Three-dimensional Cytoarchitecture of Angiogenic Blood Vessels in a Gelatin Sheet Implanted in the Rat Skeletal Muscular Layers*

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Summary. To demonstrate the structure of angiogenic blood vessels three-dimensionally, a gelatin sponge sheet immersed in a vascular endothelial growth factor (VEGF) solution was implanted in the rat dorsal muscular layer, and examined by light microscopy and scanning electron microscopy (SEM) 5 days to 2 weeks after implantation. Light microscopy of anti-collagen IV antibody immunostained specimens enabled a determination of the basement membrane tube of newly formed blood vessels in the implanted sponge sheet. The tubes were 5-40 μm in diameter, and sometimes tapered to a slender cord within the vascular network. The SEM study of 30% KOH treated tissues revealed two types of tapering ends of newly formed blood vessels. One consisted of endothelial cells with microprojections, and lacked any investment of pericytes over the length of 5-20 μm. The other type was a tapering tip of the endothelial tube covered with pericytic processes. The presence of long processes of pericytes extending beyond the tip of the endothelial tube and connecting to the adjacent vessel wall indicates that this type was produced by endothelial tube regression. Thus, the present study supports the ideas that endothelial tube formation is followed by pericyte coverage at the sprouting tip, and that endothelial tube regression precedes pericyte detachment at the regressing site.

The formation of new vessels from existent blood vessels, which occurs within both normal and pathological conditions, is called angiogenesis. Much attention has been given to the mechanism of angiogenesis as its control is important for tissue repair and regeneration, and is effective in regulating the progress of tumors and inflammation (CARMELIET and JAIN, 2000).

Previous studies have shown that sprouting from pre-existing vessels is a main process in angiogenesis (SCHOEFL, 1963; AUSPRUNK and FOLKMAN, 1976; WA-KUI et al., 1987; HASHIZUME et al., 2000), although the insertion of the columnar interstitium into the dilated vessels has also been reported in certain tissues (BURN and TAREK, 1990; PATAN et al., 1992). As for sprouting in angiogenesis, two major hypotheses have been proposed in relation to the role of endothelial cells and pericytes at the sprouting tip: one is that endothelial cell proliferation is followed by the pericyte coverage that promotes endothelial tube formation and maturation (BLOOD and ZETTER, 1990), while the other is that the sprouting tip is composed of a pericyte that guides extending endothelial cells (NEHLS et al., 1992). Consequently, more precise studies have been awaited on the three-dimensional structure of endothelial cells and pericytes at the sprouting tip.

In the present study, we implanted gelatin sponges containing human vascular endothelial cell growth factor (VEGF) to the dorsal skeletal muscle of rats, and investigated the morphology of newly formed blood vessels in the sponge by both light microscopy and scanning electron microscopy (SEM). Special attention was focused on the three-dimensional ultrastructure of endothelial cells and pericytes at the sprouting tip of angiogenic blood vessels.

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MATERIALS AND METHODS

Model for angiogenesis in a gelatin sponge sheet
Thirty adult male Wistar rats, weighing 180-300 g, were used in this study. Under deep anesthesia by intraperitoneal injection with sodium pentobarbital (Nembutal, 30 mg/kg body weight), an incision was made in the dorsal skin of the rat and an implant sheet was inserted into a musculus elecor spinae. This implant was a 5 mm square sheet of gelatin sponge (Gelfoam; Upjohn Japan, Tokyo) sandwiched between two sheets of cellophane paper, and permeated with a VEGF solution which was made by the dilution of 100 µg VEGF (Immunobiological Laboratory, Fujioke) in 100 ml phosphate buffered saline (PBS). After the fascia and skin were sutured with a nylon thread, the animals were allowed to survive for 1, 3, 5, 7 and 14 days after surgery, and treated as follows.

Light microscopy including immunohistochemistry for type IV collagen
The rats were perfused through the heart with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4), and the implanted gelatin sheet with surrounding tissues was removed from the body, followed by immersion in the same fixative for 1 or 2 days. The samples were dehydrated in a graded series of ethanol, embedded in paraffin, sectioned serially at a 5-20 µm thickness, and stained with hematoxylin and eosin.

