Effect of hypoxia upon the growth and sprouting activity of cultured aortic endothelium from the rat

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Summary

Endothelial cells were obtained from the aortas of Wistar rats by collagenase digestion. Cells were grown to confluence in medium 199 enriched with L-glutamine but without specific growth factors. Cells were subcultured into 35 mm dishes or 25 cm² flasks coated with fibronectin. For cell growth studies, cells were seeded onto multiwell plates or 35 mm dishes. In two experiments the cells were grown in an hypoxic atmosphere of 5-3 % O₂ and in a third the level of oxygen was 2-5 %. Control cultures for each experiment were grown in 5 % CO₂ and air. Cell populations were counted at 2-day intervals and at the termination of each experiment the cells were fixed and the area of each plate or flask occupied by sprouting cells was assessed by point counting.

Endothelial cells grown in 5-3 % O₂ grew more rapidly and attained confluence earlier than in the controls. An atmosphere of 2-5 % O₂ did not accelerate growth but neither did it inhibit it, so after 9 days there were as many hypoxic cells as there were controls. Hypoxia also stimulated sprouting activity to occur earlier and to become much more extensive than in control cultures. Under the influence of hypoxia, sprouting consisted of complex anastomotic or arborizing patterns forming syncytium-like masses beneath the monolayer of oval cells. This process appeared to originate from foci of altered endothelial cells that had become retracted, smaller, elongated and migratory, and which displayed increased immunoreactivity for factor VIII antigen. It was concluded that a level of hypoxia, similar to that in systemic veins, stimulates growth of arterial endothelium and provokes enhanced sprouting activity. This behaviour may be relevant to the process of vascularization of wounds or neoplasms.

Key words: tissue culture, endothelium, hypoxia, sprouting, rat.

Introduction

It has been recognized for the last 10 years that confluent cultures of endothelial cells frequently undergo a curious transformation with the appearance of elongated cells amongst the more usual polygonal ones. These elongated cells extend over the surface or underneath the monolayer and join up with one another to produce a network resembling a mycelium. This process has been referred to as 'sprouting' (Schwartz, 1978) and the term 'endothelial sprouts' is now commonly applied to these variant cells. Sprouting activity has been described most commonly in cultures of bovine aorta (Schwartz, 1978; Cotta-Pereira et al. 1980; Duthu & Smith, 1980; McAuslan et al. 1980, 1982; Delvos et al. 1982; Feder et al. 1983; Schor et al. 1983) but it also occurs in endothelial cells from bovine capillaries (Folkman & Haudenschild, 1980; Montesano et al. 1983), human umbilical vein (Maciag et al. 1982; Montesano & Orci, 1987), human capillaries (Folkman & Haudenschild, 1980) and capillaries from the rat (Madri et al. 1983). In some instances endothelial sprouts form tubular structures closely resembling capillaries (Folkman & Haudenschild, 1980; Maciag et al. 1982; Feder et al. 1983; Madri et al. 1983; Montesano et al. 1983; Schor et al. 1983; Montesano & Orci, 1987) and they are now generally regarded as representing a process of vascularization. A knowledge of the factors that provoke sprouting may, therefore, help our understanding of the process of vascularization of wounds or neoplasms.

During preliminary studies of agents that influence the growth and shape of endothelial cells from major vessels of the rat grown in vitro we noticed that hypoxia apparently accelerated their growth and caused sprouting activity earlier than was anticipated. This paper describes three experiments designed to investigate this phenomenon quantitatively. Aortic endothelium was used because in vitro it experiences the oxygen-rich environment of arterial blood. It was subjected to hypoxic conditions similar to those in systemic veins.
Materials and methods

