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Angiogenesis at the site of neuroma formation in transected peripheral nerve

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ABSTRACT

Investiture of new microvessels within an injured peripheral nerve trunk may determine the success that the local environment has in promoting axonal sprouting and regeneration. We therefore examined microvessel investment of 24 h–14 d proximal nerve stump preparations in rat sciatic nerves. The stumps, later destined to form neuromas, were created by sciatic nerve transection with resection of distal branches to prevent distal reinnervation. Microvessels were studied in the proximal stump in semithin whole mount sections of nerve and by analysis of India ink perfused microvessel profiles. Quantitative image analysis was made of the luminal profiles of vessels perfused with India ink from unfixed sections of the stumps, contralateral uninjured nerves and sham-exposed but uninjured nerves. Evidence of angiogenesis was observed in stumps 7 d after transection, indicated by a rise in the total numbers of perfused microvessels and in the numbers of 2–6 μm diameter perfused microvessels. There was a shift in the histogram of the percentage of perfused microvessels towards the 2–4 μm range and a reduction in the mean microvessel luminal area in the stumps. By 14 d, new microvessels were larger, indicated by an increase in total luminal area. New microvessels were prominent in the epineurial connective tissue or between layers of perineurial cells of former fascicles. Microvessels probably share a battery of trophic signals with other proliferating cellular elements in the milieu of the injured peripheral nerve trunk.

Key words: Nerve regeneration; peripheral nerve; vasculature; microvessels.

INTRODUCTION

The complex patterns of tissue changes that accompany axonal sprouting in vivo are not well understood. Several features of the local microenvironment of injured and regenerating intact peripheral nerve trunks may be highly relevant in determining the success of sprouting or regeneration: local perfusion and oxygenation, recruitment of inflammatory cells and mast cells, local production of peptides, cytokines and trophic factors and mechanical derangement of regenerative pathways, perhaps by fibroblasts and collagen. In human peripheral nerves resected by military surgeons attempting to improve functional recovery following war wounds, the role of some of these features seems stark and obvious: there is microvascular thrombosis, and large segments of the endoneurium of segments with failed regeneration are replaced by dense and perhaps impenetrable fibrosis (Lyons & Woodhall, 1949).

In previous work, we have provided evidence that the peripheral nerve trunk responds to local injury through a timetable of microvascular events. Early reactive hyperaemia appears to protect the nerve trunk from immediate ischaemia following injury (Zochodne & Ho, 1990). At 24 and 48 h following nerve crush or proximal to the site of a nerve transection there is sustained hyperaemia associated with local accumulation of and vasodilation by CGRP (calcitonin gene-related peptide) (Zochodne & Ho, 1992; Zochodne et al. 1995). CGRP may arise at the injury site from perivascular afferent peptidergic fibres, but more likely accumulates directly from transected axons. Later, at 7 and 14 d following

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injury, there is sustained hyperaemia but these persistent rises in local blood flow do not appear to be CGRP related. Local accumulations of mast cells occur at these later times (Zochodne et al. 1994). We have postulated that sustained CGRP-independent hyperaemia, presumably of benefit to the nerve trunk, might be accounted for by angiogenesis.

In the present work, we examined local microvessels of the proximal nerve stump of transected sciatic nerve preparations. To look for evidence of angiogenesis and its relationship to axonal sprouting, we prepared whole mount semithin sections of nerve. To quantitate changes in microvessels we carried out in vivo perfusion of the vasculature of intact rats with an India ink preparation and studied luminal profiles of microvessels from unfixed frozen sections of nerve by image analysis. By using a model of experimental nerve injury identical to previous work, microvessel changes could be correlated with previous investigations of local blood flow, CGRP action and mast cell accumulation.

