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Revascularization of Skeletal Muscle Transplanted into the Hamster Cheek Pouch: Electron Microscopy

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Revascularization of bundles of skeletal muscle fibers transplanted into the cheek pouch of hamsters was examined previously by intravital and light microscopy. Some blood vessels survived the ischemia of transplantation and revascularization resulted from the growth of surviving vessels. We have now examined the cellular events of graft revascularization by electron microscopy (EM). Approximately 20 skeletal muscle fibers were autografted into the hamster cheek pouch. Segments of the graft were prepared for EM from 0.5 to 5 days after transplantation. At 0.5 day, the endothelial cells of surviving blood vessels had short processes extending from them. By 1.0 day, some endothelial cells had separated. At 1.5 days, many endothelial cells were undergoing mitosis. Blood vessels at 2 to 2.5 days were of narrow diameter and most were densely packed with erythrocytes. Circulation was reestablished between 2.5 and 3 days. The fine structure of the blood vessels changed little after 3 days except for a decrease in the number of vesicles in the endothelial cell cytoplasm and the formation of normal endothelial cell injections. After 5 days, blood vessels were larger in diameter, but otherwise similar to control capillaries. We conclude that, despite the initial absence of circulation, surviving blood vessels underwent a rapid, orderly sequence of growth.

INTRODUCTION

Previous studies of revascularization of whole-muscle grafts have described survival of some morphological elements of blood vessels and a process of revascularization by which most, if not all, blood vessels grow into grafts from the adjacent host tissue (Hansen-Smith *et al.*, 1980). In contrast to the whole-muscle grafts, observations with intravital and light microscopy (Faulkner *et al.*, 1983) supported the hypothesis that some blood vessels in small bundles of skeletal muscle fibers transplanted into the hamster cheek pouch survive and grow out of the graft. The purpose of the present study was to describe the ultrastructural features of: (1) the blood vessels that survive in free grafts, and (2) the sprouts that grow out of the graft and anastomose with blood vessels in the adjacent tissue of the host.

MATERIALS AND METHODS

Small bundles, approximately 15–20 fibers, were dissected from the extensor digitorum longus (EDL) muscles of Syrian golden hamsters. The bundles of fibers

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were immersed in Bupivacaine, washed in mammalian Krebs–Ringer solution and then transplanted into the hamster cheek pouch by the procedure described previously (Faulkner *et al.*, 1983). Animals were anesthetized at half-day intervals post-transplantation and grafts fixed for 30 min *in situ* by filling the cheek pouch chamber with 4% cacodylate-buffered glutaraldehyde (pH 7.2). Segments of grafts and the attached cheek pouch were removed and placed in individual vials containing fresh glutaraldehyde for an additional 30 min. For controls, EDLs were removed and fixed for 1 hr in glutaraldehyde. After fixation and washing in buffer, samples were postfixed in room-temperature, cacodylate-buffered 1% osmium tetroxide to which 40.2 mg/ml of sucrose had been added. Tissues were rinsed in distilled water and then dehydrated in an alcohol series. In some cases *en bloc* staining for 1 hr with a 10% solution of uranyl acetate in 100% ethanol was done after the first dehydration. After ethanol dehydration, propylene oxide was used as a transitional solvent. Samples were embedded in Polybed 812.

One-micrometer sections were cut and collected at 5-μm intervals until the blood vessel of interest was observed. Blood vessels in longitudinal section were traced by taking several thin sections, a thick section, and then several more thin sections. The process of serial thin–thick sectioning was repeated until the blood vessel was no longer visible. Blood vessels in cross section were traced using the same procedure except the length of the vessels required interspersing gaps of 5 to 10 μm between sets of sections. The thin sections were stained in uranyl acetate except for samples that had been stained *en bloc* with uranyl acetate. Staining in lead citrate followed the uranyl acetate staining. All observations were made on a Philips 400 electron microscope.

