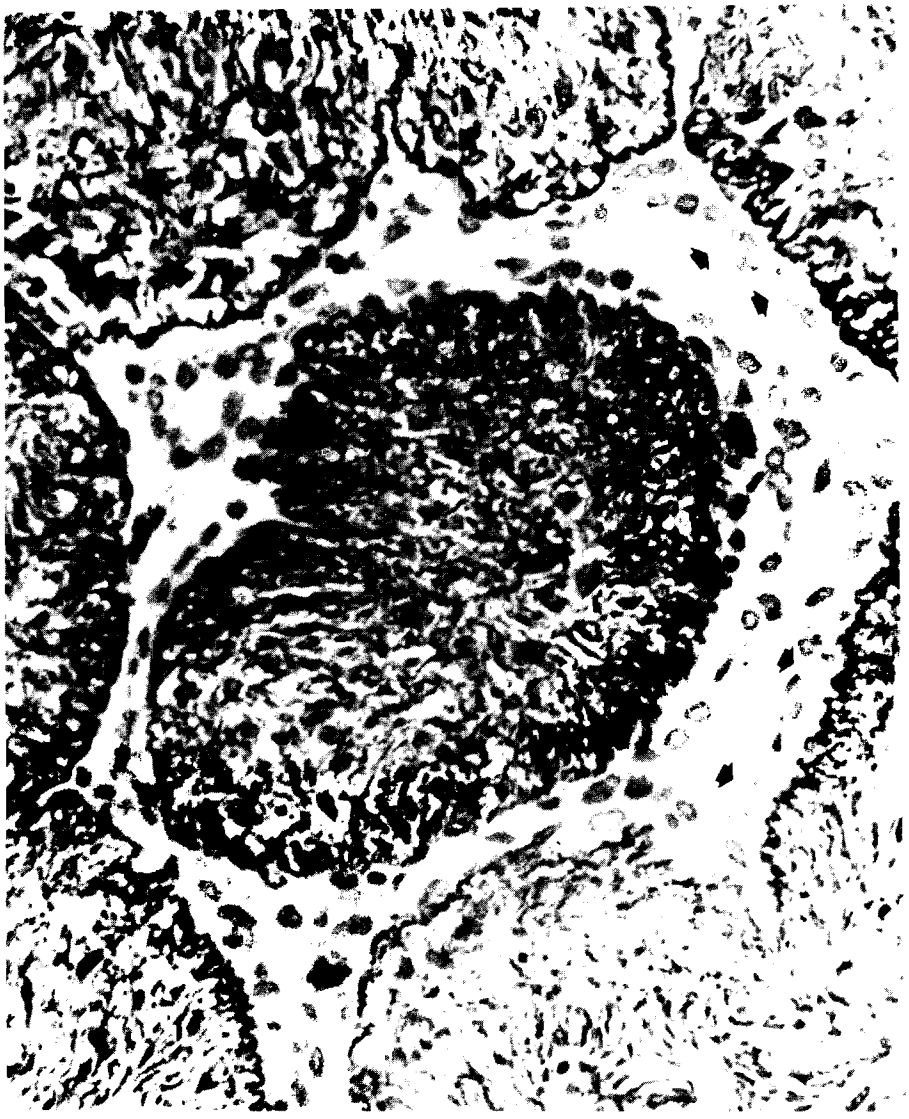

Fig. 5 Positive staining of fibronectin in the stroma and basement membrane of fibroadenoma. No staining could be detected in the epithelial cells (arrows). Anti fibronectin × 200

