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Role of multipotent fibroblasts in the healing colonic mucosa of rabbits. Ultrastructural and immunocytochemical study

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Summary. Light- and electron microscopy and immunocytochemistry were used to study the healing colonic mucosa of rabbits after experimental excision.

Between 3 and 5 days, abundant young fibroblasts which retained many features of mesenchymal cells invaded the growing capillaries into the loose connective tissue of the healing colonic mucosa. Our electron microscopy revealed the transformation of these young fibroblasts into smooth muscle cells, into histiocyte-like cells involved in phagocytotic activity, and into vasoformative cells incorporated into the growing capillaries. The mitotic proliferation of pre-existing smooth muscle cells at the ulcer margin did not seem to be the major reason for re-establishment of the muscular tissue.

The present immunocytochemistry revealed an active production of fibronectin in rough endoplasmic reticulum in the young fibroblasts. This may mean that this glycoprotein is involved in the re-establishment of both connective and muscular tissues by enhancement of adhesion and chemoattractant activities of such cells. In addition, the immunoreaction of endothelial cells of the growing capillaries suggests a role of this glycoprotein in the acceleration of the neocapillarization.

Key words: Ultrastructure, Immunocytochemistry, Wound healing, Fibronectin, Colonic mucosa, Pre-embedding method

Introduction

It is now widely accepted that fibronectin, a class of adhesive, high molecular weight glycoprotein, plays important roles in cell to cell and cell to matrix interactions (Yamada and Weston, 1974; Pearstein, 1976; Vaheri and Mosher, 1978; Yamada and Olden, 1978). Since then, considerable numbers of studies which indicate that fibroblasts release this glycoprotein utilizing for cell adhesion and migration during connective tissues re-constitution (Linder et al., 1978; Weiss et al., 1979; Gauss-Müller et al., 1980; Kurkinen et al., 1980; Clark et al., 1981; Postlethwaite et al., 1981; Grinnell, 1984) as well as embryonic development (Codogno et al., 1987) have been reported. The role of this glycoprotein in the connective tissue remodelling (Brownell et al. 1981; Clark et al. 1982b) suggests a role of mesenchyme-derived fibronectin in the epithelium-mesenchyme interaction and the constitution of the epithelial basal lamina during the epithelial tissue differentiation, and Fujikawa et al. (1981) reported a role in epithelial migration and temporary adhesion of the migrated epithelial cells to the epithelial basal lamina during corneal wound healing.

Our previous study revealed that young fibroblasts, which retain many features of mesenchymal cells, invaded the healing colonic mucosa in association with the growing capillaries from the intact underlying layer and had the ability to be transformed into new muscle cells in the regenerating muscular tissue (Mori et al., 1989). Since fibronectin plays an important role in the wound healing, more detailed investigations including immunostainings of fibronectin are required to elucidate what kind of cells release this glycoprotein and what a role it plays in the process of re-establishment of muscular and connective tissues of the healing colonic mucosa.

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Materials and methods

Six male rabbits weighing 2-3 kg were used for the present study. The animals were fasted for 24 hr before anesthetization by nembutal. The mucosae of the sigmoidal region (about 6 cm apart from the anal ring) were excised up to the tunica muscularis by a rectal biopsy forceps using a proctosigmoidscope. The intestinal walls of the wound region were isolated at 3, 5 and 7 days after operation (2 animals for each day) and utilized for both electron microscopy and immunocytochemistry.

For electron microscopy, specimens were fixed in a cacodylate buffered Karnovsky solution for

2 hr, postfixed in 1% osmium tetroxide in the same buffer, dehydrated in graded series of acetone, and embedded in epoxy resin. Semithin sections of approximately 1 μm thick were made on a Porter-Blum microtome and stained with 1% phosphate buffered toluidine blue. Ultrathin sections were obtained from the areas adjacent to those used for light microscopy, stained with uranyl acetate and lead citrate, and observed with a JEM 100 CX electron microscope.

For light microscope immunocytochemistry, specimens were fixed in a Bouin's solution for 24 hr at room temperature, dehydrated in graded concentrations of acetone, embedded in paraffin, and sectioned at approximately 4 μm thick. The immunocytochemical procedure was carried out using the biotin-streptavidin (B-SA) kits (Biogenex Laboratories, San Ramon, CA, U.S.A.). After deparaffinization, the sections were treated with 0.3% H_2O_2 in absolute methyl alcohol for 5 min to reduce the endogenous peroxidase activity, and immunoreacted to goat anti-rabbit fibronectin serum (Cappel, West Chester, PA, U.S.A.) at a dilution of 1:500 for 1 hr. The sections were rinsed in phosphate buffered saline (PBS), and reacted to both biotinylated rabbit anti-goat immunoglobulin (IgG) and peroxidase conjugated streptavidin for 20 min each. After rinsing in PBS, the sections were developed in a mixture of 0.05% diaminobenzidine hydrogen peroxidase and 0.01% H_2O_2 solution. The adjacent sections were stained with haematoxylin and eosin.

