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Neovascularization in the splenic autograft transplanted into rat omentum as studied by scanning electron microscopy of vascular casts

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Summary. Neovascularization during regenerating process in splenic tissue autotransplanted into rat omentum was studied by means of scanning electron microscopy of vascular casts. The results obtained indicated that neovascularization is classified into the following four steps; (1) capillarization in the connective tissue surrounding the degenerating autograft, (2) connection of blood vessels between the still surviving splenic cords and capillaries in the surrounding tissue, (3) rebuilding of the splenic sinuses and (4) remodelling of regenerated blood vessels. This neovascularization process is specific in that the preexisting splenic cords play an important role in angiogenesis during regenerative process in the autograft.

Key words: Neovascularization – Spleen – Autotransplantation – Rat

Introduction

Since the autotransplantation of the splenic autografts was first studied by Marine and Manley (1920), it has been intensively carried out by transplantation of splenic tissues into various tissues of many mammals (Tavassoli et al. 1973; Pabst and Reiman 1980; Patel et al. 1981; Sasaki 1984). These studies revealed that a splenic autograft can regenerate more easily than autotransplantats of other tissues which is considered to be due mainly to specific features of the neovascularization process in the splenic tissue. However many of these studies have dealt with histological data at the light microscopic level. As far as the author knows, no electron microscopic analyses of neovascularization in the splenic autograft have been reported. The represent study deals with the results of three-dimensional analysis obtained from scanning electron microscopy of vascular casts of the splenic autografts during regeneration.

Materials and methods

Twenty-six adult male rats of the Wistar strain, weighing between 180 to 200 g, were used in this study. Under anesthesia produced by an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight), splenectomy was carried out via a median incision. The spleen was cut into small slices ($2 \times 5 \times 5$ m/m). Three or four sliced tissue blocks were autotransplanted into the omentum of the same rat, and then the abdominal walls were sewed up with 2–0 silk.

1, 3, 5, 7, 10, 14, 28, and 84 days after autotransplantation, the abdominal aorta was cannulated retrogressively with a vinyl tube (1.2 mm in diameter) 15 mm below the level of the left renal artery, and perfusion fixation with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) was carried out following the following procedures. The thoracic aorta was clamped first just above the aortic hiatus of the diaphragm and the inferior vena cava was cut off above the foramen venae cavae, soon after starting rinsing with buffer solution containing heparin (0.25 g/1,000 ml) and procaine HCl (5.0 g/1,000 ml). After rinsing for 1 min, the fixative was introduced into the aorta for 2–3 min, and then the tissue blocks containing the autograft were excised and cut into small blocks in the same fixing solution. For scanning electron microscopy, the tissue blocks were fixed with 2% glutaraldehyde at 4° C for 2 h in total, washed in 3 changes of buffer solution and postfixed with OsO_4 in buffer for 2 h. The samples were dehydrated in a series of graded concentrations of cold ethanol, replaced with isoamyl acetate, and dried by the critical point drying method with Hitachi HCP-1. They were coated by gold about 20 nm in thickness with Eiko IB-3 sputter coater and examined with a Hitachi S450-LB6 scanning electron microscope at an accelerating voltage of 15 or 25 kV. For light microscopy, the samples were fixed further with 2% glutaraldehyde for 2–3 days. Paraffin sections about 3 to 4 μm in thickness were stained with haematoxylin and eosin.

For casting the vasculature, the abdominal aorta was cannulated retrogressively with a vinyl tube, similarly to the perfusion fixation. Prior to resin injection, the vascular lumen was washed with phosphate buffer alone and 40 ml of Mercox (Oken Shoji, Tokyo, Japan) were introduced into the aorta by speed of 10 ml/min. The animals injected with resin mixture were left at room temperature for 20 min and the samples were removed, kept in hot water (50° C) for 20 min and then cut into halves along the long axis of autograft before maceration. They were bathed in 18% NaOH in water one overnight and washed gently by running water for 10 h. This maceration procedure was repeated for 5–6 days until the tissue debris was completely washed out. After drying in air, the casts were mounted on an aluminum disk with an adhesive, coated by gold about 40 nm in thickness with a sputter coater and examined by scanning electron microscopy.

