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Electron and immunoelectron microscopy on healing process of the rat anterior cruciate ligament after partial transection: the roles of multipotent fibroblasts in the synovial tissue

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Summary. The healing process of the rat anterior cruciate ligament (ACL) after partial transection was examined. In sham operated samples, the synovial tissue near the infrapatellar fat pad includes an abundance of young fibroblasts which can be classified into two types: A type cells often exist along the surface of the synovial tissue and contain numerous lysosomes, while B type cells are often associated with small vessels and are actively involved in the production of fibronectin and laminin. At 1 week after transection of the ACL, B type cells frequently undergo mitotic proliferations and are eventually incorporated into the endothelium of the growing capillaries extending from the proximal remnants of the synovial tissue to the transected lesion. The transformation of B to A type cells is indicated by our electron micrographs. After 2 weeks, the replacement of the transected lesion by regenerated soft tissue becomes pronounced. After 4 weeks, B type cells in the deeper layer of the regenerated tissue are first involved in the production of type III and then in that of type I collagens as revealed by the immunocytochemistry. The present study indicates that B type cells are a kind of stem cell: they possess the ability to transform to vasoformative cells involved in a manner suggestive of vasculogenesis, to phagocytic A type cells and to the synthetic fibroblasts in the regeneration of the ACL.

Key words: ACL, Fibronectin, Healing process, Immunocytochemistry, Synovial tissue

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Introduction

The spontaneous healing process of the knee ligaments including the anterior cruciate ligament (ACL) after incomplete transection has been histologically investigated in various experimental animals (O'Donoghue et al., 1966, 1971; Bohr, 1976; Arnoczky et al., 1979; Potenza and Herte, 1982; Frank et al., 1985; Hefti et al., 1991). In these reports, the healing process at first includes inflammatory cell infiltration in the lesion, subsequent aggregation of fibroblasts to the lesion, and synthesis of collagens by these cells. In some cases, the regenerated ligament can be said to become histologically, biomechanically and biochemically normal (Arnoczky et al., 1979; Hefti et al., 1991). Since these authors have estimated extrinsic roles of the synovial tissue which induces an enhancement of vascular responses in the wound region, more detailed histological investigations including electron and immunoelectron microscopy are necessary to elucidate the synovial tissue properties involved in the spontaneous healing processes. However, descriptions as to the histology of the synovial tissue are mainly confined to the light microscopic studies (Kennedy et al., 1974; Arnoczky, 1983; Butler et al., 1985; Strocchi et al., 1992), except for a scanning electron microscopic study by Danylchuck et al. (1978).

«Vasculogenesis» is a neovascularization manner by which endothelial precursor cells are successively incorporated into the pre-existing capillaries and predominantly occurs in the morphogenesis of early embryonic vasculatures, while «angiogenesis» is another neovascularization manner which includes mitotic proliferations of endothelial cells of the pre-existing capillaries and their migration to the vascular tips forming so-called «endothelial buds» (Wagner, 1980;

Díaz-Flores et al., 1994). The extension of newly-formed capillaries in a manner suggestive of vasculogenesis in various wound healing processes has been reported in the local x-ray irradiation (Grillo, 1963), in the experimental excision of the small intestinal mucosa (Mori et al., 1989, 1992), in the chronic colitis (Hirata et al., 1992) and in the spontaneous traumatic lesion of the ligament flavum (Hijioka et al., 1994). Especially, Mori et al. (1989, 1992) proposed an active involvement of «multipotent young fibroblasts» in the wound healing process: such cells aggregate to the wound region in association with growing capillaries and transform to vasoformative cells that are involved in the formation of new vessels, or to histiocyte-like cells involved in phagocytic activities.

It is now widely accepted that fibronectin (FN) and laminin (LN) play crucial roles in cell to cell and in cell to matrix interactions. The role of FN in evoking the endothelial cell chemotaxis was first proposed by Bowersox and Sorgente (1982). Clark et al. (1982), Wakui et al. (1990), and Mori et al. (1992) described that FN increases the capillary ingrowth during the wound healing.

The present study was designed on the basis of these backgrounds. The main purpose of this study is to throw ultrastructural and immunocytochemical light on the roles of fibroblasts in the synovial tissue during the healing process of the rat ACL after partial transection.

Materials and methods

The present experiment was carried out on 50 male Wistar rats, each weighing 420 to 480 g, at 12 weeks after birth. They were housed (4 animals per each cage in an air conditioned room) and supplied with rat cubes and water ad-libitum throughout the experiment.

The partial transection of the ACL, approximately one-thirds of the mid-substance, was done at the anteromedial aspect of the mid-portion in one limb of each rodent under general anesthesia by an inhalation of ether. The location and direction of the transection was almost the same in all animals. Each contralateral limb was used for a sham operation in a similar excision and exposure manner to that used for partial transection in the paired control. The wound was closed by catgut sutures in both facial layer and skin in all experimental animals.

The postoperated limbs were not immobilized and allowed to bear weight as tolerated. The ligaments were isolated from both limbs at 1 (10 animals), 2 (10), 3 (10), 4 (10), and 6 weeks (10) after operation.

Light microscopy

Specimens were fixed in 4% paraformaldehyde in 0.1M phosphate buffered-saline (PBS) at room temperature for 24 hrs, dehydrated in graded series of ethanol, and embedded in paraffin. Sections of approximately 10 µm in thickness were stained with hematoxylin and eosin. Some sections were utilized for the light microscopic immunocytochemistry.

Electron microscopy

Specimens were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS at room temperature for 2 hrs, postfixed in 2% osmium tetroxide in 0.1M PBS, dehydrated in graded series of acetone, and embedded in epoxy resin. Semithin sections of approximately 1 µm in thickness were used for light microscopy after staining with toluidine blue. Ultrathin sections were examined in a JEM 1200 EX electron microscope.

Light microscopic immunocytochemistry

Paraffin-embedded sections of approximately 3 µm in thickness were deparaffinized, digested with 0.4% pepsin in a mixture of 0.01N HCl and 0.1M PBS for 2 hrs at 37 °C, and treated with 0.3% H_2O_2 in absolute methyl alcohol for 20 min to reduce endogenous peroxidase activities. After non-specific bindings were blocked with egg albumin in 0.1M PBS for 5 min, sections were reacted to rabbit anti-bovine FN (LSL, Japan), rabbit anti-rat LN, rabbit anti-rat type I collagen and rabbit anti-rat type III collagen (Chemicon, Temecula, Ca, USA) at a dilution of 1:100 in 0.1M PBS for 1 hr at room temperature, respectively. Sections that were rinsed in 0.1M PBS were reacted to both biotinylated goat anti-rabbit IgG and peroxidase conjugated streptavidin for 40 min each (BSA method), and were developed in a mixture of 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2.