For immunoelectron microscopy, the B-SA technique was used. Specimens were fixed in a PLP solution (McLean and Nakane, 1974) for 24 hr at 4° C in 0.1M phosphate buffer containing 10% sucrose the overnight at 4° C, and embedded in OCT compound (Miles Laboratories, Naperill, ILL, U.S.A.). Frozen sections of approximately 10 μm thick were treated with 0.3% H_2O_2 in absolute methyl alcohol and immunoreacted to goat anti-rabbit fibronectin serum (Cappel) at a dilution of 1:500 for 1 hr. After rinsing in PBS, immunostaining using this antibody was carried out by a biotin streptavidin peroxidase technique, and after rinsing in PBS, colour was developed with a mixture of 0.05% diaminobenzidine hydrogen peroxidase and 0.01% H_2O_2 solution for 10-15 min. They were then postfixed in osmium tetroxide in 0.1M phosphate buffer for 10 min, dehydrated in graded concentrations of acetone, and embedded in epoxy resin. Ultrathin sections stained with uranyl acetate were observed in a JEM 2000 EX electron microscope.

The specificity of the immunoreactions was confirmed by substitution of normal goat serum or PBS for the first antisera.

Results

1) Light and electron microscopy of the wound healing

At 3 days after operation, the wound region was still occupied with fibrin clots and various kinds of inflammatorily exudative cells such as heterophilic leucocytes, lymphocytes and macrophages. However, the ulcer margin of the excised region was considerably replaced by loose connective tissue which contained abundant young fibroblasts which retained many features of mesenchymal cells and newly-formed blood vessels (Fig. 1). At 5 days, profiles of such young fibroblasts increased in number in the connective tissue. These fibroblasts were occasionally associated with the neocapillaries and basically identical in morphology to the basophilic young fibroblasts described by Mall (1896). The discontinuous layer of the tunica muscularis mucosa concomitant with these young fibroblasts was partially re-established in the healing colonic mucosa at 3 days (Fig. 1).

In the electron microscopic survey, these fibroblasts possess a large nucleus with prominent nucleoli in comparison with paucity of the cytoplasm which developed rough endoplasmic cisternae occasionally dilating their lumen and containing electron dense substance in the interior (Fig. 2).

Differentiating smooth muscle cells often existed in aggregations of the fibroblasts (Figs. 2, 3). These muscle cells were characterized by the presence of myofilament bundles in their cytoplasm and enclosed by the discontinuous basal lamina. Many intermediate forms between these two cell groups were found especially in regions of the tunica muscularis mucosa and tunica muscularis.

Histiocyte-like cells were often encountered in the healing colonic mucosa. These cells were characterized by presence of abundant thin cytoplasmic projections and considerable numbers of lysosomes including cell debris and elastin fragments. They were often in a close association to each other and enclosed large elastin fragments. In some cases, they formed multinuclear giant cells as shown in Fig. 4. Intermediate forms between the fibroblasts and the histiocyte-like cells also existed.

2) Immunocytochemistry

At 3 days after operation, light microscopic immunoreactions for anti-fibronectin were preferentially found on the exudative region and pre-existing connective and muscular tissue of the ulcer margin (Fig. 5). Between 5 and 7 days after operation, the wound region had been completely covered with the regenerating epithelial cell layer which developed crypts of Lieberkühn. Both tunica muscularis mucosa and tunica muscularis were remarkably regenerating and abundant fibroblasts existed in the regenerating muscular tissues (Fig. 6). The light microscopic immunoreaction at this stage was preferentially found on the tunica muscularis mucosa and tunica muscularis (Fig. 7), while the adjacent control section stained by normal goat serum for the first antibody did not show any specific reactions (Fig. 8). The epithelial layer also showed a slight immunoreaction.

By immunoelectron microscopy, the immunoreaction of the young fibroblast cells was localized preferentially

