Localization of Ang-1, -2, Tie-2, and VEGF expression at endothelial-pericyte interdigitation in rat angiogenesis

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Endothelial cells and pericytes play critical role in angiogenesis, which is controlled, in part, by the angiopoietin (Ang)/Tie-2 system and vascular endothelial growth factor (VEGF). Here, we investigated Ang, Tie-2, and VEGF expression within endothelial cells and pericyte interdigitations (EPI), which consist of cytoplasmic projections of pericytes and corresponding endothelial indentations. After subcutaneous implantation of a thermoreversible gelation polymer disc in rats, the capillary density was low on day 5, increased to a peak on day 7, and then decreased on days 10–20. A small number of EPI were observed on day 5, then increased sharply to a peak on day 10, but had decreased on day 20. Light and electron microscopy immunohistochemical and RNA *in situ* hybridization analyses revealed that Tie-2 localized at endothelial cells, and Ang-2 localized at endothelial cells and pericytes, while Ang-1 and VEGF localized at pericytes, and Ang-1 was most intensely observed at EPI of pericytes. Conventional quantitative RT-PCR and Western blot analyses revealed that the level of Ang-1 was low on days 5–7, then increased on days 10–20, while the level of VEGF was high on days 5–10, but had decreased on day 20. The level of Ang-2 remained high and Tie-2 remained at the level of the control on days 5–20. The present study showed that the angiogenic phase might be initiated by increases in Ang-2 and VEGF, while the microvessel maturation phase might be initiated by a relative increase in Ang-1 and a decrease in VEGF. Moreover, EPI might serve as a pathway for the Ang-1/Tie-2 system, with VEGF promoting pericyte recruitment for microvascular integrity.

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Angiogenesis, the growth of new vessels from pre-existing blood vessels, requires complex multistep signaling pathways and a high degree of spatial and temporal coordination among endothelial cells and pericytes. The precise mechanisms at the cellular and molecular levels are not completely defined.^{1,2} Despite their recognized importance in angiogenesis, pericytes have received less attention than endothelial cells, and their functions are only now beginning to be understood.^{3–7} Pericytes share the

basement membrane with endothelial cells and come into contact with them through holes in the basement membrane. Large numbers of ultrastructural endothelial cell and pericyte cytoplasmic interdigitations (EPI) are frequently demonstrated in angiogenic sites of human and murine tissues.^{8–15} These consist of a combination of pericyte cytoplasmic projections and corresponding indentations in the adjacent endothelial cells.^{8–15} In EPI, there is a distinctive gap measuring from 15 to 30 nm in width between endothelial cells and pericytes.^{8,9,14} While it has been suggested that EPI might be a mechanical anchoring system for the two cells,^{16–18} we have shown that the intercellular EPI space lacks adhesive glycoproteins, including fibronectin and laminin.^{9,11} Moreover, we have shown that plasmalemmal pits and vesicles are frequently present in the endothelial cells at EPI,⁹ and have demonstrated

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that epidermal growth factor,¹² transforming growth factor-beta, plasminogen, and urokinase plasminogen activator are present in the intercellular EPI space.¹⁹

The intercellular signaling mechanisms that govern the formation of blood vessels have only recently begun to be studied at the molecular level. Many growth factors are proposed to play a role in angiogenesis. Two types of vascular regulatory molecules that have been the subject of intense investigation in both physiological and pathological blood vessel generation are the angiopoietin (Ang)/Tie-2 system and vascular endothelial growth factor (VEGF).^{20–30} Tie-2 is one of the receptor tyrosine kinases that are expressed by vascular endothelial cells,^{21,31,32} and Ang-1 and -2, the ligands for Tie-2, have been reported to be important in the development of microvessels.^{33–35} Ang-1 induces the autophosphorylation of Tie-2 in cultured endothelial cells,²² and it has been proposed that Ang-1 recruits pericytes and potentiates microvessel stabilization and maturation by supporting interactions between endothelial cells and pericytes; however, the detailed biological character of pericytes is unclear.^{33–36} Ang-2 acts as an antagonist and inhibits Ang-1-induced phosphorylation of Tie-2 receptors in endothelial cells; as a result, Ang-2 initiates extensive angiogenesis resulting in pericyte drop-off and vascular destabilization.^{22–25,29,33,37–39}

The nature of cell–cell interactions such as EPI that occur during vessel formation and their mediators is difficult to discern from *in vivo* studies. Based on the distinct functions of Ang-1 and -2, we hypothesized that EPI are involved in the Ang/Tie2 system at angiogenic sites. In the present study, as an initial step toward the goal of understanding angiogenesis, we examined the formation of EPI, and the expression and localization of Ang-1, -2, Tie-2, and VEGF in rats subcutaneously implanted with a thermoreversible gelation polymer (TGP) disc, a three-dimensional culture medium gel⁴⁰ angiogenic disc model. The present study gives the first indication that Ang-1 may play a role in EPI by recruiting pericytes to support the primitive endothelial tubule and enhance microvessel maturation.