METHODS

Experimental model

Proximal stumps of the sciatic nerve of male Sprague–Dawley rats (250–300 g) were created by left mid-sciatic transection and resection of the distal nerve and its branches under pentobarbital anaesthesia (65 mg/kg). At early (24 h, 48 h) time points the nerve stump above the line of transection was not enlarged. We specifically did not examine the very distal portions of the specimens containing either only connective tissue (at 24 and 48 h) or tissue outgrowth (at 7 and 14 d). At later (7 and 14 d) time points it might theoretically be difficult to distinguish areas of completely new tissue outgrowth from distorted but intact distal sections of the proximal nerve trunk. We suggest that the tissues we examined were from the 'original' proximal nerve trunk rather than from portions of new outgrowth because (1) frozen sections were always taken proximal to the specimen tip; (2) the sections studied had recognisable, albeit distorted anatomical features of the nerve trunk with distinct fascicles and perineurial cells; (3) intact nonregenerating myelinated fibre profiles of 'normal' calibre and distribution were always present; (4) our samples were early, when a large mass of distal tissue outgrowth was not present. Microvessels were examined 24 h, 48 h, 7 d and 14 d later. Sham-operated rats underwent exposure of the sciatic nerve without transection or resection. Rats were reared on shavings covered plastic cages and had free access to rat chow and water.

Microvessels

At the endpoints, the distal aorta was catheterised through the right femoral artery (PE 50) and the rats perfused with 20 ml of a solution of India ink (25%), gelatin (4%) and mannitol (5%) over 20 mins. Blood was drained from the contralateral femoral vein. After perfusion, the rats were euthanised and placed in a freezer at -20 °C for 1 h before removal of the stump and the contralateral normal nerve at the same level. The technique, adapted from the work of Bray et al. (1990) who studied ligament perfusion, allowed us to outline vascular lumina without appreciable leakage of India ink. Specimens were then fast frozen (isopentane in dry ice) in OCT (Optimum Cutting Temperature, Miles Laboratories) compound and sectioned at 10 μm, with sections placed (without fixative) on slides and mounted with glycerol. Using a computerised image analysis apparatus (Java, Jandel Scientific), numbers of perfused microvessels and their individual transverse areas were measured to allow quantitation of microvessel profile numbers, mean microvessel luminal areas, total microvessel luminal area and size distributions of microvessels. Additional work involved calculation of microvessel fibre density by relating microvessel numbers, in each section, to their total nerve area. For each nerve specimen 6–9 individual sections underwent individual quantitative analysis allowing a mean value of each measurement (including the number and percentage of vessels in each size category) to be calculated. For each time point, 3–6 rats were studied.

Additional sections of the nerve stumps were fixed in cacodylate buffered glutaraldehyde, dehydrated with alcohols, embedded in Epon and sectioned at 1 μm for nerve whole mounts.

Analysis

Means and standard errors of the mean were calculated for proximal stumps, contralateral nerves and sham exposed nerve at each of the 24 h, 48 h, 7 d and 14 d time points for total microvessel number, mean luminal area, total summed luminal area, number of microvessels per size category, percentage of microvessels per size category and transverse nerve area. At each time point the measurements for proximal stumps, contralateral controls and sham exposed nerves were compared by a 1-way analysis of variance (ANOVA) with post-ANOVA Student's t tests applied as appropriate.

RESULTS

General findings

Perfused stumps at 24 and 48 h had vessels that were indistinguishable qualitatively from contralateral normal nerves or sham-exposed controls. Occasional 'closed' microvessels were associated with areas of endoneurial haemorrhage and axonal degeneration, probably related to the trauma of nerve transection. All 3 vascular compartments of nerve were well perfused with the technique: endoneurium, perineurium and epineurium. No evidence of leakage of India ink from epineurial and perineurial vessels was identified. Sections from 7 and 14 d stumps were dramatically different. The whole nerve sections were enlarged with distortion of the normal tissue compartments of nerve (distortion of perineurial boundaries) and striking proliferation of perfused vascular profiles. Examples of perfused 7 and 14 d proximal sciatic stumps illustrating new microvessels are given in Figure 1. Nascent vascular profiles appeared very small in the 7 d stumps suggesting that these vessels had only recently developed lumina. The identity of these profiles as proliferating vessels was confirmed by identifying endothelial wall elements around them under oil immersion light microscopy and by examining separate but similar specimens embedded in Epon and stained with toluidine blue. The vessel profiles had the appearance of capillaries and erythrocytes could be identified within their lumina. Most of these angiogenic profiles were found together, in areas of the nerve that formerly represented the epineurial space. Groups of vessels were also observed within the layers of the perineurium of previous fascicles. By 14 d the profiles were distributed in a similar topographic pattern but fewer 'nascent' profiles were evident. Mast cells were frequently encountered adjacent to vascular profiles. In 14 d stumps both microvessels and clusters of regenerating myelinated fibres were noted, splaying apart perineurial cells (Fig. 2).

