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The intermediate filament cytoskeleton of myofibroblasts: an immunofluorescence and ultrastructural study

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Summary. The intermediate filament cytoskeleton of stromal myofibroblasts from a series of twenty-eight infiltrating ductal breast carcinomas was examined by transmission electron microscopy (TEM) and indirect immunofluorescence (IF), the latter using antibodies to desmin, vimentin and prekeratin. Three cases of fibromatoses, selected as an additional source of myofibroblasts, were processed in the same manner. Stromal myofibroblasts from invasive ductal breast carcinomas rich in actin and readily identified by IF, were most numerous in the "young" edematous mesenchyme, areas corresponding to early stromal invasion or the peripheral invasive cellular front. Within the central sclerotic zone wherein clusters of neoplastic epithelial cells were surrounded by abundant collagen, most stromal cells corresponded by TEM to fibroblasts. In like fashion, myofibroblasts were most numerous in cellular, poorly collagenized portions of fibromatoses. By IF the only detectable intermediate filament protein of myofibroblasts in these two settings was vimentin.

Since the appearance of stromal myofibroblasts appears to be associated with stromal invasion by malignant epithelium, their development by modulation of pre-existent periductal fibroblasts is postulated. With the exception of vascular smooth muscle cells and endothelial cells, the only periductal mesenchymal cells shown to contain vimentin were fibroblasts. The lack of desmin in myofibroblasts constitutes evidence against an origin from vascular smooth muscle cells. Because the molecular markers (intermediate filament proteins) of stromal cell differentiation presented quantitative but not qualitative modifications, the transformation of fibroblasts into myofibroblasts is quite likely, suggesting that

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myofibroblasts may be more closely related to fibroblasts than to smooth muscle cells.

Key words: Stroma – Myofibroblast – Breast Carcinoma – Fibromatosis – Vimentin

Myofibroblasts were originally described in granulation tissue of healing wounds from experimental animals (Gabbiani et al. 1971). The fundamental mechanism of wound contraction was subsequently attributed to this unique cell, which was found to share features in common with fibroblasts and smooth muscle cells; hence, the term myofibroblast. It is well known that myofibroblasts contain substantial amounts of actomyosin (Hirschel et al. 1971; Gabbiani et al. 1972), that myofibroblasts produce collagen (Gabbiani et al. 1976), and that strips of granulation tissue contract and relax in vitro in response to pharmacological agents active upon smooth muscle (Majno et al. 1971). Following their demonstration in granulation tissue, myofibroblasts have been observed in a wide assortment of conditions. Three basic settings emerged in which myofibroblasts represented the principal cellular components: responses to injury and repair phenomena, quasineoplastic proliferative conditions (Seemayer et al. 1980 and 1981) and the stroma of malignant neoplasms (Lagacé et al. 1980; Schürch et al. 1981 and 1982; Seemayer et al. 1979 and 1980). Despite the wide occurrence of myofibroblasts and numerous studies detailing their morphological, immunohistochemical, biochemical and pharmacological characterization, the origin of this unique cell remains an unsettled issue.

Myofibroblasts are particularly abundant in the "young" mesenchymal stroma of primary invasive and metastatic carcinoma, especially in infiltrating ductal mammary carcinomas (Seemayer et al. 1979; Schürch et al. 1981). This tissue, therefore, constitutes an excellent source of myofibroblasts, one which served as material for our study of the intermediate filament cytoskeleton of these cells. Previous immunohistochemical analyses of various cells and tissues have provided evidence that, at least in vivo, intermediate filaments serve as molecular markers of cell differentiation (Lazarides 1980; Franke et al. 1982). Our study demonstrates that myofibroblasts lack desmin and prekeratin and contain only one type of intermediate filament, vimentin. This finding suggests that these cells may be more closely related to fibroblasts than to smooth muscle cells.

Materials and methods

Myofibroblasts from the stroma of twenty-eight infiltrating ductal mammary carcinomas were examined for the purpose of characterizing their intermediate filament cytoskeleton. Normal breast tissue contiguous to the neoplasms served as control material for epithelial, myoepithelial, stromal and vascular cells. Immediately after removal, a sample from each neoplasm was divided into three parts and processed for light microscopy, transmission electron microscopy and indirect immunofluorescence. Three cases of fibromatoses, including one Dupuytren type fibromatosis and two desmoid fobromatoses (selected as an additional source of myofibroblasts), were processed in the same manner.

Light microscopy

A sample from each specimen was fixed in 10% neutral buffered formalin, followed by postfixation in Bouin's solution and embedded in paraffin. Sections cut at 3–5 μm were stained with hematoxylin-phloxine-saffron (HPS). In addition, frozen sections from samples processed for immunofluorescence were also stained with HPS in order to correlate histological and immunological findings.

Transmission electron microscopy

Tissue samples were cut into mm^3 cubes, fixed for 2 h in 3% glutaraldehyde buffered with 0.1 M phosphate buffer at pH 7.4, and postfixed for 1 h in 2% osmium tetroxide in the same buffer. The cubes were then dehydrated in graded acetones and embedded in Epon 812. In selected cases, semithin sections were stained with toluidine blue and thin sections were double stained on copper grids with uranyl acetate and lead citrate and examined in a Philips 201 electron microscope.

