

Vascular endothelial growth factor reduces mural cell coverage of endothelial cells and induces sprouting rather than luminal division in an HT1080 tumour angiogenesis model

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Summary

Vascular endothelial growth factor (VEGF) plays a central role in tumour angiogenesis. In a mouse intramuscular tumour model using VEGF-transfected HT1080 human fibrosarcoma, we investigated the morphological features and patterns of remodelling in size-matched tumours. Compared with the control tumours (C group), the VEGF-transfected tumours (V group) showed vigorous neovascularization with larger vessels. Fenestrations and disruptions of endothelia were specific to the V group. Three types of vascular remodelling, i.e. sprouting, luminal division and intussusceptive microvascular growth, were present in both groups. Morphometric analyses revealed that mural cell coverage of the endothelial cells was significantly smaller in the V group compared with that in the C group (V group, $28.2 \pm 18.6\%$; C group, $41.6 \pm 21.1\%$; $P < 0.0001$). To determine the prevalence of remodelling patterns, the occurrences of abluminal and luminal processes on endothelial cell surfaces were quantified. Abluminal processes are defined as cytoplasmic protrusions of the abluminal membrane of endothelial cells, which can vary from tiny spurs to solid sprouts of the cell. On the other hand, luminal processes are defined as intraluminal protrusions of the endothelial cell membrane, including various membranous changes from filiform processes to rather thick cytoplasmic bulges. An abluminal process is thought to represent an initial morphological change in sprouting type angiogenesis, and a luminal process to be a sign of implementation of luminal division. The frequency of abluminal processes was significantly higher in the V group than in the C group (V group, $0.243 \pm 0.138/\mu\text{m}$; C group, $0.114 \pm 0.101/\mu\text{m}$; $P < 0.0001$). In contrast, the number of luminal processes on the endothelial cells per micrometre was statistically comparable between the groups (V group, $0.285 \pm 0.252/\mu\text{m}$; C group, $0.309 \pm 0.236/\mu\text{m}$, $P = 0.381$). These results indicate that sprouting is the main mode of VEGF-induced tumour angiogenesis.

Keywords

tumour angiogenesis, VEGF, remodelling, pericyte coverage, electron microscopy

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As angiogenesis plays a critical role in tumour growth, a precise understanding of the morphological features as well as the molecular mechanisms involved in tumour angiogenesis is necessary to develop better therapeutic strategies.

Morphologically, two aspects are of great importance in angiogenesis: (1) the contact between pericytes and endothelial cells and (2) the types of vascular remodelling, i.e. sprouting, intussusception and luminal division.

There have been many reports suggesting that pericytes play an important role in angiogenesis and vascular maturity. Withdrawal of pericytes occurs prior to endothelial cell proliferation in an adult rat cardiac and skeletal muscle angiogenesis model, suggesting that pericytes exert an inhibitory effect on endothelial cell proliferation (Egginton *et al.* 1996). Similarly, pericyte coverage of the vascular endothelium brings vessel maturation and marks the end of the plastic state of immature vessels (Benjamin *et al.* 1998).

Currently, three distinct models of vascular morphogenesis and remodelling have been recognized in various settings of angiogenesis. Sprouting is the most conventional type of angiogenesis and most frequently used to evaluate angiogenesis both *in vitro* and *in vivo*. Capillary sprouts originate as small endothelial spurs, which eventually grow to form anastomoses with other sprouts and create functional capillary loops (Rhodin & Fujita 1989). The other two modes of angiogenesis basically involve intraluminal splitting of the blood vessels that expand the blood flow by increasing the number of blood vessels *in situ*. Intussusceptive microvascular growth (IMG) refers to vascular remodelling by insertion of tissue pillars or interstitial tissue structures (ITSs) into the vascular lumen, resulting in partitioning of the vessel lumen (Caduff *et al.* 1986; Patan *et al.* 1996a). Despite having a similar remodelling pattern to IMG, luminal division is another distinct type of intraluminal remodelling in that this does not require interstitial tissue intrusion from outside the vessel wall at the early phase of implementation. Luminal division begins with small intraluminal processes or vacuolization of endothelial cells without any morphological change of the abluminal surface that grows to divide the vessel lumen into segments (Zhou *et al.* 1998).

There are several factors that affect the pattern of angiogenesis. For example, the polarity of mechanical stimuli to the endothelial cells, i.e. intraluminal or abluminal stimulation, determines which mode of angiogenesis occurs (Egginton *et al.* 2001). In relation to growth factors, little is known as to which type of angiogenesis is preferentially induced under a certain kind of growth factor regime.