Results

Histological characteristics indicating regeneration of splenic autograft were recognized more clearly after the 7th day post-transplantation, although the initial signs of the regenerative process were detected as early as the third day after operation. Thus, the results obtained will be described here in regard to those 7, 28, and 84 days after autotransplantation, in which the regenerative process of the splenic autografts was typically represented.

Splenic autograft 7 days after transplantation. In light microscopy, the outer and inner zones were distinguished with histological characteristics as shown in Figure 1 a. The outer zone (out.) was rich in blood vessels, and numerous erythrocytes and other cell types were dispersed throughout the interstitial spaces, while the inner zone (in.) contained few blood vessels and interstitial spaces were filled with only the debris of dead cells. Figure 1 b shows a transitional area between outer and inner zones in a higher magnification. In the upper half, the outer zone which contained a blood capillary and

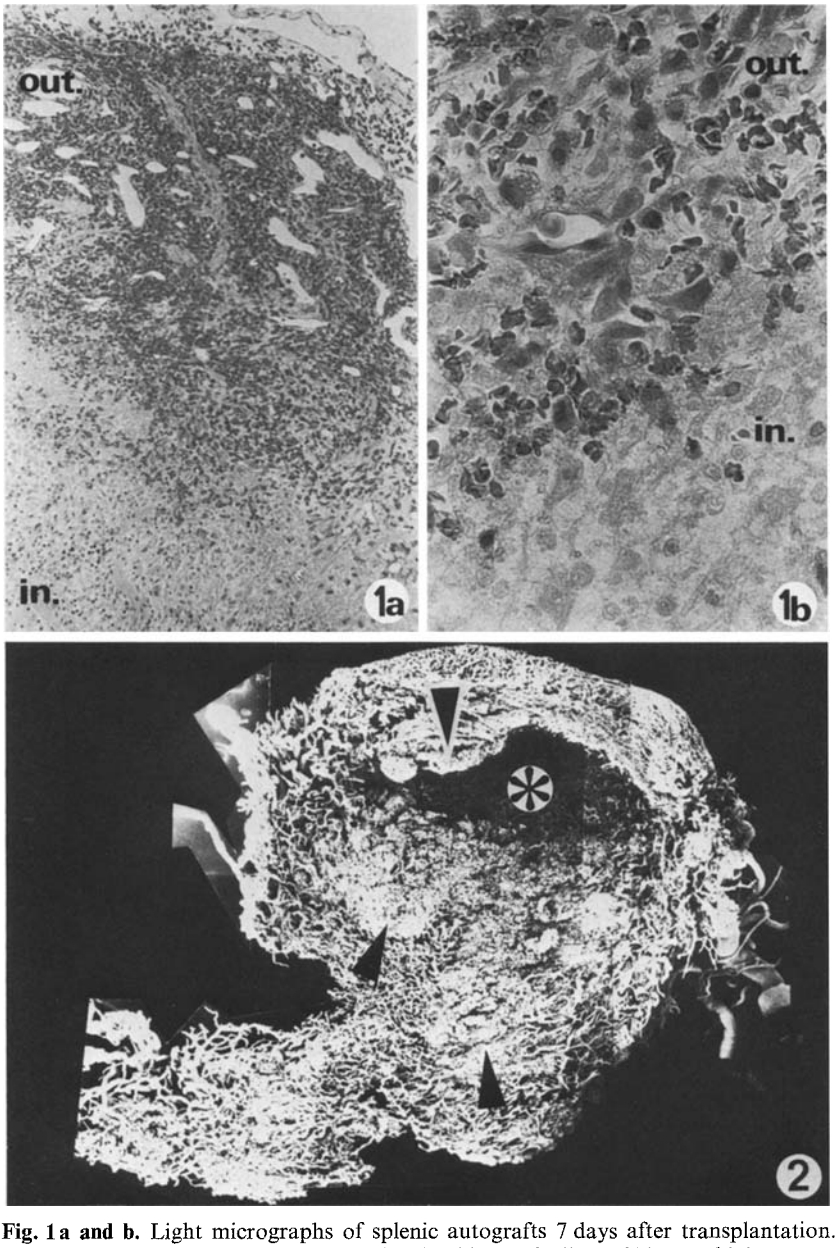