Some of the paraffin sections were deparaffinized, treated with a trypsin solution (Sigma, St Louis, USA) at a concentration of 1 mg for 1 ml of 0.05 M phosphate buffered saline (PBS) for 15 min, washed with PBS, and incubated in 10% normal goat serum for blocking non-specific reactions of secondary antibodies. Then the sections were reacted with a rabbit anti-human type IV collagen antibody (1:500 or 1000; LSL Co., Tokyo) in PBS with 2% bovine serum albumin (BSA) in a humidified chamber for either 2 h at room temperature or overnight at 4°C. The sections were then rinsed in PBS and immersed in a horseradish peroxidase conjugated goat anti-rabbit IgG antibody (1:100; Dako, Denmark) in 0.2% PBS-BSA solution. The sections were reacted with 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in PBS containing 0.006% H₂O₂ for visualization of the immunohistochemical reaction. They were dehydrated in a graded series of ethanol, transferred in xylene and mounted in Malinol (Muto Kagaku, Japan).

For immunohistochemistry of whole mount specimens, the implanted sheets were fixed with 4% paraformaldehyde overnight, rinsed briefly with PBS, and immersed in the anti-type IV collagen antibody solution for 2 h at room temperature. They were rinsed in PBS, immersed in the secondary antibody solution described above for 1 or 2 h and colored with DAB, dehydrated in a graded series of ethanol, transferred in xylene, and mounted on slides and in Malinol.

Scanning electron microscopy
One week after the implantation, the rats were perfused from the left ventricle with 2% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4). The implanted sheet taken from the body was immersed in the same fixative for more than 3 days, rinsed in a 0.1 M phosphate buffer for 10 min and treated with 30% KOH solution at 60°C for 8 min to dissolve connective tissue matrices around vessels. Some of the implanted sheets were additionally reacted with collagenase (type II Sigma, 1 µg/ml in 0.1 M phosphate buffer) for 6-8 h (Ushiwata and Ushiki, 1990; Hashizume et al., 1998; Higuchi et al., 2000).

After rinsing in a 0.1 M phosphate buffer overnight, the tissues were stained with 2% tannic acid and 1% osmium tetroxide, dehydrated with ethanol, transferred to isomyl acetate, and critical point dried in liquid CO₂. The dried specimens were put on aluminum stubs and dissected under a binocular to expose vessels of interest, coated with platinum-palladium in an ion coater, and observed in a scanning electron microscope (S-2380N, Hitachi) under an accelerating voltage of 10 or 15 kV.

RESULTS

Light microscopy
From 5 days to 1 week after implantation, the periphery of the implanted gelatin sheet was substituted with connective tissues which proliferated from the muscular layer (Fig. 1a, b). However, any invasion of connective tissues at the upper and lower side of the sheet was prevented by cellophane papers (Fig. 1a). Immunohistochemically, the basement membranes of the angiogenic vessels were clearly determined in the connective tissue by staining with the anti-type IV collagen antibody (Fig. 1c, d). The basement membranes of the larger vessels were often doubled, suggesting the presence of periendothelial cells (i.e., pericytes or smooth muscle cells) around the endothelial tube (Fig. 1d).

In whole mount specimens immunostained with anti-type IV collagen antibody, basement membranes of vessels were observed as tubes of various sizes...
which formed an extensive and irregular network in the implanted sheet (Fig. 2a). These vascular basement membrane tubes were 8–40 μm in diameter and usually single-walled, although they were sometimes split into two to form an oval or round space, probably for accommodating the cell body of pericytes (Fig. 2b). There were also tubes of basement membranes which tapered to slender and tortuous cords more than 50 μm in length; they ended freely or connected with large tubes of adjacent vessels (Fig. 2a, c).