Microvessel quantitation

Microvessel numbers were comparable between proximal stumps, contralateral control nerves and

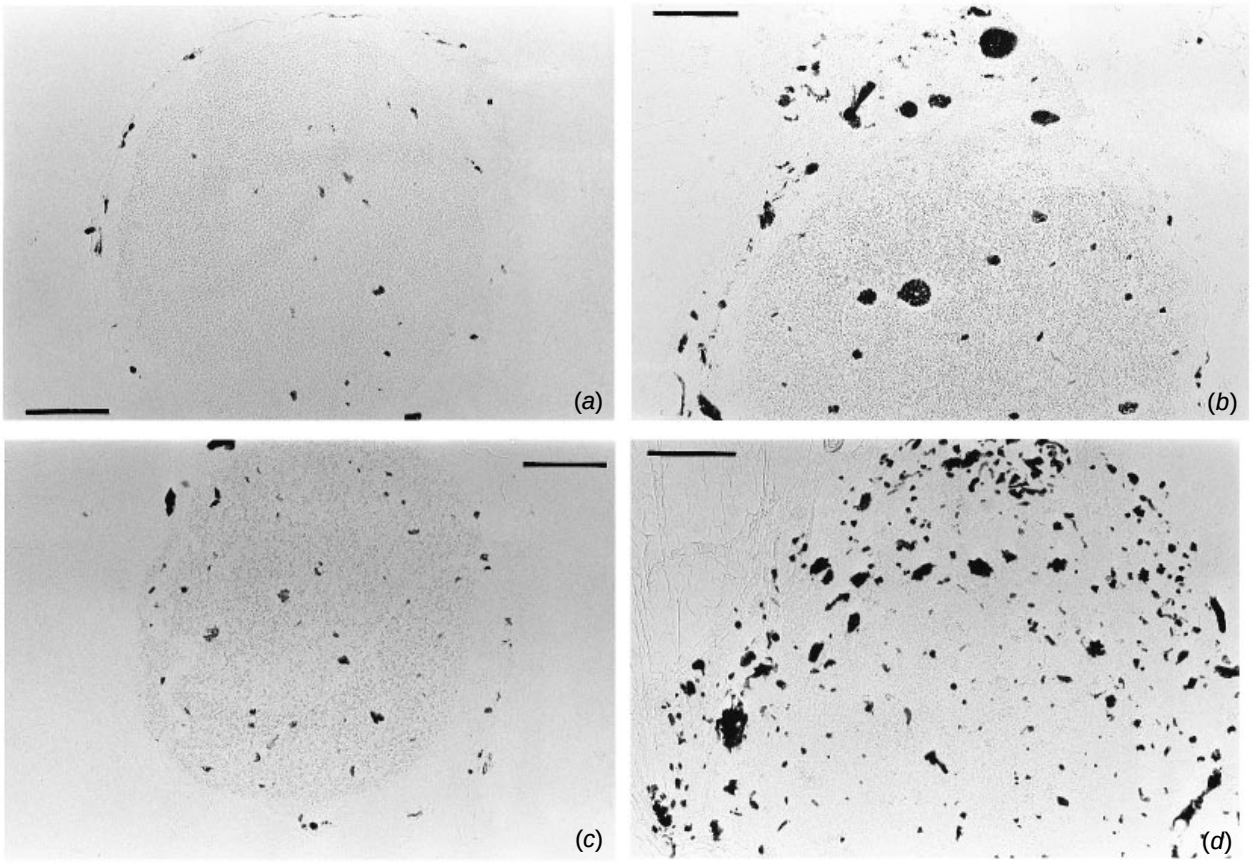

Fig. 1. Unfixed transverse sections of contralateral 7 d control nerve (*a*, left upper) 14 d control nerve (*c*, left lower) and proximal sciatic stump: 7 d (*b*, right upper; (*d*) 14 d (right lower) with microvessels perfused by India Ink. There is dramatic evidence of angiogenesis, indicated by a large number of new perfused vessel lumina. Bar, 200 μm.