RESULTS

Although graft blood vessels at 0.5 day had a fairly normal appearance when observed by light microscopy, electron microscopy showed some blood vessels were indistinguishable from control vessels (Figs. 1, 3), whereas others were somewhat abnormal or were undergoing degeneration (Fig. 2). Serial sections showed that some blood vessels, both of the simple capillary type and of the arteriole and venule type, had normal-appearing segments alternating with areas that were severely vacuolated. Beyond the vacuolated area, the blood vessels terminated (Fig. 4). This occurred when the vessel was located in the central portion of the graft as well as when vessels were located near the periphery of the graft and were probably cut during the transplantation process. Vacuolated areas and blind-ended vessels were often associated with branching vessels. The cross section of the blood vessels appeared normal as did the portion of the branches closest to the cross section, but more distal areas of the branches showed vacuolation and then the branches ended (Fig. 4). Whereas control endothelial cells had a smooth surface, endothelial cells in grafts had irregular, wavy outer surfaces. Some endothelial cells had short processes extending out from them (Fig. 5). All blood vessels observed had many vesicles in the endothelial cell cytoplasm. Some vesicles were attached to the outer wall and others to the inner wall, whereas others were free in the cytoplasm. Endothelial cell cytoplasm also contained rough endoplasmic reticulum, free ribosomes, contractile filaments, and mitochondria.

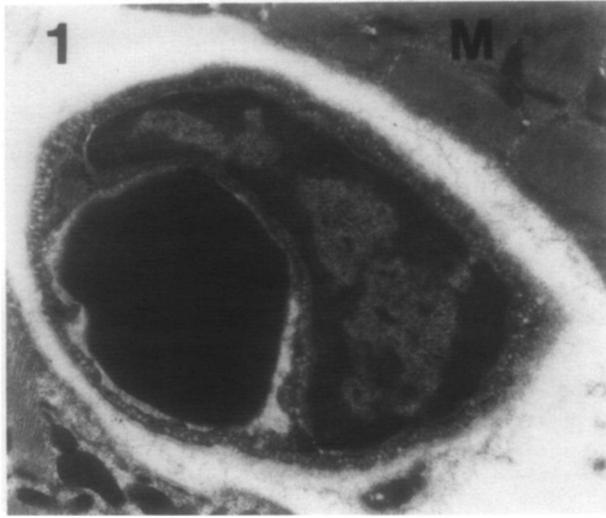

FIG. 1. A typical control capillary is situated next to a muscle fiber (M). ×12,500.

By 1.0 day, endothelial cells in blood vessels separated from each other. This was especially apparent in blood vessels viewed in longitudinal section. Some endothelial cells had no visible attachment with each other. All the endothelial cells had irregular surfaces and many had pseudopod-like processes (Figs. 5, 6) In addition, most blood vessels showed perivascular cells which were outside

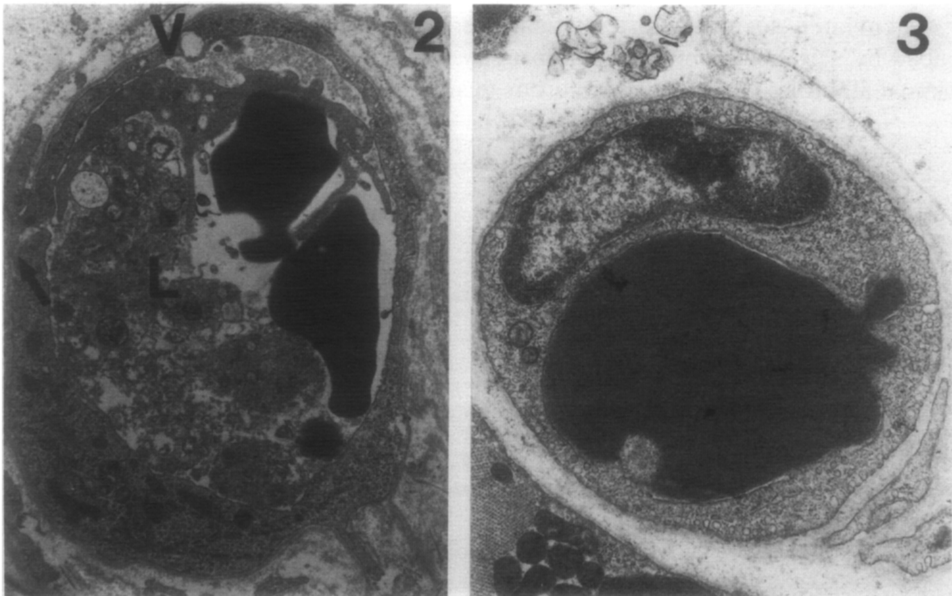

FIG. 2. At 0.5 day postgrafting, the lumen of this capillary (L) is filled with cellular debris. There are vacuoles in the endothelial cells (V) as well as gaps at endothelial cell boundaries (arrow). Cytoplasm is filled with bound and unbound ribosomes. ×7500.