Vascular endothelial growth factor (VEGF) is a potent modulator of vascular endothelial cells. There are a vast amount of data reported to date, showing that VEGF acts

as a vascular permeability factor as well as a mitogen and a survival factor of endothelial cells. Although it has been taken for granted that capillary sprouting is the prime mode of VEGF-induced angiogenesis, no such quantitative data have been published so far that compared the frequency of sprouting and other types of angiogenesis on the basis of the intensity of VEGF expression in the same tumour model.

Previously, we reported that VEGF promoted tumorigenicity and metastasis by inducing tumour angiogenesis in hepatocellular carcinoma and colorectal tumours (Mise *et al.* 1996; Ishigami *et al.* 2000; Kondo *et al.* 2000a) as well as in VEGF-overexpressing tumour cell models in mice (Mori *et al.* 1999; Kondo *et al.* 2000b). In the present study, focusing on the role of VEGF in the tumour angiogenesis, we evaluated the morphological interaction between endothelial cells and mural cells, and quantified the frequency of abluminal and luminal processes on the plasmalemmal membrane of endothelial cells that potentially lead to sprouting or luminal division in a VEGF-induced tumour angiogenesis model.

Methods

Cell culture and preparation

The human fibrosarcoma cell line HT1080 was transfected with pCAG-BSD-hVEGF₁₂₁ or pCAG-BSD to generate stable transfectants and was cultured as described previously (Mori *et al.* 1999). Briefly, the full-length hVEGF₁₂₁ cDNA (505 bp) was isolated from a human colon cancer specimen by reverse-transcriptase polymerase chain reaction. An eukaryotic expression vector pCAG-BSD was constructed from two parts of pCAGGS (Niwa *et al.* 1991) and pMAM2-BSD (Kaken, Tokyo, Japan). The hVEGF₁₂₁ cDNA was cloned into the XhoI restriction site of pCAG-BSD to create the plasmid pCAG-BSD-VEGF, in which the transcription of VEGF was constitutively driven by the CAG-enhancer promoter, and the drug-resistant selection gene *BSD* (Blasticidin S deaminase) was present (Kimura *et al.* 1994). HT1080 cells were transfected with the pCAG-BSD-hVEGF₁₂₁ or pCAG-BSD by Lipofectamine (Gibco-BRL, Gaithersburg, MD, USA) and the stable transfectants, HT1080-VEGF and HT1080-mock, were established. The expression levels of VEGF mRNA and the secreted VEGF protein were confirmed by Northern blot analysis and Western blot analysis, respectively. Of the nine clones of the HT1080-VEGF transfectant, the clone that showed the strongest expression of VEGF was labelled 'V cell', and was used as the VEGF transfectant in this study. Although both Northern blot and Western blot

analysis detected a certain amount of expression of VEGF in the HT1080-mock transfectant (labelled 'C cell'), the V cells produced additional amount of human VEGF both *in vitro* and *in vivo*. In this study, the exact values of the secreted VEGF by the tumour cells were not measured. The transfectants were cultured in minimum essential medium (MEM; Nissui, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (BioWhittaker, Walkersville, MD, USA), 100 units/ml penicillin, 100 µg/ml streptomycin and 10 µg/ml Blastidicin S (Kaken) in a humidified atmosphere of 5% CO₂. A subconfluent monolayer of each cell line was harvested with trypsin and washed twice in phosphate-buffered saline (PBS), and 1×10^7 cells were suspended in 0.2 ml of PBS for tumour injection.

Intramuscular tumour inoculation model

Five-week-old male BALB/cAnCrj-nu/nu mice were purchased from Charles River Japan (Yokohama, Japan). After 1 week of acclimatization, mice were given an intramuscular injection of the tumour cell suspension into the left hindlimb (six mice for the V group and five for the C group). The mice were sacrificed and the tumours were excised and prepared for further examination when they reached approximately 10 mm in diameter, which was 5–10 days after inoculation depending on the cell type. All *in vivo* experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto University.

Immunohistochemistry of tumour vessels

The peripheral regions of the tumours were cut into blocks and fixed in PBS containing 4% paraformaldehyde and processed for paraffin embedding. Sections of 5 µm thickness were cut and subjected to haematoxylin and eosin staining and immunohistochemistry. To visualize the tumour vessels, the sections were immunostained with antimouse CD34 monoclonal antibody (RAM34; PharMingen International, San Diego, CA, USA). In brief, the sections were deparaffinized and rehydrated in a descending series of ethanol. After antigen retrieval treatment by autoclaving in Target Retrieval solution (S1699, DAKO, Carpinteria, CA, USA) for 15 min and blocking of endogenous peroxidase with 3% hydrogen peroxide, the sections were incubated with rat antimouse CD34 monoclonal antibody (dilution; 1:50) for 1 h at room temperature. Following the incubation with the second antibody (E0468; biotinylated rabbit polyclonal antirat antibody; DAKO; dilution 1:250) for 20 min, the sections were reacted with streptavidin peroxidase (DAKO CSA System) for 20 min. Diaminobenzidine (DAB) was used as chromogen and the sections were counterstained with haematoxylin.