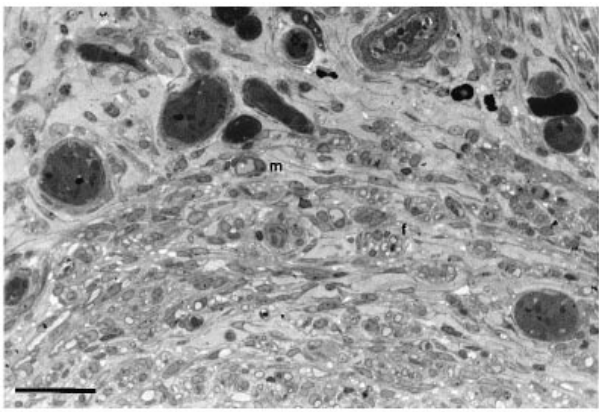

Fig. 2. Toluidine blue stained semithin transverse section of a 14 d-proximal stump. Numerous microvessels (m) are observed, some splaying apart layers of the former perineurium. There is also evidence of axonal sprouting (f). Bar, 50 μm.

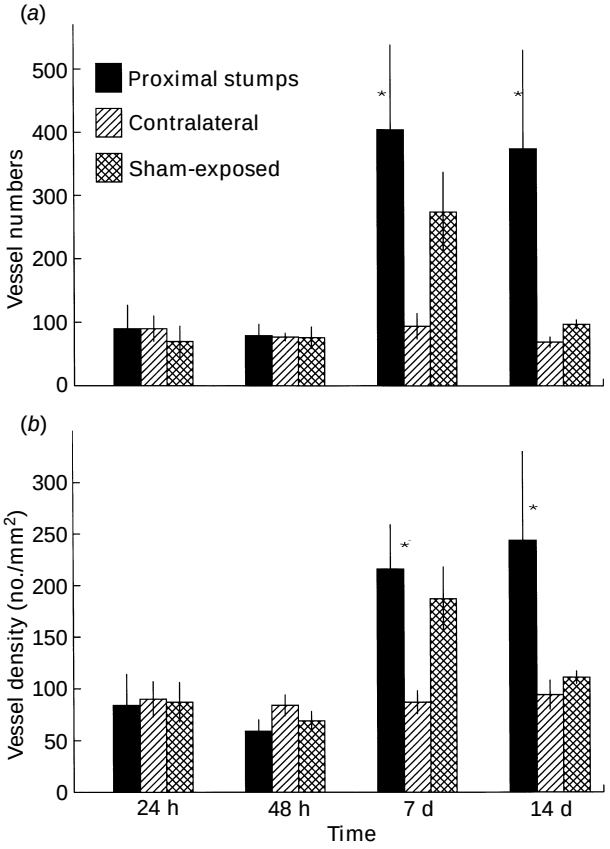

Fig. 3. Numbers (total) (*a*) and density (*b*) of perfused microvessel India ink profiles in proximal stumps, contralateral controls and sham-exposed controls. There is a significant (*) rise in microvessel numbers at 7 and 14 d, and microvessel density at 7 and 14 d.

sham-exposed nerves at 24 and 48 h (Fig. 3). There was a nonsignificant trend towards a higher percentage of larger diameter vessels and a larger mean microvessel luminal area in 48 h stumps compared with controls (Figs 4, 5; Table). At 7 d there was a dramatic rise in microvessel numbers, with a shift in the size histogram towards smaller vessels. This shift was documented as a rise in the number of microvessels in the histogram categories of 2–4 and 4–6 μm and a rise in the percentage of total vessels of diameter 2–4 μm (Figs 4, 5). In addition, the mean luminal area of microvessels in the 7 d stumps was reduced (Table). At 14 d, microvessel numbers remained high, but there was no longer a shift in the percentage of small microvessels to the smaller range (Figs 4, 5). Sham-exposed nerves had a rise in microvessel numbers at 7 d (less than that of the stumps) but numbers were equivalent to contralateral controls at 14 d. Total microvessel luminal area was increased at 14 d compared with respective controls from contralateral uninjured nerves, and sham-exposed nerves (Table). Transverse total nerve areas were increased in 48 h–14 d stumps (Table).

