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Stromal cell changes in human colorectal adenomas and carcinomas

An ultrastructural study of fibroblasts, myofibroblasts, and smooth muscle cells*

Haruo Ohtani and Nobuaki Sasano

Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Sendai 980, Japan

Summary. Structural changes in stromal cells during the development of human colorectal carcinomas were studied by light and electron microscopy. The results were as follows:

1. Stromal cells of the lamina propria in control subjects consisted principally of resting fibroblasts.

2. Stromal fibroblasts were mildly activated in adenomas with mild – moderate atypia, and more markedly in adenomas with severe atypia (carcinoma in situ).

3. In invasive adenocarcinomas, (a) desmoplastic reaction was induced, (b) stromal fibroblasts proliferated significantly and were activated showing enlarged nuclei and abundant rough endoplasmic reticulum, and (c) some smooth muscle cells were endowed with well-developed rough endoplasmic reticulum in their axial cytoplasm, resulting in a similar appearance to "myofibroblasts".

4. Stromal fibroblasts in ulcerative colitis and proctitis were also activated.

Morphometric analysis revealed that activated fibroblasts significantly increased the areas of their nuclei and cytoplasm, and the perimeter of rough endoplasmic reticulum. These activated fibroblasts suggested a higher productin of collagen and other connective tissue proteins. Bundles of microfilaments of actin type were readily found in fibroblasts in all cases examined. These filaments were most remarkable in the fibroblasts in the desmoplastic stroma of invasive adenocarcinoma and were considered to be one of the basic components of these cells. Rela-

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Offprint request to: H. Ohtani at the above address

tionships between fibroblasts, "myofibroblasts", and smooth muscle cells are discussed.

Key words: Colorectal epithelial tumors – Stroma – Fibroblast – Myofibroblast – Smooth muscle cell

In recent years myofibroblasts have been detected in the stroma of invasive and metastatic carcinoma (Seemayer et al. 1979 and 1980; Schürch et al. 1981). We have also studied the relationship of myofibroblasts and myoepithelial cells in human breast carcinomas (1980) and extended the investigation into the stroma of human colorectal tumors.

The present paper concerns the gradual activation of stromal fibroblasts with the progress of malignancy of epithelial cells and also the mutual relationship among fibroblasts, "myofibroblasts", and smooth muscle cells. Because the distinctions between these cells are sometimes so subtle, a definition of each cell type is given here.

Definition of terms used in this paper

Fibroblast. A spindle-shaped cell which has varying amounts of short anastomosing rough endoplasmic reticulum. Basal lamina is usually absent or fragmentary. Lysosomes are infrequently present.

Activated fibroblast. A fibroblast with enlarged nucleus and well-developed rough endoplasmic reticulum.

Smooth muscle cell. A spindle-shaped cell which has abundant microfilaments, 5–7 nm in diameter, with focal densities, and is surrounded by a continuous basal lamina. Pinocytotic vesicles (caveollae) are observed.

Myofibroblast. Currently defined as an intermediate cell between a fibroblast and a smooth muscle cell, being characterized by rough endoplasmic reticulum in the axial cytoplasm and narrow bundles of microfilaments of smooth muscle type in the peripheral cytoplasm. However, as we will discuss later, these subplasmalemmal narrow bundles of microfilaments of smooth muscle type are thought to be one of the basic components of a fibroblast. Therefore we use the term *fibroblast* in a wider sense including myofibroblast. The term *myofibroblast* is usually restricted to use in comparison with other papers.

Myofibroblastic change of smooth muscle cell. A change where smooth muscle cell is transformed, developing a well-developed rough endoplasmic reticulum and other cell orgenelles in the axial cytoplasm, so that the appearance of the cell becomes similar to a "myofibroblast".

This paper is part of a study of stromal reactions to carcinoma growth. Capillary reactions to the growth of carcinoma will be described in the second part of the study.

Materials and methods

Materials

Specimens were obtained from surgical and biopsy material. The normal colorectal mucosa was from 5 cases (average age 45.0 years). For histological typing of tumors, the WHO classification (Morson 1976) was adopted. Colorectal tumors were composed of 4 adenomas with mild – moderate atypia (3 solitary tubular adenomas and 1 case of adenomatosis, average age 46.8 years), 2 adenomas with severe atypia (carcinoma in situ or adenoma with focal malignancy) (average age 45.5 years) and 15 invasive adenocarcinomas (average age 57.9 years). For controls, 9 cases of colitis (6 ulcerative colitis and 3 idiopathic proctitis) (average age 36.2 years) were examined. 3 cases received glucocorticoid derivatives prior to operation or biopsy. All the cases of invasive carcinoma were histologically well or moderately differentiated adenocarcinoma. 10 lesions were ulcerating form, and 5 lesions were polypoid. Depth of invasion was submucosal in 3 lesions, muscularis propria in 3 lesions, and the subserosa or adventitia in 9 lesions.