Immunoelectron microscopy

Specimens were fixed in a solution of periodate-lysine-paraformaldehyde (McLean and Nakane, 1974) in 0.1M PBS containing 10% sucrose for 12 hrs at room temperature. Approximately 20 µm-thick frozen sections were made on a cryocut (Microm, Heidelberg, Germany), treated with 0.3% H_2O_2 in absolute methyl alcohol, and reacted to the above-mentioned antibodies at a dilution of 1:100 to 1:200 in 0.1M PBS for 1 hr at room temperature. After rinsing in 0.1M PBS, immunostaining was carried out using the BSA method. Sections were postfixed in 0.1% osmium tetroxide in 0.1M PBS for 5 min, dehydrated in graded concentrations of acetone, embedded in epoxy resin, cut into ultrathin sections, and examined in the electron microscope without staining.

Controls

The specificity for the immunoreactions was confirmed by substituting the normal rabbit sera for the primary antisera.

Results

1. Histology and immunocytochemistry of sham operated ACL

No intraarticular effusion nor vascularized inflammatory cell infiltration were observed in the joint throughout the stages that were examined. The anterior aspect of the ACL, which extends from the posterior inlet of the intercondylar notch of the femur to the intercondylar eminence of the tibia, was completely covered with a fold of the synovial tissue enclosing the infrapatellar fat pad near the tibial insertion (Fig. 1). An abundance of fibroblasts with a large nucleus in comparison with the paucity of the cytoplasm, basically identical in morphology to basophilic young fibroblasts first described by Mall (1896), existed around small vessels derived from the middle genicular artery.

By electron microscopy, these fibroblasts could be divided broadly into two cell types as previously described by Barland et al. (1962), Linck and Porte (1978) and Okada et al. (1981), although there existed many intermediate forms: A type cell (phagocytic one) that was smaller and slenderer in size and shape, had numerous cytoplasmic projections which were connected to those of the adjacent cells, occasionally contained lysosomes and existed along the surface of the synovial tissue in one or more rows, while B type cell (fibroblast-like one) was larger in size, possessed less numerous cytoplasmic projections, developed the rough endoplasmic reticulum (r-ER)-Golgi system and occasionally existed near small vessels (Figs. 2, 3). B type cells showed immunoreactions of FN and LN in the dilated rER (Figs. 4a,b).

The posterior aspect of the ACL, especially at the portion that crosses with the posterior cruciate ligament, was ensheathed by the thin synovial tissue which possessed few fibroblasts and lacked small vessels.

2. Histology and immunocytochemistry of partially transected ACL

A) Regeneration of synovial tissue

At 1 week after operation, the transected lesion in 7 of 10 operated animals was already covered in part with the regenerated loose connective tissue similar in morphology to the synovial tissue near the infrapatellar fat pad (Fig. 5). These regenerated tissues included an abundance of small vessels occasionally associated with B type cells. The surface of the regenerated tissue was covered in part with a layer of A type cells (Fig. 5). However, the other 3 experimental animals did not show such a regeneration in the lesion.

In the regenerated tissue, the mitotic proliferation of B type cells (Fig. 6a) and the transformation of B to A type cells possessing numerous lysosomes and cytoplasmic projections (Fig. 6b) were indicated. Growing capillaries in the regenerated tissue were occasionally enclosed by round endothelial cells, similar in morphology to B type cells around the vessels (Fig. 6c). By electron microscopy, the close association of cytoplasmic projections of B type cells with the endothelium of the capillaries was confirmed: B type

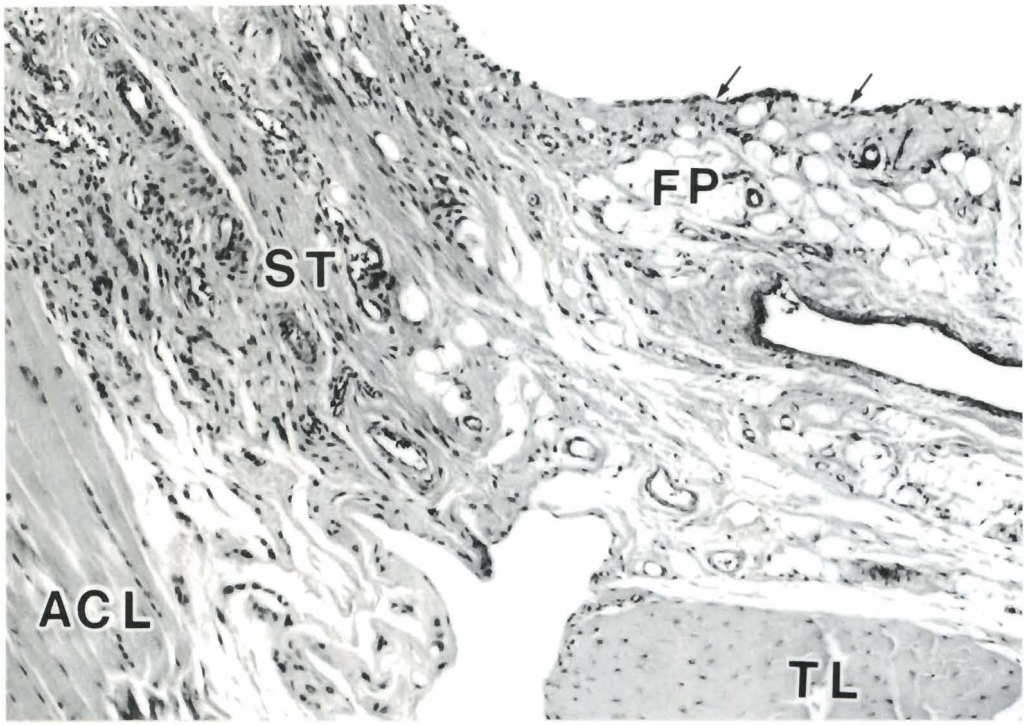


Fig. 1. The synovial tissue (ST) near the tibial insertion at 1 week after sham operation. The synovial tissue is richly endowed with microvessels associated with abundant B type cells. The surface of the synovial tissue is lined with one or more layers of A type cells (arrows). ACL: anterior cruciate ligament.
FP: infrapatellar fat pad.
TL: transverse ligament. x 130

cells extended their cytoplasmic projections to the endothelium (Fig. 7a) which are eventually conjugated with the neighboring endothelial cells by simple attachment devices. Such contact areas between a B type cell and an endothelial one showed intense immunoreactions of FN (Fig. 7b). Some B type cells near the capillaries possessed numerous small cytoplasmic vesicles similar in morphology to pinocytotic vesicles along the cell surface and were enclosed by the well-developed basal lamina in continuation with that of the