Materials and methods

Experimental Model

Forty male 7-week-old Sprague-Dawley (slc) rats (Japan SLC, Shizuoka, Japan) were housed, two per plastic cage, on hardwood-chip bedding in an environment-controlled room on a 12-h light/12-h dark cycle at $22 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ relative humidity with a conventional diet (MF, Oriental Yeast, Tokyo, Japan). All experimental procedures were conducted following approval of the Animal Care and Use Committee of the Azabu University School of Veterinary Medicine. Guidelines set by the National Institute of Health and Public Health Service Policy

on the Humane Use and Care of Laboratory Animals were followed at all times. We used TGP (Mebiol Inc., Kanagawa, Japan) without additional any growth factors for the experimental angiogenic model.⁴⁰ TGP is a thermoreversible hydrogel comprising a copolymer of poly(*N*-isopropylacrylamide) and poly(ethylene glycol).^{41,42} The sterilized TGP discs were cut into circles measuring 14 mm in diameter and 2 mm in thickness, and were implanted subcutaneously in the rat through a far away skin incision. The discs were removed from 10 rats each 5, 7, 10, and 20 days after implantation and examined.

Electron Microscopy

Specimens were fixed in 0.1 M phosphate-buffered 1.2% glutaraldehyde for 2 h and then postfixed in 1.0% osmium tetroxide for 2 h. After dehydration in graded alcohols, the specimens were embedded in Epon 812. Thin sections were cut on a Porter-Blum MT-II ultramicrotome and mounted on formvar-coated slit grids. After double staining with uranyl acetate and lead citrate, the sections were observed with a Hitachi H-500H electron microscope. In order to limit our studies to newly formed capillaries within each sample, only the centers of the discs were sampled. The morphometry of cross-sectioned vessels was analyzed, and those having a length-to-width ratio of less than two were used.¹⁴ Ten to 20 blood capillaries (not larger than $15\ \mu\text{m}$ diameter, following the description by Leeson *et al*)⁴³ were photographed randomly from each of 10 different specimen blocks from each disc. The number of EPI per capillary, the number of capillaries per $1\ \text{mm}^2$, the outer diameters of the capillaries, and the number of endothelial cells and pericytes per capillary on the electron micrographs were counted. The number of nuclei defined the endothelial cells and pericytes.

Immunohistochemistry for Light Microscopy

Immunohistochemical expressions of Ang-1, -2, Tie-2, and VEGF were analyzed using the avidin–biotin complex (ABC) method. After deparaffinization, $4\text{-}\mu\text{m}$ -thick sections were treated sequentially with 0.3% H_2O_2 for 10 min, then blocked with 10% goat serum or rabbit serum in PBS for 20 min. Frozen sections were thawed, rinsed in PBS, and treated with primary antibodies of Ang-1 (N-18) sc-6319 goat polyclonal IgG (Santa Cruz Biotech, CA, USA; diluted 1:500), Ang-2 (F-18) sc-7017 goat polyclonal IgG (Santa Cruz Biotech; diluted 1:500), Tie-2 (H-167) sc-9026 rabbit polyclonal IgG (Santa Cruz Biotech; diluted 1:500), or VEGF (147) sc-507 rabbit polyclonal IgG (Santa Cruz Biotech; diluted 1:500). Bound IgG was detected with biotinylated goat anti-goat IgG (Vector Lab., CA, USA; diluted 1:100) or anti-rabbit IgG (Vector Lab.; diluted 1:100), followed

by ABC-peroxidase (Vector Lab.) and diaminobenzidine (Sigma-Aldrich, MO, USA). Sections were then counterstained with hematoxylin. As a negative control, nonimmunized rabbit serum was substituted for the primary antibody.