Endothelial cells were harvested from the aortas of male Wistar albino rats by a modification of the technique of Jaffe et al. (1973). Briefly, a length of the aorta from the descending portion of the arch to the origin of the left renal artery was removed and stripped of excess fat, and the proximal end was tied to a fine plastic cannula. It was flushed out with phosphate-buffered saline (PBS) and then filled with 0.2% crude collagenase. The distal end of the vessel was ligated and the whole assembly was transferred to a sterile test tube and incubated at 37°C for 30 min. The aorta was re-filled with collagenase through the cannula at 5 min intervals to replace fluid that oozed through the intercostal arteries. After incubation, the aorta was gently kneaded with fine forceps and vigorously flushed out with 5 ml Earle's balanced salt solution (EBS) into a conical centrifuge tube. Following gentle centrifugation the cells were washed with EBS, recentrifuged and the supernatant was replaced with 3 ml of complete medium. Cells were resuspended by gentle pipetting and plated onto 35 mm tissue culture dishes previously coated with bovine fibronectin (30 μg/ml). All cultures were fed thrice weekly with medium 199 and Hepes buffer at a pH of 7.4 supplemented with 20% foetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 2 mM glutamine. We have found previously that endothelial cell growth supplementation is not required for adequate growth of rat endothelium. Primary cultures were incubated at 37°C in 5% CO₂ in a humid atmosphere until confluence was reached, approximately 3 weeks after initial plating. The cells were then subcultured for the experimental procedures described below. All media and chemicals were obtained from Sigma Chemical Co., and plastic tissue-culture dishes and flasks from Corning glass works.

Experiment A

Two primary cultures of rat aortic endothelium were subcultured by trypsin/EDTA digestion into 12 35-mm fibronectin-coated dishes at a density of 0.9 x 10⁶ cells cm⁻². Six of these dishes were designated as controls and incubated in a conventional CO₂ incubator in which the level of carbon dioxide was 5% and of oxygen 19.6%. This concentration of oxygen is not the same as that in arterial blood at between 13 and 14%, but it is the same as that normally employed for growing endothelium from all locations. It was chosen for that reason. The remaining six plates comprised the hypoxic group and were placed in a Queue 2711 incubator in which the levels of both oxygen and CO₂ could be regulated. After allowing the cells to settle for 2h the gases in the incubator were changed to 5.3% oxygen. Group 1 comprised cells that had had no previous exposure to hypoxia but were now grown in 2.5% oxygen. Group 3 comprised cells that had previously been grown in hypoxia and were now grown in 2.5% oxygen. Group 4 comprised cells that had been previously grown in hypoxia and were now grown in 19.6% oxygen. By this means not only could the effect of severe hypoxia on the morphology of endothelial cells be studied, but also the effects of any legacy of previous hypoxia on this subsequent hypoxic episode could be determined.

Primary cultures from two different aortas from those used in experiment A were grown to confluence for 3 weeks. Both were then subcultured, each into 25 cm² tissue-culture flasks coated with fibronectin. These first-passage cultures were nearly confluent by 1 week. One of them was split into four similar flasks. Two of these were grown in the same hypoxic gas mixture as used above, the other two were gassed with a 5% CO₂ and air mixture, their stoppers were screwed on tightly and the flasks were placed in a separate incubator in ambient air.

Another flask was subcultured into two multiwell plates with 24 wells per plate. Each well received 0.7 x 10⁶ cells cm⁻². One plate was put into the hypoxic incubator, the other into a standard incubator with a humidified atmosphere containing 5% CO₂. Each day two wells from each plate were trypsinized to remove all the cells. These were centrifuged and the supernatant was replaced with 1 ml of complete medium. The cells were then counted using a haemocytometer slide. Four separate counts were performed on each cell suspension and the average count of the two wells for the hypoxic and control plates was calculated. Cell counts were repeated using 32 tissue-culture flasks that had been seeded with aortic endothelial cells in their fifth passage at a density of 0.4 x 10⁶ cells cm⁻². These were equally divided into hypoxic and control groups and treated as described above. On approximately alternate days, two flasks from each group were trypsinized and 10 separate counts of the cell population were made on each flask. Results were expressed as cells cm⁻².

Tissue-culture flasks were examined each day and the morphology of the endothelium was recorded. After 12 days the experiment was terminated and the cells from one hypoxic and one control flask were fixed with 3% buffered glutaraldehyde. The two remaining flasks were retained for the subsequent experiment.

Experiment C

In this experiment one flask containing cells that had been grown in hypoxia and one control flask from experiment B were used. Both of these were subcultured into two flasks, each at a split ratio of 1:4. These attained confluence by 5 days and were then subcultured into four more flasks to produce a fourth passage.

Endothelial cells derived from one of the control flasks were also seeded into eight 35-mm cell culture dishes at a density of 0.9 x 10⁶ cells cm⁻² for estimates of cellular growth. Both the flasks and the plates had been coated with bovine fibronectin.