Transmission electron microscopy

The excised tumour was superfused with 2.5% glutaraldehyde in 0.1 M PBS. The peripheral regions of the tumour were sliced into blocks with faces of 1–2 mm² and fixed by immersion in the same fixative for 4 h at 4 °C. The blocks were then rinsed twice in PBS containing 0.2 M sucrose and postfixed in 1% osmium tetroxide, dehydrated in an ascending series of ethanol, cleared in propylene oxide and embedded in resin (Spurr Resin Embedding Kit, Taab, Berkshire, UK). At least 10 blocks per tumour were chosen at random, and semithin sections of 1 µm thickness were cut and stained with toluidine blue to lightmicroscopically orientate the tumour vessels in the block. The vascular-rich area of each block was further trimmed to ultrathin sections of 80-nm thickness and stained with uranyl acetate and lead citrate. The sections were observed with a transmission electron microscope (Hitachi, Ibaraki, Japan) at an accelerating voltage of 75 kV.

Morphometric quantitative analysis

Tumour microvessels with complete circumferences in cross-section that are smaller than 40 µm were photographed randomly by electron microscopy at a magnification of $\times 4000$ –12,000. On these photographs, three morphological aspects were quantified using a wire-measuring method. A wire was placed along the line to be measured, and the actual length was calculated in terms of the magnification. As it is not easy to identify cells in close apposition to a capillary as pericytes, those cells in close contact with endothelial cells are termed mural cells in this paper. Mural cell coverage was defined as the percentage of the abluminal circumference of the vessel covered by mural cells and calculated as (the length of the abluminal surface of the vessel directly covered by mural cells)/(the length of the abluminal circumference of the vessel) $\times 100$. For the purpose of clarifying the predominant type of angiogenesis occurring in the sample, abluminal processes and luminal processes on the endothelial cell plasma membrane were counted and the frequency of occurrence on the circumference were calculated. Abluminal processes were defined as cytoplasmic protrusions of abluminal membrane of the endothelial cell, which varied from tiny spurs to solid sprouts of the cell. On the other hand, luminal processes were defined as intraluminal protrusions of the endothelial cell membrane including various membranous changes from filiform processes to rather thick cytoplasmic bulges. Abluminal processes are considered to be an initial morphological change in sprouting type angiogenesis (Rhodin & Fujita 1989), while luminal processes are thought to be a sign of implementation of luminal division (Zhou *et al.* 1998).

Statistical analysis

All results are expressed as mean \pm standard deviation. Differences between groups were analysed by the unpaired Student's *t*-test or the χ^2 test. *P*-values <0.05 were considered significant.

Results

Light microscopic observation of tumour vessels

Haematoxylin and eosin staining and immunohistochemistry for CD34 revealed more vigorous and larger tumour vessels in the V group than in the C group. From a morphological point of view, it was noteworthy that 54% of the large tumour vessels in the V group (107/198 vessels of six tumours) featured tissue pillars or ITSs, which are landmarks of IMG, whereas in the C group, such structures were rarely seen, probably because of the lack of large vessels (Figure 1).

Transmission electron microscopic observation of tumour vessels

To further analyse the effects of VEGF on the morphological aspects of tumour angiogenesis, tumour vessels of each group were observed by transmission electron microscopy (TEM). The

tumour vessels of the peripheral region, especially in the VEGF-transfected tumours, varied in size and shape; however, the basic components of the microvessel, i.e. vascular endothelial cells, mural cells and basement membrane remained in place in both groups. A close observation of endothelial cells revealed plasmalemmal processes on the abluminal and luminal surfaces. Particularly in the V group, endothelial fenestrations were frequently observed. Most of the vessels with fenestrations had elongated and sometimes disrupted endothelia. Another specific feature of tumour vessels in the V group was the appearance of enlarged nuclei indicative of activated endothelial cells (Figure 2).