Immunohistochemistry for Electron Microscopy

An indirect enzyme immunohistochemical of pre-embedded method was used.⁴⁴ Specimens were fixed in PLP solution⁴⁴ for 6 h, washed in graded sucrose solutions in 0.1 M phosphate-buffered saline, and frozen. Frozen sections were cut on a cryostat at a thickness of 6 μ m and mounted on PLL-coated glass slides. The sections were incubated at 4°C in a humidified chamber for 12 h with a primary antibody for Ang-1 (N-18) sc-6319 goat polyclonal IgG (Santa Cruz Biotech; diluted 1:100), Ang-2 (F-18) sc-7017 goat polyclonal IgG (Santa Cruz Biotech; diluted 1:100), Tie-2 (H-167) sc-9026 rabbit polyclonal IgG (Santa Cruz Biotech; diluted 1:200), or VEGF (147) sc-507 rabbit polyclonal IgG (Santa Cruz Biotech; diluted 1:200). Peroxidase-conjugated rabbit F(ab')₂ to goat IgG (ICN/Cappel Inc., OH, USA; diluted 1:100) or peroxidase-conjugated rabbit F(ab')₂ to rabbit IgG (ICN/Cappel Inc.; diluted 1:100) was used as the secondary antibody and incubated for 8 h. Then sections were immersed in Tris-HCl-buffered 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) containing 0.1 M sodium azide and 0.005% hydrogen peroxidase for 3–6 min. A control section was stained using rabbit or goat preimmune serum without the corresponding primary antibody and processed as described above. For electron microscopy, sections were postfixed with 1.0% osmium tetroxide for 1 h and embedded in Epon 812. Ultrathin sections were cut on a Porter-Blum ultramicrotome and were mounted on Formvar-coated slit grids. The sections were observed with a Hitachi H-2000 electron microscope without uranyl acetate and lead citrate staining.

RNA *In Situ* Hybridization

mRNA detection was used to prepare digoxigenin (DIG)-labeled antisense and sense probes. DIG-labeled sense probes were used as a negative control. Frozen sections were cut at a thickness of 10 μ m on a cryostat, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and permeabilized with 0.01 M sodium citrate and 0.2 M HCl for 30 min, phosphate-buffered 1% sodium dodecyl sulfate (SDS) for 5 min, 1 μ g/ml proteinase K for 30 min at 37°C, and acetylated with 0.1 M triethanolamine containing 0.25% (v/v) acetic anhydride. Sections were incubated in hybridization solution, consisting of 1 $\times$ Denhardt's solution, 2 $\times$ SSC, 10% dextran sulfate, 50% formamide, 50 μ g/ml yeast tRNA, 1 M DTT, and 1 mg/ml salmon sperm DNA for 16 h at 55°C. After hybridization, sections

were rinsed in 2 $\times$ SSC at room temperature, then sequentially washed in 2 $\times$ SSC/50% formamide at 55°C, rinsed in 2 $\times$ SSC at 37°C, washed twice in 2 $\times$ SSC/50% formamide at 55°C, and washed three times in 2 $\times$ SSC at room temperature. For signal amplification, a horseradish peroxidase-conjugated rabbit anti-digoxigenin antibody (Dako, CA, USA) was used to catalyze the deposition of biotinyltyramide, followed by a secondary streptavidin complex Gen Point kit (Dako). The final signal was visualized by incubation with DAB and 0.03% (v/v) hydrogen peroxide in 0.05 M Tris-HCl for 20 min, and the sections were counterstained in hematoxylin for 5 s. Antisense probes used for *in situ* hybridization were Ang-1 (5'-AGCATGGTGGCCGTGTGGTTTTGAACCGCATTCTGTTGTATCT-3'), Ang-2 (5'-CTTGTGCTCTGGTTTAGTACTTGGGCTTCCACATCAGTCAGTTTCCGAGTTTG-3'), Tie-2 (5'-GCAACATAATCAGAAACGCCAATAGCACCGTGATGCAAGTCATTCCAG-3'), and VEGF (5'-TGCGCTGGTAGACGTCATGAACCTTCACCACTTCATGGGCTTTCTGCT-3'); these antisense sequences were complementary to nucleotide mRNA specific to rat.⁴⁵ Probes were chemically synthesized by and DIG-labeled oligonucleotide was obtained from Sigma Genosys (Sigma-Aldrich).

RNA Isolation

Total RNA was extracted from frozen tissue using a QIA shredding homogenizer and a Qiagen Rneasy Mini Kit (Qiagen Inc., CA, USA). After isolation, the quality of RNA samples was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies Inc., CA, USA) and a RNA 6000 LabChip kit (Agilent Technologies Inc.).

Semiquantitative RT-PCR

cDNA synthesis was performed using random hexamers with the Super Script First-Strand Synthesis kit according to the instructions of the manufacturer (Invitrogen Co., CA, USA). Then, 1 μ l of the reverse transcription reaction was used for PCR amplification in a volume of 50 μ l containing gene-specific primers for Ang-1, Ang-2, VEGF, Tie-2, and GAPDH. Aliquots of the PCR reaction were removed at different cycles for agarose gel analysis to determine the linear range of amplification. All reactions were run on 2% agarose gel. The following primers were used: Ang-1 (GenBank AB080023) (fw: 5'-AGCATGTGATGGAAAATTATACT-3'), (rv: 5'-AGTACCTGGGTCTCCACATC-3'); Ang-2 (XM_344544) (fw: 5'-AATACATCCAGGACAACAT-3'), (rv: 5'-TATATTGTAGTTGTTTATTTCACTG-3'); VEGF (NM_031836) (fw: 5'-CTGCTCTCTTGGGTGCACT-3'), (rv: 5'-ATACACTATCTCATCGGGGTACT-3'); Tie-2 (AF030423) (fw: 5'-GCTGAGAACAAACATAGGAT-3'), (rv: 5'-CTGAGTTGAAGTGAACAGC-3'); and GAPDH (AF106860) (fw: 5'-CTCTACCCACGGCAAGTTC-3'), (rv: 5'-ACGATGC