Fig. 1 a and b. Light micrographs of splenic autografts 7 days after transplantation. **a** Outer (*out.*) and inner (*in.*) zones are distinguishable histologically. × 200. **b** In higher magnification, the outer zone is seen to be composed of degenerated reticular cells and numerous erythrocytes inbetween. × 1,000

Fig. 2. A vascular cast of the autograft at the same stage as in Fig. 1. A deep excavation in the central region of the cast indicated a necrotic area of the autograft (*). A number of small conglomeration of vascular casts which are called vascular plaques (*arrow heads*) in this study for convenience of description, are observed to protrude into the necrotic tissue areas

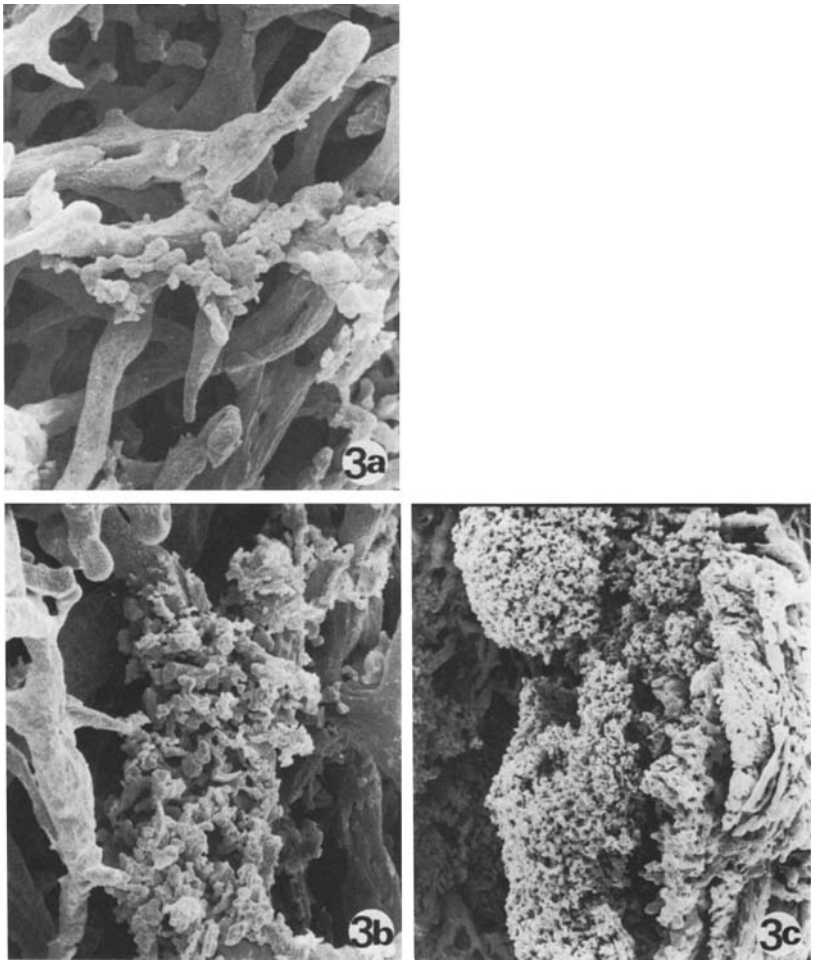


Fig. 3a–c. Vascular plaques shown in Fig. 2 in higher magnifications. **a** In early stage of plaque formation, a bead-like chain is formed between capillaries and arborized to form small networks. × 500. **b** As regeneration proceeds, a vascular plaque becomes larger in size and more complicated in structure. × 500. **c** In a later stage of vascular plaque formation, each individual plaque has a coral-like appearance and they fuse with each other to make a broad plate among capillary networks. × 200

