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Original Articles

Stromal Myofibroblasts in Primary Invasive and Metastatic Carcinomas

A Combined Immunological, Light and Electron Microscopic Study*

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Summary. A series of 23 primary invasive and 7 metastatic carcinomas was examined by light microscopy (LM), transmission electron microscopy (TEM) and immunofluorescence (IF), the latter employing an anti-actin antibody. The results were correlated with macroscopic features such as retraction and consistency. Stromal cells rich in actin, readily identified by IF in firm and retracted carcinomas, were rare or absent in neoplasms lacking these features. TEM established the myofibroblastic nature of these stromal cells. Alternate sections (LM, IF) of each neoplasm demonstrated that myofibroblasts were more numerous in "young" mesenchymal stroma than in densely sclerotic areas. The connective tissue adjacent to intraductal mammary carcinoma lacked myofibroblasts, suggesting that epithelial stromal invasion is required to evoke a myofibroblastic stromal response. Myofibroblasts which possess synthetic (type III collagen) and contractile properties may well contribute to the firm consistency and retraction which characterize many carcinomas. The induction of myofibroblasts might represent an important host stromal response directed toward containment of invasive and/or metastatic carcinoma. This response may be especially important in neoplasms with weak antigenicity and/or slow doubling times.

Key words: Myofibroblast – Immunofluorescence – Stroma – Carcinoma

Introduction

Pathobiologic and clinical effects of neoplasia result from a variety of factors, some contributed by neoplastic cells, others by the host. Proliferation, invasion and the expression of fetal, embryonic and other cellular markers are features of neoplastic cells whereas stroma vascularization, desmoplasia and complex

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immunologic mechanisms constitute host cellular responses to neoplasia (Wolf 1979; Smuckler 1979). Recent investigators have directed considerable attention to the neoplastic cellular population. Studies related to the stromal reaction of neoplasms are less numerous and deal largely with cellular immunologic responses (Ioachim 1976; Klein 1973) and tumor angiogenesis (Folkman et al. 1971; Folkman 1975; Folkman and Cotran 1976).

The richness of the supporting connective tissue determines to a considerable extent the consistency of most neoplasms. Many carcinomas are firm or hard, retracted and replete with collagen. In contrast, others contain little collagen, are soft and bulging on cut-surface.

A recent ultrastructural study documented myofibroblasts in scirrhous breast carcinoma (Tremblay 1979). The author proposed that the retraction which characterized these neoplasms might be related to the presence of this contractile cell. This finding led us to examine by transmission electron microscopy a series of various invasive and metastatic carcinomas to determine in which neoplasms and with what frequency myofibroblasts might be demonstrated (Seemayer et al. 1979). Myofibroblasts were identified in each instance and were most apparent in neoplasms characterized by desmoplasia, firmness and retraction. It was, therefore, suggested that myofibroblasts contribute to the desmoplasia and retraction of many carcinomas and that this stromal reaction might constitute a host response to invasive and metastatic carcinoma.

In the present work, a prospective extension of prior investigation, a series of primary invasive and metastatic carcinomas was examined by immunofluorescence with an anti-actin antibody. Since myofibroblasts cannot be unequivocally identified by conventional light microscopy, this study was undertaken to determine, semi-quantitatively, both the number and spatial relation of stromal myofibroblasts to epithelial components of various invasive and metastatic carcinomas. The immunofluorescent results in each instance were then correlated with conventional light and transmission electron microscopy.

The purpose of this manuscript is to describe the results of this study which strongly suggest that the stromal myofibroblast response to many carcinomas (invasive and metastatic) constitutes a common pathobiological event and is especially demonstrable in the cellular phase of invasion and/or metastasis characterized by a "young" mesenchymal stroma. Moreover, the intensity of this response appears more striking with immunofluorescence than heretofore appreciated. This technique would appear to possess considerable value in prospective pathomorphologic studies of neoplasia.

Material and Methods

The neoplasms included in the study were obtained from surgical pathology material and consisted of 23 primary invasive and 7 metastatic carcinomas. The primary invasive carcinomas originated in the breast, lung, stomach, colon and uterine cervix (Table 1). The metastatic carcinomas were located in the breast, lymph node, liver, bone and ovary (Table 2). The macroscopic features of each neoplasm, i.e. texture, cut-surface, were recorded. Immediately after removal a sample from each neoplasm was divided into three parts and processed for light microscopy (LM), immunofluorescence (IF) and transmission electron microscopy (TEM).

Light Microscopy. The samples were fixed in 10% neutral buffered formalin, postfixed in Bouin's solution and embedded in paraffin. Sections cut at 3–5 μm were stained with haematoxylin-phloxine-

saffron (HPS). In addition sections from specimens processed for immunofluorescence were stained with HPS in order to correlate the histological and immunological results.

Immunofluorescence. The anti-actin antibody (AAA) employed in this study, a generous gift of Professor Shao-Nan Huang, Faculty of Medicine, Memorial University of Newfoundland was produced in rabbits with purified actin from chicken gizzards according to the technique described by Lazarides (Lazarides 1975). The titer of the AAA tested on human smooth muscle from myometrium, stomach and leiomyomas varied from 1:640 to 1:1,280. After absorption with lysed platelets, a rich source of actin, (Gabbiani et al. 1973) the titer decreased and varied from 1:80 to 1:160.

Tissue samples from primary invasive and metastatic carcinomas 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 phosphate buffered saline (PBS) and fixed in 95% ethanol for 30 min. After several washings in PBS, the sections were incubated with the AAA serum at dilutions of 1:20 to 1:40 for 45 min at room temperature, washed several times in PBS and then incubated with a 1:30 dilution of FITC-conjugated goat anti-rabbit gammaglobulin (Behringwerke, Marburg, West Germany) for 45 min. The sections were mounted in buffered glycerine. Fluorescence was compared with sections treated with normal rabbit serum in lieu of the rabbit AAA serum followed by FITC-conjugated goat anti-rabbit gammaglobulin. Photographs were taken on a Zeiss UV photomicroscope using daylight Ektachrome high speed color film.