CAAAGTTTCATG-3'). PCR was performed using a 9700 GeneAmp (Applied Biosystems, CA, USA) at 94°C for 5 min, followed by 28 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and finally 72°C for 7 min. PCR products were visualized on 2% agarose gels stained with ethidium bromide. Relative mRNA levels were compared with the corresponding levels of GAPDH.

Quantitative RT-PCR

Real-time RT-PCR reactions were carried out using the cDNA equivalent of 100 ng total RNA for each sample in a total volume of 25 μ l of TaqMan Universal PCR Master Mix (Applied Biosystems). The thermal cycling program for PCR was 50°C for 2 min, 95°C for 10 min, and 40 cycles of amplification comprising 95°C for 15 s, and 60°C for 1 min. PCR assay was performed using Assays on Demand Gene Expression Products in an ABI Prism 7700 sequence detector (Applied Biosystems). Gene expressions of Ang-2 (Rn01756774_m1), Tie-2 (Rn01433337_m1), and VEGF (Rn01511607_m1) (Applied Biosystems) were assayed. As a gene expression assay for Ang-1 has not been developed for Assays on Demand (Applied Biosystems), we applied a TaqMan rodent Ang-1 assay using the primers (fw: 5'-CCATGCTTGAGATAGGAACCAG-3'), (rv: 5'-TTCAAGTCGGGATGTTTGATTT-3').⁴⁶ The GAPDH primer was obtained from TaqMan rodent control reagents (Applied Biosystems). Amplification of GAPDH mRNA was performed as the internal gene control to account for variations in RNA levels between different samples. Data were collected and analyzed with Sequence detector v.1.7. The relative quantification method of $\Delta\Delta C_T$ was used as described in the manufacturer's manual (User Bulletin #2; P/N 4303859, pp 35).⁴⁷ Moreover, normal subcutaneous tissue was designated as the calibrator, and the normalized target gene values were compared to the calibration value according to the manufacturer's instructions for quantification of relative gene expression (User Bulletin #2; P/N 4303859, pp 15, 34).⁴⁸

Western Blot Analysis

Specimens were homogenized in 50 mM Tris-HCl, 150 mM KCl (pH 7.4), 1% Triton X-100, and 0.25 mM phenylmethylsulfonyl fluoride and centrifuged at 8000 *g* for 30 min at 4°C. The pellet was lysed with lysis buffer (10 mM Tris-HCl, 1% SDS, 1 mM EDTA, 10% glycerol, and 5% 2-mercaptoethanol). The protein concentrations of these lysates were quantified using 4 μ l in a Protein 200 Lab-chip kit (Agilent Technologies Inc.) and run on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc.). An equal amount of protein (10 μ g) from each lysate was resolved on 10% SDS polyacrylamide gels under denaturing conditions and then transferred

to Immuno-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad Lab., CA, USA). After overnight blocking by immersion in 5% non-fat dried milk in phosphate-buffered saline with 0.1% (v/v) Tween 20 (PBS-T), Western blot analysis was performed using antibodies to Ang-1 (N-18) sc-6319 goat polyclonal IgG (Santa Cruz Biotech; diluted 1:1000), Ang-2 (F-18) sc-7017 goat polyclonal IgG (Santa Cruz Biotech; diluted 1:1500), Tie-2 (H-167) sc-9026 rabbit polyclonal IgG (Santa Cruz Biotech; diluted 1:2000), VEGF (147) sc-507 rabbit polyclonal IgG (Santa Cruz Biotech; diluted 1:1000), and beta-actin polyclonal IgG (Santa Cruz Biotech; diluted 1:4000) in PBS-T and incubated 1 h at room temperature on an orbital shaker. After being washed three times with PBS-T, the membranes were incubated with a 1:2500 dilution of peroxidase-conjugated goat IgG (ICN/Cappel Inc.) or peroxidase-conjugated rabbit IgG (ICN/Cappel Inc.) for 1 h on an orbital shaker. After being washed three times with PBS-T, the membranes were detected using the ECL Plus Western Blotting Detection System (Applied Biosystems). To verify the relative amounts of protein in each lane, the levels of beta-actin were determined as an internal control.