Light microscopy

Specimens were fixed in 10% formalin solution and embedded in paraffin. Sections, 3–4 μm in thickness, were stained with haematoxylin-eosin, elastica-Masson trichrome and Gomori's reticulin stains.

Electron microscopy

Specimens were fixed in 2.5% glutaraldehyde-2 % paraformaldehyde in 0.1 M Na cacodylate buffer, pH 7.2–7.4. After postfixation in 1% osmium tetroxide for 2 h at 4° C, specimens were dehydrated and embedded in Epok 812. Semithin sections, 1 μm in thickness, were stained with toluidine blue for light microscopy to select proper areas. Thin sections of silver-gold interference color were stained with uranyl acetate and lead citrate, and examined with a JEOL 100B electron microscope accelerated at 80 kV.

For electron microscopical investigation of stromal cells, only the blocks containing epithelial components were used. Areas infiltrated with numerous inflammatory cells were excluded.

Electron microscopic morphometry

The exact magnification of electron microscope was measured by a carbon grating space (Oken Ltd, Tokyo). The photographs were taken from stromal areas at radon, and the negative films were printed in A4 projection paper, × 5,400 at final magnification. The original size of print was 53.7 μm × 38.1 μm. The average numbers of prints per one case in each group were 11.4 in normal, 14 in adenoma, 13 in carcinoma in situ, 13.9 in invasive carcinoma, and 11.7 in colitis. All stromal cells (fibroblasts and so-called myofibroblasts) with a view of a nearly central cut surface in the prints were measured by a semiautomatic image analyser (Videoplan-system of Kontron). The numbers of cells measured in each group were 86 (average; 17.2) in normal, 87 (average; 21.8) in adenoma, 45 (average; 22.5) in carcinoma in situ, 407 (average; 27.1) in invasive carcinoma, and 195 (average; 21.7) in ulcerative colitis. The variables measured were the area of the nuclei and cells, and the sum of the perimeters of rough endoplasmic reticulum per cell, of which cross sections were measured and oblique sections excluded. All the crude data of the same group were accumulated, and the mean values were compared.

Light microscopic morphometry

A square, 25 × 25 mm in size, was randomly placed in the area of stroma near the epithelial components using × 400 field, and spindle-shaped cells in the square were selected for counting the number. Plasma cells, lymphocytes, endothelial cells and smooth muscle cells were carefully excluded.

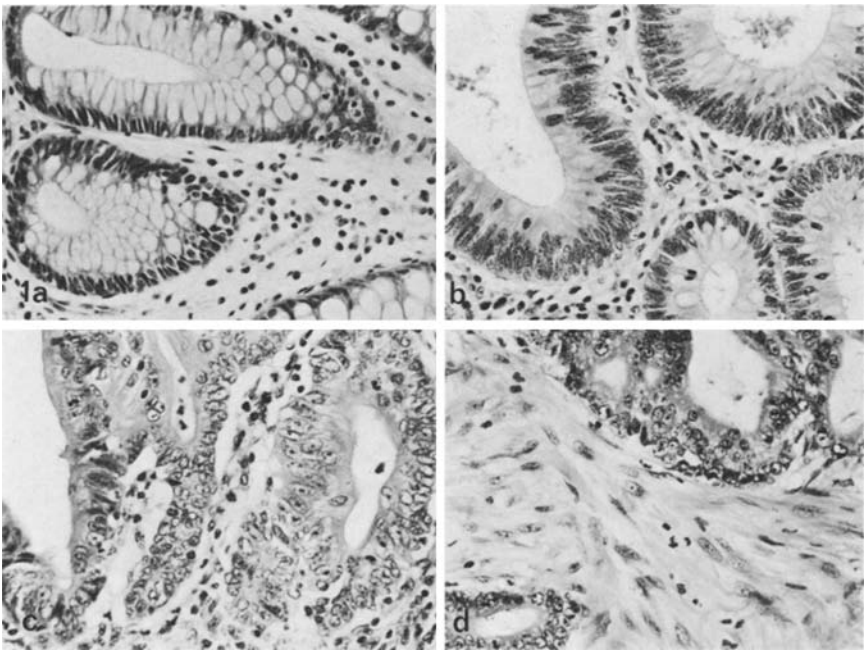

Fig. 1 a–d. Light microscopy of each lesion (H–E). **a** Normal rectal mucosa. × 200. **b** Adenoma with mild atypia. × 200. **c** Carcinoma in situ (adenoma with severe atypia). × 200. **d** Invasive adenocarcinoma with desmoplastic stroma. × 200

Result

1. Control colorectal mucosa

The lamina propria consisted of loose connective tissue containing spindle-shaped fibroblasts and capillaries (Fig. 1 a).