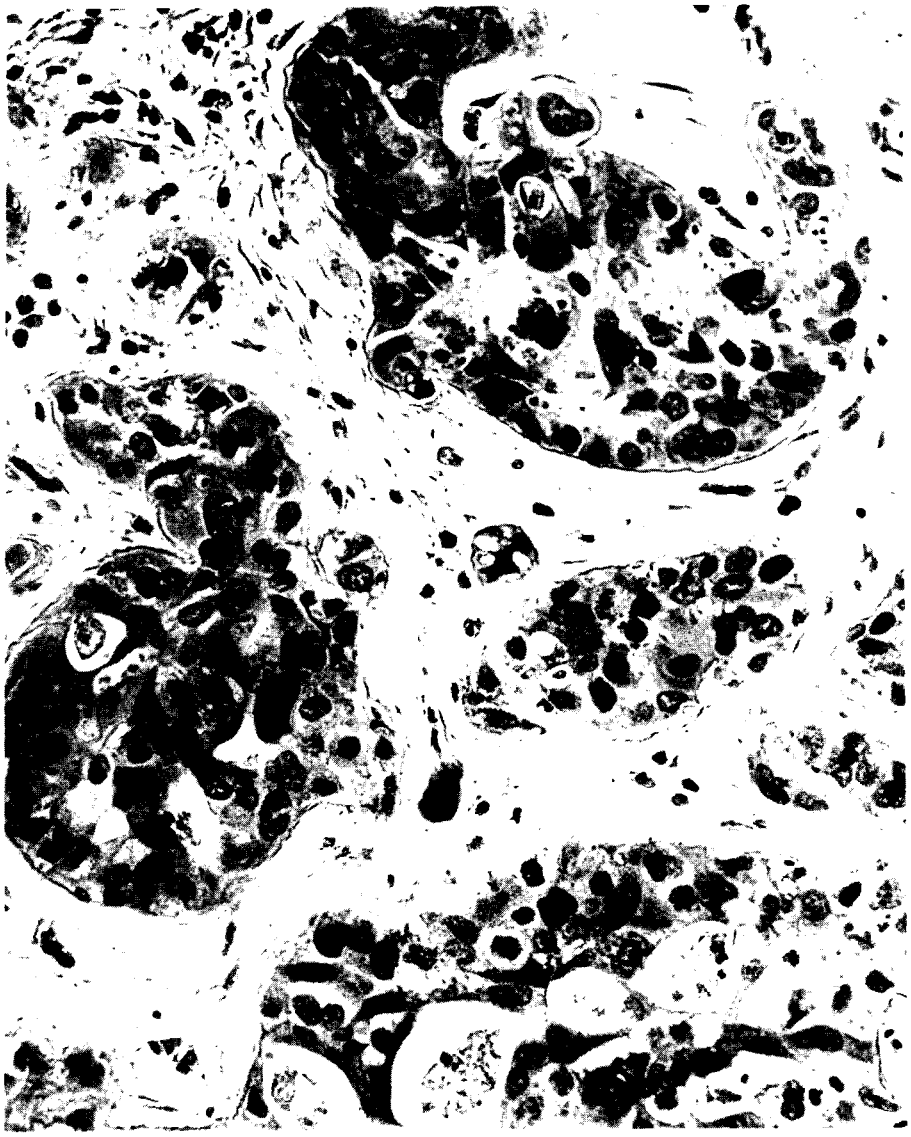

Fig. 6. Intraductal carcinoma of the breast showing positive staining of variable intensity for type IV collagen in the cytoplasm of the malignant cells. Anti type IV collagen × 250