Antisera

Rabbit antisera against actin, desmin, prekeratin and vimentin were utilized. The anti-actin antibody, a gift from Professor Shao-Nan Huang, Faculty of Medicine, Memorial University of Newfoundland was produced with purified actin from chicken gizzards according to the technique described by Lazarides (1975). The properties of this antibody have been previously described (Schürch et al. 1981). Desmin antibodies were raised in rabbits against the 53 KD band of a 7% preparative and a $DodSO_4$-polyacrylamide gel loaded with chicken gizzard acetone powder highly enriched in desmin (Hubbard and Lazarides 1979). An IgG fraction of the antibody-containing serum was obtained by separation on a column of sepharose, covalently linked with protein A (protein-A sepharose CL-4B Pharmacia, Zürich, Switzerland). Affinity purified antibodies were then prepared by passing this IgG fraction through a column of ultrogel AcA22 (LKB Company, Lucerne, Switzerland) covalently linked (Nagle et al. 1983) with desmin purified from chicken gizzard (Geisler and Weber 1980). The anti-prekeratin antibody and antivimentin antibody, gifts from Dr. Reuben Baumal, Department of Pathology, Hospital for Sick Children, University of Toronto, Toronto, Ontario, were prepared respectively from human plantar scrapings by the method described by Sun and Green (1978) and from human embryonic lung fibroblast cultures by the method of Franke et al. (1979). The detailed preparation and the specificity controls of each antibody are described by Kahn et al. (1982 and 1983).

Indirect immunofluorescence

Tissue samples were snap frozen in liquid isopentane stored at −70° C. Cryostat sections were cut at 3–5 μm, air dried at room temperature for 30–45 min, washed in tris buffered saline (TBS) and fixed in 95% ethanol for 30 min. After several washings in TBS, the sections were incubated with the anti-actin, anti-desmin, anti-prekeratin and anti-vimentin antibodies at the dilution of 1:20 for 45 min at room temperature, washed several times in TBS and then incubated with a 1:30 dilution of FITC-conjugated goat anti-rabbit gammaglobulin (Behringwerke, Marburg, FRG) for 45 min. The sections were mounted in buffered glycerine. The intensity of fluorescence was compared with sections treated with normal rabbit serum in lieu of the rabbit anti-actin, anti-desmin, anti-prekeratin and anti-vimentin antibodies, followed by FITC conjugated goat anti-rabbit gammaglobulin. Photographs were taken with a Zeiss photomicroscope (Carl Zeiss Inc. Oberkochen, FRG) equipped with epiillumination and specific filters for fluorescein using a daylight Ektachrome high speed color film. Black and white prints were prepared from color slides.

Morphologic definitions

Light microscopic, immunofluorescence and ultrastructural criteria judged essential to establish the presence of myofibroblasts, fibroblasts and myoepithelial cells have been previously defined (Schürch et al. 1981).

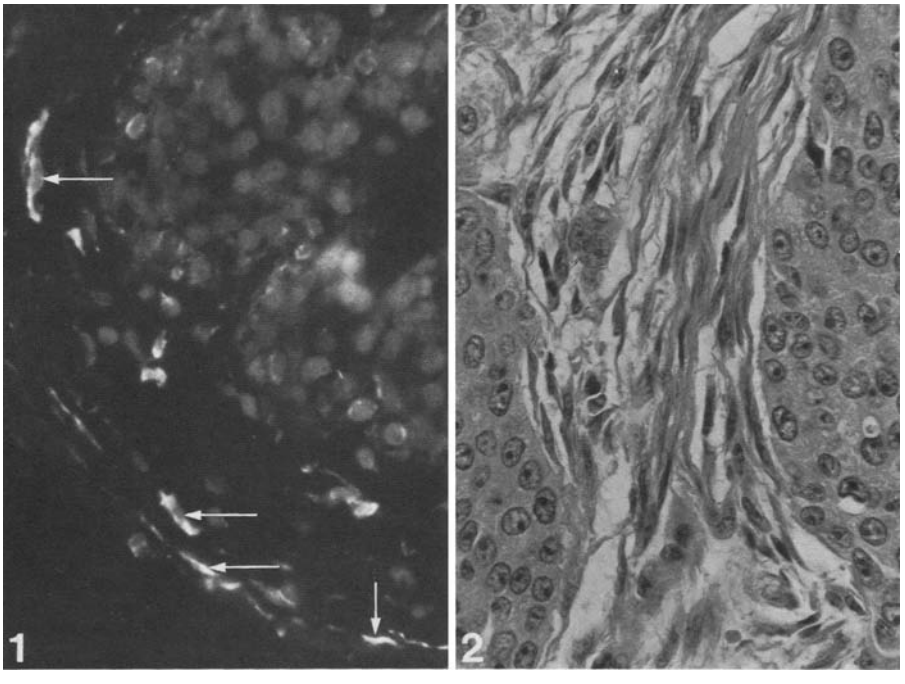


Fig. 1. Immunofluorescence micrograph of normal breast demonstrating a mammary duct. Several elongated periductal stromal cells reveal distinct cytoplasmic fluorescence with the anti-vimentin antibody (*arrows*). × 600