As for the types of angiogenesis, i.e. sprouting, luminal division and IMG, all three modes were observed in the tumours from both groups (Figure 3). As described in the light microscopic observation, the fact that IMG occurred only in large vessels hampered the comparison of the frequency of IMG between the two groups due to the scarcity of large tumour vessels in the C group. The length of abluminal circumference of the vessels analysed in this study was comparable between the groups (V group, $24.9 \pm 8.0 \mu\text{m}$; C group, $24.0 \pm 6.9 \mu\text{m}$, $P = 0.27$). In these small tumour vessels, endothelial fenestrations were present in 22.5% (40/178) of the vessels in the V group and 1.3% (2/153) in the C group ($P < 0.0001$). Mural cell coverage of the endothelial cells was significantly smaller in the VEGF-transfected tumours compared to that in the control tumours (V group, $28.2 \pm 18.6\%$; C group, $41.6 \pm 21.1\%$;

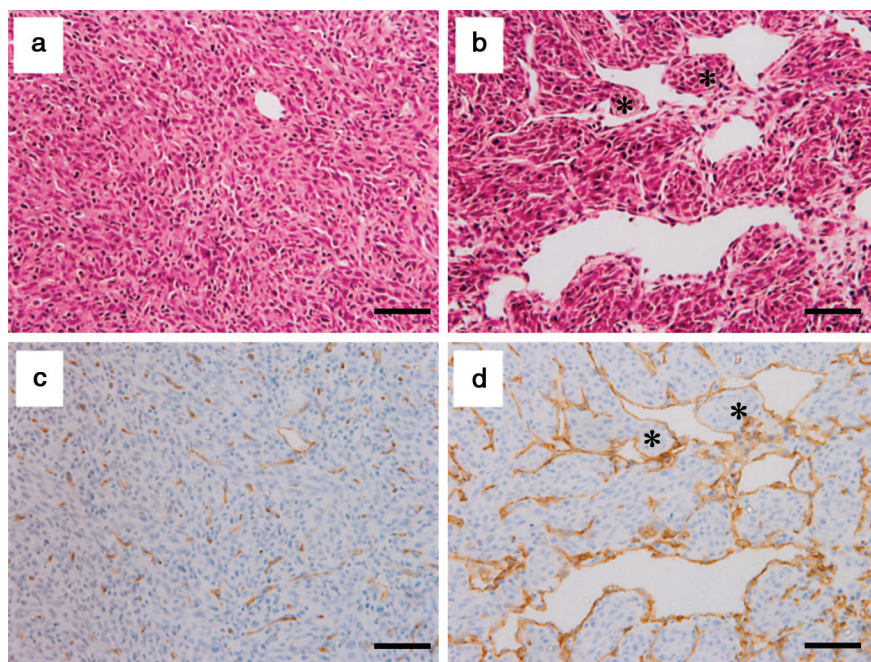
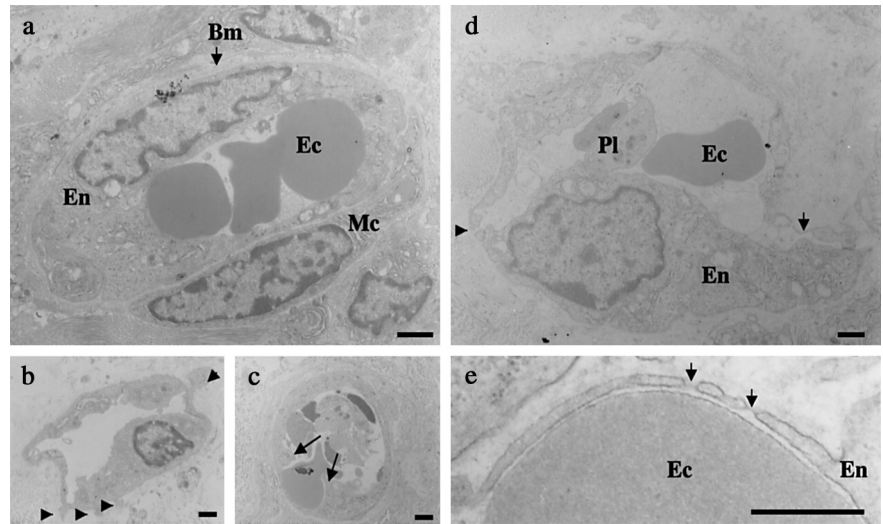


Figure 1 Tumour vessels in HT1080 intramuscular tumours from the vascular endothelial growth factor-transfected (V) group and the mock-transfected (C) group. The peripheral regions of the tumours were observed by haematoxylin and eosin staining (a, b) and immunohistochemistry with anti-CD34 monoclonal antibody (c, d). The tumours from the V group showed a more vigorous and intense vascularization than the control tumours. In addition to the appearance of large sinusoidal vessels in the V group, immunostaining for CD34 revealed fine networks of tumour vessels, reflecting perpetuating tumour angiogenesis (d). Notably, interstitial tissue structures suggestive of intussusceptive microvascular growth were frequently present in large sinusoidal vessels in the V group (asterisks in b and d). a, c: tumours from C group; b, d: tumours from V group. Scale bars: 50 μm .