Transmission Electron Microscopy. Tissue samples were cut into mm^3 fragments, 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 fragments were then dehydrated in graded acetones and embedded in Epon 812. Semi-thin sections were stained with toluidine blue; thin sections were double stained on copper grids with uranyl acetate and lead citrate and examined in a Philips 201 transmission electron microscope.

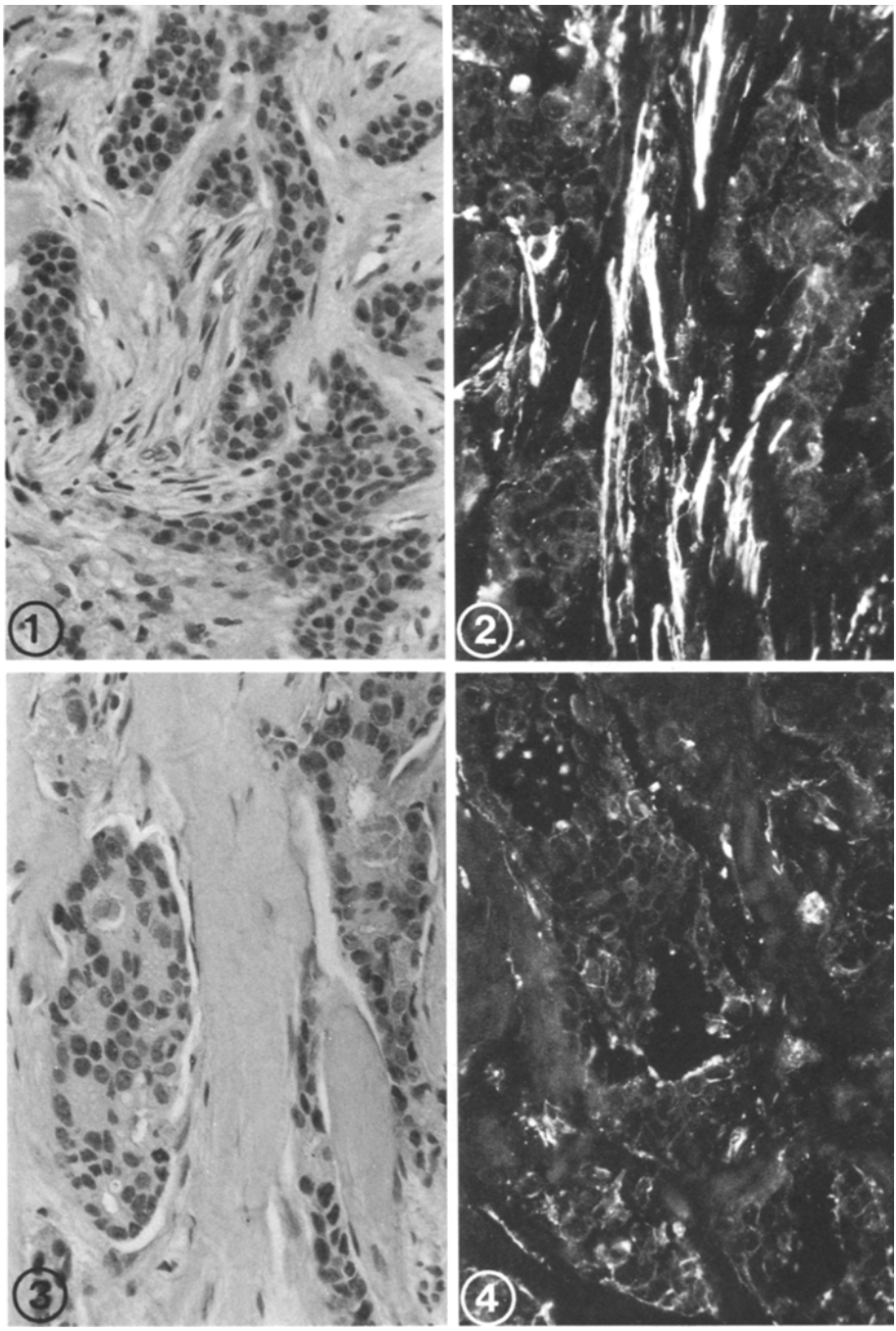
Quantitation of Findings. The over-all number and distribution of myofibroblasts in the stroma of each neoplasm were semi-quantitatively evaluated (+ to ++++) by IF. The results obtained were then correlated with LM using alternate sections (IF, LM), TEM and macroscopic features such as consistency and the nature of the cut-surface.

Morphologic Criteria. The criteria judged essential to establish the presence of stromal myofibroblasts included: a) LM: Elongated cells with distinct acidophilic cytoplasm; b) IF: Elongated cells with bright cytoplasmic fluorescence using the AAA-containing serum; c) TEM: Elongated cells with

Table 1. Primary invasive carcinomas (23 cases)

Site	Type	No. of cases	Consistency	Retraction of cut surface	No. of stromal Myofibroblasts[a]
Breast	Ductal, infiltrating	11	Firm to Hard	present	+++ to ++++
	Lobular, infiltrating	1	Firm	present	++
	Medullary	1	Soft	absent	+
	Papillary (adeno)	1	Soft	absent	±
Lung	Adeno	2	Semi-Firm	present	++
	Small cell	1	Soft	absent	+
Stomach	Adeno	1	Firm	present	++
	Linitis plastica	1	Firm to Hard	present	+++
Colon	Adeno	3	Firm to Hard	present	+++ to ++++
Cervix	Small cell (carcinoid)	1	Soft	absent	±

[a] ±, equivocal; +, rare; ++, few; +++, moderate; ++++, abundant



Figs. 1–6. Infiltrating ductal mammary carcinoma with "young" mesenchymal stroma (Figs. 1, 2, 5) and densely sclerotic stroma (Figs. 3, 4, 6)