Fig. 2. Photomicrograph of infiltrating ductal mammary carcinoma demonstrating loose ("young") stroma within peripheral invading cellular front. Numerous elongated stromal cells are visible between clusters of epithelial carcinoma cells. HPS × 450

Results

A. Normal breast parenchyma. Normal ductal and acinar epithelium and myoepithelium showed a bright cytoplasmic fluorescence with the anti-prekeratin antibody. In addition, myoepithelial cells were readily identified by their strong cytoplasmic fluorescence using the anti-actin serum. In glandular epithelium, cytoplasmic fluorescence staining with the anti-actin serum was weak and usually limited to the luminal border. Stromal cells, endothelial and vascular smooth muscle cells were stained by the anti-vimentin antibody (Fig. 1). The only cells stained by the anti-desmin antibody were a few scattered vascular smooth muscle cells.

B. Infiltrating ductal mammary carcinoma. Many of the neoplasms contained foci of intraductal in association with infiltrating carcinoma. By light microscopy, most had a sclerotic center in which neoplastic epithelial clusters were surrounded by thick bands of collagen and a few spindle-shaped stromal cells. By immunofluorescence, using the anti-actin antibody, myofibroblasts were most numerous and best developed in the "young" mesenchymal

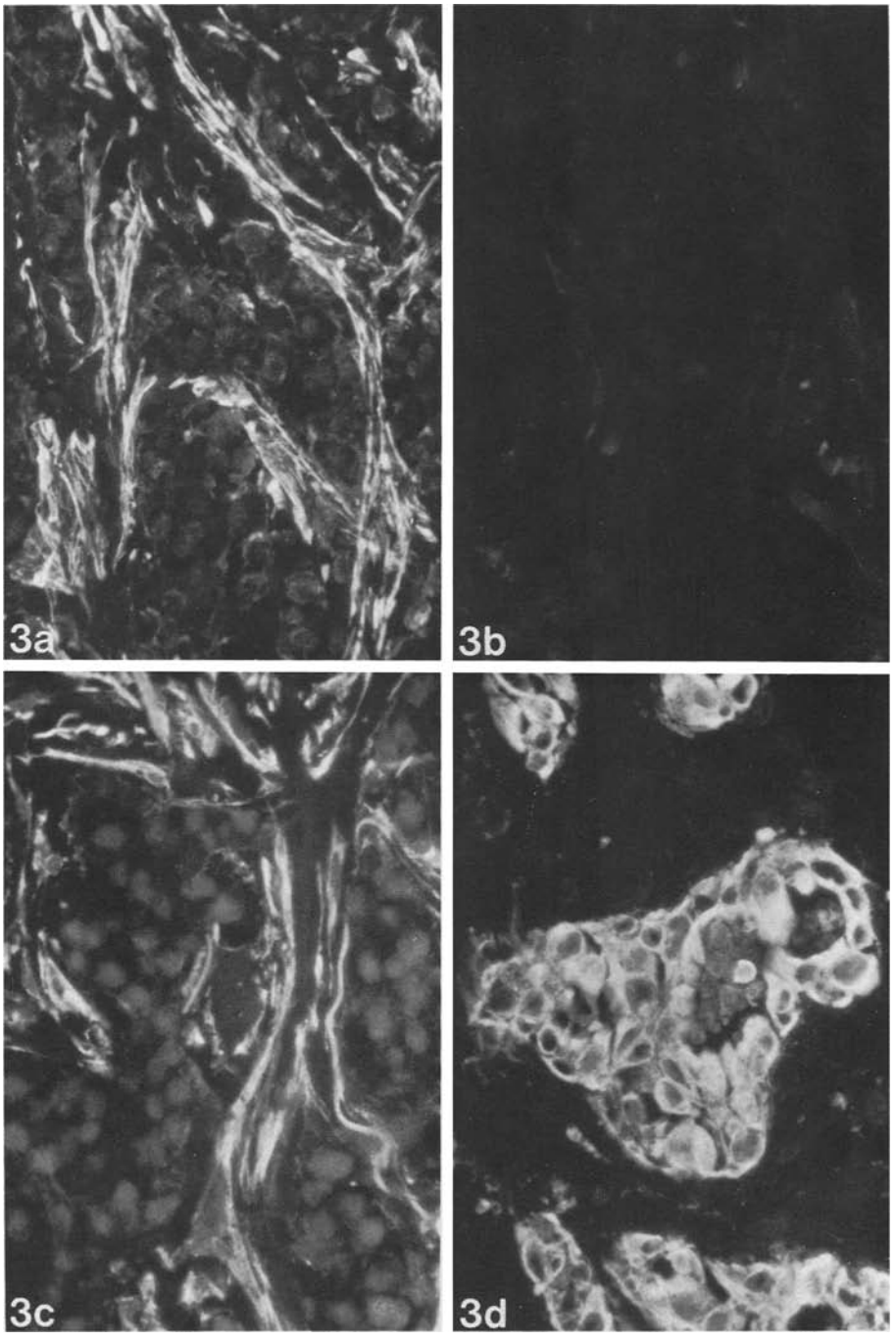


Fig. 3a–d. Immunofluorescence micrographs of infiltrating ductal breast carcinoma illustrating "young" mesenchymal stroma within peripheral invading cellular front. **a** Using the anti-actin antibody, numerous elongated stromal cells reveal a bright cytoplasmic fluorescence. **b** With the anti-desmin antibody, no cytoplasmic fluorescent staining of stromal cells is visible. **c** The anti-vimentin antibody demonstrates an intense cytoplasmic fluorescence of stromal cells. **d** The anti-prekeratin antibody stains intensely only the carcinoma cells. **a**, **b**, **c** and **d** $\times 350$