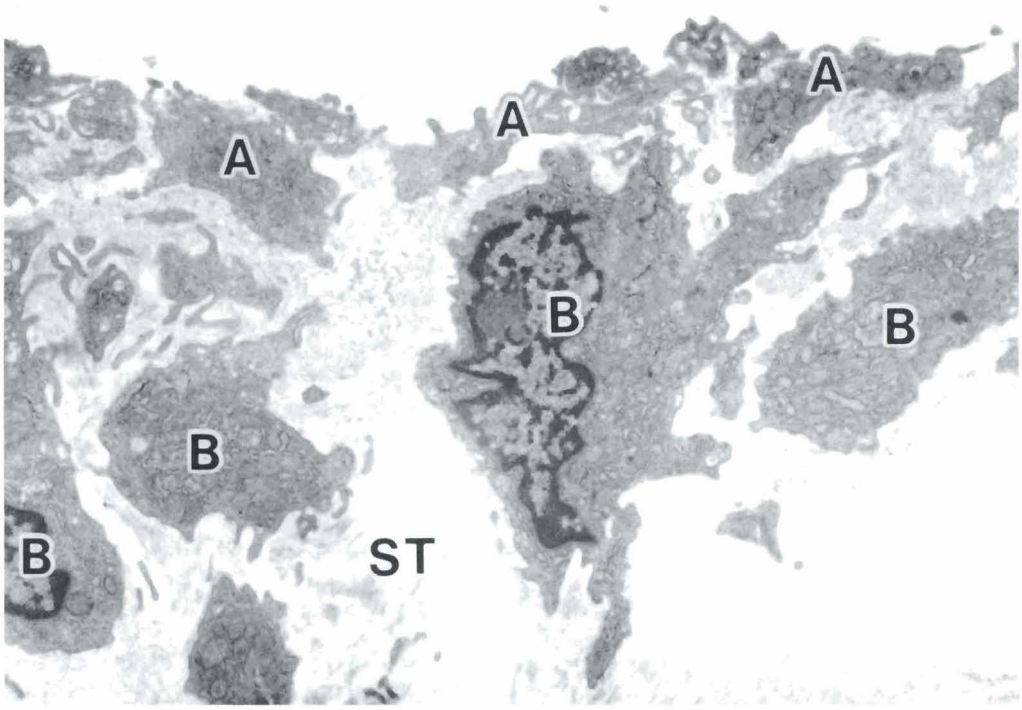


Fig. 2. Electron micrograph of A and B type cells in the synovial tissue (ST) at 1 week after sham operation. x 8,000

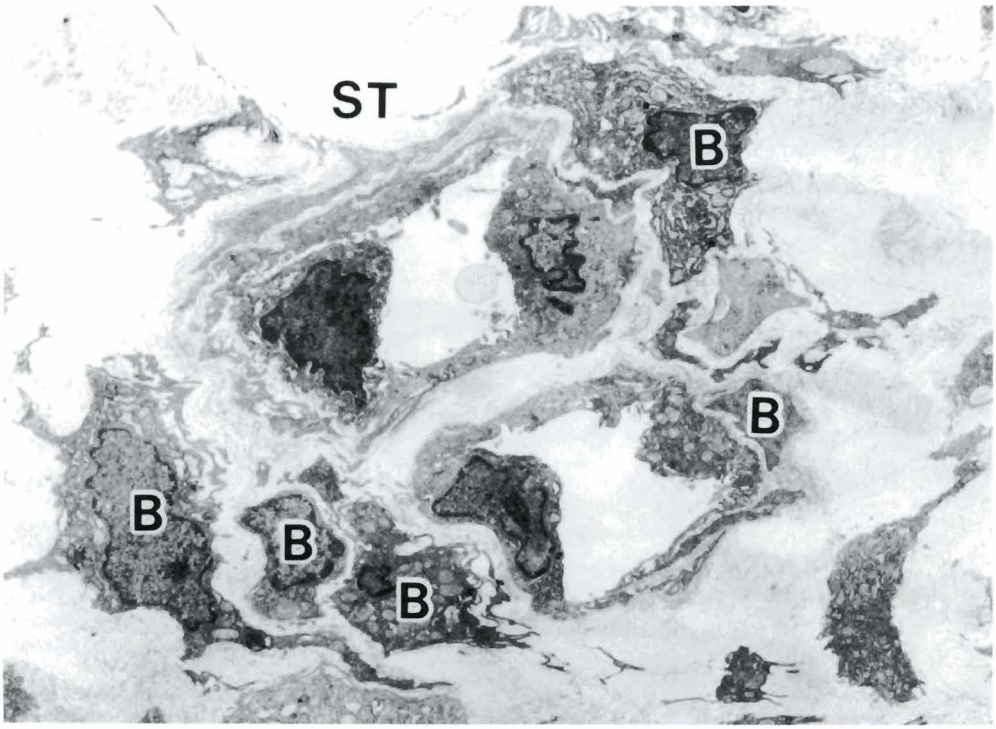


Fig. 3. Electron micrograph of capillary profiles associated with abundant B type cells in the synovial tissue (ST) at 1 week after sham operation. x 4,000

pre-existing capillaries (Fig. 7c). A type cells which exist along the surface of the wound margin as shown in Fig. 5, often increased in the number of secondary lysosomes containing a variety of cell debris (Fig. 8).

At 4 weeks after operation, the replacement of the transected lesion by the regenerated loose connective tissue became more pronounced in 8 of the 10 experimental animals. The regenerated tissue was filled with an abundance of B type cells, rich in capillaries, and were almost completely covered with a layer of A type cells (Fig. 9). By immunoelectron microscopy, the rER and the cell surface of B type cells showed intense immunoreactions of FN (Fig. 10). Since the antibody did not completely penetrate through 20 µm-thick sections mounted on the slide glass, the rER in the center of the cytoplasm often showed little or no immunoreactivities.

B) Regeneration of ACL

Between 2 and 4 weeks after operation, the regeneration of the ACL began to appear in the deeper layer of the regenerated tissue covering the transected lesion (Fig. 9). This process was first evidenced by the expression of type III collagen in the extracellular matrix (ECM) components by immunocytochemistry (Fig. 11a). We found little or no immunoreactivity in the ECM for type I collagen in the adjacent sections during this period (Fig. 11b).