Statistical Analysis

For each data set, the mean value, s.d., and s.e.m. were calculated and compared by Scheffé's *F*-test using the statistical analysis program Stat View-J 5.0 (Abacus Concepts, NC, USA).

Results

General Characteristics of Newly Formed Capillaries in TGP Discs

Microvascular growth in the TGP disc proceeded in a centripetal direction. The angiogenic process was preceded by fibrovascular granulation tissues, consisting of capillary endothelial cells, pericytes, fibroblasts, and vacuolated fibroblasts, and was almost free from migrating inflammatory cells (Figures 1 and 2).

On days 5–7, newly formed capillaries were morphologically composed of immature endothelial cells partially surrounded by pericytes (Figures 1 and 2). The cytoplasm of endothelial cells showed relative lucency with numerous free ribosomes, abundant intermediate filaments, a few rER, and a few mitochondria. The nuclei of the endothelial cells were large in comparison to the cytoplasm. These capillaries had relatively immature cell-to-cell junctions near the lumen that form tight or gap junctions. The basement membrane was discontinuous. On day 10, more developed capillaries showing premature morphology were predominant (Figures 1 and 2). These had relatively large

Figure 1 Light micrographs of the granulation tissues in subcutaneously implanted TGF discs. H&E stain. Bars indicate 50 μ m. (a) Day 5 after implantation: fibroblasts have migrated with a small number of newly formed capillaries. (b) Day 7 after implantation: many newly formed capillaries are observed with many fibroblasts. (c) Day 10 after implantation: many capillaries are observed. (d) Day 20 after implantation: many large capillaries are observed. Some vacuolated degenerating fibroblasts are also present.

endothelial cells containing a greater amount of rER, well-developed Golgi complexes, many ovoid or spherical mitochondria, and abundant plasmalemmal pits and vesicles. The basement membrane was discontinuous to continuous. Mature capillaries were observed on days 10–20 (Figures 1 and 2). These capillaries consisted of a relatively thin wall of endothelial cells, and Golgi apparatus and cytoplasmic vesicles had increased proportionately to the cell volume. Degenerated capillaries were also observed on day 20 (Figures 1 and 2). These capillaries consisted of quite thin and twisting walls of endothelial cells with decreased rER.

In the various developmental stages of the newly formed capillaries, many EPI were observed. An EPI is a combination of a pericyte cytoplasmic projection and a corresponding endothelial cytoplasmic indentation; the intercellular spaces of EPI lacked a basement membrane and plasmalemmal pits and vesicles located at the endothelial indentation cell membrane (Figures 1 and 2).

Quantification of EPI

The number of EPI per endothelial cell and pericyte was similar during the course of angiogenesis (Figure 3). The number of EPI per endothelial cell and pericyte was low on day 5, and comparable to those of normal subcutaneous tissue (control). The

number of EPI per endothelial cell then gradually increased in the following days to reach a peak on day 10, when it was significantly higher than that of control. The number of EPI per pericyte also significantly increased on days 5–10 compared to that of control and reached a peak on day 10. The maximum number of EPI per endothelial cell and pericyte was about two to three times that of control. The number of EPI per endothelial cell and pericyte subsequently decreased and was similar to that of control by day 20 (Figure 3).

Quantitative Study of Density and Size of Newly Formed Capillaries

The number of capillaries per 1 mm² was taken as the capillary density. The capillary density was low on day 5, but it was significantly higher than that of control (Figure 3). After day 5, the capillary density increased sharply to reach a peak on day 7. The maximum values of the capillary density were about four to five times that of control. Then, the density showed a significant decline on days 10–20. The capillary density on day 20 was similar to that of control, but the density on other days was significantly higher than that of the control (Figure 3). The outer diameter of the newly formed capillary was interpreted as the capillary size. Capillaries at days 5–7 were significantly smaller than those of control. Capillary size increased on days 10–20, and it was similar to that of control on day 20 (Figure 3).

Quantification of Endothelial Cells and Pericytes

The numbers of endothelial cells and pericytes per capillary cross-section were counted. The numbers changed significantly during the course of angiogenesis. The change in the number of endothelial cells during angiogenesis was found to be the inverse of the change in the number of pericytes (Figure 3). Endothelial cell numbers on days 5–7 were similar to those of control. The endothelial cell number had significantly decreased by day 10, when it increased and on day 20 was similar to those of control (Figure 3). The number of pericytes on days 5–7 was small and similar to those of control. By day 10, the pericyte number had significantly increased, while it had decreased by day 20 and was similar to those of control (Figure 3).