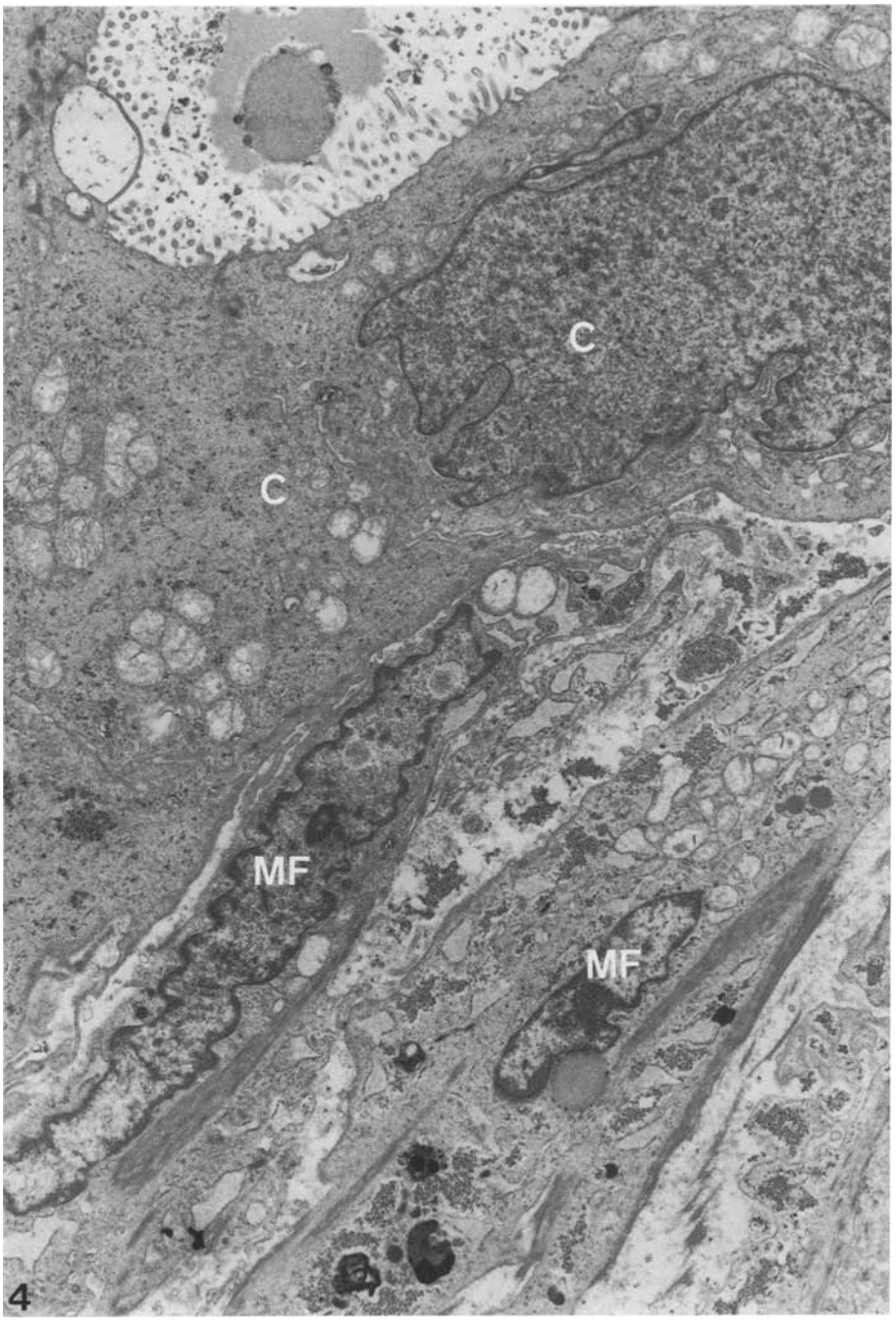


Fig. 4. Electron micrograph demonstrating character of "young" mesenchymal stroma of infiltrating ductal breast carcinoma. Carcinoma cells (*C*) are surrounded by several typical myofibroblasts with notched nuclei (*MF*) laden with bundles of 6 nm filaments and associated dense bodies. Uranyl acetate and lead citrate, ×8,100