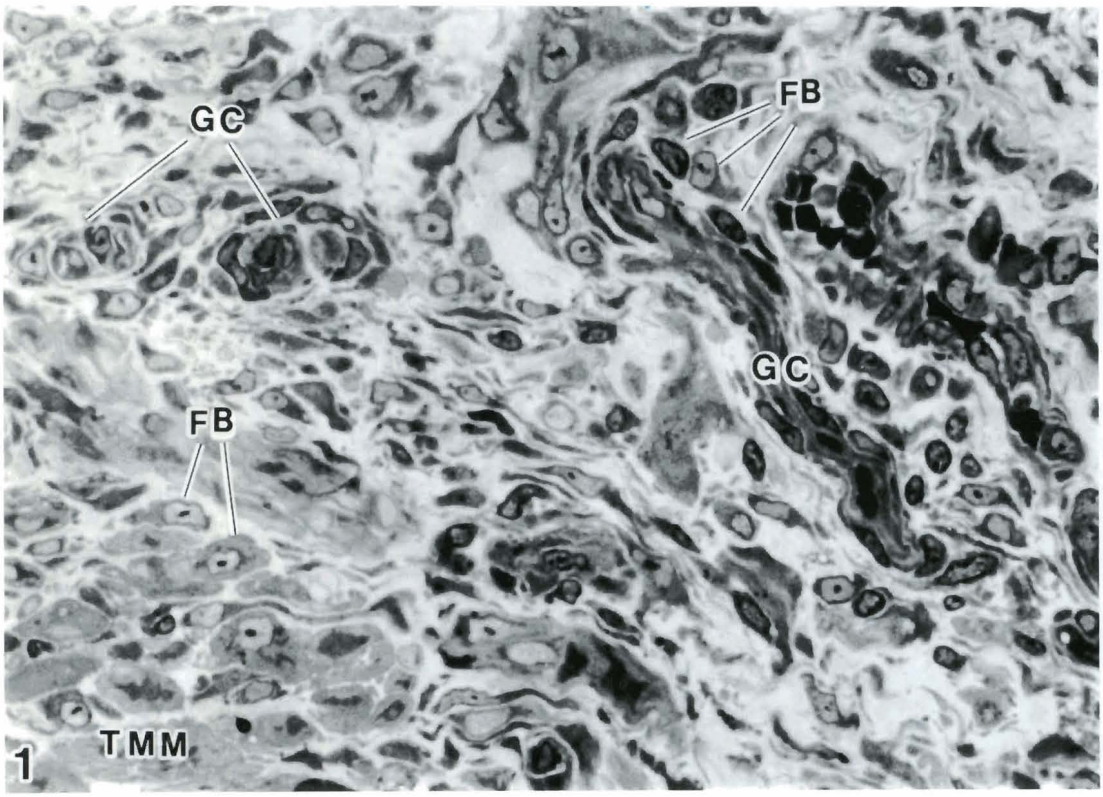


Fig. 1. Abundant young fibroblasts (FB) aggregate to loose connective tissue of the healing colonic mucosa and are occasionally associated with the growing capillaries (GC). Abundant young fibroblasts are found in the regenerating tunica muscularis mucosa (TMM). At 3 days. x 500

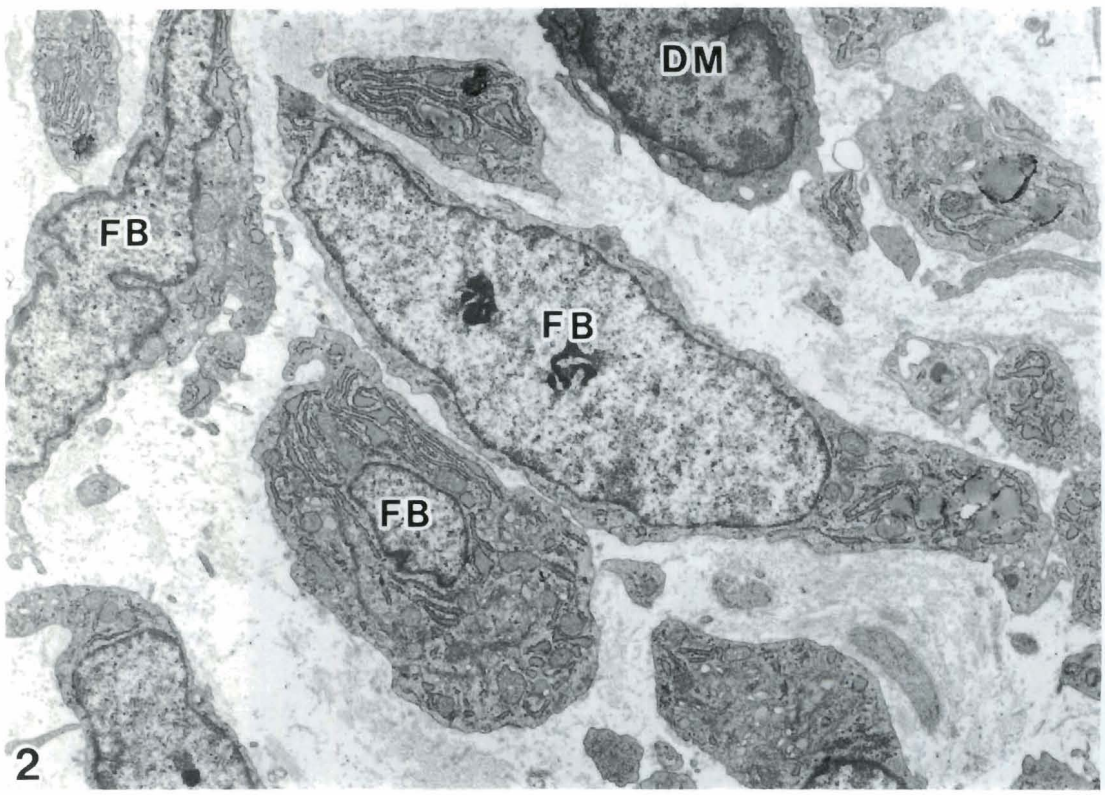


Fig. 2. Young fibroblasts (FB) develop rough endoplasmic cisternae often containing electron dense substance. A differentiating muscle cell (DM) is seen in the aggregation of the fibroblasts. At 5 days. x 4,000