**Scanning electron microscopy**

With a KOH-collagenase digestion method, all connective tissue matrices—except for a few elastic fibers—were dissolved and angiogenic blood vessels in the implanted sheet were successfully observed from the adventitial side. These newly formed vessels were 5–40 μm in diameter and took irregular and winding courses to form a widespread network (Fig. 3a). The vessels were usually densely covered with pericytes on their wall. These pericytes had oval or spherical cell bodies with a few slender processes (Fig. 3b), which further issued secondary and tertiary processes with irregular outlines, thereby surrounding densely endothelial tubes circularly or obliquely (Fig. 3b). Fibroblastic cells also associated with the vessel network (Fig. 3a), lacking any direct connection with pericytes and endothelial cells. These cells were apparently different from the pericytes because...
Fig. 2 a. A whole mount specimen of the gelatin sponge sheet stained with the anti-type IV collagen antibody. Tubular basement membranes of angiogenic vessels formed an extended network. Arrows indicate the sites where the basement membrane tubes tapered to a slender cord and arrowheads show the connecting points of the thin cords with an adjacent vessel wall. ×250. b. Closer view of the tubular basement membrane. Profiles of an oval shape are visible on the tube (arrowheads). ×500. c. Closer view of the tapering portion of the basement membrane tube, its thin branches ending freely. ×500

of their large size and flat shape (Fig. 3a, c).

In the network of newly formed vessels, tapering tips were occasionally observed. They were divided into two types from their morphology: the first type was composed of endothelial cells without a pericyte coverage (Fig. 4), and the second type was a pericyte covering a conical endothelial tube (Figs. 5, 6).

In the first type, a conical tip appeared to be produced as an extension of endothelial tubes in angiogenic vessels. Some of these tips extended vertically from the sidewall of the endothelial tube (Fig. 4a), while others were observed as a simple elongation of the tapering end of endothelial tubes (Fig. 4b). The extremity of these vessel tips was characterized by the presence of finger-like or fine tortuous microprojections extending irregularly (Fig. 4a, c). Junctions between endothelial cells were sometimes noticed near the extreme portion (Fig. 4c). Pericytes were often found at the base of the tip, but were not observed at the extreme portion of this type (Fig. 4b).
Fig. 3a. Scanning electron micrograph of an angiogenic vessel running a tortuous course. *Arrowheads* point newly formed vessels. Large flattened fibroblastic cells are associated with the vessel (*arrows*). ×700. b. A pericyte surrounding the endothelial tube of the angiogenic vessel. The pericyte has a relatively flat cell body (*P*) which extends irregularly branched processes. These processes are tape-like and tightly surround the endothelial tube. ×3,000. c. Closer view of fibroblastic cells (*F*) associated with the blood vessel. These cells differ in size and shape from the pericyte (*P*). ×1,700
**Fig. 4 a.** A blood vessel showing its conical tip with microprojections (*arrowhead*). ×3,000. **b.** A tapering tip of a blood vessel. The vessel is covered with a pericyte (*P*) at the base. The extreme portion of the vessel is indicated by *arrowheads*. ×2,000. **c.** A closer view of the tip indicated by the *arrowheads* in b. The extreme portion is composed of the endothelial cells with microprojections. *Arrowhead* shows a cleft between endothelial cells. ×3,500
The second type was a tip composed of the conical tip of the endothelial tube covered with pericytes (Figs. 5, 6). The pericyte processes were observed as flat tapes with somewhat irregular outlines, sometimes branching to secondary and tertiary projections that surrounded the conical endothelial tip (Fig. 5b). These processes often extended as one or two slender cords beyond the endothelial tip and ran independently from the endothelial tube to some extent (Fig. 5b). The pericyte processes beyond the endothelial tip occasionally connected with the adjacent vessel wall (Fig. 6).

DISCUSSION

The present study has documented the three-dimensional ultrastructure of angiogenic blood vessels in relation to the arrangement of such cellular components as endothelial cells and pericytes. Because the implanted gelatin is easily substituted by vascularized connective tissues without any severe inflammatory reaction (Thompson et al., 1988), a gelatin sponge sheet was used as an implant for inducing angiogenesis in the tissues. The addition of VEGF to the sponge sheet is useful for accelerating vessel formation (Ferrara et al., 1995). Using this
Fig. 6 a. A tapering vessel connected with an adjacent vessel wall (arrow). ×1,800. b. Closer view of a portion shown in a. The tapering site is composed of pericyte processes extending beyond the conical endothelial tip (arrowhead). A few of these processes connect with an adjacent vessel wall (arrow). ×7,000
Fig. 7. A schematic diagram showing the process of vessel sprouting (a) and regression (b) suggested in this study. a. Endothelial cell protrusion from a preexistent vessel is a first step of sprouting (A). The endothelial cells proliferate, become covered by pericytes, and form a tubular structure (B, C). The tip of the endothelial cells is exposed without any investment of pericytes (B). Some microprojections are present at the sprouting tip (A, B). b. Vessel regression proceeds with retraction of the endothelial tube (D), and is followed by that of the pericytes (E, F). A tapered basement membrane remains to cover this type of vascular tip (arrow).

method, we succeeded in consistently and efficiently observing newly formed vessels.