Fig. 1. Photomicrograph demonstrating "loose" character of stroma which contains numerous elongated cells and scant collagen. (HPS × 300)

Fig. 2. Immunofluorescence of a corresponding area illustrates elongated stromal cells with bright cytoplasmic fluorescence. (Rabbit AAA, FITC-conjugated goat anti-rabbit gammaglobulin, × 300)

Fig. 3. Photomicrograph demonstrating densely sclerotic area with few stromal cells. (HPS × 300)

Fig. 4. Immunofluorescence of corresponding sclerotic area demonstrates very little stromal fluorescence. (Rabbit AAA, FITC-conjugated goat anti-rabbit gammaglobulin, × 300)

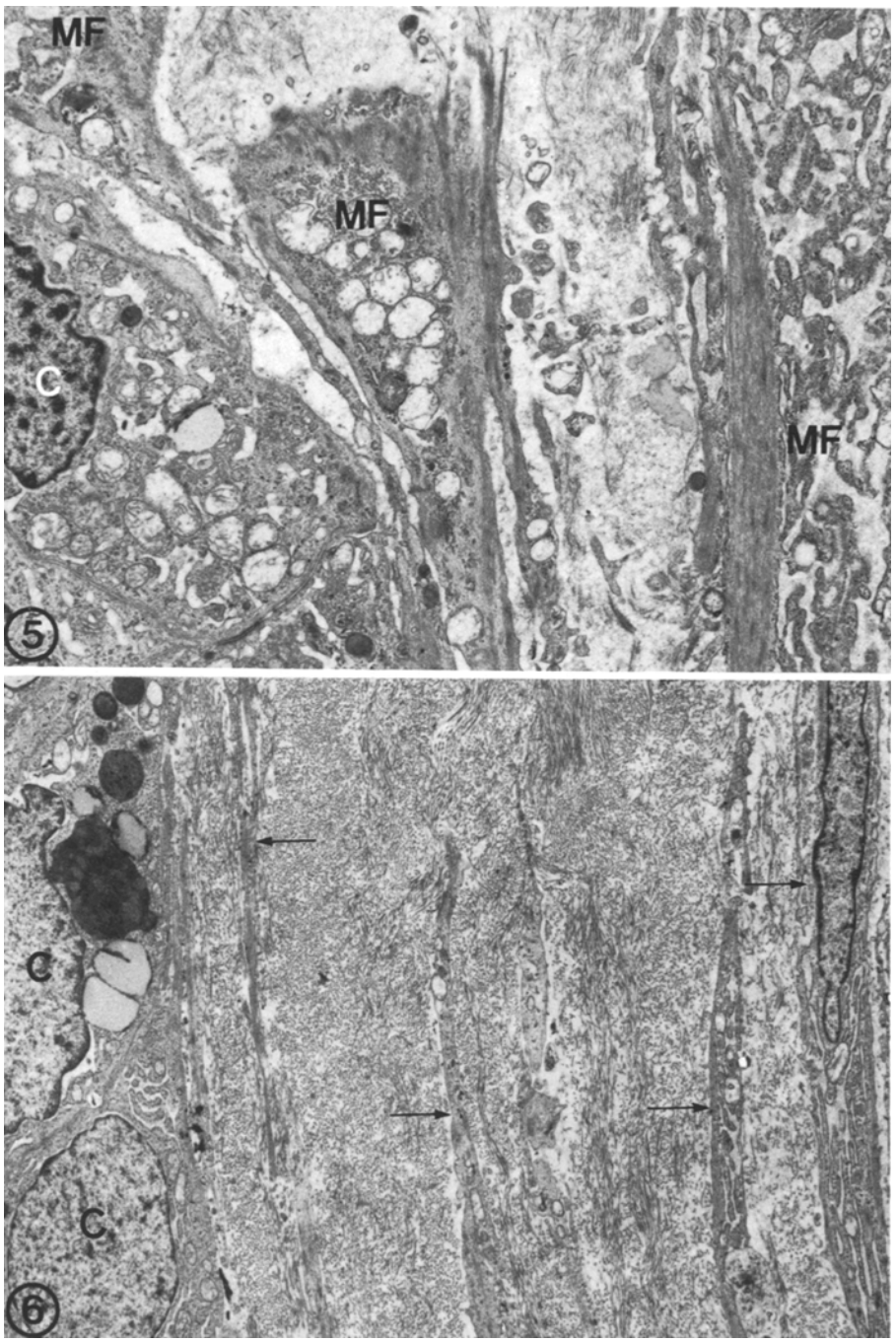
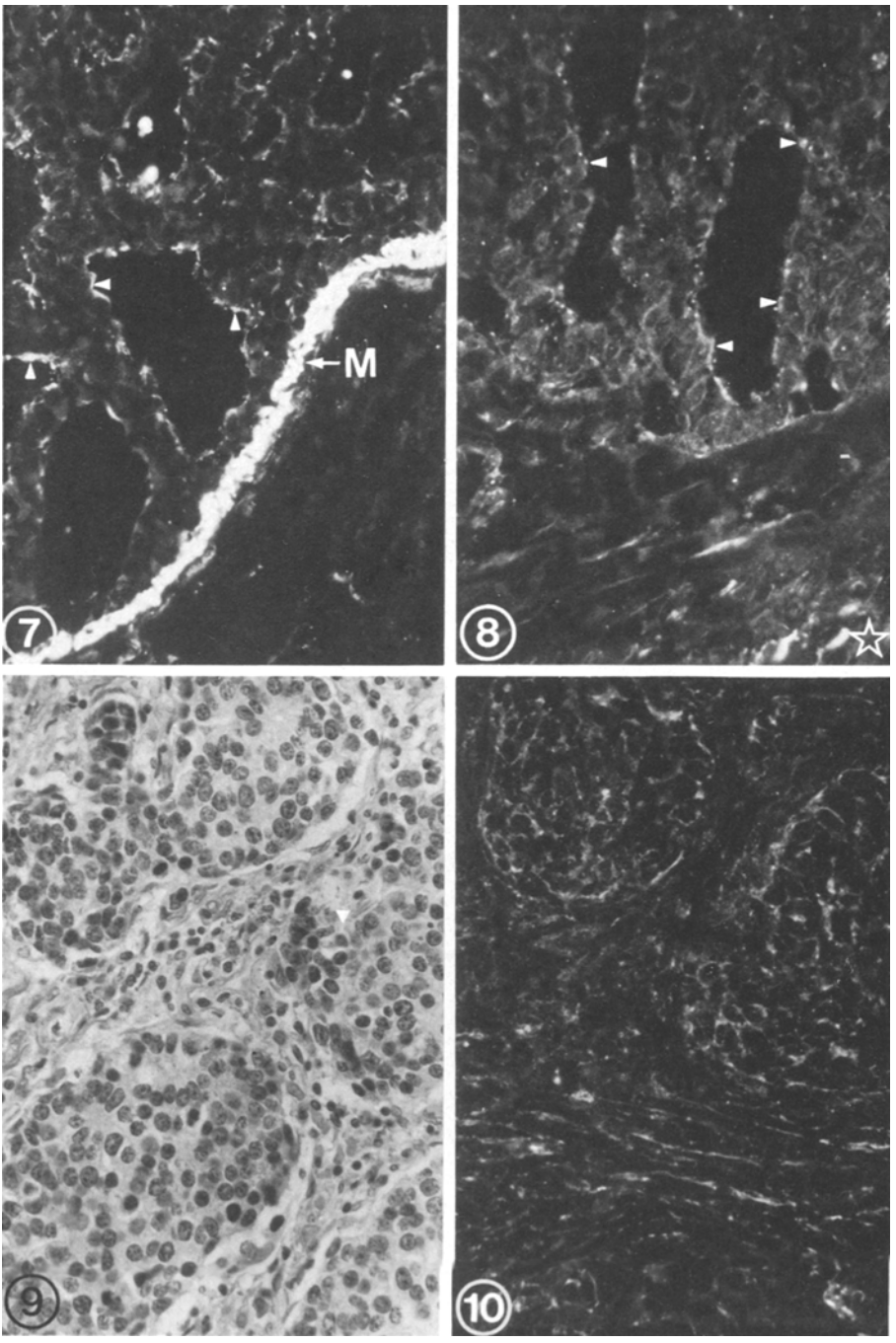