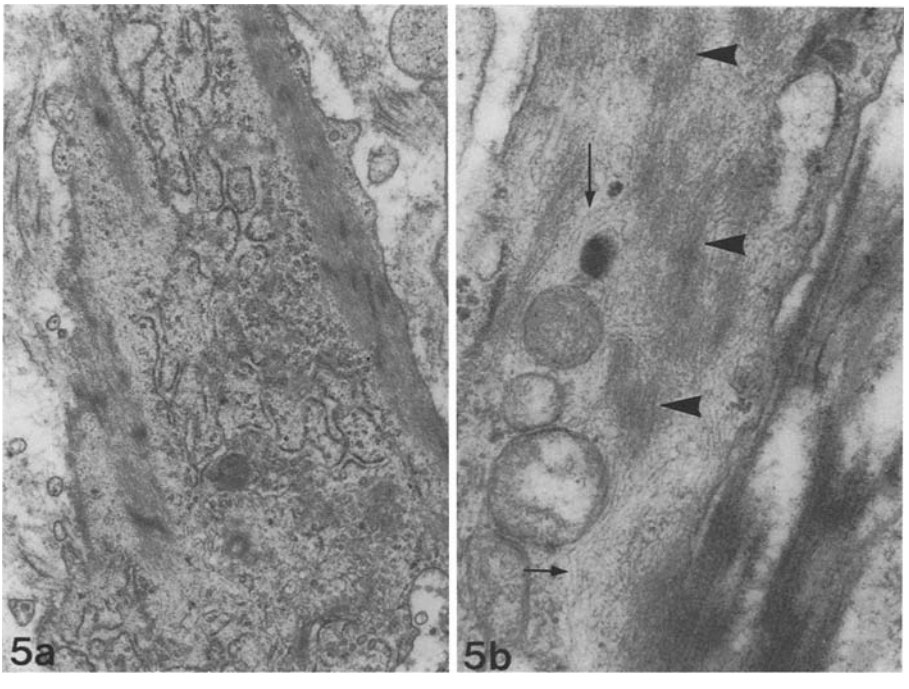


Fig. 5a, b. Electron micrographs showing details of stromal myofibroblast from infiltrating ductal breast carcinoma. **a** Several bundles of 6 nm filaments and associated dense bodies are arranged parallel to the long axis of the cytoplasmic extension. **b** Cytoplasmic extension of myofibroblast illustrating 6 nm filaments and associated dense bodies (*arrow heads*) as well as dispersed 10 nm intermediate filaments (*arrows*). Uranyl acetate and lead citrate, **a** ×14,400, **b** ×40,000

stroma, i.e., areas corresponding to early stromal invasion or, more consistently, the peripheral invasive cellular front of the neoplasm (Fig. 2). In the densely sclerotic center and around foci of intraductal carcinoma, myofibroblasts were poorly developed or absent. Myofibroblasts, readily identified by immunofluorescence with the anti-actin serum, showed an equally intense cytoplasmic fluorescence with the anti-vimentin antibody. Using the anti-desmin and anti-prekeratin antibodies, however, no fluorescent reaction was observed within myofibroblasts, although carcinoma cells were intensely stained by the anti-prekeratin serum (Fig. 3a–d). By transmission electron microscopy, "young" mesenchymal stroma contained numerous large myofibroblasts with several cytoplasmic extensions laden with 6 nm filaments and associated dense bodies, often arranged parallel to the long axis of the cells and their cytoplasmic extensions (Fig. 4 and 5a). Intermediate filaments, i.e., 10 nm filaments, were identified in most myofibroblasts and were characterized by their dispersed and often curvilinear arrangement (Fig. 5b). Myofibroblasts were surrounded by a few mature collagen fibers, aggregates of non-distinct fibrils and granular or basal lamina-like material. In the sclerotic central areas, myofibroblasts were few in number, slender, and contained but a scant number of 6 nm filaments; intermediate filaments