By electron microscopy, fibroblasts had elongated nuclei with condensed chromatin (heterochromatin), and cytoplasmic organelles such as rough endoplasmic reticulum and Golgi complexes were poorly developed (Fig. 2 a). Sometimes a short region of basal (external) lamina like substance was observed, but continuous basal lamina was never seen. Judged by these features, these cells were resting (quiescent) fibroblasts. Some of them had narrow bundles of microfilaments, 5–7 nm in diameter, with focal densities (dense bodies) beneath the cell membrane (Fig. 2 b, c). Despite their small quantity, these filaments were very similar to those in smooth muscle cells (actin-type filaments) (Fig. 6 a, b).

In the upper-most portion of lamina propria, fibroblasts with fairly well-developed rough endoplasmic reticulum were observed (Kaye et al. 1968).

Smooth muscle cells were also observed in the lamina propria around crypts. Most of them had smaller amounts of microfilaments than the usual smooth muscle cells found in the muscularis mucosae or muscularis propria.

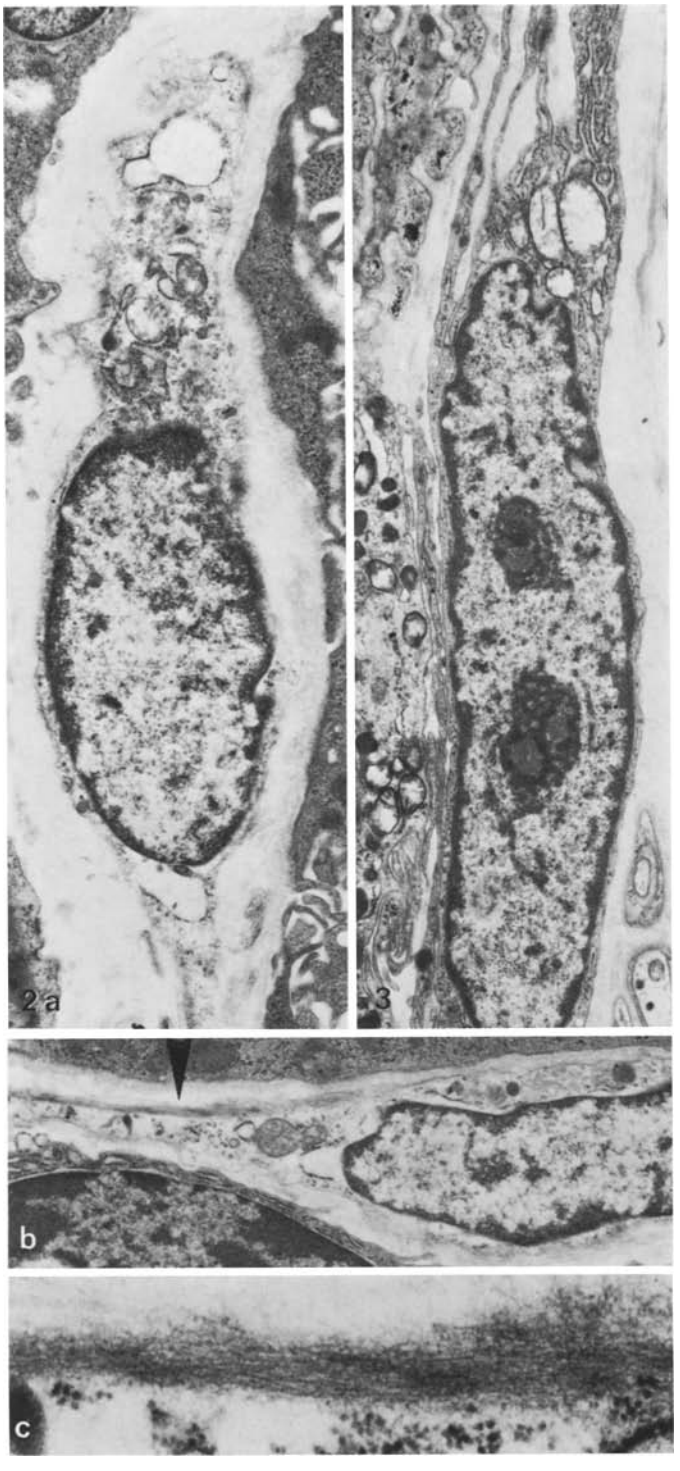

Fig. 2a–c. Fibroblasts in the lamina propria of normal mucosa. **a** A resting fibroblast. × 10,200. **b** A resting fibroblast which has a narrow bundle of microfilaments beneath plasma membrane of luminal side (*arrow head*). × 8,840. **c** Higher magnification of filaments in Fig. 2b. × 45,000