Fig. 5. Electronmicrograph of "young" mesenchymal stroma illustrates carcinoma cells (*C*) enveloped by myofibroblasts (*MF*) laden with cytofilaments and dense bodies. (× 7,500)

Fig. 6. Electronmicrograph of densely sclerotic area illustrates carcinoma cells (*C*) surrounded by slender stromal cells (arrows) with a few cytofilaments and abundant collagen. (× 6,000)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cytoplasmic extensions, a fusiform, often notched nucleus, numerous cisternae of granular endoplasmic reticulum, well-developed Golgi areas, and abundant cytofilaments (40–60 Å and less frequently 100–120 Å in diameter) often grouped in bundles and concentrated peripherally forming dense bodies. Additional findings deemed helpful but not essential included focal deposition of basal lamina-like material and cell-to-cell or cell-to-stroma attachment sites.

Myoepithelial cells, constituents of the normal mammary gland, also contain actin and thus might represent a source of confusion in data interpretation. Therefore, an ultrastructural definition of myoepithelial cells is deemed important. Features judged essential to establish the presence of myoepithelial cells are numerous bundles of microfilaments, 40–60 Å in diameter and associated dense bodies; bundles of thicker filaments, 80–100 Å in diameter (tonofilaments) and above all, well-developed cellular junctions in the form of desmosomes with adjacent myoepithelial or carcinoma cells. An additional attribute of myoepithelial cells resides in their intimate juxtaposition to epithelia, i.e. within the confines of a basal lamina, which may be discontinuous in carcinomas, and formation of hemidesmosomes on the basal plasma membrane. Thus, myoepithelial cells are defined both by their localization and their ultrastructural composition.

Results

Primary Carcinomas (Table 1)