a number of erythrocytes among irregularly shaped, large cells were seen, while in the lower half, the cellular components appeared to be degraded and fragmented to become debris. Figure 2 shows a vascular cast of 7-day autograft. A deep excavatation was observed in the upper central region of the cast. This area seems to correspond to the necrotic inner zone of the autograft, because the tissue had no blood vessels in this area which was dissolved during maceration leaving a defect in the cast. The peripheral areas surrounding the defect were the outer zone of the autograft and consisted of a capsular layer of meshworks of blood vessels. On its concave

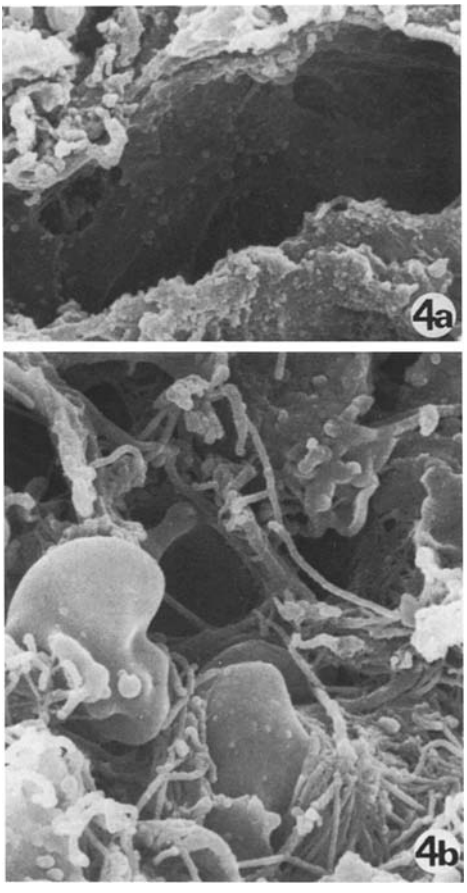
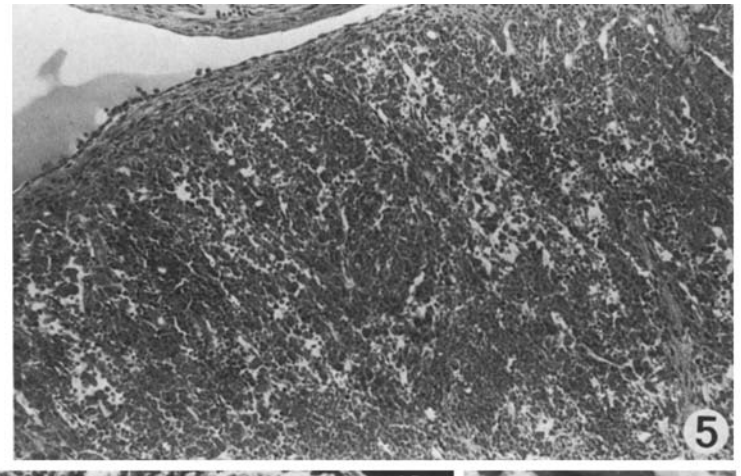
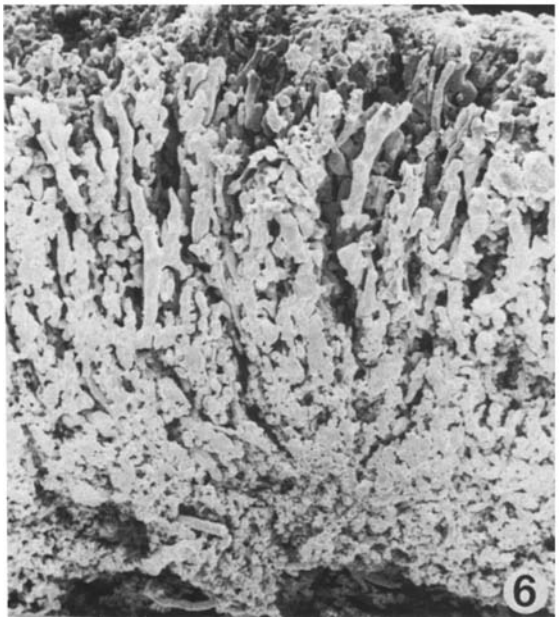