Fig. 3. A fibroblast in the stroma of adenoma with mild atypia manifesting mild activation. × 8,840

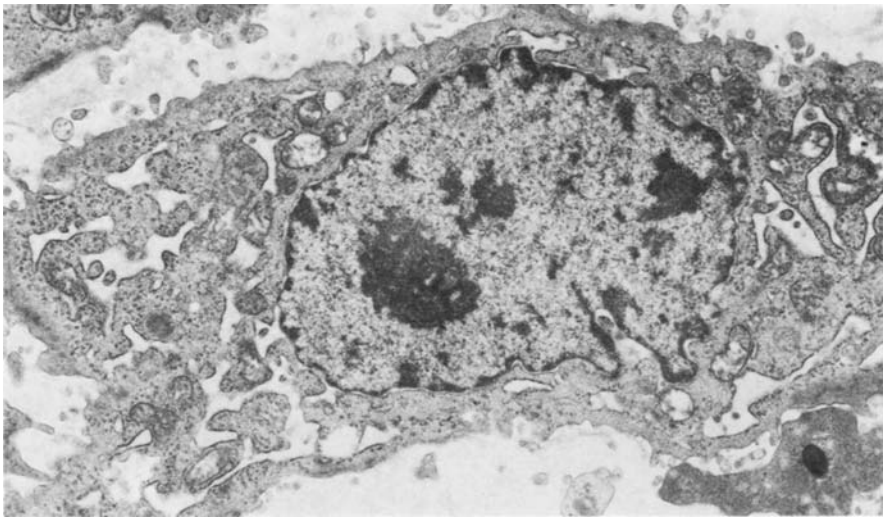

Fig. 4. An activated fibroblast in the stroma of carcinoma in situ (adenoma with severe atypia). Bundles of microfilaments are discernible in the peripheral cytoplasm. × 8,840

2. Sequential stromal cell changes from adenoma to invasive adenocarcinoma

Adenoma (Fig. 1b), carcinoma in situ (adenoma with severa atypia) (Fig. 1c), and some of invasive adenocarcinomata with polypoid shape had stroma of loose connective tissue, while sites of invasion of invasive adenocarcinoma had desmoplastic stroma (Fig. 1d). The desmoplastic stroma was stained brown and black by Gomori's silver impregnation and showed high cellularity (Fig. 8). In the present series, however, no case had sclerotic stroma.

Electron microscopy revealed a gradual activation of stromal fibroblasts from resting to active stages with the progress of malignancy of epithelial components. The active fibroblasts had enlarged nuclei with dispersed chromatin (euchromatin) and occasional prominent nucleoli, and well-developed rough endoplasmic reticulum and Golgi complex in the cytoplasm. The results of our observations are summarized as follows:

1. Adenoma with mild to moderate atypia had stromal fibroblasts showing mild activation (Fig. 3).

2. Adenoma with severe atypia (carcinoma in situ) had stromal fibroblasts with advanced activation (Fig. 4).

3. In invasive carcinoma stromal fibroblasts were usually activated with a remarkable variation of activity in individual cases (Fig. 5a).

This sequential activation of fibroblasts was confirmed by morphometric analysis. Nuclear and cellular areas and the sum of perimeters of rough endoplasmic reticulum increased gradually with the progress of malignancy (Fig. 9, Fig. 10, Fig. 11, and Table 2). Of the three variables, the sum of the perimeters of rough endoplasmic reticulum showed a most remarkable change; the mean value in invasive carcinoma group was 5.0 times larger

Table 1. Cellularity of stromal cells. Values are expressed as means ± 1SD. Asterisks indicate the size of critical region of *t*-test with the normal mean value

Group	Lesions	No. of squares	Cell count
Normal	4	80	7.3 ±3.0
Adenoma	4	80	7.5 ±3.4
Carcinoma in situ	2	40	8.8 ±3.4
Invasive carcinoma	15	300	14.2* ±5.6
Ulcerative colitis	6	120	6.1** ±3.3
Non-treated	3	60	7.5 ±3.3
Treated with corticosteroid	3	60	4.7 ±2.5

* = $p<0.001$, ** = $p<0.01$, and no sign = not significant. The ulcerative colitis group is subdivided into non-treated group and group treated with corticosteroid

Table 2. Summary of data of electron microscopic morphometry. Values are expressed as means ± 1SD. Figures in parenthesis indicate ratio to normal. The size of critical region of *t*-test between mean values of neighboring groups are indicated in the table between each group. The ulcerative colitis group is subdivided into non-treated group and group treated with corticosteroid