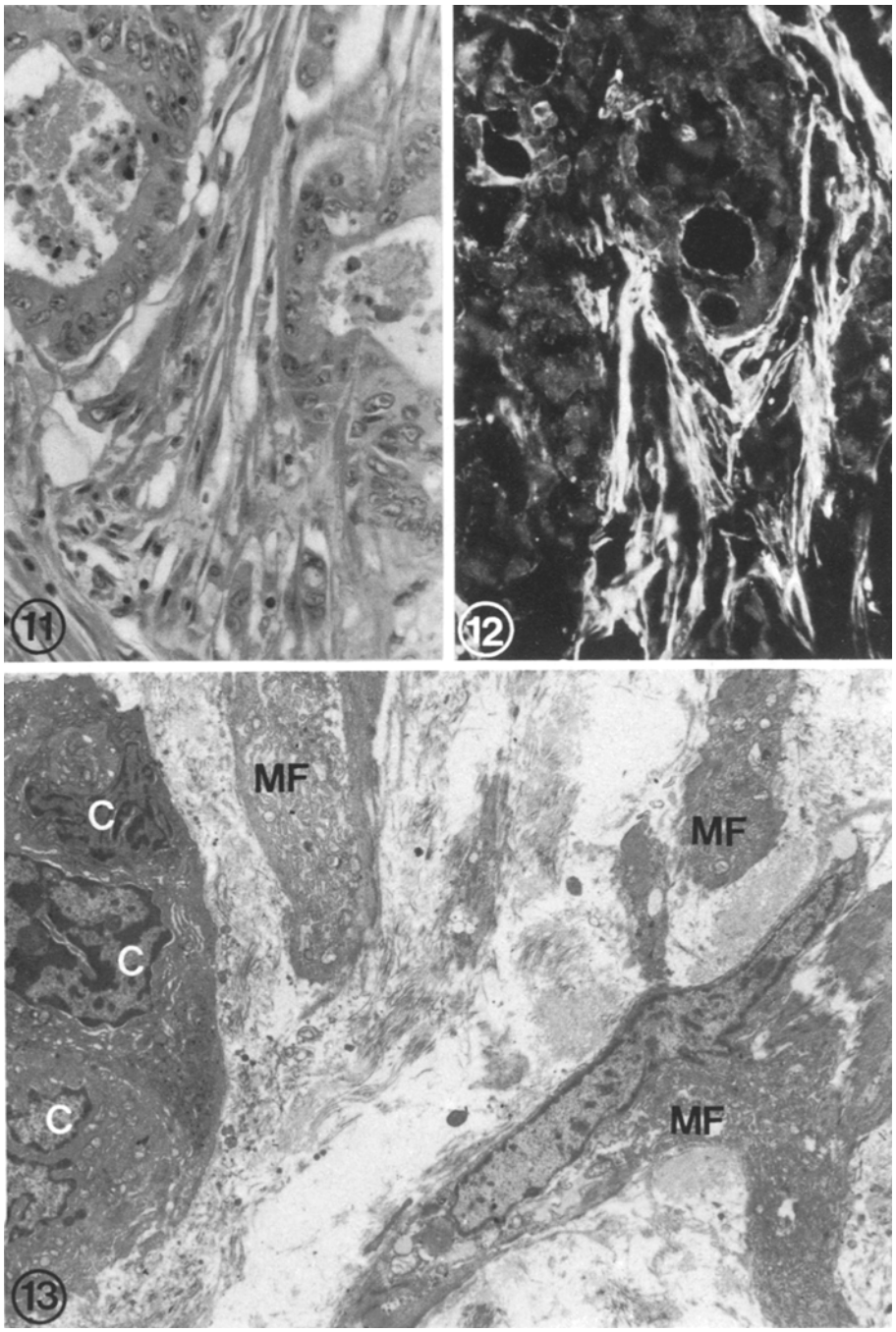
a) Breast Carcinomas. The carcinomas of mammary ducts generally demonstrated both intraductal and infiltrating components. On macroscopic examination they were firm to hard an retracted. The number of stromal myofibroblasts as judged by IF in infiltrating carcinoma was moderate to abundant. Their distribution varied considerably within the same infiltrating ductal carcinoma. Alternate sections (IF, LM) revealed many myofibroblasts in "young" mesenchymal stroma (Figs. 1 and 2) containing little or no collagen. In densely sclerotic zones wherein clusters of carcinoma cells were enveloped by thick bands of collagen, myofibroblasts, although generally present, were distinctly less numerous (Figs. 3 and 4). By TEM areas corresponding to "young" mesenchymal stroma contained numerous myofibroblasts laden with cytofilaments (Fig. 5) usually surrounded by a few well-formed collagen fibers, microfibrils and granular or basal lamina-like material. In densely sclerotic zones myofibroblasts,

Fig. 7. Immunofluorescence of cribriform component of intraductal mammary carcinoma. Stromal cells with cytoplasmic fluorescence are absent. There is a continuous layer of strongly fluorescent myoepithelial cells (*M*). Note faint staining along luminal border of intraductal carcinoma (arrow heads). (Rabbit AAA, FITC-conjugated goat anti-rabbit gammaglobulin, × 300)

Fig. 8. Immunofluorescence of papillary adenocarcinoma of the breast. Stroma contains few elongated cells with weak cytoplasmic fluorescence. Portion of a blood vessel is visible in lower right corner.* A very faint granular pattern of fluorescence is evident along the luminal border of the carcinoma (arrow heads). (Rabbit AAA, FITC-conjugated goat anti-rabbit gammaglobulin, × 300)

Fig. 9. Photomicrograph of small cell carcinoma of lung illustrating numerous lymphocytes in stroma. (HPS × 300)

Fig. 10. Immunofluorescence of corresponding area of small cell pulmonary carcinoma illustrates a few elongated stromal cells with weak cytoplasmic fluorescence. Note weak fluorescence along membranes of neoplastic cells. (Rabbit AAA, FITC-conjugated goat anti-rabbit gammaglobulin, × 300)



Figs. 11–13. Adenocarcinoma of colon invading pericolic fat.

Fig. 11. Photomicrograph of neoplasm illustrates numerous elongated stromal cells. (HPS × 300)

Fig. 12. Immunofluorescence of a corresponding area reveals numerous elongated cells with intense cytoplasmic fluorescence. (Rabbit AAA. FITC-conjugated goat anti-rabbit gammaglobulin, × 300)

Fig. 13. Electronmicrograph of neoplasm illustrating several myofibroblasts (*MF*) in vicinity of carcinoma cells (*C*). (× 5,000)

Table 2. Metastatic carcinomas (7 cases)

Site of metastasis	Primary neoplasm (type)	No. of cases	Consistency	Retraction of cut surface	No. of stromal myofibroblasts[a]
Breast	Ileum (carcinoid)	1	Firm	present	+++
Lymph node	Larynx (epidermoid)	1	Semi-Firm	present	++
Liver	Colon (adeno)	1	Firm	present	+++
	Unknown (adeno)	1	Firm	present	++++
	Kidney (clear cell)	1	Soft	absent	±
Bone	Unknown (adeno)	1	Firm	present	++
Ovary	Colon (adeno)	1	Soft	absent	±