FIG. 3. Some capillaries at 0.5 day, capillaries appear completely normal. ×13,500.

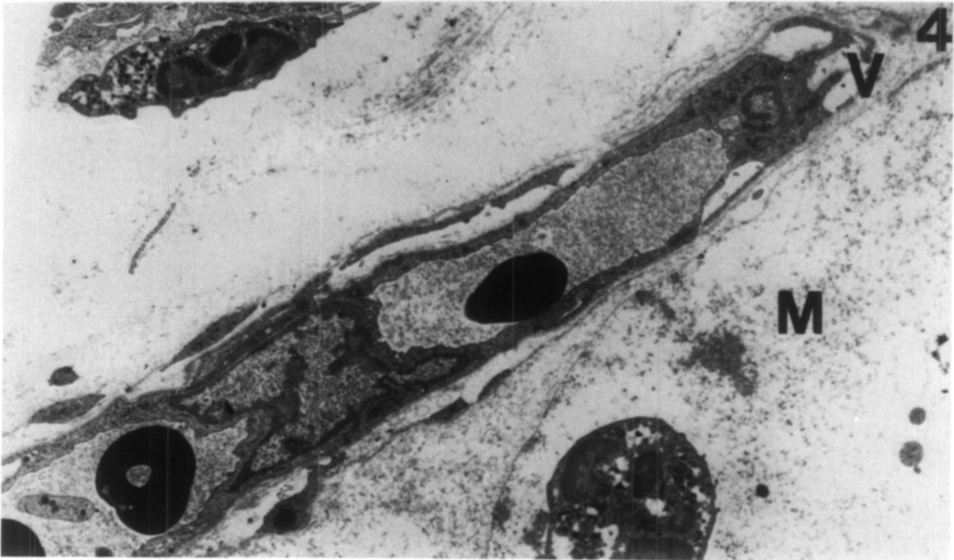

FIG. 4. A capillary at 0.5 day shows vacuolation (V), but is normal in some areas. In contrast, the muscle (M) has degenerated. Serial sections showed this to be the end of the blood vessel. ×4000.

the endothelial cells and were often only loosely attached to the endothelial cells. Some of these cells were smooth muscle cells (Figs. 5, 6). As at 0.5 day, serial sectioning showed there were blood vessels which had normal segments, followed by vacuolated segments, after which the vessel ended (Fig. 7). Some vessels traced by serial sectioning ended abruptly without any evidence of vacuolation. Almost all of the vessels had numerous vesicles in the endothelial cell cytoplasm.