Fig. 4a, b. Scanning electron micrographs of 7-day autograft. **a** Inner surface of a vascular wall. A number of fenestrae with various sizes are found and closely apposed to reticular fibers outside. Microprojections of the endothelial cell surface were well-developed. × 10,000. **b.** One erythrocyte is passing through a mesh of reticular fiber network outside the vessel. × 10,000

surface, massive protrusions of dense cast meshworks which varied in size and form were seen (arrow heads in Fig. 2). In higher magnification, the blood vessels composed a meshwork were about 9 to 10 μm in diameter and the surface of their cast appeared mostly smooth. There were shallow irregular striations running longitudinally to its long axis which may represent the boundaries between individual endothelial cells. Massive protrusions of the vascular cast on the concave surface, as detected in Figure 2, were observed to consist of a number of irregularly shaped processes. They were connected with each other to form a bead-like chain or a small network in a simpler form (Fig. 3a), and/or to have a coral-like shape as they grow (Fig. 3b and 3c). In scanning electron microscopy of the tissue samples the capillary wall appeared to have a number of fenestrae with varying diameters and small bundles of reticular fibers were seen, through the fenestration, to be closely apposed to the outside of the capillary wall (Fig. 4a). In an "outside" view of the capillary an erythrocyte was frequently observed to emerge, passing through a narrow space between reticular cells and asso-





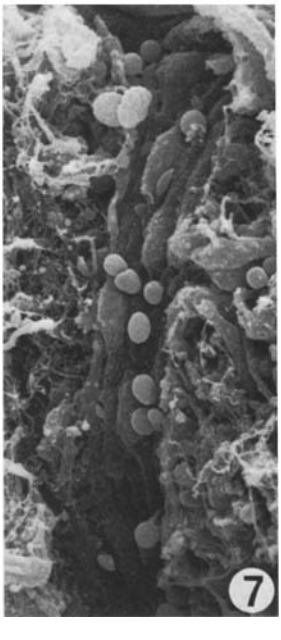


Fig. 5. A light micrograph of the splenic autograft 28 days after transplantation. Necrosis is not seen. The slight infiltration of lymphocytes are recognized. There is connective tissue of greater omentum at the left upper region. ×200

Fig. 6. A scanning electron micrograph of the autograft in the regeneration stage as in Fig. 5. A splenic sinus appears to be surrounded with endothelial cells which are arranged in parallel with the long axis of the sinus and provide interendothelial slits

Fig. 7. A vascular cast of the autograft in the same stage as in Fig. 5. Most of vessels run vertically from the outer region toward the center of the autograft, and from vascular plaques at their ends in the central area. ×200