[a] ±, equivocal; +, rare; ++, few; +++, moderate; ++++, abundant

in contrast, were few, slender, contained but scant numbers of microfilaments and were surrounded by bands of well-formed collagen fibers (Fig. 6). Some of the stromal cells demonstrated ultrastructural appearances of quiescent fibroblasts. No myoepithelial cells were identified in the stroma of invasive carcinomas. Scant numbers were identified adjacent to intraductal carcinomas juxtaposed to epithelia, always within the confines of a delineating basal lamina. By LM the connective tissue stroma adjacent to intraductal carcinomas was usually delicate; by IF myofibroblasts were absent (Fig. 7). In cribriform intraductal carcinoma a continuous peripheral layer of myoepithelial cells was identified by IF. In the single case of infiltrating lobular carcinoma the distribution of stromal myofibroblasts was similar to that in infiltrating ductal carcinomas. Myofibroblasts were numerous in infiltrating lobular carcinoma composed of "young" mesenchyme, rare or absent in densely sclerotic zones and absent in areas of in-situ lobular carcinoma. The lymphoid rich stroma of medullary carcinoma contained but a few myofibroblasts; in the papillary carcinoma myofibroblasts were not identified with certainty (Fig. 8). The latter two neoplasms were soft and bulging, rather than retracted.

b) Small Cell Carcinoma of Lung and Cervix. These neoplasms were soft and bulging. Their stroma demonstrated an inflammatory rather than a desmoplastic response. Extensive areas of necrosis were characteristically observed. Myofibroblasts were not identified with certainty (Figs. 9 and 10).

c) Adenocarcinomas of Lung, Stomach and Colon. The pulmonary adenocarcinomas were semi-firm, peripherally located and demonstrated macroscopic pleural retraction. LM revealed many inflammatory cells and a few elongated stromal cells with acidophilic cytoplasm which by IF and TEM proved to be myofibroblasts. Sections from the adenocarcinomas of the stomach and colon were selected from areas wherein the neoplasm had extended through the wall into the pericolic fat. All had a firm to hard consistency; the colonic adenocarcinomas were typically annular and stenosing. The number of myofibroblasts was moderate

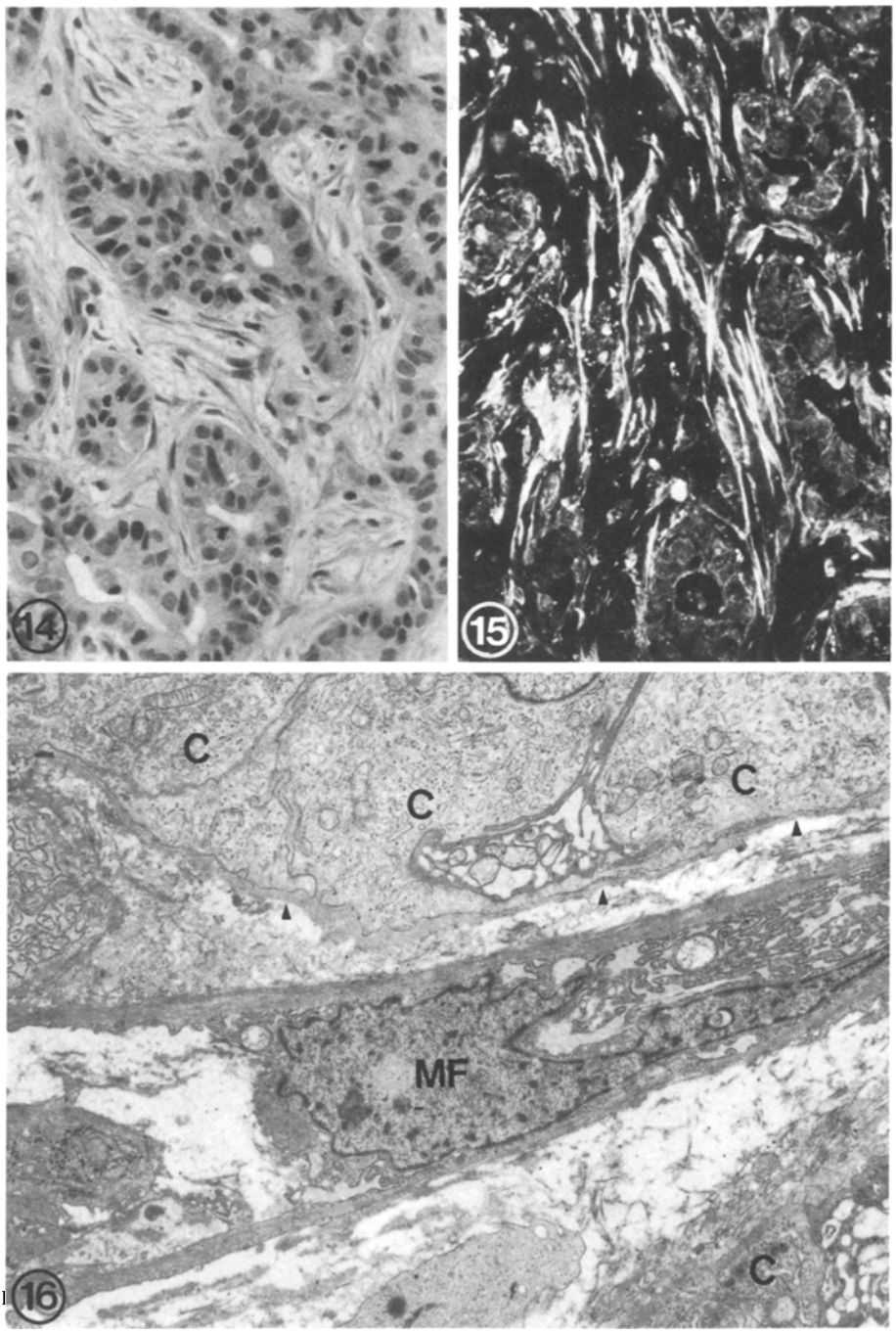
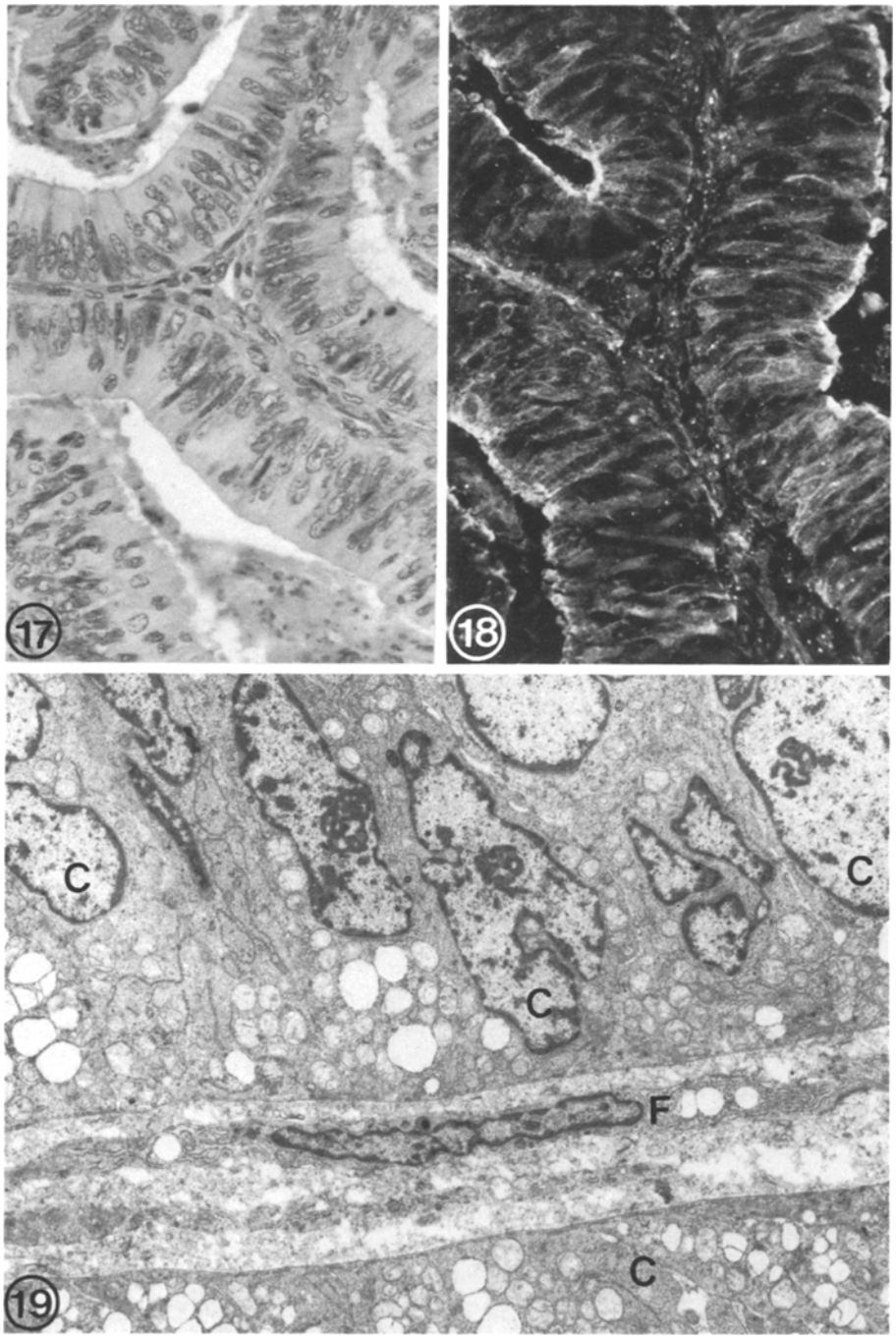