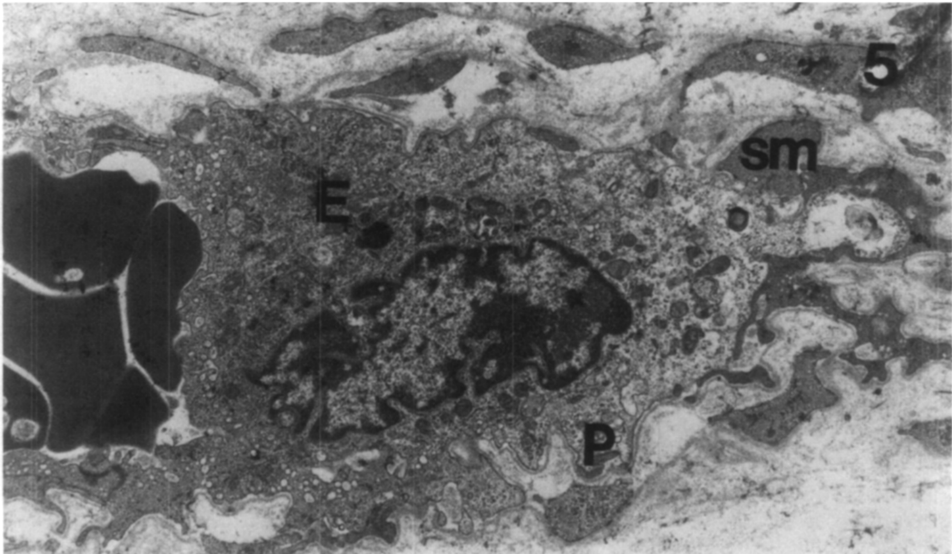

FIG. 5. The smooth muscle (sm) layer of a 0.5-day vessel has separated off, leaving gaps between the smooth muscle and the endothelial cell (E). The endothelial cell shows pseudopod-like projections (P) and is of a very irregular shape, but its cytoplasmic contents are normal. ×7500.