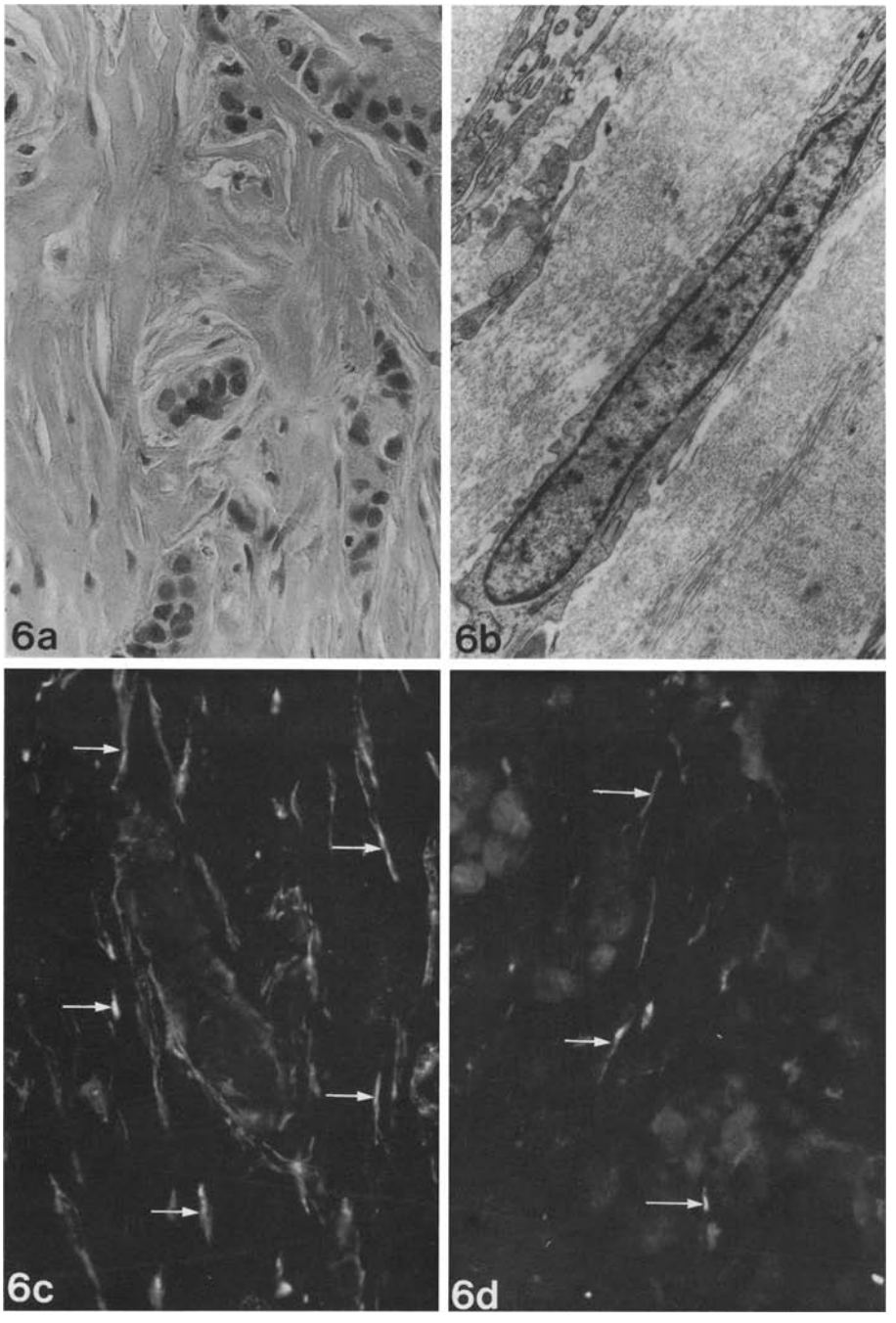
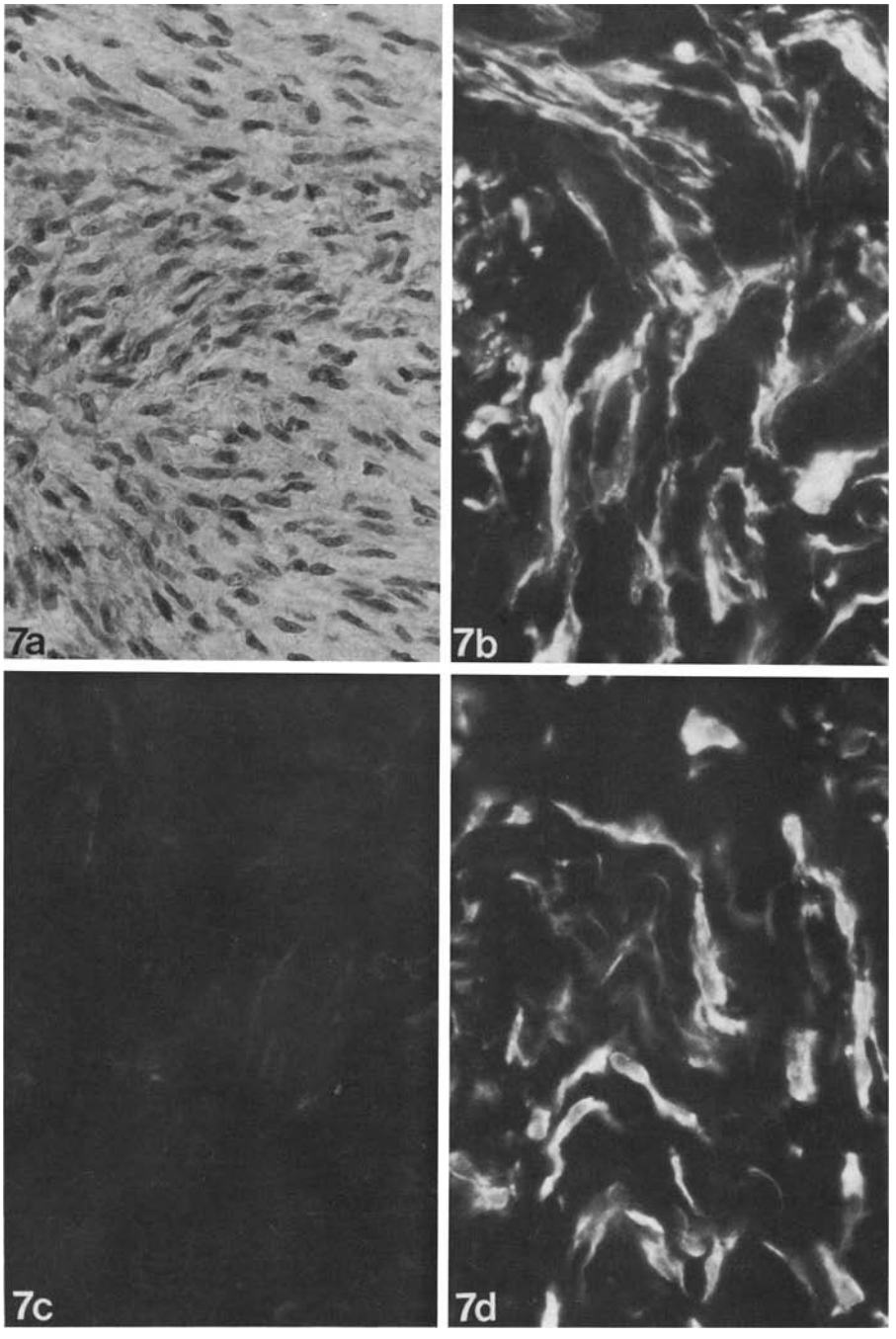