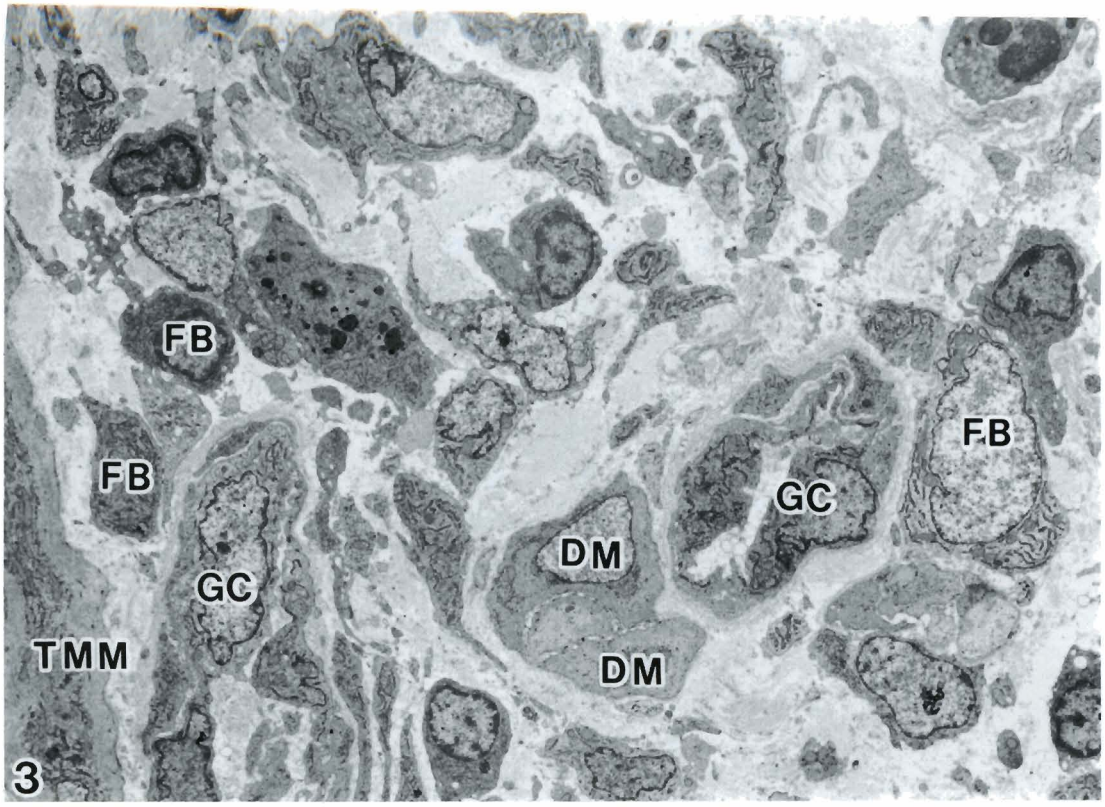


Fig. 3. In the loose connective tissue near the regenerating tunica muscularis mucosa (TMM), endothelial cells of the growing capillaries (GC) are rather round in shape and are associated with abundant young fibroblasts (FB). Differentiating muscle cells (DM) are seen among these young fibroblasts. At 5 days. x 2,500