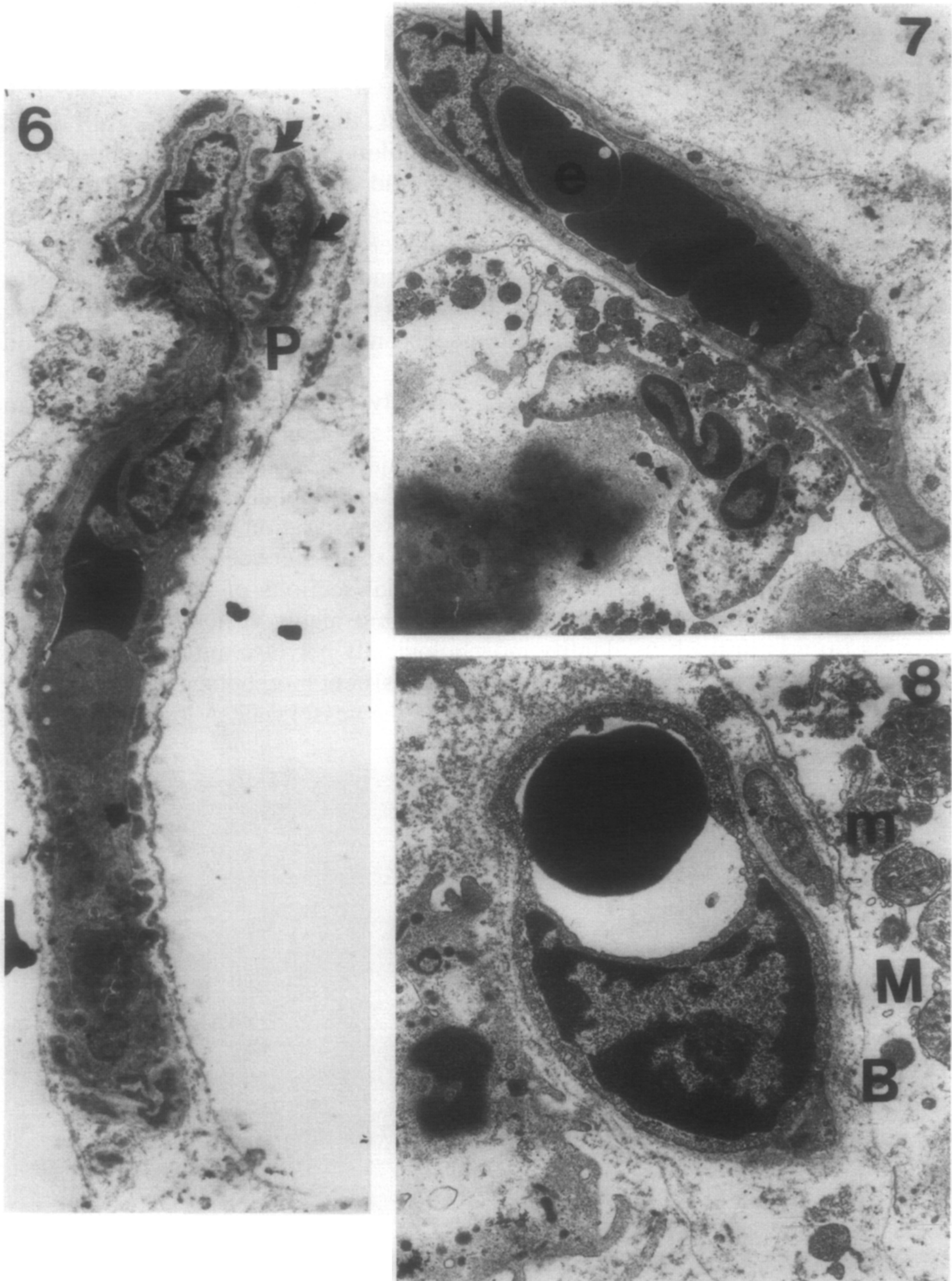

FIG. 6. At 1.0 day, this vessel has cells (arrows) which may be endothelial or smooth muscle cells next to the blood vessel, but separated from it. ×4500.

FIG. 7. At 1.0 day, a vessel appears normal at one end (N) and vacuolated (V) at the other. The tightly packed erythrocytes (e) are typical of this time period. ×4500.

FIG. 8. At 1.0 day postgrafting, a capillary appears to be normal. Muscle fibers (M) have undergone degeneration leaving only basement membrane (B) and mitochondria (m). ×11,500.

At 1.5 days, the junctional separation between endothelial cells was less conspicuous. Open intercellular spaces were seen between endothelial cells, but they were much narrower than the separations seen at 1.0 day. Except for the lack of specialized junctions between endothelial cells, the structure of the graft blood vessels was similar to that of capillaries. Many blood vessels had cells undergoing mitosis at the blind end. The mitotic activity did not always extend to the very end of the blood vessel. Sometimes a highly vesicular vascular segment or one or two endothelial cells preceded the cell undergoing mitosis (Faulkner *et al.*, 1983). In all cases, the blood vessels were surrounded by an irregular arrangement of what appeared to be collagen fibers. Loosely associated basal laminae belonging to degenerating muscle fibers were often seen on one or both sides of the blood vessel (Fig. 8).

At 2 to 2.5 days, blood vessels were typically of narrow diameter, less than 50 μm, with a thin outer membrane, an incomplete basal lamina, and most were packed with erythrocytes (Figs. 9, 10). The major change was the development of endothelial cell junctions. The appearance of endothelial cell junctions varied from fully differentiated to that in which a narrow separation between endothelial cells was present. In all cases, however, the space between endothelial cells was much less than that observed earlier. Serial sections of blind ends with 5 μm between sections showed them to be solid but highly vesiculated. The solid growing tip was difficult to identify morphologically because mitoses were much rarer at this stage, and because the tip had no consistent morphologic characteristics which distinguished it from endothelial cells of the vessel wall. A loosely attached