Localization of Ang-1, -2, Tie-2, and VEGF mRNA

RNA *in situ* hybridization analysis revealed Ang-2 mRNA on the endothelial cell- and pericyte-like capillary walls (Figure 4). Tie-2 mRNA was observed on the endothelial cell-like luminal side of capillaries composed cells (Figure 4), while Ang-1 mRNA and VEGF mRNA were observed at pericyte-like perivascular mural cells (Figure 4).

Figure 2 Electron micrographs of the newly formed capillaries in subcutaneously implanted TGF discs. E, endothelial cell; P, pericyte; L, capillary lumen; F, fibroblast; DF, vacuolated degenerated fibroblast; BM, basement membrane. **(a)** Granulation tissue on day 7 after implantation. Granulation tissue is composed of capillaries, fibroblasts, and vacuolated degenerating fibroblasts. A relatively mature capillary (arrow) composed of endothelial cells and pericytes that are surrounded by fibroblasts. A small, immature capillary is indicated by an arrowhead, and an enlargement is inserted in the lower right. Bar indicates 5 μ m. **(b)** Immature capillary observed on day 5 after implantation. A pericyte is partially surrounded by endothelial cells. Bar indicates 1 μ m. **(c)** A more-developed capillary observed on day 7 after implantation. Pericytes surround endothelial cells. EPI is indicated by an arrowhead, and an enlargement is inserted in the lower right. Bar indicates 1 μ m. **(d)** Relatively mature capillary observed on day 10 after implantation. Pericytes surround endothelial cells. Three EPI are indicated by arrowheads. Bar indicates 1 μ m. **(e)** Degenerated capillary observed on day 20 after implantation. EPI is indicated by an arrowhead. Bar indicates 5 μ m. **(f)** High-power view of EPI observed on day 10 after implantation. EPI (*) is composed of a cytoplasmic projection and corresponding endothelial indentation and the lack of a basement membrane (BM) between pericyte and endothelial cells. A plasmalemmal pit locates at the endothelial cell indentation (arrowhead). Bar indicates 12 nm.

Light and Electron Microscopy Immunohistochemical Analyses of Ang-1, -2, Tie-2, and VEGF Protein

To determine whether the localizations of Ang-1, -2, Tie-2, and VEGF mRNA correlated with the distribution of protein expression, immunohistochemical analysis was performed. Light and electron microscopy immunohistochemical analyses revealed that the expression of each protein was consistent with the pattern observed for each mRNA (Figures 5 and 6). Electron microscopy immunohistochemistry revealed that Ang-2 located at the cell membrane, plasmalemmal pits and vesicles, and cytoplasm of endothelial cells and pericytes of either immature or mature capillaries, and Tie-2 located at those of endothelial cells (Figure 6). Although Ang-1 located at pericytes in both pre-mature and mature capillaries, it was not observed in immature capillaries (Figure 6). Moreover, Ang-1 was observed to localize intensely at the pericyte

cytoplasmic projections of EPI and the cell membrane of the corresponding endothelial indentation including plasmalemmal pits and vesicles; localization was especially distinct on day 10 (Figure 6). On the other hand, VEGF was located at the cell membrane and cytoplasm of pericytes (Figure 6).

Ang-1, -2, Tie-2, and VEGF mRNA Expression in TGF Discs

The levels of Ang-1, -2, Tie-2, and VEGF mRNA expression were assessed by conventional and real-time quantitative RT-PCR. On days 5–7 after implantation, the expression of Ang-1 mRNA was significantly lower (0.4- to 0.5-fold) than that of control (Figure 7). On day 10, Ang-1 mRNA had increased significantly (33-fold), and it had decreased (16-fold) by day 20, but both were significantly higher than that of control (Figure 7). On days

Figure 3 Time course of changes in EPI number, capillary density, capillary diameter, and number of endothelial cells and pericytes in TGF after subcutaneous implantation. 'control' indicates capillaries of normal (control) subcutaneous tissue. Values represent mean $\pm$ s.e.m. EPI counts per endothelial cell (a) and pericyte (b) after implantation. The peak number of EPI is observed on day 10 after implantation (a, b). The density of newly formed capillaries per mm² after implantation shows a peak on day 7 after implantation (c). The outer diameter (μ m) of a newly formed capillary shows the peak on day 20 after implantation (d). Numbers of endothelial cells (e) and pericytes (f) after implantation. On day 10 after implantation, the number of endothelial cells decrease and that of pericytes increase (e, f). (**) Scheffé's F-test $P < 0.01$.