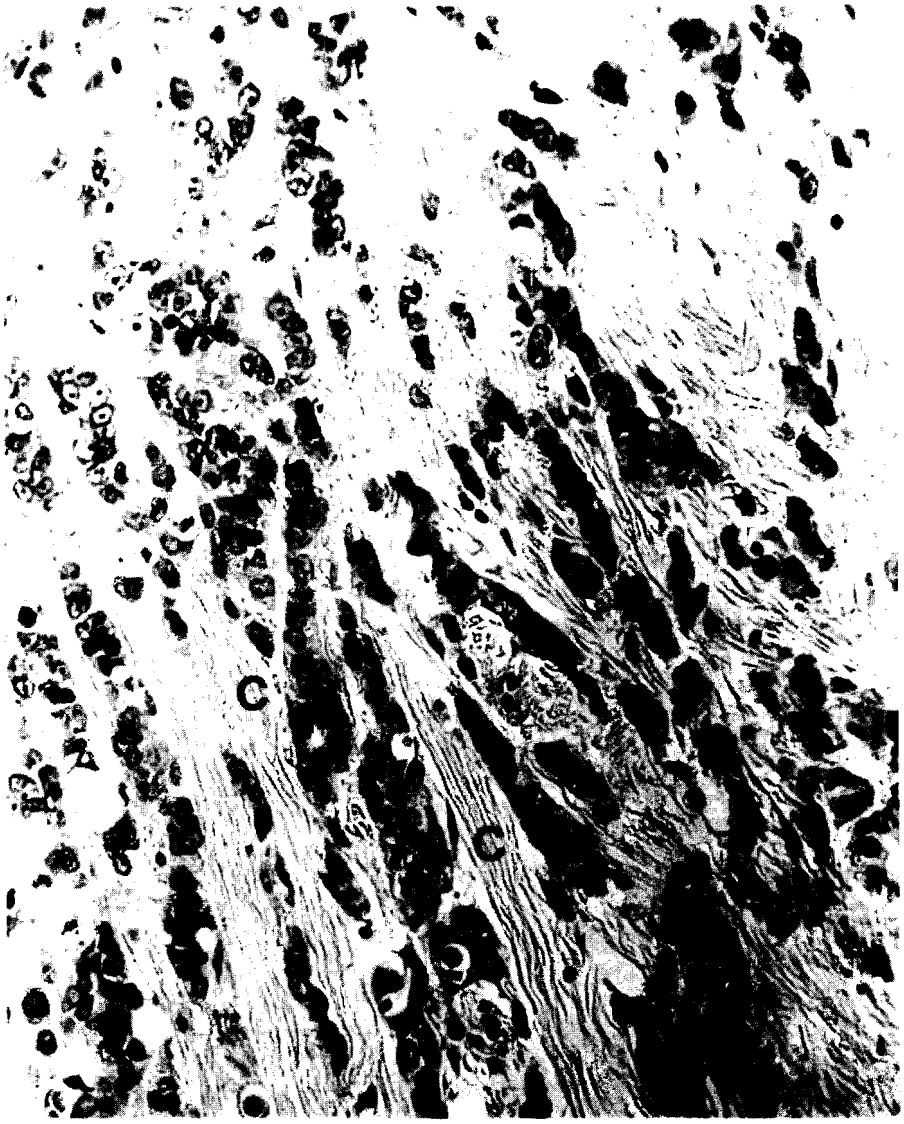

Fig. 7. Infiltrating carcinoma «scirrhous». The columns of cancer cells as well as some collagen fibers (C) in the stroma are positively stained.
Anti type I collagen × 200