Groups	Lesions	Cells	Nuclear area (μm^2)	Cellular area (μm^2)	Sum of perimeter of rER (μm)
Normal	5	86	19.8±6.9 (1) n.s.	37.1±13.0 (1) $p<0.001$	8.8±6.8 (1) $p<0.001$
Adenoma	4	87	20.7±8.4 (1.0) $p<0.01$	47.2±16.5 (1.3) $p<0.01$	15.0±12.2 (1.7) $p<0.01$
Carcinoma in situ	2	45	27.1±11.7 (1.4) n.s.	62.0±27.6 (1.7) $p<0.001$	27.6±22.2 (3.1) $p<0.001$
Invasive carcinoma	15	407	31.0±17.8 (1.6)	80.5±54.2 (2.2)	44.1±45.6 (5.0)
Ulcerative colitis	9	195	32.3±12.7 (1.6)	74.1±31.6 (2.0)	42.0±29.1 (4.8)
Non-treated	6	107	30.4±11.6	65.6±25.5	40.9±28.0
Treated with corticosteroids	3	88	34.6±13.5	84.5±35.2	43.4±30.3

than that of normal group. Statistically significant differences in mean values of cellular area and the sum of the perimeters of rough endoplasmic reticulum were confirmed between normal and adenoma, adenoma and in situ carcinoma, and in situ and invasive carcinomas.

Narrow bundles of microfilaments, 5–7 nm in diameter, with focal densities were readily observed in the peripheral cytoplasm of fibroblasts in every group. The quantity of microfilaments in fibroblasts was small in the normal group, and most remarkable in the activated fibroblasts in the desmoplastic