Between 4 and 6 weeks after operation, the regeneration of the ACL in the healing tissue became pronounced: the regenerated tissue richly endowed with capillaries and basically identical with the synovial tissue, and the regenerated ACL partially consisting of collagen bundles were clearly distinguished (Fig. 12). Immunoreactions of both type III (Fig. 13a) and type I

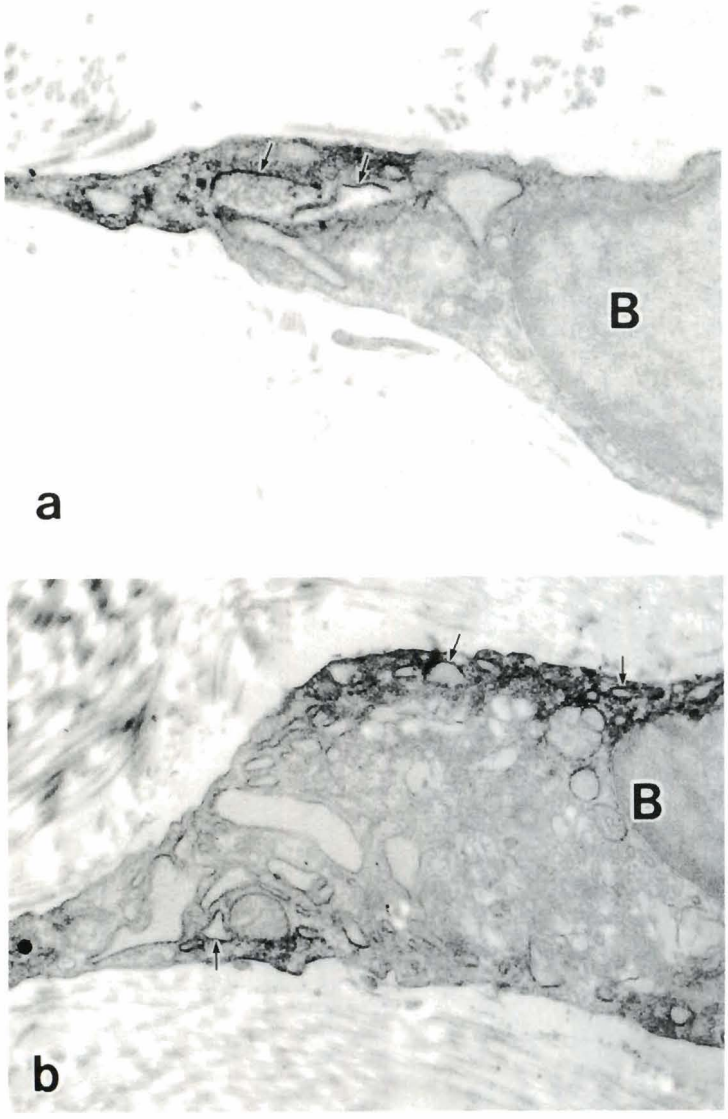


Fig. 4. Immunoreactions of FN (**a**) and LN (**b**) in the rER (arrows). Uncounterstained. x 24,000

(Fig. 13b) collagens were seen in the regenerated ACL at 6 weeks after operation.

Discussion

Nonoperative, conservative treatments of ACL injuries were apt to result in the restriction of the joint motions (Hart, 1987; Inoue et al., 1987; Indelicato et al., 1990; Lechner and Dahners, 1991), whereas several previous workers obtained favorable results in patients (McDaniel and Dameron, 1980; Giove et al., 1983; Jokl et al., 1984; Kannus and Järvinen, 1987; Sandberg et al., 1987) as well as in experimental animals under favorable conditions (O'Donoghue et al., 1966, 1971; Hefti et al., 1991). Hefti et al. (1991) described that some rabbit ACLs return to normal in stiffness as well as in histology at 3 months after partial transection. In such a healing process, extrinsic roles of the synovial tissue mainly due to the enhancement of vascular responses have been indicated (Arnoczky et al., 1979; Hefti et al., 1991).

The microvasculature of ACL has been investigated by tissue-clearing and microangiographic techniques combined with histological investigations (Alm and Strömberg, 1974; Clancy et al., 1981; Arnoczky et al., 1982; Jackson et al., 1991). The soft tissue including the infrapatellar fat pad of the knee joint are richly endowed with microvessels mainly derived from the middle genicular artery which aborizes to form a weblike network of the periligamentous vessels throughout the entire length of ACL.

In 7 of our 10 experimental animals, the transected lesion was covered with loose connective tissue which is continuous with the pre-existing synovial tissue at 1 week, and such a regenerative process became more pronounced in 8 of the 10 experimental animals at 4 weeks after operation. In such samples, the transected lesion was considerably replaced by loose connective tissue, which is similar in morphology to the adjacent synovial tissue near the infrapatellar fat pad and was bulged into the wound gap in the healing samples. The regenerated tissue included an abundance of B type cells occasionally undergoing mitotic proliferations and growing capillaries extending from the proximal remnants of the synovial tissue. The present observation indicates that the growing capillaries are always associated with an abundance of B type cells. We found basically no inconsistencies in the ultrastructure of these B type cells aggregating to the transected lesion with fibroblastic cells in the healing process of the intestinal mucosae (Mori et al., 1989, 1992; Hirata et al., 1992).

The crucial roles of these B type cells were indicated from the present observation. At first, they transform to vasoformative cells and are successively incorporated into the endothelium of the growing capillaries. This may be evidenced by our findings that these cells often extend the cytoplasmic projections to the endothelium of which endothelial cells are occasionally similar in morphology to the surrounding B type cells. Vasculogenesis is defined as the differentiation of mesenchymal cells into endothelial precursor cells in the morphogenesis of the early embryonic vasculature such as the dorsal aorta and the posterior cardinal vein (Wagner, 1980). Processes in a manner suggestive of vasculogenesis have been already cited in embryonic