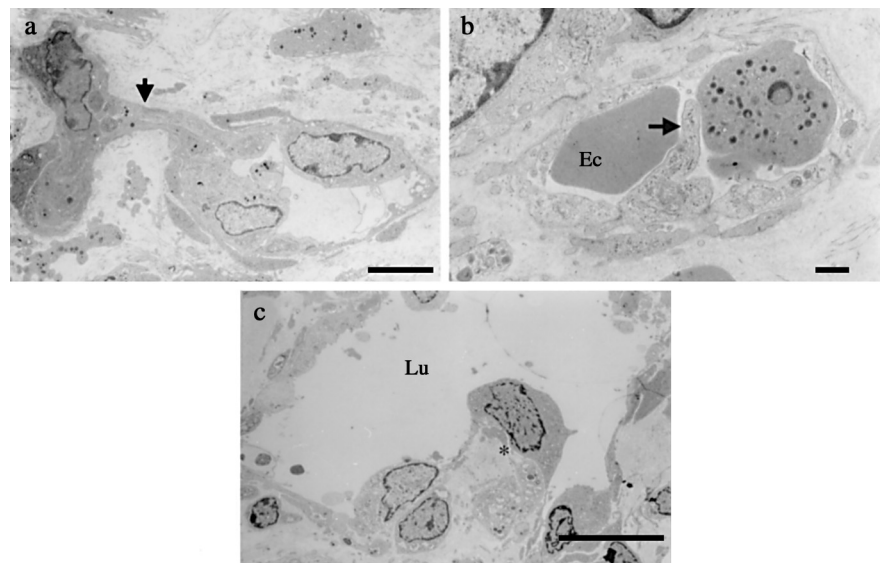
Figure 2 Transmission electron microphotographs of representative tumour microvessels. (a) Microvasculature from the control tumour showing intact vascular elements, i.e. endothelial cells, mural cells and basement membrane. (b) Abluminal processes emerging from a vascular endothelial growth factor (VEGF)-transfected tumour vessel (arrowheads). (c) Luminal processes originating from the inside wall of endothelial cells of a VEGF-transfected tumour vessel (arrows). (d) A characteristic microvessel observed in the VEGF-transfected tumour. Vascular endothelium was elongated with a plump nucleus, suggesting endothelial cell activation. Endothelial fenestrations (arrow) and a disruption (arrowhead) were specific to the VEGF-transfected tumours. (e) Higher magnification of the endothelial fenestrations with diaphragms (arrows). Bm, basement membrane; Ec, erythrocyte; En, endothelial cell; Mc, mural cell; Pl, platelet. Scale bars: 1 μm (a–d); 0.5 μm (e).



$P < 0.0001$). The number of abluminal processes on the endothelial cell surface per micrometre was significantly higher in the VEGF-transfected tumours than in the control tumours (V group, $0.243 \pm 0.138/\mu\text{m}$; C group; $0.114 \pm 0.101/\mu\text{m}$;

$P < 0.0001$). In contrast, the number of luminal processes on the luminal surface of endothelial cells per μm was comparable between the groups (V group, $0.285 \pm 0.252/\mu\text{m}$; C group, $0.309 \pm 0.236/\mu\text{m}$; $P = 0.381$) (Figure 4).

Figure 3 Three types of tumour angiogenesis observed in the vascular endothelial growth factor (VEGF)-transfected tumours. (a) Sprouting angiogenesis showing a sprout (arrow) with a slit-like lumen originating from two endothelial cells. (b) Luminal division of a tumour vessel with a luminal process (arrow) separating the vessel lumen. (c) Intussusceptive microvascular growth (IMG) was observed in large sinusoidal vessels in the VEGF-transfected tumours. The asterisk indicates interstitial tissue structures that are hallmarks of IMG. IMG was rarely seen in the control tumours probably because of the lack of large vessels. Ec, erythrocyte; Lu, lumen. Scale bars: 5 μm (a); 1 μm (b); 10 μm (c).