Fig. 14. Photomicrograph illustrates loosely arranged elongated stromal cells between groups of neoplastic cells. (HPS × 300)

Fig. 15. Immunofluorescence of a corresponding area of hepatic metastasis illustrates numerous elongated stromal cells with intense cytoplasmic fluorescence. (Rabbit AAA, FITC-conjugated goat anti-rabbit gammaglobulin, × 300)

Fig. 16. Electronmicrograph illustrating stromal myofibroblast (*MF*) rich in cytofilaments, adjacent to carcinoma cells (*C*). The stroma is composed of granular and finely fibrillar material with scant collagen. Basal lamina (arrow head) separates carcinoma cells from stroma. (× 5,000)



Figs. 17–19. Ovarian metastasis of colonic adenocarcinoma.

Fig. 17. Photomicrograph illustrates delicate connective tissue septae between neoplastic glands. (HPS × 300)

Fig. 18. Immunofluorescence fails to detect stromal cells with cytoplasmic fluorescence but does illustrate strong fluorescence along luminal border of carcinoma cells. (Rabbit AAA, FITC-conjugated goat anti-rabbit gammaglobulin, × 300)

Fig. 19. Electronmicrograph illustrates a slender fibroblast (*F*) between two rows of carcinoma cells (*C*). (× 5,000)

to abundant; again, most were identified in areas composed of "young" mesenchyme (Figs. 11–13).

Metastases (Table 2)

Stromal myofibroblasts, consistently present in firm and retracted metastases, were more numerous in those associated with "young" mesenchymal stroma. This was especially well-illustrated in two hepatic metastases, one from colonic adenocarcinoma, the other from an adenocarcinoma of undetermined origin. Both demonstrated numerous stromal myofibroblasts in the absence of notable collagen (Figs. 14–16). An ovarian metastasis from colonic adenocarcinoma was soft and neither by IF nor TEM contained stromal myofibroblasts (Figs. 17–19).

Neoplastic cells from all primary and metastatic carcinomas studied demonstrated positive immunofluorescent cytoplasmic staining with the AAA-containing serum. Similar results have been described utilizing human anti-actin autoantibodies and rabbit anti-human actin antibodies (Gabbiani et al. 1975, 1976a). This cytoplasmic fluorescence, relatively weak compared to that observed in stromal myofibroblasts, was generally distributed along the peripheral and/or apical (luminal) cell borders (Figs. 10 and 18). A similar cytoplasmic fluorescence has been described in carcinomas employing an antimyosin serum (Macartney et al. 1979).