Half of the flasks, with loosely attached lids, and half of the dishes were put into the Queue incubator and the oxygen level was reduced to 5.3% for 24h. Thereafter, the concentration of oxygen was reduced further to 2.5%. The concentration of CO₂ was 6.1% and the atmosphere was humidified. The oxygen level was monitored daily with an oxygen meter to check for any deviation from that selected on the incubator.

The remaining flasks were gassed with a 5% CO₂/air mixture, their lids were tightened and then they, and four dishes, were placed in a standard CO₂ incubator. As a result of this treatment there were four groups of flasks as follows. Group 1 comprised cells that had had no previous exposure to hypoxia and were grown in 19.6% oxygen. Group 2 comprised cells that had been previously grown in hypoxia but were now grown in 2.5% oxygen. Group 3 comprised cells that had been previously grown in hypoxia and were now grown in 2.5% oxygen.

This means not only could the effect of severe hypoxia on the morphology of endothelial cells be studied, but also the effects of any legacy of previous hypoxia on this subsequent hypoxic episode could be determined.

The incubators were opened three times weekly to feed the cultures and to record their morphology. One dish from each of the hypoxic and normoxic incubators was removed at this time to count the total number of cells as described in experiment B. The experiment was terminated after 9 days when all but two flasks were irrigated with 3% glutaraldehyde to fix the cells.
Morphometry
All the dishes from experiment A and flasks of fixed cells from experiments B and C were point counted. They were examined with a phase-contrast inverted microscope fitted with an eyepiece graticule containing 58 regularly spaced points. All points coinciding with endothelial sprouts or with cell bodies giving rise to sprouts were recorded. Fields were selected 'blindly' using a systematic random sampling system encompassing all of the area of the flask. Between 40 and 100 fields were counted in each flask using the ×20 objective. The number of points coinciding with sprouting cells was expressed as a percentage of the total number of points in order to derive an estimate of the proportion of the cultures occupied by endothelial sprouts.

Immunocytochemistry
Following point counting, representative flasks were stained for factor VIII by an immunocytochemical technique described by Goetz et al. (1985). Briefly, the cells were treated with a mixture of methanol and hydrogen peroxide for 15 min to block endogenous peroxidases and then washed with PBS and incubated with 0.1% normal swine serum and 0.1% Triton X-100 in PBS for 15 min. They were then incubated for 1 h with a rabbit antiserum to human factor VIII diluted 1:75 (Dako), containing 0.1% Triton X-100 and 0.1% normal swine serum. Normal swine serum was used for control incubations. The cells were washed with PBS and incubated with swine anti-rabbit serum (diluted 1:36) for 30 min washed with PBS and then a peroxidase/antiperoxidase complex (Dako) diluted 1:50 was added for 30 min. The chromogen used was 0.06% 3',3'-diaminobenzidine with 0.012% hydrogen peroxide for 10 min.

Results
Morphology of endothelium
After 2 days control plates from experiment A contained separate colonies of polygonal, large cells with few mitotic figures. On hypoxic plates colonies were larger and beginning to fuse. By 3 days cells were subconfluent in the central region of the plates and these continuous sheets of cells were more extensive in the hypoxic plates than in the controls. Mitotic figures were abundant and were conspicuously more common in those plates exposed to hypoxia. The cells were polygonal in the main but were tending to become oval in the centres of continuous sheets. Cells with two nuclei were common and occasionally cells with three nuclei were found. There was no difference in the appearance of endothelial cells between hypoxic and control plates, with the exception that rudimentary sprouting activity was observed in the centres of the subconfluent sheets in hypoxic cells derived from one rat but not in those derived from the other.

By the sixth and last day of this experiment most of the control plates and all of the hypoxic ones showed confluent monolayers covering all but a few small areas of each dish. Sprouting activity was very extensive in all of the hypoxic dishes. The sprouting cells were conspicuous because of their increased optical density with phase-contrast illumination. They tended to grow underneath the surrounding polygonal cells and may have appeared denser because they were being viewed through this overlying layer of cytoplasm. On the other hand the cell bodies that gave rise to sprouts were also denser than usual, despite the fact that they were not always covered by other cells. Some sprouts consisted simply of long, tapered, cytoplasmic streamers beneath the monolayer of oval cells beneath which they are growing. The sprouts terminate as a fan-like arrangement of fine cytoplasmic strands. Two endothelial sprouts can be seen intersecting each other without any apparent junction between them. Phase-contrast. ×360.