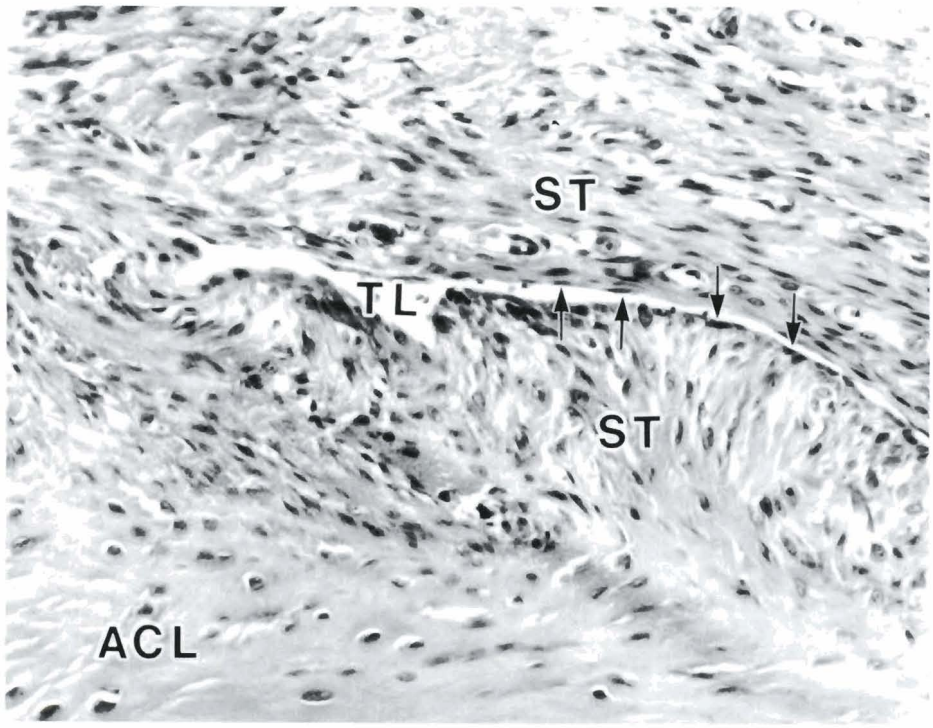


Fig. 5. The transected lesion (TL) of anterior cruciate ligament (ACL) is covered with regenerated soft tissue (ST), including abundant B type cells at 1 week after transection. The surface of the regenerated tissue is lined in part with A type cells (arrows). x 300

(Gonzalez-Crussi, 1971; Fujimoto et al., 1987; Hara et al., 1994), tumor (Hammersen et al., 1985) and wound healing (Mori et al., 1989, 1992; Hirata et al., 1992) tissues. In the present immunocytochemistry, B type cells actively produced several glycoproteins such as FN and LN in their rER and released them as components of

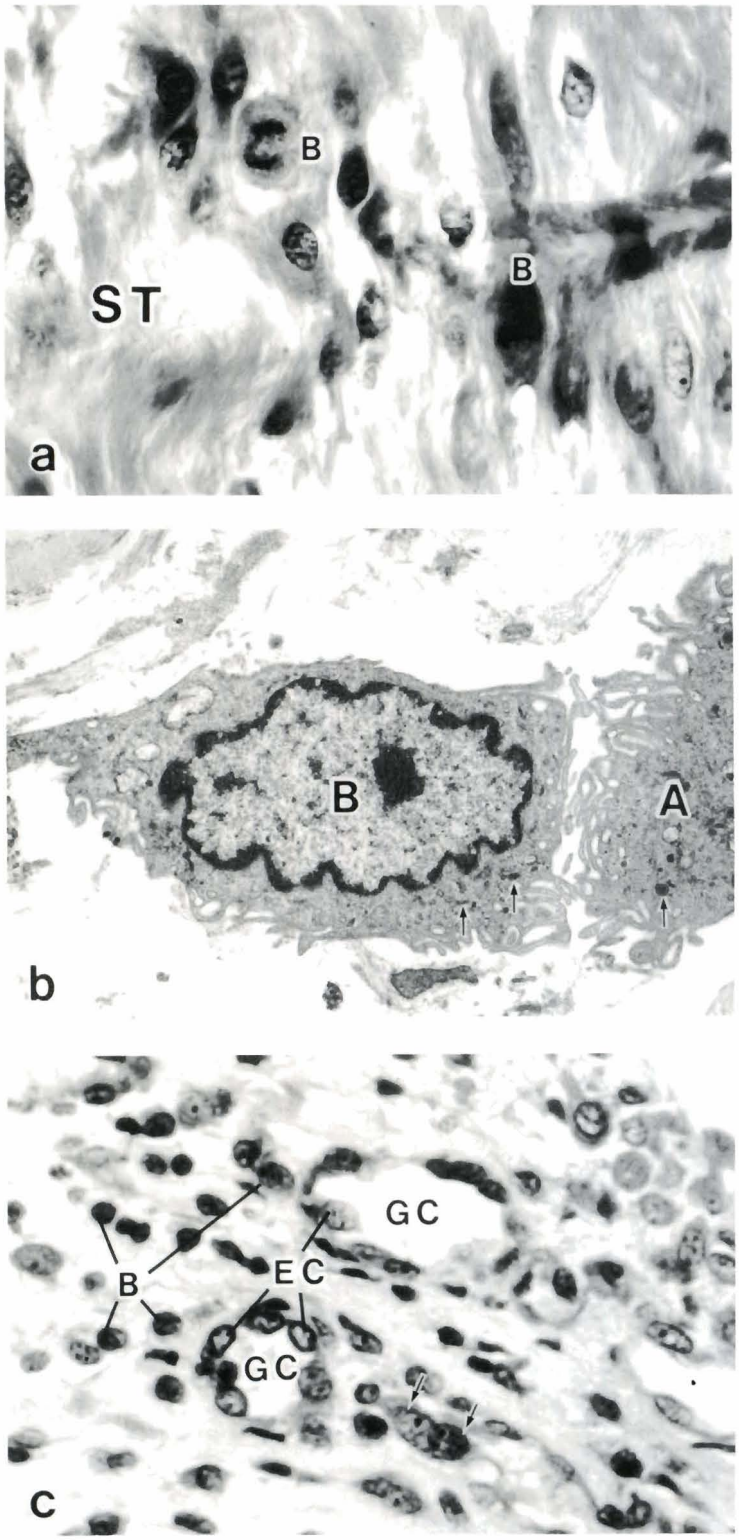


Fig. 6. a. Mitotic figures of B type cells in the synovial tissue (ST) adjacent to the transected lesion at 1 week after transection. **b.** Two kinds of B type cells at 1 week after transection. The one (B) increases in number of cytoplasmic projections and membrane-bound granules like lysosomes (arrows), and the other (A) possesses more numerous cytoplasmic projections and granules. **c.** Endothelial cells (EC) of growing capillaries (GC) in regenerated soft tissue are similar in features to the surrounding B type cells at 1 week after transection. A solid cell cord consisting of B type cells is seen (arrows). a, x 800; b, x 7,000; c, x 500