DISCUSSION

The major findings of this work were (1) angiogenesis appeared in the proximal stumps of transected sciatic nerves of rats by 7 d and was confirmed by quantitative methods: there was a dramatic rise in the number of total India ink perfused vasa nervorum, a rise in the number of 2–6 μm diameter vessels, and a shift in the size histogram towards a higher percentage of small 2–4 μm diameter vessels; (2) new microvessels were most frequently, but not exclusively, observed in the connective tissue around the former fascicular boundaries. Microvessels frequently splayed apart layers of the perineurium of previous fascicles; (3) total microvessel luminal area was increased in 14 d stumps.

In this work, we selected an in vivo perfusion method to outline microvessel areas without the influence of fixatives that might distort vessel anatomy and calibre. The India ink–gelatin–mannitol solution was perfused through the distal aorta to ensure full delineation of lower body vasculature while maintaining the rats under anaesthesia until euthanasia. Since the proximal stumps of transected nerves become distorted structures and form neuromas, the boundaries between endoneurium and epineurium are lost, and hence the analysis of microvessels in this study was of whole nerve rather than specific compartments, unlike previous reports of rat sciatic nerve studied under different circumstances (Bell & Weddell, 1984; Zochodne et al. 1990). The histograms in the present work otherwise resemble those of Marcarian & Smith (1968) of formalin-fixed and latex dye injected vasa nervorum of cat ulnar nerves.