Sprouting in hypoxic endothelium

Fig. 1. Details of sprouting cells in a confluent culture of endothelium exposed to 2.5% O₂ (experiment C). Sprouts are elongated and optically denser than the monolayer of oval cells beneath which they are growing. The sprouts terminate as a fan-like arrangement of fine cytoplasmic strands. Two endothelial sprouts can be seen intersecting each other without any apparent junction between them. Phase-contrast. ×360.
either a few, short, rather blunted cells migrating underneath the monolayer (Fig. 3), or else focal networks of sparsely distributed, fine, cytoplasmic streamers. There were no complex masses as in the hypoxic cells.

Cells that were uninvolved in sprouting in both control and hypoxic plates were polygonal or oval, slightly smaller than at 3 days and showed much reduced mitotic activity except near the few remaining growing edges.

Hypoxic endothelial cells from experiment B did not show sprouting until a considerably longer period of hypoxia had elapsed, but by the end of the experiment, on the twelfth day, the difference between sprouting activity of the control and hypoxic endothelial cells was striking.

On the ninth day of experiment C control flasks contained several foci where the endothelial cells had an altered appearance. The cells in these small groups were conspicuously denser and smaller than normal, often with an elongated profile and a dense accumulation of granules in their cytoplasm (Fig. 4). Some gave rise to short, rudimentary sprouts. In the hypoxic flasks these foci of dense, elongated cells were much larger and more numerous. They gave rise to many branching sprouts that anastomosed with those of neighbouring cells (Fig. 5). They were also associated with an accumulation of granular material resembling debris (Fig. 5). Sprouting activity associated with the more severe degree of hypoxia employed in this experiment appeared to be less extensive than in either of the two previous experiments. Also there was no difference in the configuration of cells that had or had not been previously exposed to hypoxia.

Immunohistochemistry revealed the presence of factor VIII antigen in the vast majority of cells from all cultures, confirming their endothelial nature and demonstrating homogeneity of the cultures. A culture of smooth muscle cells was used as a negative control and showed negligible immunoreactivity for factor VIII. Sprout cells were heavily labelled as were the foci of dark, elongated cells referred to above.

Cell growth studies

The growth of cells seeded onto multiwell plates is shown graphically in Fig. 6. There were insufficient measurements from which to calculate error bars for this graph, but it is included because the cells used were from the same animal and at the same passage as those used for quantifying the degree of sprouting. The graph shows that for the first 5–6 days there was no change in the number of cells, but thereafter there was a sharp increase in the cell population, which was considerably greater for those cells grown in hypoxia in contrast to the controls. After 11 days the number of cells in control wells declined, whereas those cells grown in hypoxia continued to multiply. Fig. 7 shows the more detailed measurements of the growth of endothelial cells seeded into flasks in which the growth curves are smoother. These cells were derived from a different animal and had been passaged more frequently than those used for studying sprouting, but nevertheless they show a similar trend. Thus there is a period of approximately 6 days in which there is little growth, which is followed by a period of rapid proliferation. Cells in control flasks were confluent.
by the eleventh day and thereafter their rate of growth declined. In contrast, the hypoxic cells were confluent by the seventh day at a density of $5 \times 10^4$ cells cm$^{-2}$ but continued to proliferate rapidly and linearly until the termination of the experiment on day 15.

In experiment C where the degree of hypoxia was greater there was no significant difference between the growth of hypoxic and control cultures.

**Point-counting of sprout cells**

The results of the morphometric studies of endothelial cell sprouts are summarized in Table 1. The greatest degree of sprouting was found in experiment A where sprout cells occupied 21-6% of the plates in contrast to only 2-3% in the controls, a difference that was highly significant ($P < 0.001$). The area occupied by sprouts in the hypoxic flasks from experiment B was similar to that in experiment A at 20-9%, but the value in the control flasks was considerably higher at 7-5%. Nevertheless there was a highly significant difference between these two groups in this experiment ($P < 0.001$). Sprouting was least extensive in experiment C (Table 1). Nevertheless both groups of hypoxic flasks (groups 3 and 4) showed a significantly greater degree of sprouting than both control groups (1 and 2) ($P < 0.001$). There was also a significant difference between the two groups of hypoxic flasks ($P < 0.01$). Thus, when endothelial cells with no previous exposure to hypoxia were exposed to 2-5% O$_2$ they produced a greater degree of sprouting than cells at the same level of hypoxia that had been exposed to 5-3% O$_2$ two passages previously.
flasks and exposed to the same gas mixtures as in Fig. 6. Fig. 7. Growth of aortic endothelial cells seeded into culture flasks and exposed to the same gas mixtures as in Fig. 6. Cultures were derived from a different cell line than those used for examining sprouting activity. Bar lines represent the standard error of the mean of 20 counts. (• •) controls; (▲——▲) hypoxic cells.