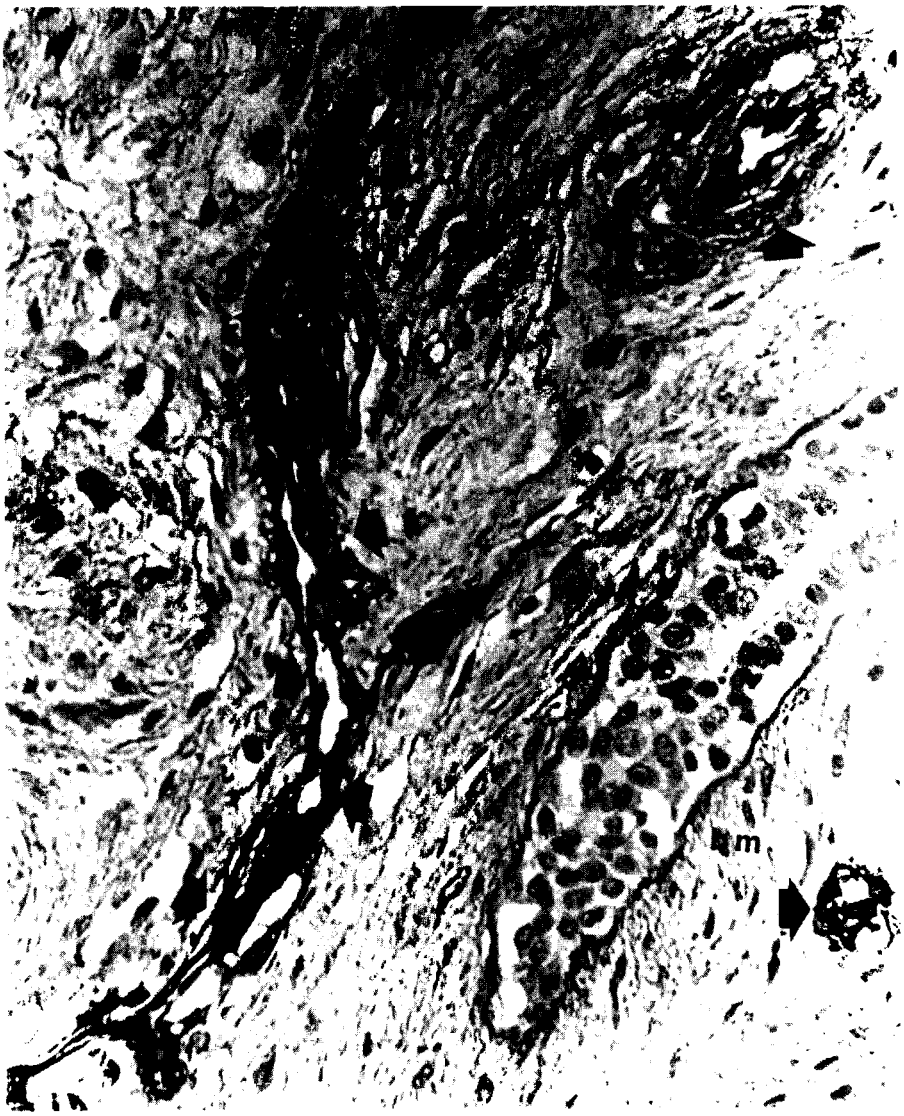

Fig. 8. Fibroadenoma showing intense positive staining of walls of blood vessels (arrows),and region of basement membrane (bm) around a duct, while the epithelial cells are negative.
Anti type III collagen × 200

Table 1. Distribution and staining intensity of collagen types, fibronectin and controls of the various components of human breast lesions examined (1)

A. Malignant lesions

No. of cases	Lesions	(2) Main tissue components	Collagens			Fibronectin	Controls	
			I	III	IV		α-1-Antitrypsin	Non-immune serum or absorbed sera
14	Infiltrating duct Ca.	Ca. C B.M. Str.	+ 0 +	+ 0 +	+ 0(3) 0	0 to + 0 +	0 0 0 to +	0 0 0
6	Infiltrating lobular Ca.	Ca.C. B.M. Str.	+ 0 +	+ 0 +	++ 0(3) 0	0 0 to + 0	0 to + 0 0	0 0 0
3	Medullary Ca.	Ca.C B.M. Str.	0 0 0	0 0 0	0to+ 0 0	0 to + 0 0	0 to + 0 0	0 0 0
3	Metastatic Ca. in lymph Nodes	Ca.C B.M. Str. Lym.	0 to + 0 + 0	0 to + 0 + 0	0to+ 0 + 0	0 to + 0 0 0	0 to + 0 0 0	0 0 0 0

B. Non-Malignant lesions

6	Fibroadenoma	Ep. B.M. Str.	0 0 +	0 0 to + +	0 ++ 0	0 to + + +++	0 to + 0 0 to +	0 0 0
6	Hyperplasia	Ep. B.M. Str.	0 0 +	0 0to+ +	0 ++ 0	0 + ++	0 to + 0 to + 0	0 0 0
3	Lactating breast	Ep. B.M. Str.	0 0 0	0 0to+ +	0 +++ 0	0 to + 0 to + +	0 to + 0 0 to +	0 0 0
3	Normal breast	Ep. B.M. Str.	0 0 +	0 0to+ +	0 + 0	0 to + 0 to + 0 to +	0 0 0	0 0 0

* The abbreviation used indicate Cancer cells (Ca.C.), basement membrane (B.M.), Stoma (Str.) and Lymphocytes (lym.).

(1) Assessment of staining intensity based on subjective grade 0 to +++

(2) The blood vessels are invariably stained for collagen type III and IV and fibronectin.

(3) Areas of intraduct Ca. or in situ lobular Ca showed intact basement membrane positively stained for collagen type IV.

Table 2. Number and percent of cancer cells or other epithelia positive for the antigens examined in the various breast lesions.