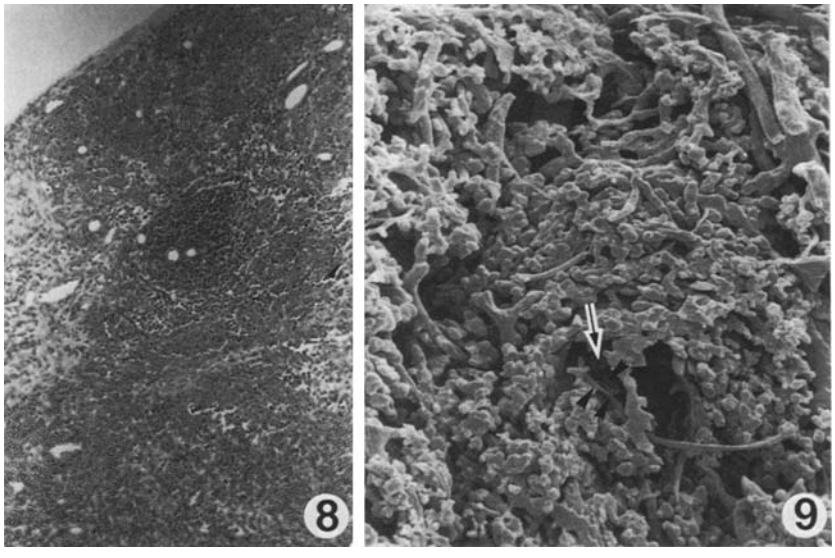


Fig. 8. A light micrograph of 84-day splenic autograft. Periarterial lymphatic sheaths are recognized, and histological characteristics appear to become similar to those in normal splenic tissue. ×200

Fig. 9. Vascular casts of the autograft in the same stage of regeneration as in Fig. 8. A central artery (*arrow*), follicular arteries (arrowheads) and splenic sinuses are distinguished. ×100

ciated fibers (Fig. 4b). These suggest that the small processes seen in the vascular cast were formed by leaking resin mixture, escaping into the perivascular space through the fenestrae of capillary wall. In addition, the fact that these processes provided such organized forms as a simple bead-like chain and/or complicated form of coral-like structure may indicate a process of neovascular formation. The author would like to call massive protrusions of the cast 'vascular plaques' in this manuscript for convenience of description.

Splenic autograft 28 days after transplantation. In light microscopic preparations, well-developed blood vessels which were lined with endothelial cells were observed to be arranged vertically from the surface of autograft toward the central region (Fig. 5). Lymphocyte infiltration was detected around vessels at this stage of regeneration process. In scanning electron micrographs of the tissue samples, a blood vessel 15–20 μm in diameter which was composed of longitudinally arranged, spindle-shaped endothelial cells was occasionally observed in the outermost to the middle parts of the outer zone of the autograft (Fig. 6). Individual cells were swollen in the center of the cell body by the nucleus and there was a narrow slit between neighboring cell boundaries. Erythrocytes appeared to have passed through the slit and come out into the perivascular space on many occasions (Fig. 6). These observations indicated a resemblance of these vessels to the normal splenic

sinus. The vascular casts gave more precise information about neovascularization in the autograft (Fig. 7). The outer zone of the autograft became thicker than in the previous stage and was mostly occupied with a number of blood vessels perpendicularly oriented to the surface of the autograft. These vessels were distinguished into three kinds depending on their diameter and smoothness of cast surface; that is, arteries with a smaller diameter and a smooth surface, veins with a larger size and indented surface and splenic sinuses with the largest diameter and an irregularly undulated surface. In a deeper part of the outer zone, a well-organized vascular structure became irregular and in conspicuous, and the vascular plaques were well developed.

Splenic autograft 84 days after transplantation. Histological features of the autografts in this stage became similar to those of the normal splenic tissue (Fig. 8). Lymphocytes infiltration developed well, surrounding a small artery to resemble formation of the white pulp. The distribution pattern of blood vessels became more complicated and the vertical orientation of vessels seen in the previous stage was no longer detected in this regenerative phase. As seen in Fig. 9, the vascular casts indicated that complexity of vascular distribution pattern was caused by formation of transverse anastomoses among longitudinally oriented vessels and remodelling.