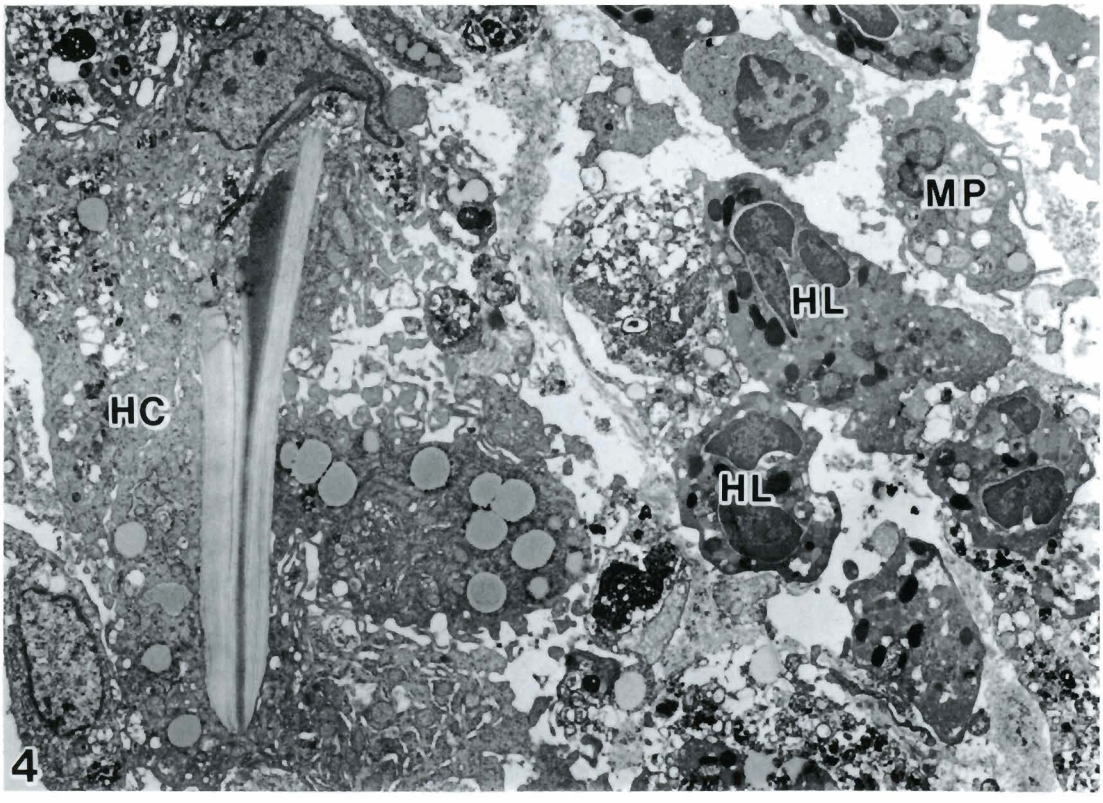
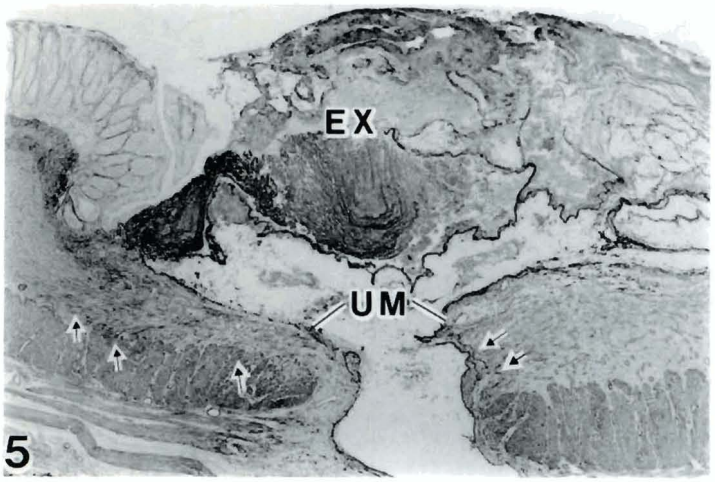
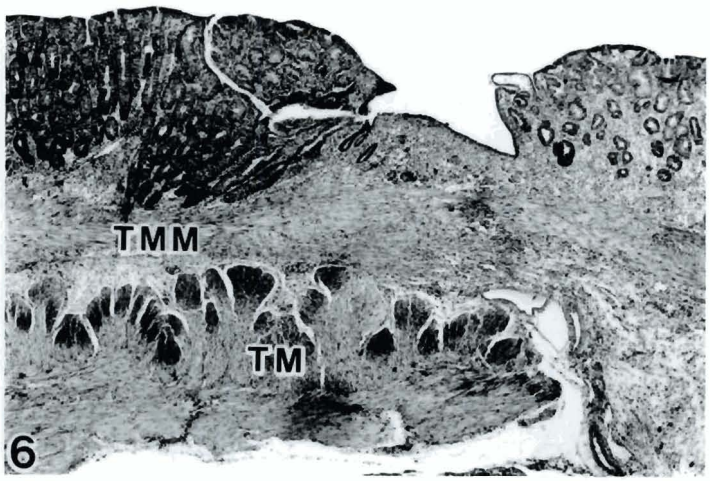
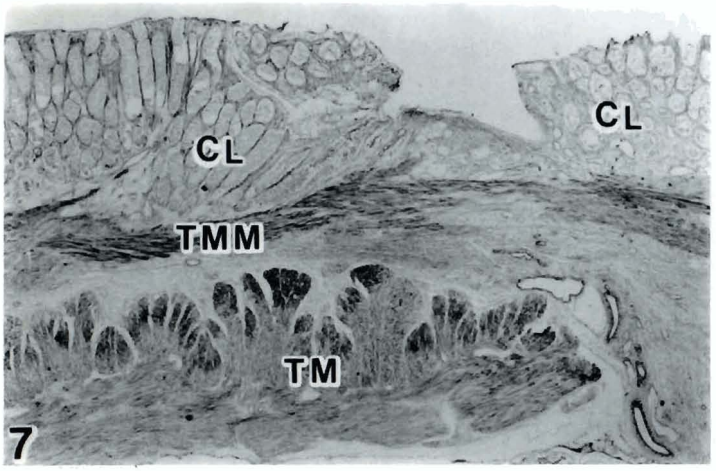


Fig. 4. A histiocyte-like cell (HC) forming a multinuclear cell and enclosing elastin fragments exists in the ulcer margin. HL: Heterophilic leucocytes. MP: Macrophage. At 3 days. x 4,000