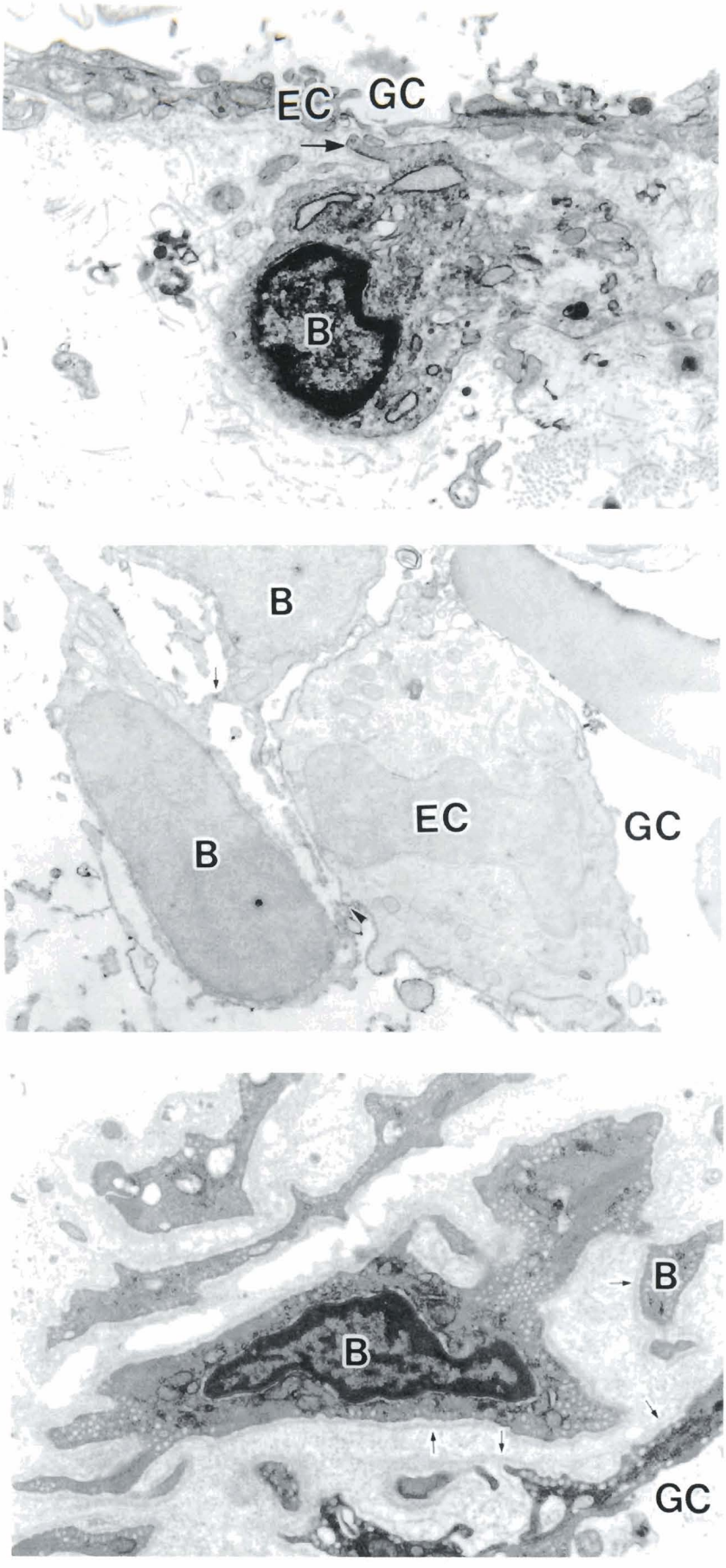


Fig. 7. a. A B type cell extends the cytoplasmic projection (arrow) to the endothelium (EC) of a growing capillary (GC) at 1 week after transection. **b.** Immunoreactions of FN on contact areas between two adjacent B type cells (arrow) and between B type and endothelial (EC) cells of a capillary (GC) (arrowhead) at 1 week after transection. Uncounterstained. **c.** B type cells associated with a capillary (GC) possess an abundance of cytoplasmic vesicles and are enclosed by the basal lamina (arrows) which is in continuation with that of the endothelium of the growing capillary at 1 week after transection. a, x 13,000; b, x 9,000; c, x 19,000

the ECM. Contact areas between such B type cells and endothelial ones of the pre-existing capillaries showed intense immunoreactions of FN. The role of FN in the endothelial cell chemotaxis has been proposed by Bowersox and Sorgente (1982). In addition, the role of FN in the acceleration of capillary ingrowth was noted in wound healing processes (Clark et al., 1982; Wakui et al., 1990; Hirata et al., 1992; Mori et al., 1992) and in embryonic organs (Hara et al., 1994). Taking these facts into consideration, FN and possibly LN may be involved

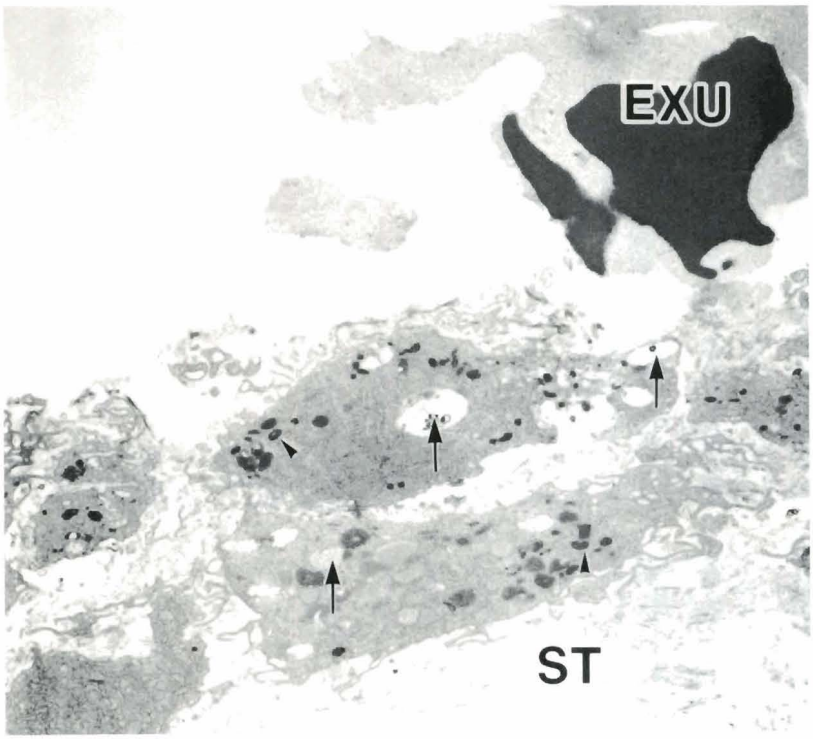


Fig. 8. A type cells along the surface of the synovial tissue (ST) near the wound margin possess primary (arrowheads) and secondary lysosomes (arrows) at 1 week after transection. EXU: Exudation. x 6,000