5–20 after implantation, the expression of Ang-2 mRNA maintained a significantly high level (12- to 15-fold) compared to that of control (Figure 8), and the expression of Tie-2 mRNA maintained a low

Figure 4 Hybridization of *in situ* Ang-1, -2, Tie-2, and VEGF mRNA in newly formed capillary in TGF disc on day 10 after subcutaneous implantation. Bars show 10 μ m. (a) Ang-2 mRNA signals are present at capillary luminal and perivascular mural cells. (b) Tie-2 mRNA signals are present at capillary luminal cells. (c) Ang-1 mRNA signals are present at perivascular mural cells. (d) VEGF mRNA signals are present at perivascular mural cells.

Figure 5 Immunohistochemical localization of Ang-1, -2, Tie-2, and VEGF in newly formed capillary in TGF disc on day 10 after subcutaneous implantation. Bars indicate 10 μ m. (a) Immunoreactive deposits of Ang-2 are present at endothelial cells and pericytes. (b) Immunoreactive deposits of Tie-2 are present at endothelial cells. (c) Immunoreactive deposits of Ang-1 are present at pericytes. (d) Immunoreactive deposits of VEGF are present at pericytes.

Figure 6 Immunoelectron micrographs of Ang-1, -2, Tie-2, and VEGF in newly formed capillaries in subcutaneously implanted TGF discs. E, endothelial cell; P, pericyte; L, capillary lumen. (a) Immunoelectron micrograph of Ang-2 on day 10 after implantation. Immunoreactive deposits are present in the cytoplasm of endothelial cells and pericytes. Bar indicates 2.5 μ m. (b) Immunoelectron micrograph of Tie-2 on day 10 after implantation. Immunoreactive deposits are present in the cytoplasm of endothelial cells. Bar indicates 3.3 μ m. (c) High power view of immunoelectron micrograph of Tie-2 on the vasolateral side of endothelial cells on day 10 after implantation. Some plasmalemmal pits and vesicles show dense immunoreactive deposits (arrowheads). Bar indicates 12 nm. (d) Immunoelectron micrograph of Ang-1 on day 7 after implantation. No immunoreactive deposits are observed at either pericytes or endothelial cells. Bar indicates 1.6 μ m. (e) Immunoelectron micrograph of Ang-1 on day 10 after implantation. Immunoreactive deposits are present in the cytoplasm of pericytes. Bar indicates 3.3 μ m. (f) Immunoelectron micrograph of Ang-1 on day 10 after implantation. Immunoreactive deposits are densely present in the pericyte cytoplasmic projection of EPI (arrowheads). Bar indicates 0.1 μ m. (g) High-power view of immunoelectron micrograph of Ang-1 at EPI. Immunoreactive deposits are observed at a pericyte cytoplasmic projection of EPI and cell membrane of the corresponding endothelial indentation (arrowhead). Bar indicates 12 nm. (h) Immunoelectron micrograph of VEGF on day 10 after implantation. Immunoreactive deposits are present in the cytoplasm of pericytes. Bar indicates 3.3 μ m.

level similar to that of control (Figure 9). On days 5–7 after implantation, the expression of VEGF mRNA was significantly higher (28- to 30-fold) than that of control,

and it reached a peak on day 10 (38-fold). The expression of VEGF mRNA had decreased sharply on day 20, when it was similar to that of control (Figure 10).

Figure 7 Time course of changes in Ang-1 mRNA expression in TGF after subcutaneous implantation. 'control' indicates capillaries of normal (control) subcutaneous tissue. (a) Semiquantitative PCR indicates high Ang-1 mRNA on days 10–20 after implantation. (b) Real-time PCR for Angi-1 mRNA. The expression level on days 10–20 is significantly higher than that on days 5–7, which is significantly lower than that of the control. Values represent mean $\pm$ s.d. (**) Scheffé's *F*-test $P < 0.01$.

Figure 9 Time course of changes in Tie-2 mRNA expression in TGF after subcutaneous implantation. 'control' indicates capillaries of normal (control) subcutaneous tissue. (a) Semiquantitative PCR showing that Tie-2 mRNA expression on days 5–20 after implantation is similar to that of control. (b) Real-time PCR for Tie-2 mRNA. The expression level on days 5–20 is similar to that of control values represent mean $\pm$ s.d.

Figure 8 Time course of changes in Ang-2 mRNA expression in TGF after subcutaneous implantation. 'control' indicates capillaries of normal (control) subcutaneous tissue. (a) Semiquantitative PCR indicates higher Ang-2 mRNA on days 5–20 after implantation compared to 'control'. (b) Real-time PCR for Angi-2 mRNA. The expression level on days 5–20 is significantly higher than that of control values represent mean $\pm$ s.d. (**) Scheffé's *F*-test $P < 0.01$.