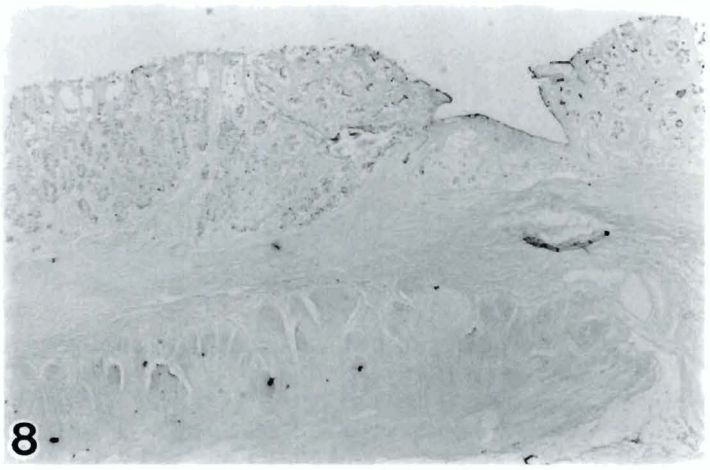


Fig. 5. Immunoreactions for anti-fibronectin are observed in the exudative region (EX) of the ulcer margin (UM). The pre-existing connective and muscular tissues of the ulcer margin are also positively stained (arrows). At 3 days. x 50

Fig. 6. Epithelium of the healing colonic mucosa is almost completely regenerated. Abundant young fibroblasts aggregate in both regenerating tunica muscularis mucosa (TMM) and tunica muscularis (TM). Stained with haematoxylin-eosin. At 5 days. x 50

Fig. 7. Immunoreactions for anti-fibronectin in the healing colonic mucosa at 5 days. The reactions are preferentially located in the basement membrane of crypts of Lieberkühn (CL), and tunica muscularis mucosa (TMM) and tunica muscularis (TM). x 50

Fig. 8. A control staining using normal goat serum in an adjacent section of that shown in Fig. 7. x 50

in their rough endoplasmic cisternae and the extracellular fibrillar matrix attached to the outer surface of the plasma membrane (Fig. 9). These cells were occasionally in contact with each other and extended long cytoplasmic projections to the neighbouring endothelium of the growing capillaries (Fig. 10).

Growing capillaries in the healing tissue were often enclosed by a slit-like lumen and were associated with abundant young fibroblasts. The endothelial cells were conjugated to each other by simple attachment devices and occasionally revealed immunoreaction in rough endoplasmic cisternae (Fig. 10). The young fibroblasts surrounding the growing capillaries occasionally extended the cytoplasmic projection to the endothelial cells (Fig. 10). The contact area between the fibroblast and the endothelial cell showed an intense immunoreaction (Fig. 10).

Discussion

Our light microscopy of young fibroblasts which become aggregate to the healing colonic mucosae after 3 days is basically the same as that of «basophilic spindle cells» first described by Mall (1896). The mitotic proliferation of these fibroblasts is very frequent. By electron microscopy, the transformation of such fibroblasts both to myogenic cells and histiocyte-like ones is apparent because of the existence of their intermediate forms. The young fibroblasts also exist near the pre-existing muscle layer and actively differentiate to smooth muscle cells in quite the same ultrastructural manner as the histogenesis of smooth muscle cells as