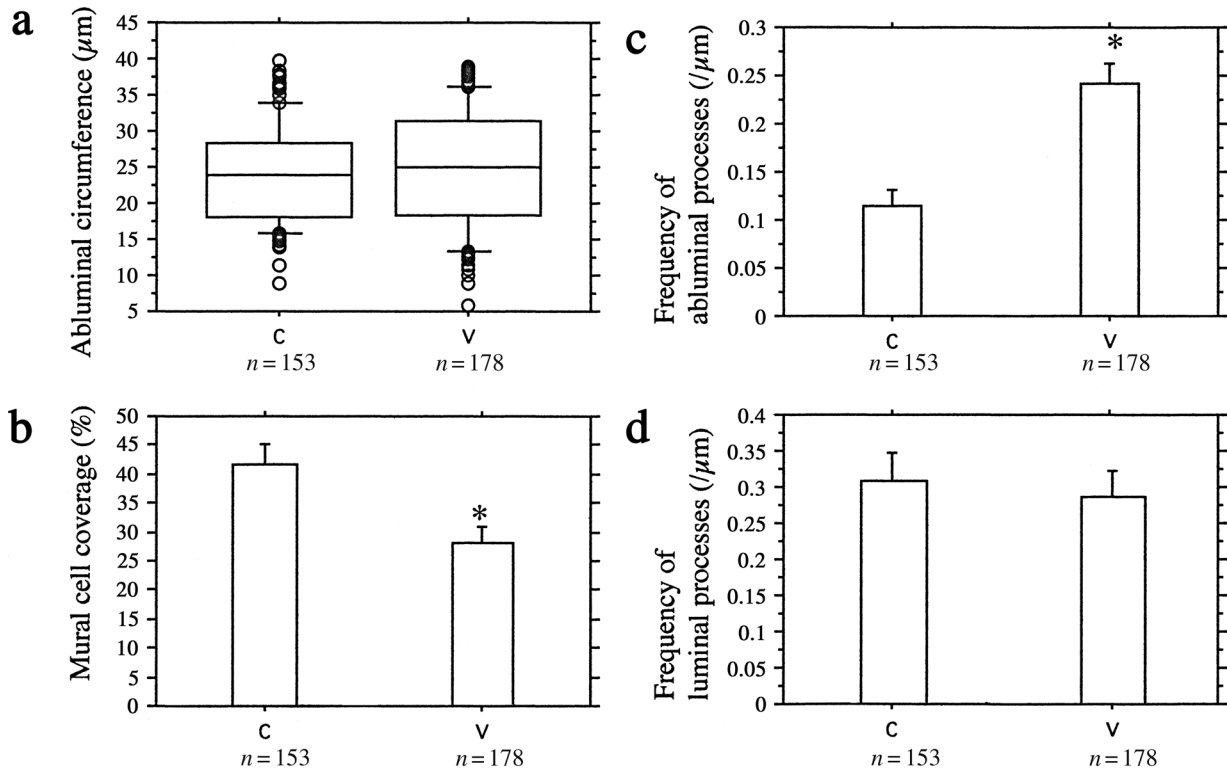


Figure 4 Quantitative analyses on the morphological changes in the tumour microvessels. (a) The length of abluminal circumference of the vessels analysed in this study was smaller than 40 µm, and there was no significant difference between the groups. (b) The percentage of mural cell coverage of endothelial cells was significantly lower in the vascular endothelial growth factor (VEGF)-transfected tumours than the control tumours. (c) The frequency of abluminal processes on endothelial cells per µm was significantly higher in the VEGF-transfected tumours than the control tumours. (d) The difference in the frequency of luminal processes was not significant. * $P < 0.0001$.

Discussion

Tumour vessels are highly heterogeneous, both morphologically and biologically within individual tumours as well as among different tumours (Paku & Lapis 1993; Eberhard *et al.* 2000). Our knowledge about the initiation of tumour neovascularization has become complex as many concepts, such as vasculogenic mimicry, vessel co-option and involvement of endothelial progenitor cells in solid tumour angiogenesis, have emerged over the last decade (Asahara *et al.* 1999; Holash *et al.* 1999; Maniotis *et al.* 1999). In addition, the coexistence of various stages of angiogenesis and different modes of remodelling in a single tumour makes analysis of tumour vasculature quite difficult, therefore only a few studies have so far presented definite quantitative data on the tumour vessel morphology. In the present study, to mitigate the complexity of tumour vasculature, we only investigated the peripheral tumour vasculature, which is less affected by factors such as hypoxia, interstitial pressure and metabolites that are often enhanced in the tumour centre (Wartenberg *et al.* 2001).

In addition, we sampled size-matched tumours around 10 mm in diameter where all kinds of remodelling are considered to be present and observed only small vessels whose perimeter in cross section was up to 40 µm. These observation limitations validated the morphometric and quantitative analyses on morphological changes and remodelling patterns in a VEGF-enhanced tumour angiogenesis model.