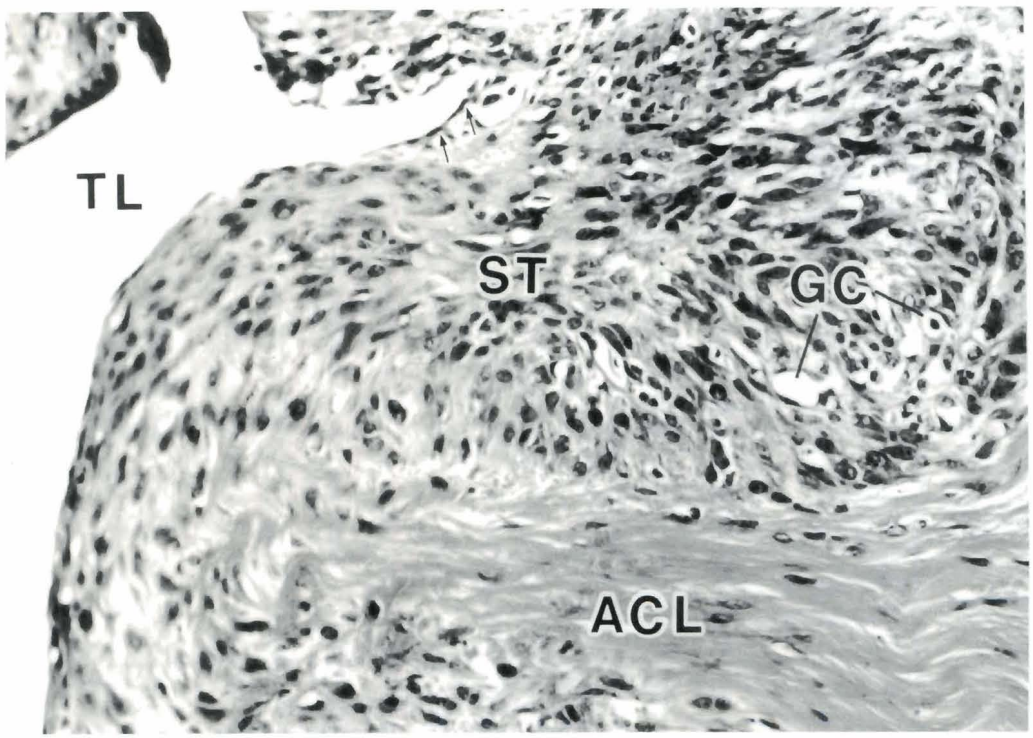


Fig. 9. The transected lesion (TL) is considerably replaced by regenerated soft tissue (ST) including abundant B type cells and growing capillaries (GC) at 4 weeks after transection. The surface of the regenerated tissue is covered in part with A type cells (arrows). The regeneration of the anterior cruciate ligament (ACL) can be seen in the deeper layer of the regenerated tissue. x 350

in a mechanical linkage between the vasoformative B type cells and endothelial ones.

The present observations indicated that B type cells transform to the phagocytic A type cells. In the present study, intermediate forms between A and B type cells were seen both in the sham and in the partially-transected samples. The appearance of the phagocytic cells has been described in the reconstruction of the monkey ACL (Clancy et al., 1981). Peach et al. (1961) reported the appearance of A (migratory) and B (synthetic) types of fibroblasts in the regenerating guinea pig tendon. They considered that the existence of

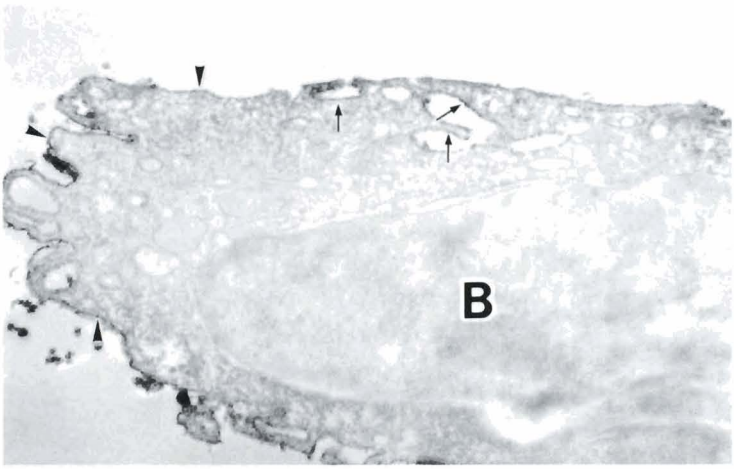


Fig. 10. Immunoreactions of FN in the rER (arrows) and cell surface (arrowheads) of a B type cell in the regenerated soft tissue at 2 weeks after transection. Uncounterstained. x 14,000

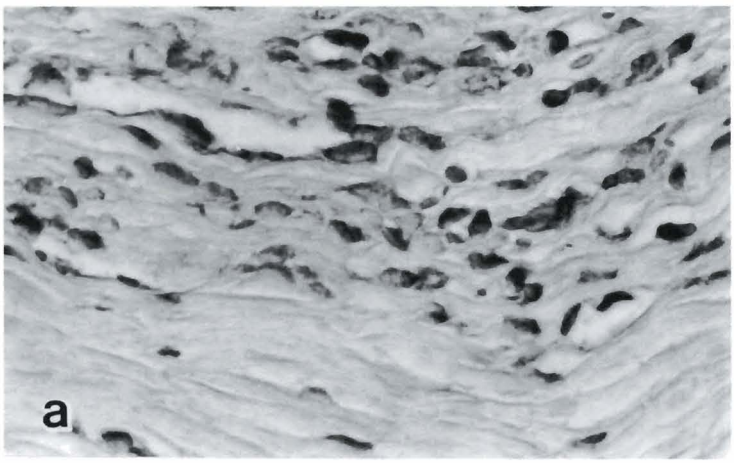


Fig. 11. a. Immunoreactions of type III collagen on B type cells and ECM around the cells in regenerated ACL at 4 weeks after transection. **b.** Less pronounced immunoreactivities of type I collagen, especially in ECM in the adjacent section. x 450