The most important finding documented in this study is the uncovering of two types of three-dimensional tip profiles of newly formed vessels in the angiogenic network. One is a tip composed of endothelial cells with or without tube formation, and the other is the tip of pericytic extension beyond the conical end of endothelial tubes. For the sake of convenience, we will refer to these two types as the first and second types, respectively. While previous reports showed one of these two types (BLOOD and ZETTER, 1990; NEHLS et al., 1992), we have proven for the first time the presence of both types in the angiogenic vessel network, and precisely documented the three-dimensional structure of these tips by SEM.

Spatial relationships between endothelial cells and pericytes at sprouting tips have been discussed together with the mechanism of vessel sprouting (CROCKER et al., 1970; BLOOD and ZETTER, 1990; NEHLS et al., 1992; MORIKAWA et al., 2002). Some researchers employing transmission electron microscopy (TEM) observed exposed endothelial cells at the sprouting site and considered that endothelial cell proliferation is followed by pericyte coverage that occurs at the end stage of vessel formation (CROCKER et al., 1970; AUSTRUNK and FOLKMAN, 1976; BLOOD and ZETTER, 1990). These previous findings and views probably belong to the first type in this study.

RHODIN and FUJITA (1989) also used TEM to analyze the structure of the vessel tip after they observed the sprouting directly by intra-vital video microscopy. According to their findings, sprouting is produced by endothelial cells without pericyte coverage, the shape of which is similar to the first type in the present study. Thus, the first type of tip is probably the true sprouting of vessels, which begins with endothelial cell projection followed by the pericyte coverage.

It is interesting that the endothelial cell had numerous microprojections at the extreme tip of the first type. A similar findings was reported by RHODIN and FUJITA (1989) in their TEM studies of the vessel tip after identification of vessel sprouting by video
microscopy. Microprojections in the sprouting tip have been also reported in tumors (AUSPRUNK and FOLKMAN, 1976) and granulation tissues (WAKUI, 1988). From these findings, it is probable that the presence of microporjections is a characteristic of the proliferating or immature endothelial cells. Figure 7a is a schematic drawing of the first type of vessel ends.

The second type has been observed mainly by immunohistochemistry and confocal laser scanning microscopy in the model of angiogenesis in tumors and inflammations (NEHLS et al., 1992; MORIKAWA et al., 2002), and in the rat tooth germ by TEM (TSUZUKI and SASA, 1994); these studies concluded that pericytes are involved in or induce endothelial cell proliferation and tube formation at the sprouting tips.

However, our findings have shown that the second type often had long and slender processes of pericytes which connected the tapering tip with an adjacent tubular vessel. This implies that the second type of tip might be a site of vessel regression, as some investigators noticed the occurrence of vessel regression during angiogenesis (FOLKMAN, 1984; HOLASH et al., 1999). Figure 7b shows the process of vessel regression which we propose in this study. This vessel regression begins with the regression of endothelial tubes followed by pericyte retraction and detachment. The slender cords of vascular basement membranes found by light microscopy were probably concerned with the covering of the second type of tip.

In the present study, we showed the occurrence of the fibroblastic flat cells around the angiogenic vessels. Previous researchers emphasized that these flat cells change their morphology to pericytes to cover the endothelial tube (CLARK and CLARK, 1925; NAKAYASU, 1988; RHODIN and FUJITA, 1989; NEHLS and DRENKHAHN, 1993). However, we did not find any transitional cells from fibroblasts to pericytes.

In conclusion, we have combined the use of light microscopic immunohistochemistry and SEM to demonstrate for the first time the three-dimensional structure of angiogenic vessels in relation to the vessel sprouting and regression tips. We propose that the vessel sprouting is produced by endothelial offshoots followed by pericyte coverage. Retraction of the endothelial tube, on the other hand, precedes pericyte regression in the process of vessel regression. We are currently investigating the time course of blood vessel formation and regression in living tissues.

REFERENCES