Basically, three essential components of the vascular walls, including endothelial cells, mural cells and the basement membrane, were found intact for the most part in both the VEGF-transfected and control tumours. Nevertheless, minor morphological differences existed between the groups. Plump nuclei of endothelial cells and enlarged vessel profile, for example, were frequently observed in the VEGF-transfected tumours. Among several morphological features induced by VEGF stimulation, induction of endothelial fenestration was very specific to the VEGF-transfected tumours, which is consistent with other reports (Roberts & Palade 1995, 1997; Esser *et al.* 1998; Feng *et al.* 1999a; Funyu *et al.* 2001; Chen *et al.* 2002). In this study, we observed that the fenestrae were

always located in extremely thin endothelia, and that occasionally the vessel walls were found to be disrupted. Attenuated endothelial walls induced by VEGF may facilitate the generation and maintenance of fenestration. Recently, it has been shown that VEGF-induced vascular permeability increase is attributed to the coalescence and reorganization of caveolae resulting in vesiculovacuolar organelles (VVOs), *trans*-endothelial cell pores and fenestrae that provide a route for macromolecular substances to translocate through the endothelial wall bidirectionally (Feng *et al.* 1999a, b; Chen *et al.* 2002). Together with our results, vascular permeability increase in VEGF-expressing tumours seems to be caused by vesicle-related structures such as fenestration and/or endothelial disruptions.

In the present study, we demonstrated the three distinct types of tumour angiogenesis, i.e. sprouting, luminal division and IMG, in an intramuscular tumour model of HT1080 in nude mice. Furthermore, we investigated the ultrastructural changes in relation to tumour angiogenesis and clarified by comparing VEGF transfectants with control tumours that VEGF induces capillary sprouting rather than luminal division in small vessels. In addition, we found that the mural cell coverage in the VEGF-transfected tumours was lower than that in the control tumours, which also supports the hypothesis that VEGF facilitates sprouting.

Alternative splicing of human VEGF mRNA gives rise to at least six isoforms consisting of 121, 145, 165, 183, 189 and 206 amino acid residues (Zhang *et al.* 2000). With exception of VEGF₁₂₁, these isoforms bind to heparin or heparan sulphate proteoglycan, which confers onto VEGF₁₂₁ its most diffusible characteristic (Küstners *et al.* 2003). Many clinical studies have demonstrated that in various cancers, VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉ are the main isoforms and that the expression and function of these isoforms vary substantially. High expression levels of VEGF₁₂₁ mRNA reportedly are associated with some clinical features such as short survival, early relapse and high intratumoural microvessel density in renal cell carcinoma, non-small-cell lung carcinoma and breast carcinoma (Yuan *et al.* 2001; Kim *et al.* 2002; Ljungberg *et al.* 2003). In osteosarcoma, on the other hand, VEGF₁₆₅ and VEGF₁₈₉, but not VEGF₁₂₁, were found to be essential for neovascularization (Lee *et al.* 1999). Likewise, some contradictory findings have been made by animal experiments regarding the functional difference of the isoforms. VEGF₁₂₁-transfected MCF-7 breast carcinoma cells were more angiogenic and tumorigenic than were VEGF₁₆₅ and VEGF₁₈₉ transfectants (Zhang *et al.* 2000), while a melanoma-transfectant model VEGF₁₆₅ or VEGF₁₈₉ expression induced intratumoural neovascularization, but VEGF₁₂₁ did not (Küstners *et al.* 2003). It was found that in a VEGF-deficient oncogenically transformed

fibroblast cell line, only VEGF₁₆₄ transfection (the murine version of VEGF₁₆₅) could fully rescue tumour growth and neovascularization. In this model, VEGF₁₂₀ (the murine version of VEGF₁₂₁) induced peripheral neovascularization due to the diffuse signal perfusion (Grunstein *et al.* 2000). In our study, we used a VEGF₁₂₁-transfected tumour model. The wild-type and mock-transfected HT1080 cells express endogenous VEGF₁₂₁ and VEGF₁₆₅ (Mori *et al.* 1999). Thus, the significance of this experimental model is to specify the tumour angiogenesis when VEGF₁₂₁ is highly enhanced and co-ordinates with VEGF₁₆₅. On the basis of these reported findings, we speculate that the biological function of VEGF *in vivo* is the result of co-ordination of various VEGF isoforms, leading us to believe that this model represents one aspect of actual tumour angiogenesis.

Although a number of *in vitro* studies have clearly demonstrated that VEGF induces angiogenic sprouting (Helmlinger *et al.* 2000; Korff *et al.* 2001), there has been only a few reports so far that have evaluated sprouting with respect to VEGF expression in tumours. Likewise, only a few studies have addressed the relationship between growth factors and patterns of vascular remodelling, e.g. VEGF and IMG (Wilting *et al.* 1996), Angiopoietin-1/TIE2 and IMG (Patan 1998). On the other hand, as for physical stimulation to the vessel endothelia, luminal division is induced by shear stress, whereas capillary sprouting is caused by abluminal stress in a physiological angiogenesis model in rats (Egginton *et al.* 2001).