Figure 10 Time course of changes in VEGF mRNA expression in TGF after subcutaneous implantation. 'Control' indicates capillaries of normal (control) subcutaneous tissue. (a) Semiquantitative PCR showing high VEGF mRNA expression on days 5–10 after implantation. (b) Real-time PCR for VEGF mRNA. The expression on days 5–10 is significantly higher than that of control, and that on day 20 is similar to that of control values represent mean $\pm$ s.d. (**) Scheffé's *F*-test $P < 0.01$.

Ang-1, -2, Tie-2, and VEGF Protein Expression in TGF Discs

To determine whether the mRNA modulation of Ang-1, -2, Tie-2, and VEGF correlated with changes in protein expression, Western blotting was performed. The protein expression was qualitatively

consistent with the patterns observed for each mRNA (Figure 11).

Discussion

Constitutive expression of Ang-2 by normal tissue stabilizes existing blood vessels^{38,39} because the ratio of Ang-1 to Ang-2 appears to control the switch to microvessel remodeling.^{49–51} Recently, it has been

Figure 11 Western blots of Ang-1, -2, Tie-2, and VEGF in TGF after subcutaneous implantation. (a) Shows representative Western blot bands, and (b) shows the density ratio to beta-actin; results were obtained by screening samples from five rats on each day. Values represent mean $\pm$ s.d.

reported that an equal amount of Ang-1 can rescue Ang-2-mediated effects, indicating that a balance between the two factors regulates the quiescence and responsiveness of microvessels.⁵² The present study shows that the angiogenic phase might be initiated by increases in Ang-2 and VEGF, while the microvessel maturation phase might be initiated by a relative increase in Ang-1 and a decrease in VEGF. It has been postulated that the angiogenic process of the present TGP *in vivo* angiogenic model depends on a change in the Ang-1 level. Further studies will be designed to test this hypothesis directly.

In this study, Tie-2 was observed to localize at the cell membrane, plasmalemmal pits and vesicles,

and cytoplasm of endothelial cells. These results agree with most of the literature published concerning this receptor system.^{53,54} Endothelial cells have been described as the primary source for Ang-2, suggesting that it might act as an autocrine regulator of endothelial cell functions,^{23,55–61} while *in vitro* data has suggested that Ang-2 expression increases in pericytes under hyperglycemic conditions.⁶¹ The present *in vivo* study revealed that Ang-2 localized at endothelial cells and pericytes of both immature and mature microvessels, while *in vitro* studies have produced evidence consistent with the hypothesis that Ang-1 is produced by pericytes and smooth muscle cells, and might act in a paracrine manner for Tie-2-expressing endothelial cells.^{22,23,55,57,62,63} Whether pericytes express Ang-1 *in vivo* has been hard to demonstrate, but cell sorting experiments suggest they do.⁶⁴ Moreover, a recent study using *in situ* hybridization demonstrated Ang-1 mRNA expression by perivascular cells during microvessel maturation, but the limited resolution of light microscopy presented made it difficult to distinguish pericytes.⁶⁵ We also observed Ang-1 mRNA expression by pericyte-like perivascular mural cells by light microscopic *in situ* hybridization, while the present study using electron microscopy immunohistochemistry clearly revealed that pericytes were the primary source of Ang-1. Moreover, recent studies have shown that pericytes are induced to express VEGF, which might contribute to development of the microvessel itself.^{7,64,66–70} In this study, it was clearly shown by electron microscopy immunohistochemistry that pericytes express VEGF.

EPI are sites of intimate contact between pericytes and endothelial cells by interdigitation.^{8–10} We have shown in the present study and a previous report that EPI are predominate following the active microvessel proliferation stage in the angiogenic process.¹⁴ Physical contact between pericytes and endothelial cells has been reported to be necessary to prevent angiogenesis with inhibition of endothelial cell proliferation.^{3,4,71,72} Further, we have demonstrated that EPIs acting as a pathway for the EGF/EGF-receptor system might activate pericytes for capillary maturation.^{12,13} We have also reported that EPI activation of latent TGF-beta might inhibit endothelial cell proliferation, and promote capillary maturation and degeneration.¹⁹ It has been reported that reduced expression of VEGF and enhanced expression of Ang-1 might contribute to the formation of leakage-resistant blood vessels.^{34,73} Recently, we demonstrated that the direct injection of an antibody neutralizing VEGF induces a significant decrease in the number of EPI and an increase in the number of nonleaky capillaries in an *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine-induced rat bladder carcinoma model.¹⁵ EPI have been difficult structures to study by light microscopy. The present study revealed that Ang-1 is expressed ultrastructurally by pericytes, especially at the pericyte cytoplasmic projections of EPI and cell membranes of the

corresponding endothelial indentation. As Ang-1 and Tie-2 appear to be required for communication of endothelial cells with pericytes, EPI might, in part, act as a pathway for the Ang-1/Tie-2 system to stabilize microvessels.

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