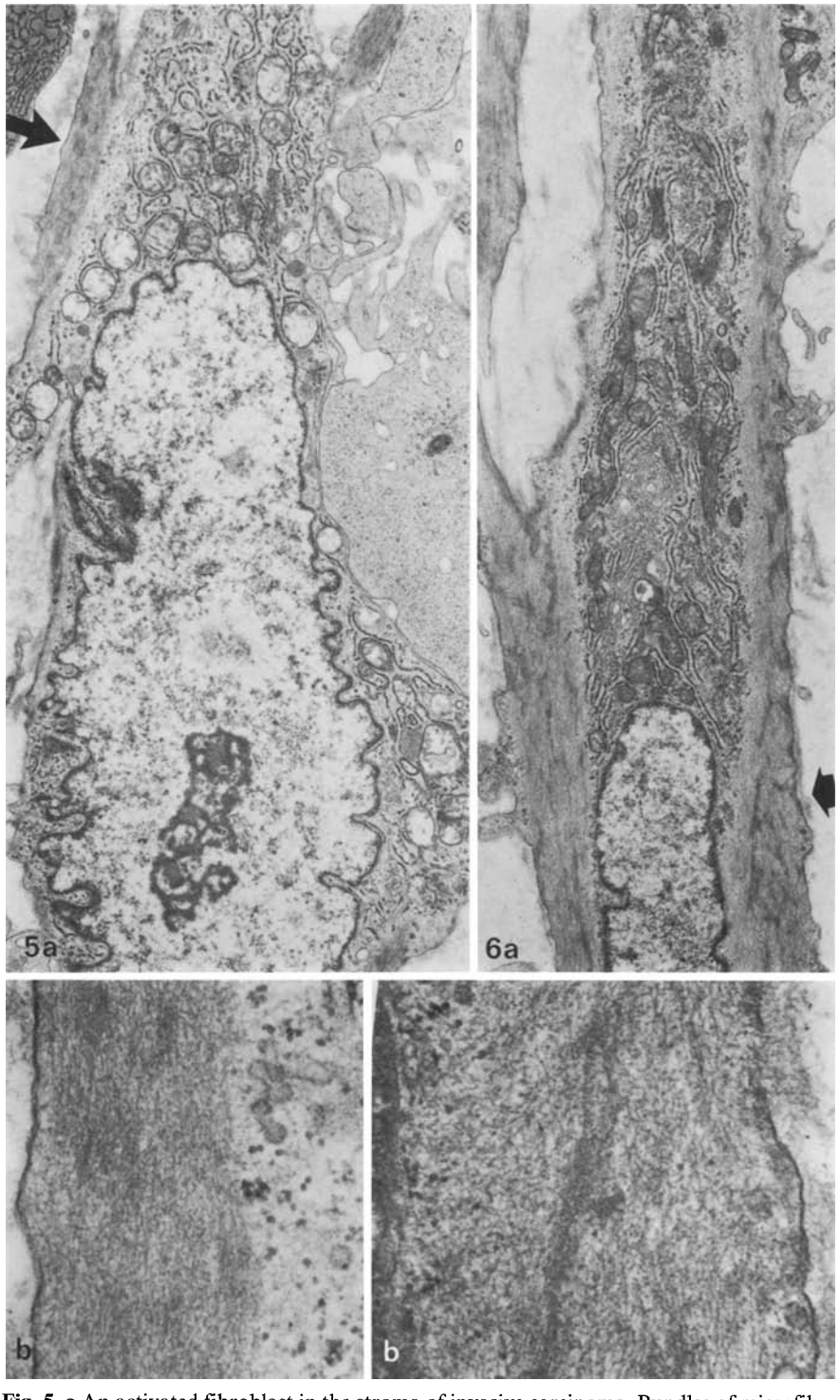

Fig. 5. a An activated fibroblast in the stroma of invasive carcinoma. Bundles of microfilaments (*arrow*) are clearly observed. A cell of this type can be designated myofibroblast. ×8,840. **b** Higher magnification of microfilaments indicated by an arrow in **a**. ×45,000

Fig. 6. a A smooth muscle cell with myofibroblastic change in the stroma of invasive carcinoma. ×8,840. **b** Higher magnification of microfilaments indicated by an arrow in **a**. ×45,000

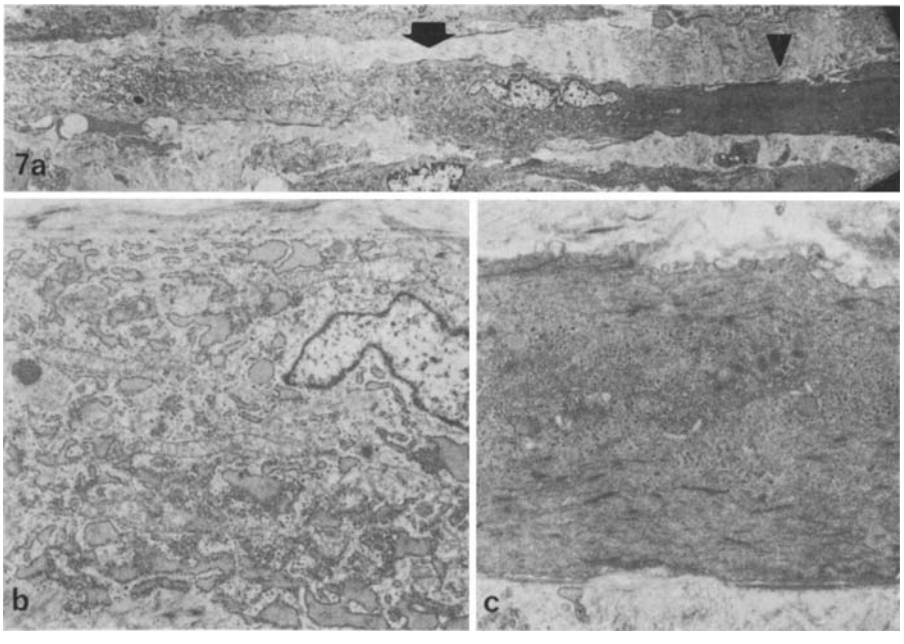

Fig. 7a–c. A cell with features of a fibroblast and a smooth muscle cell in the stroma of invasive carcinoma. **a** Lower magnification. ×1,200. **b** A part with a fibroblastic feature indicated by an arrow in **a**. ×5,500. **c** A part with smooth muscle cell feature indicated by a triangle in **a**. ×6,750

stroma of invasive carcinoma. In the latter cases, they could be also designated myofibroblasts.

3. "Myofibroblastic change" of smooth muscle cells

Well-developed smooth muscle cells which probably originated from the muscularis mucosae or muscularis propria were frequently observed in the stroma of invasive carcinoma. Some of them were typical smooth muscle cells, while some had varying amounts of rough endoplasmic reticulum and Golgi complex in their axial cytoplasm (Fig. 6a). When these organelles were fully developed, the bundle of microfilaments of actin-type remained only in the periphery of cytoplasm, and the basal (or external) lamina became discontinuous or fragmentary. These cells corresponded to the "myofibroblasts". Usually their character as smooth muscle cells was revealed by a relative abundance of microfilaments, well-developed basal lamina and pinocytotic vesicles. But in some cases we found intermediate cells and could not decide whether they were derived from a fibroblast or from a smooth muscle cell. This myofibroblastic change of smooth muscle cells was observed in 14 out of 15 cases of invasive adenocarcinoma.