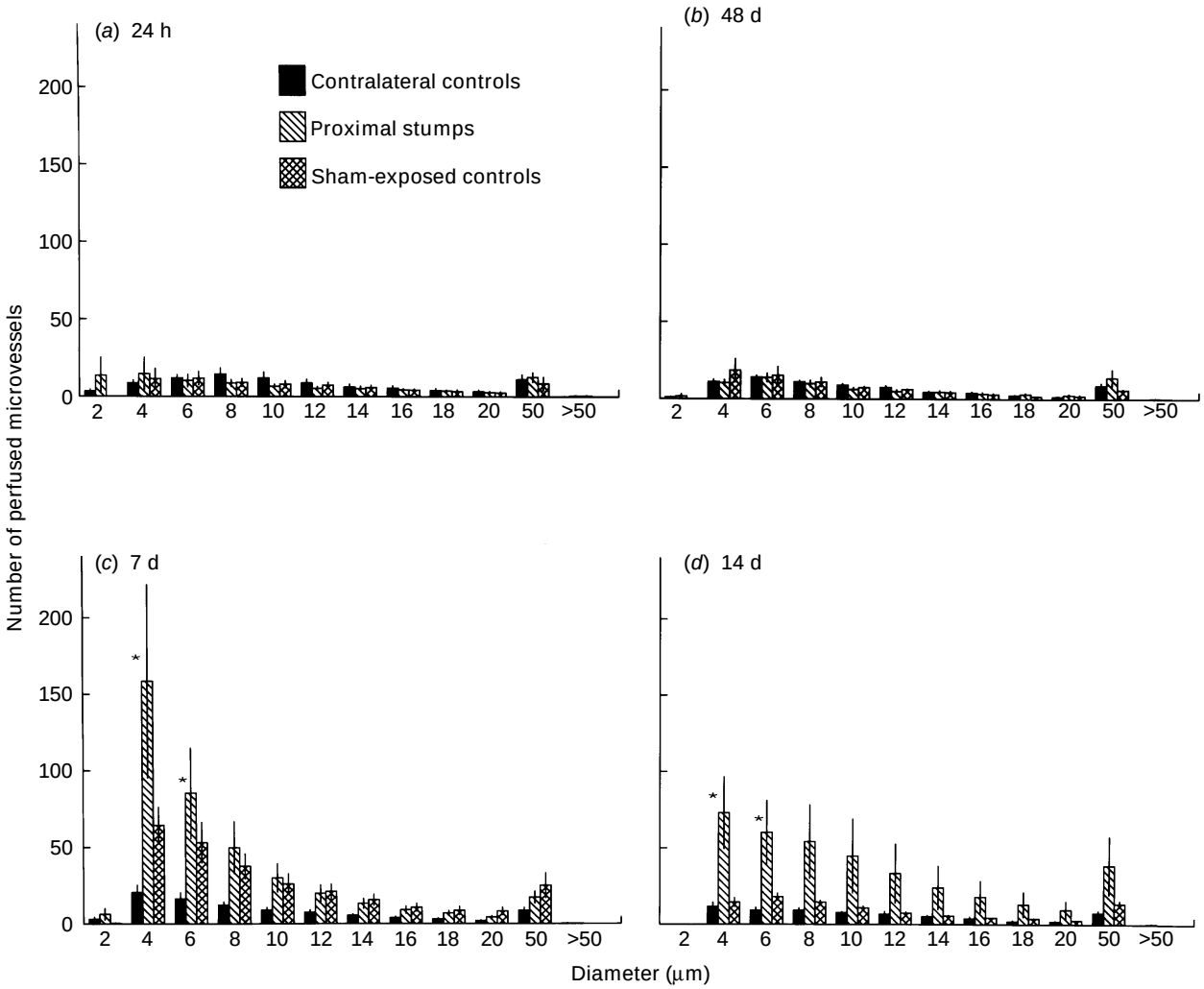

Fig. 4. Size-frequency microvessel number histograms of proximal sciatic trunks and controls. Asterisk identifies an increase ($P < 0.05$) in stump samples compared with contralateral controls.

The findings in this work are best considered in the context of our prior investigations of local blood flow in similar proximal stump preparations. The present work demonstrated some histological support for microvessel dilatation at 48 h with trends towards a larger mean luminal area and increases in the percentage of larger vessels. At 48 h, high local flow was previously linked with local accumulations of CGRP and was reversed by a CGRP antagonist (Zochodne et al. 1990). In prior work, we also noted that 7 and 14 d old proximal stumps were associated with local accumulation and degranulation of mast cells (Zochodne et al. 1994). It is interesting that angiogenesis was evident at these times in the present work. Mast cells contain several probable angiogenic and vasodilator signals including histamine, heparin tumour necrosis factor (TNF) and proteases (Meininger & Zetter, 1992). It seems clear that mast cell numbers rise at sites of new vessel growth, such as at the interface of normal tissue with implanted tumours (Dethlefsen et al. 1994). Mast cells appear to facilitate early tumour growth by stimulating angiogenesis (Dethlefsen et al. 1994). Kessler et al. (1976) suggested that mast cells potentiate, rather than initiate, angiogenesis. Applied alone on the chick chorioallantoic membrane, mast cells failed to induce angiogenesis. Other authors have argued differently, suggesting that there may be a direct effect of histamine from mast cells and perhaps heparin, on angiogenesis (Norrby & Sorbo, 1992; Norrby, 1995).

Basic fibroblast growth factor (bFGF) is expressed in human cutaneous mast cells (Reed et al. 1995). Interestingly bFGF acts as an angiogenic factor (Schweigerer et al. 1987) and also appears to promote mast cell recruitment (Gruber et al. 1995). Mast cells in turn might promote further bFGF release from extracellular matrix stores through the release of heparin (Meininger & Stein, 1992). A significant component, however, of the mast cell accumulation in nerve trunks appears to arise from proliferation rather