Discussion

The study of the cellular mechanisms involved in wound healing led to the discovery of the myofibroblast (Majno et al. 1971; Gabbiani et al. 1971). The findings assembled to support the conclusion that this unique cell contributed to the process of wound closure were morphological, pharmacological, biochemical and immunological (Gabbiani et al. 1972; Hirschel et al. 1971). Since the publication of these pivotal experiments, myofibroblasts have been described in a wide assortment of pathologic conditions and have been the subject of several recent reviews (Mussini et al. 1977; Guber and Rudolph 1978; Majno 1979; Seemayer et al. 1980b).

In a recent ultrastructural study we demonstrated variable numbers of stromal myofibroblasts in a series of primary invasive and metastatic carcinomas (Seemayer et al. 1979). Since the amount of tissue which can be surveyed in ultrastructural preparations is relatively small, it was deemed essential to employ a technique which would permit the examination of a larger area of each neoplasm. The anti-actin antibody preparation ideally served this purpose for it permitted one to identify and semi-quantitate by light microscopy the number and spatial relations of stromal myofibroblasts within different microscopic fields of a variety of invasive and metastatic carcinomas. This combined light microscopic, immunological and ultrastructural study demonstrated that myofibroblasts were most numerous in carcinomas associated with a "young" mesenchymal stroma which contained few mature collagen fibers. In densely sclerotic zones in which infiltrating carcinoma cells were enveloped by thick bands of collagen fibers, myofibroblasts were generally scant in number, often poorly

developed and occasionally were even absent. Myoepithelial cells were not observed in the "young" mesenchymal or densely sclerotic stroma of infiltrating carcinomas. Similar findings have been reported previously (Ozzello 1971a; Ohtani and Sasano 1980). The connective tissue stroma of the intraductal components of mammary carcinomas lacked myofibroblasts and contained only a few slender cells with features of resting fibroblasts. In contrast, myoepithelial cells were identified adjacent to epithelium in intraductal components of infiltrating mammary carcinoma. These findings are consonant with prior ultrastructural studies of the epithelial-stromal junction of the normal and dysplastic breast as well as intraductal mammary carcinoma (Ozzello 1970, 1971b). The absence of myofibroblasts in the delicate stroma adjacent to intraductal mammary carcinoma suggests that epithelial invasion beyond the basal lamina is required to evoke a myofibroblastic stromal response. Turning to macroscopic features, myofibroblasts were most numerous in primary and secondary carcinomas which were firm and retracted. In contrast, carcinomas of soft consistency and bulging cut-surface contained, at most, very few myofibroblasts.

Myofibroblasts possess not only contractile but also synthetic properties for type III collagen (Gabbiani et al. 1976b). In granulation tissue of healing wounds myofibroblasts are especially numerous and the collagen produced is principally type III, that characterized by plasticity. When granulation tissue is resorbed following wound closure myofibroblasts disappear (Rudolph et al. 1977) and a more rigid collagen (type I) is identified (Gabbiani et al. 1976b).

At the cellular level a number of similarities exist between the process of wound healing and the stromal response to invasive and metastatic carcinoma. The following theoretical pathobiological sequence of events is proposed to account for the findings of the present study. Epithelial invasion beyond the epithelial basal lamina into the subjacent stroma in certain carcinomas induces a transformation of quiescent stromal fibroblasts into reactive fibroblasts, i.e., myofibroblasts. The latter, endowed with contractile and synthetic properties, produce type III collagen, a non-rigid protein, which facilitates the transmission of cell-to-cell and cell-to-stroma contractile forces generated by myofibroblasts. Retraction is possible at this stage since the "young" mesenchymal stroma contains a limited amount of collagen. As the stromal response proceeds, a more rigid type I collagen is produced and myofibroblasts progressively disappear. At this point the neoplasm has many features of a scar in which clusters of carcinoma cells are enveloped by thick bands of mature collagen whereas myofibroblasts are rare and most stromal cells are fibroblasts. The retraction, desmoplasia and sclerosis observed in many carcinomas might thus result from the marked production of type I collagen at a time at which the stromal myofibroblasts were maximally contracted. It seems less likely that malignant epithelial cells contribute significantly to the retraction process since their content of actin is considerably less than that of myofibroblasts.

The factor(s) responsible for the induction of myofibroblasts in wound healing and invasive and metastatic carcinomas is unknown. In the latter setting myofibroblasts are generally numerous in carcinomas which lack morphologic evidence of a cellular immunological response. Phylogenetically, an angiofibroblastic reaction represents a principal defensive mechanism of invertebrates which

lack an immune system and, thus, adaptive immune responses (Bolande 1979). Possibly aberrant malignant epithelia release factors which initiate myofibroblast induction, somewhat analogous to tumour angiogenesis.

The magnitude of the myofibroblastic stromal reaction in carcinomas may in some way be related to the growth rate of the neoplasm. Small cell undifferentiated carcinomas which inherently possess a fast doubling time (Garland et al. 1963) demonstrate a feeble (at best) myofibroblastic response. In contrast slowly growing neoplasms. i.e., conventional ductal mammary carcinomas, (MacDonald 1966) are characterized by a brisk myofibroblastic response. The stromal reaction may even vary for the same carcinoma depending on the host tissue as illustrated by two metastases from a colonic adenocarcinoma. The hepatic metastasis contained numerous stromal myofibroblasts whereas the ovarian metastasis lacked myofibroblasts altogether.

We have previously suggested that the myofibroblastic reaction observed in carcinomas and nodular sclerosing Hodgkin's disease might represent a host response to neoplasia (Seemayer et al. 1979, 1980a). This induction of stromal myofibroblasts with subsequent collagen synthesis and tissue contraction might represent an attempt to contain the neoplasm. The sclerotic and contracted state of such stroma may retard local invasion and access to vascular channels by neoplastic cells. The immunofluorescent demonstration of this striking myofibroblast response, particularly in "*young*" *mesenchymal stroma* of invasive and metastatic carcinomas (which might represent foci of early invasion and metastasis) provides further evidence to support the original hypothesis.

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