Discussion

The present study revealed the mechanism of neovascularization in the splenic autograft transplanted into rat major omentum. That is, within a week after transplantation the major omentum reacts with autograft to form a capsular layer of the loose connective tissue rich in capillaries around implant. These capillaries proliferate and invade into the peripheral region of the autograft to form the outer zone of the transplant. They have a number of fenestrae in their wall and their lumen is open through the fenestrae to the perivascular space which has a caveola-like shape and is lined with reticular cells and their associated fibers. As time goes by, the central region of the inner zone of the autograft falls into necrosis, while the outer zone with capillary networks becomes thicker by its invasion into the inner layer of necrotic tissues. Thus, the outer and inner zones are histologically distinguished in the autograft. In an early stage of regeneration, as early as one week after transplantation, the outer zone of the autograft is fairly thin and has a number of vascular plaques at the concave face toward the inner zone. They are various in size and shape, and form a simple bead-like structure or a massive meshwork with coral-like appearance in vascular casts. In a simple form of the plaque, individual protrusions of the cast appeared to represent expansions of the vascular lumen into the perivascular space, and they are then connected with each other to form a more complicated meshwork as they grow. Thus, it is considered that the lumen of vascular plaque is not lined with endothelial cells but sur-

rounded with the reticular tissue originated from the pre-existing splenic cord of the autograft. This potential prevascular structure is considered to introduce blood into the autograft and accelerate the regenerative process of splenic tissue. As regeneration proceeds, newly-formed blood vessels were elongated toward the inner zone of the autograft and arranged vertically to the outer surface of the autograft as seen in the samples 28 days after transplantation. Well-developed vascular plaques were still detected in the transitional areas between the outer and inner zones. These observations suggest that angiogenesis occurs at the transitional part between the vascular plaques and the blood vessels. Newly-formed splenic sinuses were first detected at this stage. The origin of endothelial cells of splenic sinuses is not clear. The question whether they originated from mitosis of pre-existing endothelial cells or transitional of reticular cells lining the caveola-like perivascular space remains to be elucidated. The vascular structure lost its vertical arrangements and became more complicated in distribution pattern, similar to that in the normal spleen. This change appears to be carried out mainly by formation of transverse anastomosis among vertically running vessels.

Many studies on neovascularization in inflammatory or neoplastic tissues have been reported (Schoefl 1963; Greenblatt 1972; Folkman 1974; Polverini 1977; McCracken et al. 1979; McAuslan et al. 1979; Fenselau et al. 1981; Burger et al. 1983; Sholley et al. 1984; Polverini et al. 1984). Schoefl (1963) reported that newly-formed vessels in inflammation produced by muscle wounding and corneal lesions were fragile at their tips which were surrounded by extravasated red blood cells. Burger et al. (1983) have recently investigated angiogenesis in the inflammatory cornea by means of a vascular casting method. They showed the three-dimensional structure of these fragil regenerating capillaries which consists of projections with irregular surface from capillary network, but they have not detected the structure identical to the vascular plaque in the present study. In an early growth of the neoplastic tissue, a budding of host venous capillaries and small veins infiltrates into the tumour fragments without an inflammatory reaction (Greenblatt 1972). This neoplastic angiogenesis is brought about by the proliferation of endothelial cells which are activated by angiogenic factors (Folkman 1974; McAuslan 1979; Fenselau 1981) and/or macrophages (Polverini 1977, 1978). The neovascularization in the regenerative process of the splenic autograft seems to take a different form from that seen in these previous studies. Our, vascular plaques appeared to be massive protrusions of the cast with various shapes and seem to play an important role in neovascularization in the splenic autograft. The fact that these plaques were considered to be formed by leaking of resin mixture into the perivascular space through the fenestrae of capillary walls suggests the existence of potentiality for angiogenesis in the perivascular space. In *other words,* it seems that the reticular cells in the perivascular space may be closely related to neovascularization, rather than proliferation of pre-existing endothelial cells. This may be one of the reasons why autotransplanted splenic tissue can regenerate more easily than other tissues.

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