In the present study, we provided evidence that VEGF generated by tumour cells induces microvascular sprouting rather than luminal division *in vivo*. In addition, although the frequency of IMG could not be compared between the groups due to the scarcity of large vessels in the control tumours, quite a few ITSs were observed in large vessels in the VEGF transfectants, suggesting that VEGF plays an important role in IMG as well as in sprouting. IMG, indeed, is a type of angiogenic remodelling that eventually separates a vessel lumen, thus resembling luminal division. However, quintessentially, the cells most involved in IMG especially in the early phase of remodelling are mesenchymal cells that lie abluminal to vessels (Burri & Tarek 1990; Patan *et al.* 1996a). Therefore, luminal division and IMG are quite different in the process, and IMG could be characterized, in a sense, as an abluminal type of angiogenesis that has an inward directionality. As observed in the present study, sprouting and IMG could be the main processes of tumour angiogenesis. In fact, the coexistence of IMG and sprouting has been reported in an LS174T human colon adenocarcinoma model (Patan *et al.* 1996b, 2001a). In the article, the authors observed that vascular growth occurred by both IMG and sprouting at the leading edge of the tumour.

On the other hand, luminal division in tumour angiogenesis has been documented in a couple of papers in which luminal division is termed as transluminal endothelial bridging or vessel segmentation (Nagy *et al.* 1995; Patan *et al.* 2001a). As tumour-derived VEGF does not induce luminal division, it may be induced by other growth factors or shear stress generated by increased blood flow in tumours.

The interaction between vascular endothelial cells and mural cells has been a matter of interest for several decades (Shepro & Morel 1993). In physiological models of adult rat cardiac and skeletal muscle angiogenesis, the pericyte coverage area of vascular endothelia was reduced by physical angiogenic stimuli (Egginton *et al.* 1996). On the other hand, the degree of pericyte recruitment in a variety of human tumours appears to vary substantially (Eberhard *et al.* 2000). In the current study, the overexpression of VEGF caused a significant reduction of the mural cell coverage. There are two plausible explanations for this phenomenon. First, VEGF serves as a survival factor for endothelial cells (Reinmuth *et al.* 2001), therefore more endothelial cells could survive without pericyte coverage in the VEGF-transfected tumours, leading to a higher population of endothelial cells with a relatively smaller degree of pericyte coverage. Second, VEGF stimulated endothelial cells to upregulate Angiopoietin-2, resulting in the separation of pericytes from the vessel walls. However, in an *in vitro* experiment using cocultured spheroids of endothelial cells and smooth muscle cells, exogenous administration of VEGF did not affect the relative levels of Angiopoietin-1 and Angiopoietin-2 of the endothelial cells (Korff *et al.* 2001). Taken together, although the mechanism of VEGF-induced reduction of mural cell coverage still remains to be elucidated, it is most likely that abundant VEGF in concert with the reduction of pericyte coverage accelerated the proliferation of endothelial cells and contributed to the formation of capillary sprouting.

We reported previously that the VEGF-transfected tumours grew faster than the wild type in a subcutaneous tumour model (Mori *et al.* 1999). In that report, it was speculated that the growth advantage of the VEGF transfectants was attributed to the enhanced tumour vascularity as well as the suppression of apoptosis in the tumour cells. Also, in the current study, we observed the VEGF transfectants grew faster in the intramuscular tumour model, even though the number of inoculated cells and the injection site were different from those in the subcutaneous model. We speculate that the same mechanism is at work in this model.

The tumours analysed in this study were sampled on day 5 for the V group, and on day 10 for the C group to obtain samples from tumours of an appropriate size. It is therefore possible that the differences in the morphological features between the groups were the result of the difference in the

times at which the tumours were sampled. However, additional studies comparing day 5 and day 10 tumours from both groups showed that the findings were very similar for the two time points (data not shown). Hence, we conclude that the data in our study were not affected by the time factor.

In conclusion, this study demonstrated the morphological characteristics of VEGF-induced tumour angiogenesis. Most importantly, we showed the three types of remodelling in a single tumour and presented clear evidence that VEGF promotes sprouting rather than luminal division and reduces the mural cell coverage. The vast majority of cancers express VEGF (Kerbel 2000). Moreover, we reported that the VEGF expression in tumours promotes tumour progression and metastasis by enhancing angiogenesis, leading to poor survival in many clinical settings as well as in animal experiments (Mise *et al.* 1996; Ishigami *et al.* 2000; Mori *et al.* 1999; Kondo *et al.* 2000a, b). We therefore believe that the findings presented here provide a rationale for anti-angiogenic cancer therapies that inhibit tumour vessel sprouting by blocking the degradation of the extracellular matrix or the withdrawal of pericytes.

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