Discussion

This investigation has demonstrated that arterial endothelial cells from the rat grow faster in an atmosphere containing 5.3 % O₂ in contrast to the growth at 19.6 % O₂, used in conventional CO₂ incubators. This difference could be detected simply by observing the size of colonies and time of attainment of confluence as in experiment A, an impression that was confirmed by counting the cells in experiment B. Here there was little change in cell numbers for the first 6 days, presumably whilst the cells were migrating and expanding. From 5 days onwards mitotic activity became prominent, especially in the hypoxic cultures and this was associated with a disproportionate increase in the total number of cells cm⁻². It is of interest that after confluence was reached at a density of 5 × 10⁴ cells cm⁻², cells in both control and hypoxic cultures continued to proliferate, the increase in number being accommodated by a more compact grouping rather than cells growing over one another. However, control cultures showed a reduced rate of growth after attaining confluence whereas hypoxic cultures continued dividing, so that on day 15 there were 11 × 10⁴ cells cm⁻² in contrast to 7.4 × 10⁴ cells cm⁻² in the controls (Fig. 7). It is possible that at least part of this disproportionate increase in cell population in hypoxic cultures could be accounted for by the formation of sprouting cells. The more severe hypoxia employed in experiment C induced no change in the growth of endothelial cells. However, the level of hypoxia used here (2.5 % O₂) was extreme and, far from being inhibited by it, the endothelial cells grew as rapidly as those in control flasks. It is of interest to note that Kan et al. (1985) routinely grew human umbilical vein endothelial cells in 7 % O₂ because they found growth to be more rapid. They did not perform any systematic study of this phenomenon but the experiments described here confirm their impression.

Other workers have shown that some cultured cells of non-endothelial origin, such as fibroblasts (Taylor et al. 1978) and neoplastic cells (Courtenay, 1976; Gupta & Eberle, 1984), will grow more rapidly in a hypoxic environment. In vitro these cells normally grow where there is a very low partial pressure of oxygen. It is perhaps not surprising, therefore, that they grow better in vivo in conditions that more closely mimic their environment in vivo. Arterial endothelium is unusual in that it is bathed directly by arterial blood with a pO₂ of 100 mm Hg. It is interesting, therefore, that these well-oxygenated cells should also grow faster in a hypoxic environment with a pO₂ of 40 mmHg.

The levels of oxygen and carbon dioxide employed in hypoxic cultures for the first two experiments were chosen because they were approximately equal to the partial pressures of these gases in venous blood. Thus it is possible that endothelium in veins can grow more rapidly than that in arteries if it responds to hypoxia in vivo in the same way as it does in culture. This could explain why atherosclerosis is a disease of arteries and not veins. One of the factors implicated in the aetiology of atherosclerosis is focal endothelial damage that permits lipoproteins to pass through into the subendothelial space. Regeneration of endothelium in veins, stimulated by the hypoxic environment, may be so rapid that lipid accumulation cannot occur.

Hypoxia also acted as a stimulus for the process of sprouting. This process has been referred to in several papers describing confluent cultures of human or bovine endothelium, as referred to in the Introduction. It also occurs spontaneously in confluent cultures of rat aortic endothelium, as shown by its appearance in control cultures in the present paper. However, under the influence of hypoxia, sprouting occurred earlier and became much more extensive. Thus in experiment A the area of hypoxic dishes occupied by sprout cells was nine times that in control dishes. There was also a large difference in the degree of sprouting in experiment B, but not as pronounced as in the first experiment, despite the fact that the level of hypoxia was the same and its duration longer. The reason for this is unclear, but it has been noted that there is variation in the extent and time of onset of sprouting between cell strains (Schwartz, 1978; Cotta-Pereira et al. 1980). It may also be related to the fact that in experiment B cells were grown in flasks rather than dishes.