An extreme case is presented in Fig. 7. Half the cytoplasm is filled with numerous rough endoplasmic reticulum, typical for a fibroblast, and the

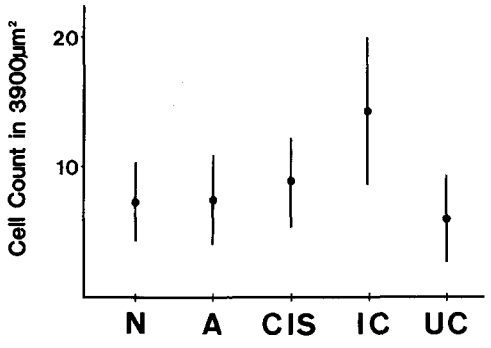

Fig. 8. Cellularity of stromal spindle shaped cells. Values are expressed as mean counts of spindle cells in areas of 3,900 $\mu m^2 \pm 1SD$. (Abbreviations used in Fig. 8–11: *N*=normal, *A*=adenoma, *CIS*=carcinoma in situ, *IC*=invasive carcinoma, and *UC*=ulcerative colitis)

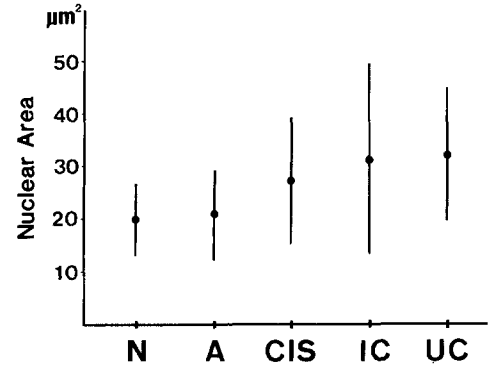

Fig. 9. Nuclear area of stromal cells in each group. Values are expressed as mean $\pm 1SD$

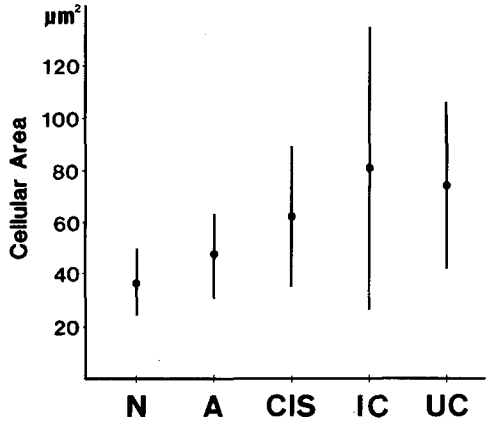

Fig. 10. Cellular area of stromal cells in each group. Values are expressed as mean $\pm 1SD$

other half contains myofilaments with few rough endoplasmic reticulum, showing a typical feature of smooth muscle cells.

Smooth muscle cells were occasionally observed in the stroma of adenoma and carcinoma in situ, but those cells did not manifest myofibroblastic change.

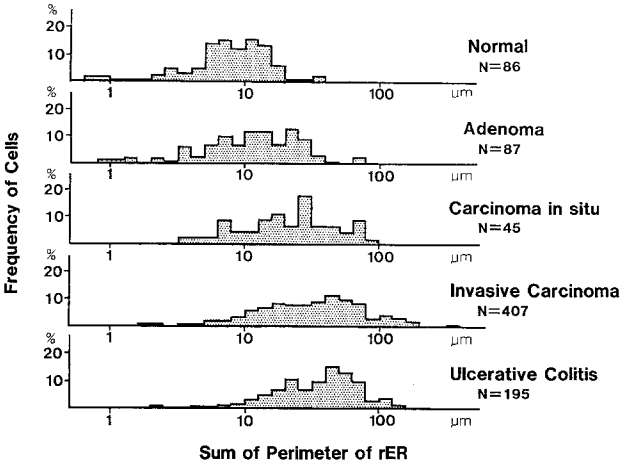

Fig. 11. Sum of perimeter of rough endoplasmic reticulum in stromal cells in each group after logarithmic conversion

4. Stromal cells in ulcerative colitis

Severely inflammed lamina propria and granulation tissue were observed. No case showed a vigorous desmoplastic reaction. Fibroblasts in those areas were active in features. Microfilaments were usually scanty but occasionally as many were seen as were found in typical "myofibroblasts".

Three cases received glucocorticoid derivatives before operation. Those cases showed decreased cellularity of the stroma when compared with that of non-treated cases, but the development of rough endoplasmic reticulum was not altered (Table 1 and 2).

In two cases, fibrotic submucosal layers were studied. Histologically collagenous fibers were mature and stained brown with Gomori's silver impregnation. Few or scanty microfilaments of actin-type were observed in the fibroblasts.