Fig. 6a–d. Densely sclerotic central area of infiltrating ductal breast carcinoma. **a** Photomicrograph showing clusters of carcinoma cells surrounded by a few slender elongated stromal cells and thick bands of collagen. **b** Electron micrograph demonstrating a slender stromal cell with a smooth contoured nucleus and few cytoplasmic filaments, enveloped by abundant collagen. **c** The immunofluorescence reveals a weak cytoplasmic staining reaction of the stromal cells with the anti-actin antibody (*arrows*) and **d** the anti-vimentin antibody (arrows). **a** HPS, $\times 350$; **b** uranyl acetate and lead citrate, $\times 11{,}200$; **c** and **d** $\times 350$



Figs. 7a–d. Cellular area of fibromatosis. **a** Photomicrograph illustrating numerous spindle cells and scant collagen. **b** The anti-actin antibody demonstrates a bright cytoplasmic fluorescence of spindle cells. **c** With the anti-desmin antibody, no cytoplasmic fluorescent reaction is visible. **d** The anti-vimentin antibody stains intensely the cytoplasm of spindle cells. **a** HPS $\times 350$; **b**, **c** and **d** $\times 360$

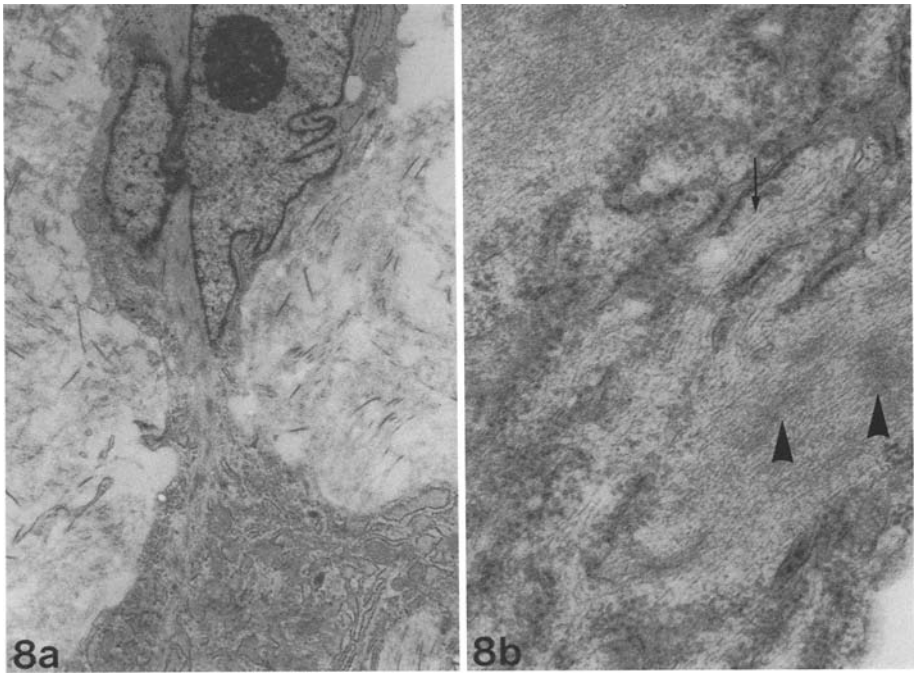


Fig. 8a, b. Electron micrograph of a typical myofibroblast from cellular area of fibromatosis. **a** Myofibroblast with notched nucleus and bundle of 6 nm filaments and associated dense bodies dispersed through the cytoplasm. **b** Detail of cytoplasmic extension of a myofibroblast demonstrating bundles of 6 nm filaments with dense bodies (*arrow heads*) and dispersed 10 nm intermediate filaments (*arrows*). Uranyl acetate and lead citrate, **a** ×7,200, **b** ×46,500

were rarely observed. Most of the stromal cells in the sclerotic center showed ultrastructural features of resting fibroblasts and were surrounded by abundant collagen. Using the anti-actin and anti-vimentin antibodies, a weak cytoplasmic staining reaction was observed, corresponding to the small amount of cytoplasm and the poorly developed cytoskeleton of these slender stromal cells (Fig. 6a–d).