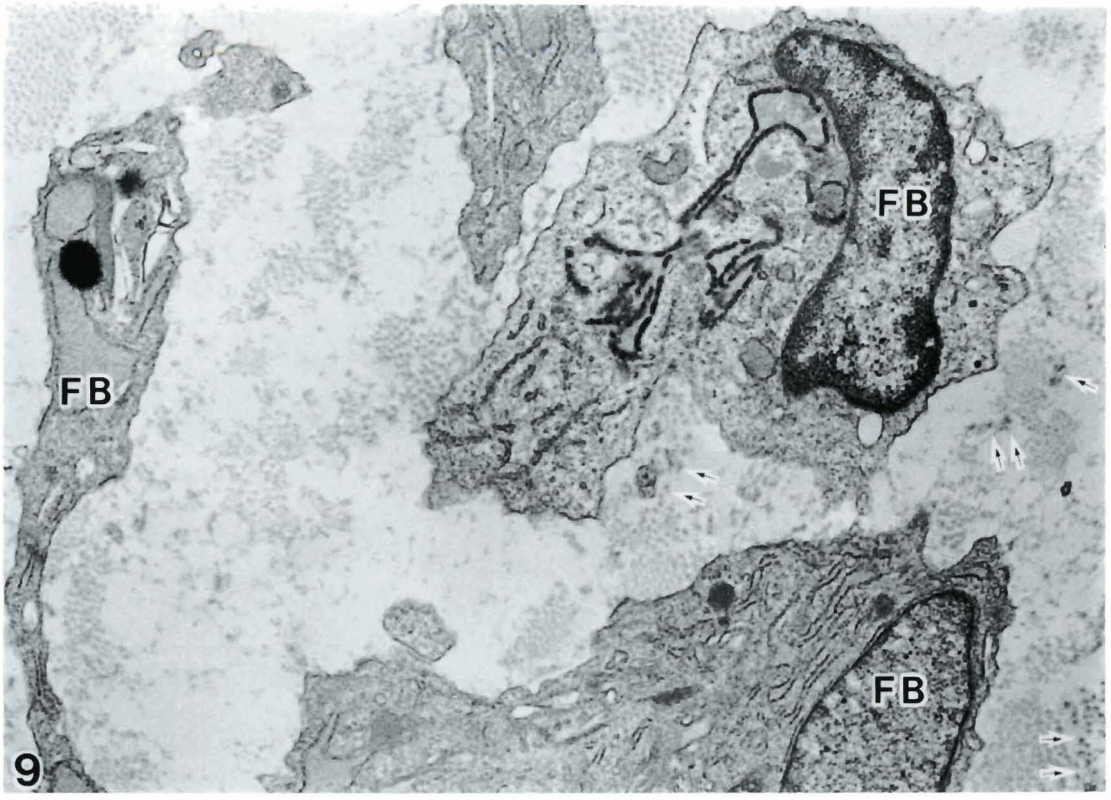


Fig. 9. Rough endoplasmic cisternae of young fibroblasts (FB) are reacted to anti-fibronectin. Collagenous fibrils around the cell surface (arrows) show a slight positive reaction. At 5 days. Unstained. x 12,000

extensively observed by Yamauchi and Burnstock (1969) and Campbell et al. (1971).

According to the previous workers (Zetterlund, 1967; McGeachie, 1971; Jurukova and Atanassova, 1974), the mitotic proliferation of smooth muscle cells in areas adjacent to injury foci and their migration to the reparative region is the major reason for regeneration of the muscular tissue. However, the present observations reveal neither mitotic proliferation of highly differentiated smooth muscle cells nor their de-differentiation, as reported by Jurukova and Atanassova (1974) in the pre-existing muscular tissue. On the contrary, our observations rather indicate that these fibroblasts migrate to the healing colonic mucosa in association with the growing capillaries from the intact tunica submucosa and actively transform to new smooth muscle cells. These findings argue for the previous reports that the true muscle cell proliferation must play a negligible role in the muscular tissue of the reconstitution of the visceral wall (Ross et al., 1969).

The histiocyte-like cells also appear to be originated from the young fibroblasts. Their monocytic origin is unlikely because of a much lower number of lysosomes in their cytoplasm when compared to those in monocyte-derived histiocytes. The multinuclear cells formed by fusions of these histiocyte-like cells may be involved in the enclosure of large tissue debris such as elastin fragments. Recently, Fujita et al. (1988) reported a role of the corneal fibroblasts in endocytotic activity of small foreign bodies. Thus, the appearance of phagocytotic cells from the fibroblasts in the healing colonic mucosa may argue for the concept that certain fibroblasts may be involved in non-inflammatory defence mechanism.

In the present immunocytochemistry, an intense reaction for anti-fibronectin in the exudate region at 3 days may represent a chemoattractant role of this glycoprotein in inflammatory cells such as neutrophils, lymphocytes and monocytes as previously described (Sobel and Gallin, 1979; Bevilacqua et al., 1981; Norris et al., 1982; Yonemasu et al., 1983).

The present study clearly reveals that the young fibroblasts actively produce fibronectin in their rough endoplasmic cisternae. These ultrastructural findings are basically consistent with the previous immunoelectron microscopy of the cultured fibroblasts (Hedman, 1980; Yamada et al., 1980; Fromme et al., 1982) and mesenchymal cells (Brownell et al., 1981). Since these fibroblasts associated with the neocapillaries have an ability of multipotent cells in the re-establishment of the connective and muscular tissues in the healing colonic mucosa, it seems reasonable to consider that fibronectin released from these cells may have a chemotactic function for promotion of their migration to the wound bed as suggested by Gauss-Müller et al. (1980) and Postlethwaite et al. (1981), and for the re-organization of the matrix by co-deposition with collagenous fibrils as suggested by Bornstein and Ash (1977) and Codogno et al. (1987). In addition, although entirely in a speculative way, a role of this glycoprotein in developmental instructions from fibroblasts to smooth muscle cells might be proposed.