Discussion

1. Stromal cell changes with cancerization

Histological typing of epithelial tumors is generally based on findings on epithelial cells and not so much attention has been paid to the stromal elements. In this paper we confirmed gradual activation of stromal cells with the progress of neoplastic growth from the normal mucosa – adenoma – carcinoma in situ – to invasive carcinoma. This is generally consistent with our previous report on human breast carcinoma (1979). Normal intralobular fibroblasts in the breast were in resting stages as were most fibroblasts in the lamina propria of colorectal mucosa, and stromal cells of invasive ductal carcinoma were active, usually with the presence of microfilaments of actin-type. This activation of fibroblasts suggests a higher produc-

tion of collagen and other connective tissue proteins by these cells. Although the stromal cells became activated at morphological level when typical or suspected carcinoma cells were in the lamina propria,vigorous desmoplastic reaction with proliferation of stromal cells was induced only after carcinoma cells invaded beyond the muscularis mucosae. This fact indicates a special environmental property of the lamina propria in that a desmoplastic reaction is not induced, even though the fibroblasts in it become activated. This is consistent with our observations that signet ring cells confined within the lamina propria of gastric mucosa did not induce desmoplastic reactions, while they induce remarkable fibrosis when they invade beyond the muscularis mucosae.

Although the true mechanism(s) of this stromal change is not known, it is probably due to humoral factor(s) secreted by cancer cells. Naito and Kino (1982) reported that the production of collagen fibers by fibroblasts was enhanced by gastric cancer cells using a parabiotic culture bottle, where the media could pass freely between fibroblasts and gastric cancer cells but the cellular passage was blocked by membrane filter. They suggested that the production of collagen fiber was promoted by some factor(s) produced by gastric cancer cells. Our observations on colorectal and breast tumors are consistent with their experiments. A mild activation of fibroblasts in adenomas suggested that this benign epithelial lesion may cause some effects on stromal cells. When all these factors are considered, stromal fibroblastic reaction is thought to be determined by both tumor cell factor(s) and environmental factor(s) of the host.

The presence of actin-type filaments in fibroblasts was most remarkable in desmoplastic stroma of invasive carcinoma, and not so remarkable in active fibroblasts in preinvasive carcinoma and ulcerative colitis where a desmoplastic reaction was not induced. These findings were fundamentally consistent with "myofibroblastic reactions" to the carcinoma invasion described by Seemayer et al. (1980). This coincidence of "myofibroblasts" and desmoplasia in invasive carcinoma is in favor of a tissue contraction mechanism theory of myofibroblasts (Ryan et al. 1974). But this theory does not explain the presence of microfilaments of actin-type in fibroblasts in loose connective tissue-stroma in other lesions.

2. The relationship between fibroblasts – myofibroblasts – and smooth muscle cells

Since Majno et al. (1971) and Gabbiani et al. (1972) proposed the name of myofibroblasts, these cells were reported in many tissues (reviewed by Lipper et al. 1980 and Seemayer et al. 1980) as a distinct entity. But microfilaments of actin-type are found in fibroblasts in most cases, indicating that these filaments are one of the basic components of fibroblasts. This is the main reason why we use the term *fibroblasts* to include myofibroblasts in this paper; we could not make a clear distinction between fibroblasts and myofibroblasts since they constituted a continuous cell spectrum (refer to our previous paper for further discussion, 1980). We have confirmed marked

activation of fibroblasts with the progress of malignancy in this paper. These facts lead us to consider that we should assess fibroblastic cells also on the standpoint of the secretory activity and that the development of rough endoplasmic reticulum is probably the best variable, at the ultrastructural level, for cellular secretory activity.

Lipper et al. (1980) suggested a close relationship between myofibroblasts and smooth muscle cells. However, no definite evidence for their view has been reported so far. In this paper we have shown that smooth muscle cells have a potentiality for a well-developed rough endoplasmic reticulum and other cell organelles, and the presence of a continuous cell spectrum between smooth muscle cells and "myofibroblasts" with various kinds of intermediate stages is also suggested. This change of smooth muscle cells in the stroma of invasive carcinoma suggested that they were incorporated into "active connective tissue" and then transformed into protein secreting cells. This agrees with the report of Ross (1971), who showed that smooth muscle cells could produce connective tissue proteins in vivo and in vitro. This potentiality of smooth muscle cells could explain changes consistent with myofibroblastic change of smooth muscle cells in tissue repair (Haudenschild and Schwartz 1979), and in hypoxia-induced change of vascular smooth muscle cells (Meyrick and Reid 1980). For the diagnosis of spindle shaped cell tumors, especially malignant ones, the close relationship between fibroblasts and smooth muscle cells is important because an occurrence of intermediate cells or mixed occurrence of both cell types may be expected (cf. Böcker and Streker 1975).

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