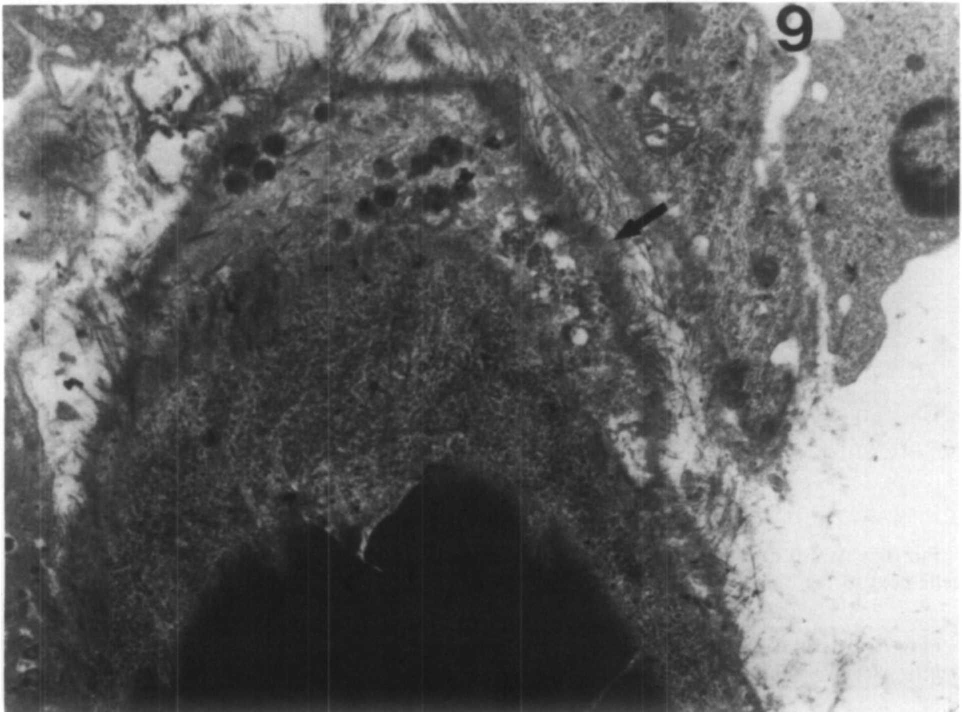

FIG. 9. By 2.5 days, basement-membrane material (arrow) is observed around the blood vessel. Erythrocytes were still densely packed. ×9500.

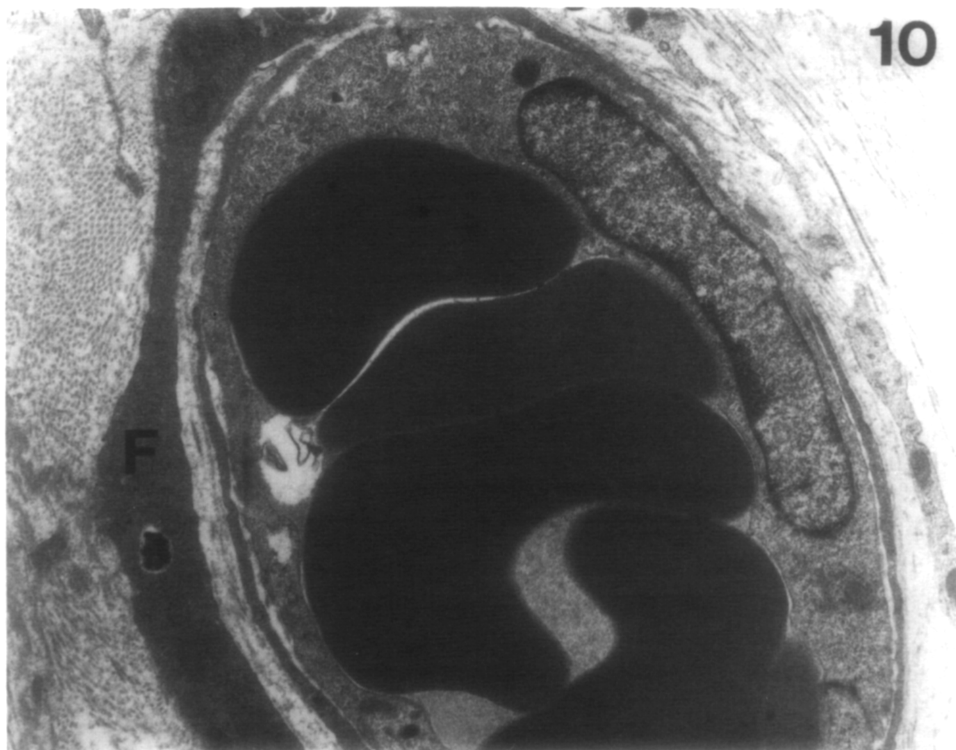

FIG. 10. By 5 days, the vessels have no smooth muscle layer, but are otherwise normal in appearance. A fibroblast-like cell (F) is closely associated with the blood vessel. ×9500.

cell, probably a pericyte, was often observed ahead of the growing tip. These cells lacked vesicles and fibers.

The fine structure of the blood vessels changed little from 3 to 5 days except for a decrease in the number of cell vesicles and the presence at 5 days of endothelial cell junctions in almost all endothelial cells. After 3 days, the blood vessels contained less densely packed erythrocytes within the lumen than were seen previously. Well-spaced erythrocytes were typical of vessels in which flow had been documented by intravital microscopy (Faulkner *et al.*, 1983). Blind-ended vessels, as determined by serial sectioning, were rare except for short branches seen growing out from the middle of blood vessels. Many vesicles and cytoplasmic filaments were still observed in the cytoplasm of the endothelial cells. By the end of 5 days, none of the blood vessels showed any evidence of a smooth-muscle layer. They were indistinguishable from normal capillaries except for size. Blood vessels in control muscle always had a smooth muscle layer when larger than 20 μm in diameter. Many graft blood vessels were of this size, but had no smooth muscle layer (Fig. 10).