Our histiocyte-like cells are confirmed to be derived

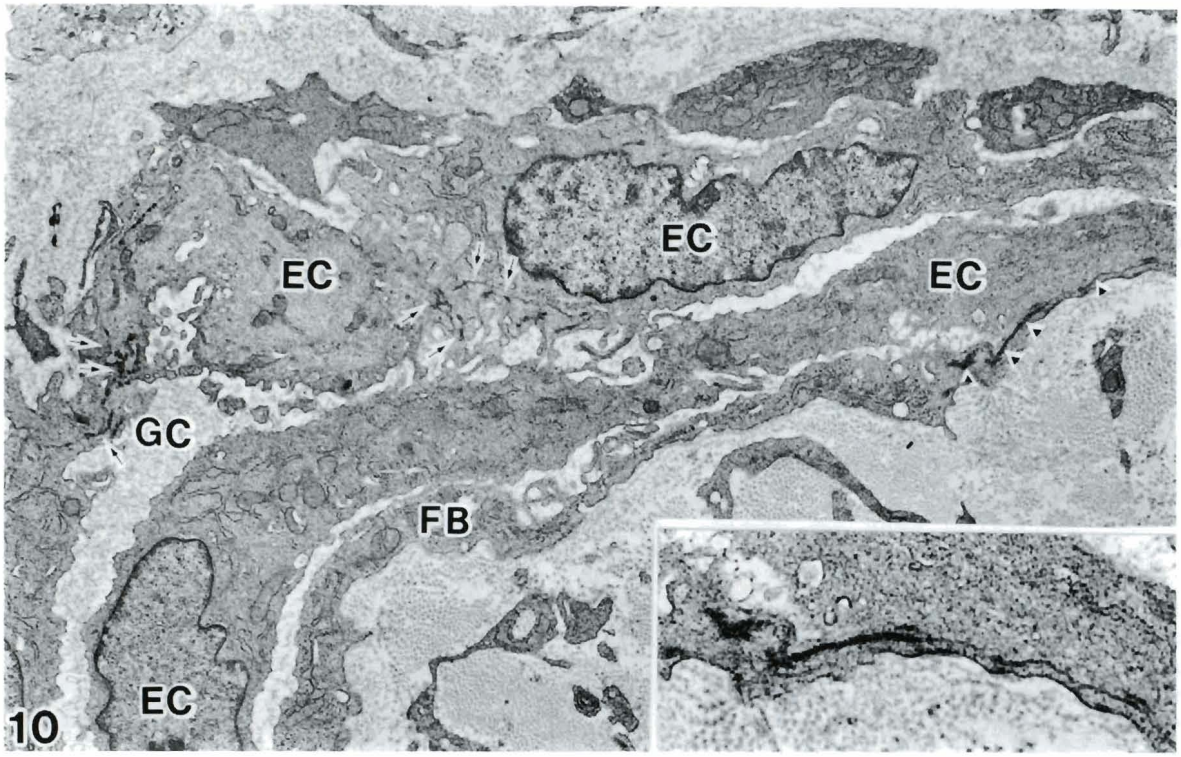


Fig. 10. Immunoreactions for anti-fibronectin (arrows) are localized in rough endoplasmic cisternae of an endothelial cell (EC) of the growing capillary (GC). Young fibroblasts (FB) associated with the growing capillary extend the cytoplasmic projection to the endothelium. The contact area (arrowheads) shows an intense immunoreaction (insert). At 5 days. Unstained. x 5,000. Insert, x 15,000

from the young fibroblasts. They also show the immunoreaction in their rough endoplasmic cisternae. The production of fibronectin in monocytes and monocyte-derived macrophages has been described by several workers (Alitalo et al., 1980; Bevilacqua et al., 1981; Tsukamoto et al., 1981; Norris et al., 1982; Yonemasu et al., 1983). They suggested roles of nonspecific opsonin, promotion of macrophage migration, and chemoattractant for fibroblasts. Since our histocyte-like cells occasionally formed the multinuclear cells enclosing cell debrises such as elastin fragments, fibronectin may act in vivo as promotion of the cell migration and adhesion in the healing conolic mucosa.

The transformation of fibroblasts to vessel-forming cells has already been cited in embryonic (González-Crussi, 1971; Fujimoto et al., 1987) and tumor (Hammersen et al., 1985) tissues. In the present observation, a close association of the young fibroblasts with endothelial cells near tips of the growing capillaries through each cytoplasmic projection was frequent as in the case of the pre- and postnatal rabbit corpora cavernosa penis (Fujimoto et al., 1987). Thus, we consider that abundant young fibroblasts which aggregate to the healing colonic mucosae between 3 and 7 days are a kind of multipotent fibroblasts involved not only in the supply of new smooth muscle cells but also in the phagocytotic activity as histiocyte-like cells and in the acceleration of neocapillarization as vasoformative cells in the regenerating colonic mucosa.

It is interesting that the vasoformative fibroblasts are densely enclosed by fibrillar strands which show an intense immunoreaction. A role of fibronectin in evoking the endothelial cell chemotaxis has been proposed by Bowersox and Sorgente (1982). Clark et al. (1982a,c) and Wakui et al. (1990) described that fibronectin increases capillary ingrowth during the wound healing. Taking all this into consideration, fibronectin may be involved in a mechanical linkage between endothelial cells and vasoformative fibroblasts. Since our immunoelectron micrographs reveal that endothelial cells in the neocapillaries actively produce fibronectin in their rough endoplasmic cisternae, a role of this glycoprotein in the acceleration of the neocapillarization is necessary for further investigation.

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