Flasks with loose lids take longer to equilibrate with the atmosphere in the incubator than do dishes and hence may, on average, have contained a slightly higher level of oxygen. A test with chemical indicators revealed that dishes equilibrate within 15 min whereas flasks take 1 h. Since cultures were removed for feeding only thrice weekly, this difference is unlikely to have any real influence.

Sprouting activity was also significantly more extensive in cell cultures exposed to 2.5 % O₂ for 9 days. However, the area of the flasks occupied by sprouts, although two to three times as great as in control cultures, was substantially less than in the other two experiments where 5.3 % O₂ was used. It is likely that there is an optimum
concentration of oxygen that stimulates sprouting, below which inhibition of oxygen-dependent metabolic processes tends to slow it down. Also, cells used in experiment C had been subcultured more often than those used in the other two, and it is possible that the ability to form sprouts declines with repeated passaging.

The precise nature of endothelial sprouting is unclear. It frequently develops spontaneously in post-confluent cultures as in the control plates in the present study. However, some workers have found this particular circumstance to be important in the genesis of sprouting (McAuslan et al. 1982). To this list can now be added hypoxia. Of the stimulus for sprouting in these experiments may also be induced by covering the cells with gels.

However, some workers have found this particular circumstance to be important in the genesis of sprouting (McAuslan et al. 1982), phorbol esters (Montesano et al. 1982), basement membrane collagen as a substratum (Madri et al. 1983), phorbol esters (Montesano & Orci, 1987) and growth within collagen gels (Montesano & Orci, 1983; Delvos et al. 1982). Unidentified factors within serum have also been suggested to be important in the genesis of sprouting (McAuslan et al. 1982). To this list can now be added hypoxia. Of particular interest in this last respect are the experiments in which confluent cultures of endothelial cells were overlaid with collagen gels. This provoked the rapid proliferation of sprouting cells (Delvos et al. 1982; Montesano et al. 1983; Schor et al. 1983). Furthermore, the same effect could be induced by covering the cells with a glass coverslip (Schor et al. 1983). It is likely that the stimulus for sprouting in these experiments may also have been hypoxia brought about by the increased diffusion distance for oxygen. Since sprout cells grow beneath the monolayer they become further removed from their oxygen supply even without covering them with gels.

The process of sprouting appears to be entirely reversible, so that passaging cultures with extensive sprouting leads to growth of polygonal or slightly elongated cells typical of the rat aortic endothelium at least until confluence is reached. Thus one set of control flasks in experiment C contained cells that had previously been hypoxic and displayed extensive sprouting. However, their descendents showed no more sprouting than did similar control flasks in which the cells had had no previous hypoxic episode (Table 1). Furthermore, hypoxic cells in this experiment showed a greater degree of sprouting when they had received no previous exposure to hypoxia than those that had (Table 1). An interchange of phenotype of cultured bovine aortic endothelium was found by McAuslan et al. (1982). Reversibility of sprouting was also elegantly demonstrated by Schor et al. (1983) in which the upper layer of a collagen gel covering sprouting cultures was removed, whereupon the sprouts rapidly reverted to a polygonal morphology.

Sprouting activity in both hypoxic and control endothelial cultures was heralded by a curious change in morphology of the cells. Thus, focal collections of them became smaller and shrank away from their neighbours. They also became elongated and optically denser with a greater concentration of cytoplasmic granules. These changes were associated with increased immunoreactivity for factor VIII. It is likely that these foci of dark cells were destined to become sprouts. By losing their attachment to their neighbours and becoming elongated they would be able to migrate beneath the surrounding polygonal cells, become attenuated and hence form the characteristic sprouting pattern. A similar retraction, elongation and migration of endothelial cells from human umbilical vein was noted by Maciag et al. (1982) immediately prior to the formation of capillary tubes.

If sprouting is indeed a process of vascularization that is being accelerated by hypoxia, it may partly explain why capillaries grow into a wound, haematoma or inflammatory exudate, or why tumours become vascularized. All of these lesions are hypoxic and may thus stimulate division of adjacent endothelial cells, followed by their ingrowth as capillary sprouts, presumably guided by other growth factors and trophic substances within the lesions themselves.

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