DISCUSSION

As early as 0.5 day after grafting, the surviving blood vessels were undergoing changes that would contribute to revascularization of the graft. Why some segments of blood vessels survived, while others degenerated, is not clear. Regardless of

the mechanism of survival, the growth in surviving blood vessels occurred in a rapid, directional, and very orderly sequence.

Smooth muscle cells began separating from the normal-appearing endothelial cells at 0.5 day. As no surviving blood vessels were seen to have a smooth muscle layer at 1.5 days or anytime thereafter, dedifferentiation of the blood vessel is concluded to be a prerequisite to growth in this system. Clark and Clark (1940) have shown separation of smooth muscle cells to be part of the process of dedifferentiation which precedes blood vessel growth into injured areas.

Separation of endothelial cells was observed between 0.5 and 1.0 day. Endothelial cells are capable of separation and migration during angiogenesis (Ausprunk and Folkman, 1977; Cliff, 1965; Schoefl, 1963) and during repair after endothelial cell injury (Reidy and Schwartz, 1981). Such a process may be occurring in our system. Endothelial cells located at or near the blind end of blood vessels underwent mitosis. Growth was directional toward the host and especially toward host blood vessels. Large increases in length appeared to result from a migration and mitosis of endothelial cells. Additionally, growth might occur by lengthwise stretching of the endothelial cells. Attenuation of the cytoplasm of endothelial cells yielding an increase in blood vessel length and a concomitant increase in luminal diameter may occur during angiogenesis in some embryonic systems (P. Jokelainen, personal communication). Attenuation of endothelial cell cytoplasm could also be responsible for the large increase in diameter of blood vessels seen at 3 days.

Intercalation of vesicles into the endothelial cell membrane might also contribute to the increase in blood vessel diameter. Wolff (1967, 1980) has shown that the presence of vesicles in endothelial cell cytoplasm is associated with the increase in the diameter of blood vessels due to inflammation. Vesicles were present in the blood vessels of the graft in larger quantities before the increase in diameter and in smaller quantities after the blood vessels returned to a smaller diameter by 4–5 days. In addition, vesicles seemed to join to the outer membrane. Whether the vesicles were empty or whether they expelled their contents during the process of joining the membrane, is unclear. Numerous vesicles are characteristic of blood vessels and they may be involved in blood vessel permeability (Bruns and Palade, 1968; Johansson, 1978).

In his theoretical article on blood vessel growth, Waxman (1981) hypothesized that a blood vessel connected at both ends to other vessels will show an increase in diameter as a result of endothelial cell mitosis. Eventually, the growth will lead to buckling, after which sprouting occurs. This is the sequence observed in the surviving blood vessels of the graft. Before blood vessels were attached to the circulation, they showed straight-line, unbranched growth. There was nothing to restrain that type of growth until the vessels established continuity with the host circulation. When circulation was reestablished, the continued growth of the vessels led to an increase in diameter. Subsequently, buckling of vessels produced a sinusoidal shape. Finally, sprouting occurred usually at the crests. Further studies will be necessary to determine how much of the diameter increase is due to the intercalation of vesicles and how much to the growth of vessels after circulation has been reestablished, and how much to attenuation of endothelial cell cytoplasm.

Once surviving blood vessels attached to the circulation, they grew just as other blood vessels do (Ausprunk and Folkman, 1977; Yamagami, 1970). The

difference between this system and others was that before blood vessels were attached to the circulation, they were capable of linear growth and this linear growth occurred without any pressure from the circulatory system. Endothelial cells are capable of separation and mitosis in the grafts without circulation just as they are in tissue culture (Glaser *et al.*, 1980). We conclude that blood pressure is not a required stimulus for blood vessel growth *in situ*.

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