C. Fibromatoses. Similar to the "young" mesenchymal stroma of infiltrating ductal breast carcinomas, myofibroblasts were most numerous and best developed in cellular areas which contained scant collagen. Using the anti-actin and anti-vimentin sera, myofibroblasts displayed a strong cytoplasmic fluorescence. Again, the anti-desmin antibody did not reveal any cytoplasmic immunofluorescent reaction in myofibroblasts (Fig. 7a–d). By transmission electron microscopy, the myofibroblasts contained numerous bundles of 6 nm filaments and associated dense bodies as well as 10 nm intermediate filaments (Fig. 8a and b). Endothelial and vascular smooth muscle cells were stained by the anti-actin and anti-vimentin antibodies. The sole anti-

desmin positive staining reaction was observed in occasional vascular smooth muscle cells[1].

Discussion

This immunohistochemical and ultrastructural study demonstrates that myofibroblasts from the stroma of invasive ductal mammary carcinomas and from fibromatoses are devoid of desmin and prekeratin but contain vimentin as their sole detectable intermediate filament protein. Vimentin filaments are reported to be typical for cells derived from the embryonic mesenchyme such as fibroblasts (Franke et al. 1978), thereby suggesting that myofibroblasts might develop from pre-existent fibroblasts through a mechanism of modulation. In a previous immunofluorescence and ultrastructural study, we reported that the appearance and development of myofibroblasts in invasive carcinomas might be related directly to epithelial invasion beyond the epithelial basal lamina into the subjacent stroma (Schürch et al. 1981). The delicate connective tissue stroma of intraductal mammary carcinomas lacks myofibroblasts and contains but a few slender stromal cells with ultrastructural features of resting fibroblasts (Schürch et al. 1982). These findings are also consonant with prior ultrastructural studies of the epithelial-stromal junction of the normal and dysplastic breast, as well as intraductal mammary carcinomas (Ozzello 1971). In the present study, myofibroblasts were again determined to be most numerous and best developed in the "young" edematous mesenchyme of infiltrating ductal breast carcinomas, areas corresponding to early stromal invasion or to the peripheral invading cellular front. Within the central sclerotic areas wherein clusters of neoplastic epithelial cells were surrounded by abundant collagen and around foci of intraductal carcinoma, most stromal cells corresponded ultrastructurally to resting fibroblasts. These cells revealed a weak cytoplasmic fluorescent staining reaction with the anti-actin and anti-vimentin antibodies compared to the bright cytoplasmic fluorescence of myofibroblasts from the "young" mesenchymal stroma. In like fashion, myofibroblasts were most numerous and best developed in the cellular, poorly collagenized foci of fibromatoses and less numerous and developed within densely collagenized areas. As suggested earlier, these findings denote morphological and possibly pathophysiological similarities between the myofibroblastic stromal reaction in invasive carcinomas and in wound healing (Schürch et al. 1981).

Since the presence of myofibroblasts seems to be associated with stromal invasion by malignant epithelium, the following sequence of events is proposed to account for their development in this setting. Invasion of carcinoma cells beyond the basal lamina of mammary ducts into the subadjacent stroma induces a transformation of resting preexistent fibroblasts into myofibroblasts, i.e., reactive or modified fibroblasts. From the peripheral invasive

1 At the time of completion of this study, monoclonal antibodies against desmin, prekeratin and vimentin became commercially available (Ortho Diagnostic Systems Inc., Don Mills, Ontario, Canada). The results obtained with the monoclonal antibodies used in a limited number of cases were identical to those described with the polyclonal antibodies

front of carcinomas, where myofibroblasts are most numerous, there is a progressive decrease in the number and development of myofibroblasts toward the sclerotic center, wherein most cells reveal features of poorly developed myofibroblasts or resting fibroblasts surrounded by abundant collagen (Schürch et al. 1982). Since the molecular markers of stromal cell differentiation as judged by immunofluorescence reactions for actin, desmin, and vimentin, presented quantitative rather than qualitative modifications, the transformation (modulation) of fibroblasts into myofibroblasts is quite plausible.

Previously it was suggested that in breast carcinoma myoepithelial cells might transform into myofibroblasts (Ahmed 1978). Study of the intermediate filament cytoskeleton of myoepithelial cells demonstrates that these cells contain prekeratin as their sole intermediate filament (Franke et al. 1980). A transformation of myoepithelial cells into myofibroblasts is, therefore, quite unlikely since the latter cells contain vimentin and lack prekeratin.

One of the purposes of this study was to determine whether or not myofibroblasts in the stroma of invasive carcinomas might originate from vascular smooth muscle cells. In such cells vimentin constitutes the principal intermediate filament (Gabbiani et al. 1981 and 1982), although approximately 50% of vascular smooth muscle cells also contain variable amounts of desmin (Frank and Warren 1981; Schmid et al. 1982), indicating heterogeneity in regard to their intermediate filament content. Since proliferating (reactive or neoplastic) cells usually maintain their original complement of intermediate filaments (Bannasch et al. 1980; Gabbiani et al. 1981; Osborn and Weber 1983), one would expect that a proportion of myofibroblasts would be stained with anti-desmin antibodies if they were derived from stromal vascular smooth muscle cells. In this study myofibroblasts, both from invasive mammary carcinomas and fibromatoses, lacked demonstrable desmin but were uniformly positively stained with vimentin antibodies, indicating a homogenous cell population with respect to their intermediate filament proteins. These findings provide a measure of evidence that myofibroblasts may not be derived from vascular smooth muscle cells, thereby further supporting their proposed origin from fibroblasts.

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