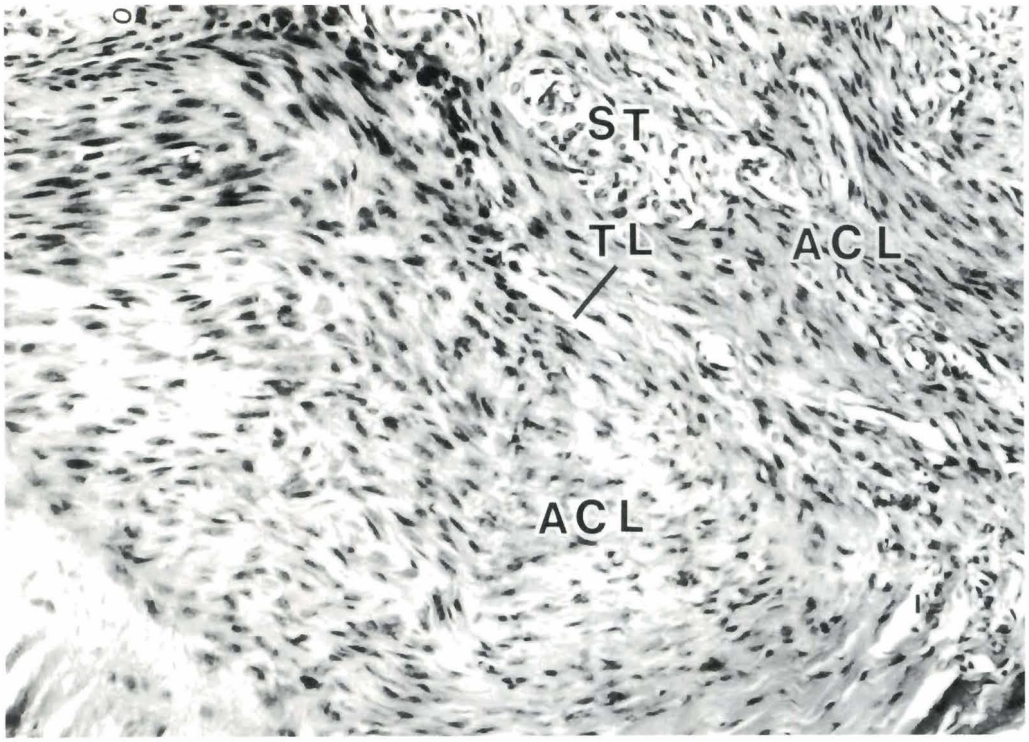
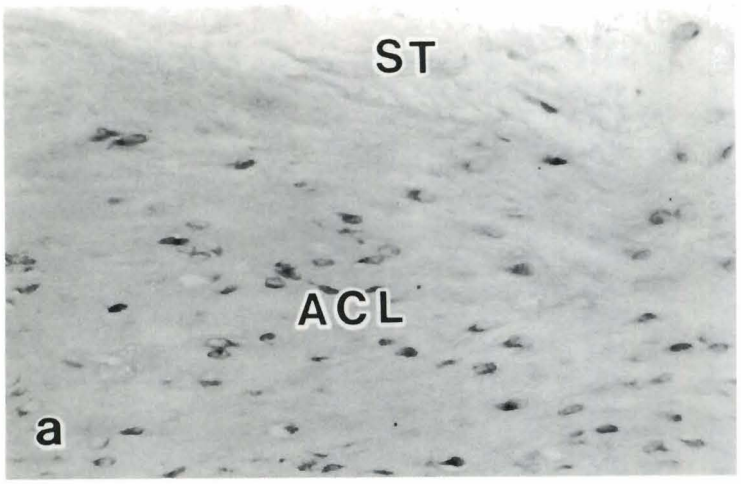


Fig. 12. The transected lesion (TL) is almost completely replaced by proliferative soft tissue which is distinguishable between the regenerated synovial tissue (ST) and regenerated anterior cruciate ligament (ACL) at 6 weeks after transection. x 200



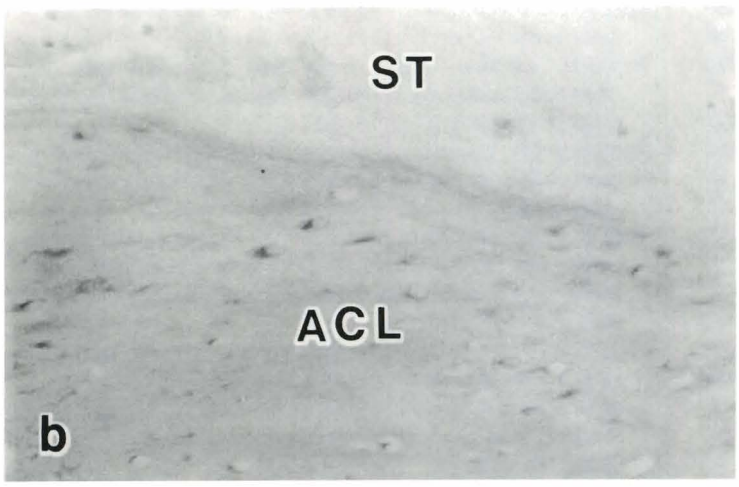


Fig. 13. a. Immunoreactions of type III collagen on B type cells and ECM around the cells at 6 weeks after transection in the regenerated anterior cruciate ligament (ACL). **b.** Immunoreactions of type I collagen in the adjacent section. ST: Regenerated synovial tissue. x 250

intermediate forms reflects a cyclical change between migrating and synthesizing phases of these cells. In our samples, some B type cells located at the advancing edge of the regenerated connective tissue between 2 and 4 weeks after operation possessed numerous secondary lysosomes and cytoplasmic projections. The origin of histiocyte-like cells from fibroblastic ones has already been described by Mori et al. in the wound healing process (Mori et al., 1989). Thus, the appearance of the phagocytic A type cells, transformed from B type cells in the healing process of the synovial tissue, may argue for the above-concept that A type cells are involved in the non-inflammatory defense mechanism under the effects of certain stimuli. A monocytic origin of A type cells seems to be unlikely in our samples since their ultrastructure is quite different from monocyte-derived histiocytes in regard to the much lesser numbers of lysosomes.

The surface of the regenerated synovial tissue was covered in part by a layer of A type cells occasionally involving phagocytic functions between 2 and 4 weeks after operation. B type cells in the deeper layer of the regenerated tissue were mainly involved in the production of type III collagen during this stage as revealed by our light immunocytochemical studies. This result argues for the data reported by Forrest (1983). Frank et al. (1983) have also described that the ligament scars of the middle cruciate ligament contain a considerable amount of type III collagen by the SDS gel electrophoresis, while the normal ligament is composed primarily of type I collagen. Our immunocytochemical analyses indicate that B type cells in the deeper layer of regenerated tissue begin to produce type I collagen at 6 weeks after operation. This may be a sign of the onset of the regeneration of the ACL since this period is almost in accordance with the appearance of collagen bundles by our electron micrographs. The question why and how small vessels decrease in number in the regenerated ACL is now under investigation.

In conclusion, B type cells are a kind of multipotent fibroblast that preserves the ability to transform to the vasoformative cell involved in the extension of growing capillaries to the transected lesion, to the phagocytic cell involved in the cleaning of cell debris in the transected lesion, and to the synthetic fibroblast involved in the regeneration of ACL.

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