DIAGNOSIS	No. of cases	I	III	IV	FN	α_1–Anti Trypsin
Infiltrating duct Ca	14	12 (86)	12 (86)	14 (100)	4 (29)	2 (14)
Infiltrating lobular Ca	6	5 (83)	5 (83)	5 (83)	2 (33)	2 (33)
Medullary Ca	3	0	0	1 (33)	1 (33)	2 (66)
Metastatic Ca in Lymph node	3	1 (33)	1 (33)	1 (33)	1 (33)	1 (33)
Fibroadenoma	6	0	0	0	2 (33)	2 (33)
Hyperplasia	6	0	0	0	0	1 (17)
Lactating breast	3	0	0	0	2 (66)	2 (66)
Normal breast	3	0	0	0	1 (33)	0
Total	44					

Discussion

The present results confirm our previous report that breast carcinoma cells especially the infiltrating «scirrhous» type contain collagen antigen(s) (Al Adnani et al., 1975). In this study, however, we have utilized specific polyclonal antibodies to genetically distinct types of collagens (Bornstein and Sage, 1980). We were able to demonstrate clearly that infiltrating carcinomas of the breast either of ductal or lobular origin contain interstitial collagen type I and III as well as basement membrane collagen type IV antigens. The pre-invasive intra duct carcinomas also showed positive staining for the three collagen antigens with a prominent focal pattern, although the basement membrane was intact. Our results strongly argue against the old idea that the stroma in many tumours is a mere host desmoplastic reaction (Willis, 1952). This is supported by the negative staining for collagens in most medullary carcinoma and some metastatic tumours which lack a significant stromal component. Many reports have shown that invasive carcinoma lack basement membrane while in intra duct tumours, the basement membrane is preserved (Ekbolm et al., 1984; Gusterson et al., 1982). Gusterson et al., 1984, however reported that some invading squamous cell carcinoma can retain a basal lamina in the primary, metastatic and tumour grown as xenografts in nude mice. None of these reports clearly suggest a cell of origin for the basement membrane components. Others occasionally detected cytoplasmic immunoreactivity for laminin and collagen type IV in breast cancer cells (Alrechtsen et al., 1981; Barsky et al., 1982). Loss of basement membrane was mainly attributed to degradation by collagenases detected in invasive breast carcinomas (Barsky et al., 1983) and other malignant neoplasms (Harris et al., 1972). Our results however, favour the view that various types of collagens and other stromal components are continuously produced by the neoplastic cells but not organized as intact tissue in the invasive tumours. This hypothesis is supported by the detection of type I and III collagens and fibronectin in the stroma and in areas of «elastosis» and by ultrastructural evidence. Shivas and Mackenzie (1974), noted heterogeneous mixture of elastin, collagen and indeterminant fibrillar material in the stroma of scirrhous breast cancer.

The variable localisation of fibronectin and its diversity in benign and malignant neoplasms of the breast and other neoplasms confirms previous reports (Taylor-Papadimitriou et al., 1981; D'Ardenne and McGee, 1984).

Detection of α_1-antitrypsin in some types of breast tumours particularly in medullary carcinoma is interesting. Although α_1-antitrypsin is normally produced by hepatocytes and could be detected in hepatocellular carcinoma (Al Adnani and Ali, 1983), it is increasingly used as a marker for histiocytic cells (Isaacson et al., 1983). Our observation argues against the specificity of this enzyme as a specific cell marker.

Focal detection of collagens and other antigens is similar to the distribution of other markers previously reported such as CaI (McGee et al., 1982) and prostatic acid phosphatase (Nadji et al., 1980). The focal pattern cannot be due only to technical reasons such as fixation but to the cancer cell populations being phenotypically heterogeneous.

In conclusion therefore, our results show that most breast cancer cells contain collagen antigens (types I, III and IV) and are capable of producing collagen types and inconsistently other proteins such as fibronectin. It is most likely, therefore, that cancer cells contribute to the production of their own stromal components. This could be regarded as inappropriate products of the cancer cells in contrast to the normal, hyperplastic and benign epithelia counterpart of the human breast. This is in line with a recent study of gastric carcinoma by Sakakibara et al. (1982). These workers reported biosynthesis of collagen type I in vivo and in cell lines after transplantation into nude mice.

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