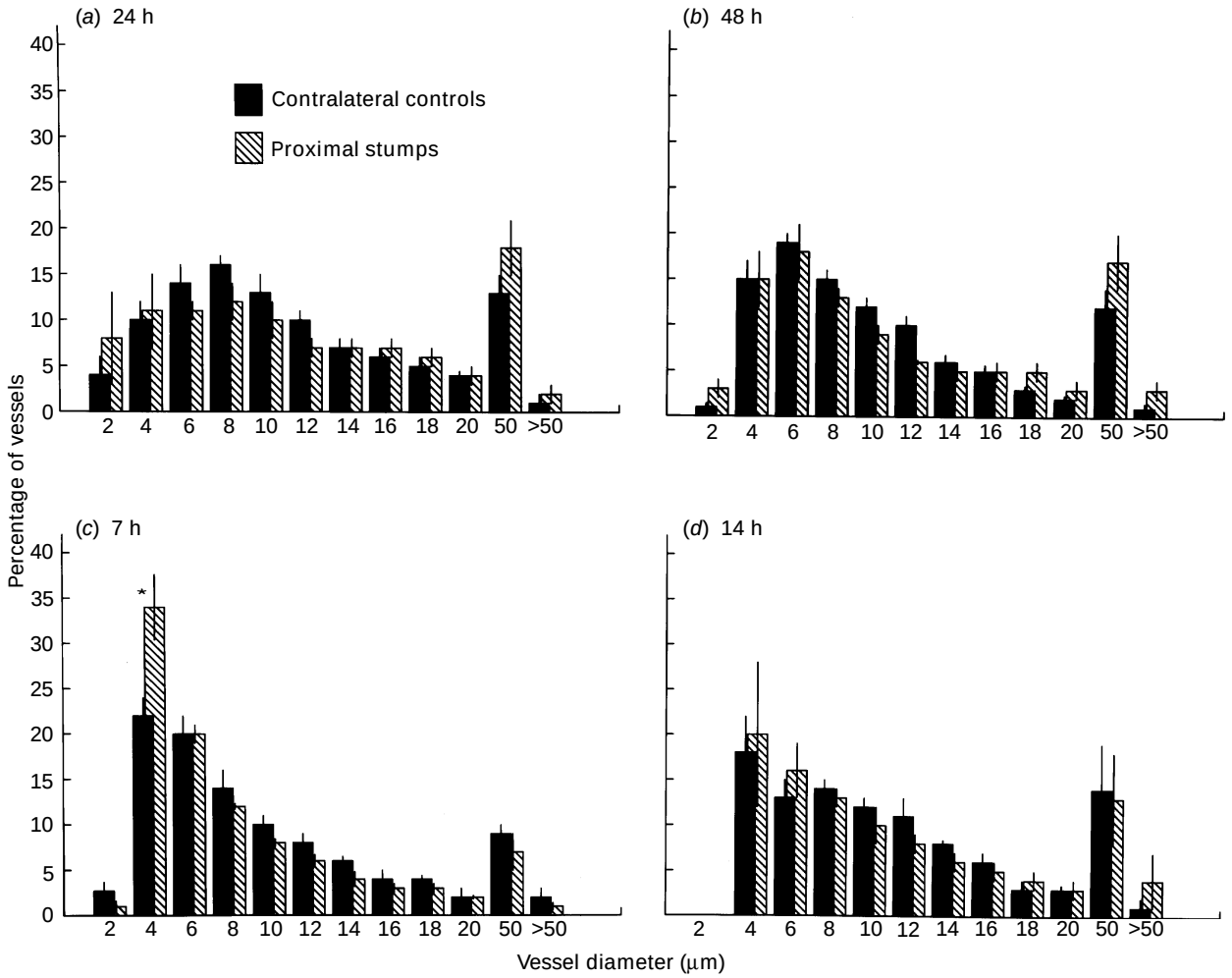

Fig. 5. Size-frequency microvessel percentage histograms of proximal sciatic trunks and contralateral controls. Asterisk identifies an increase ($P < 0.05$) in stump samples compared with contralateral controls.

than recruitment (Olsson, 1967, 1968). Thus it appears there are complex interrelationships between mast cell proliferation, recruitment, degranulation and new vessel growth.

Angiogenesis is an expected component of general wound repair. In this sense, peripheral nerve may not differ a great deal from injury reactions of skin and other tissues. The findings do, however, differ from the CNS where neither this degree of angiogenesis nor axonal regeneration are demonstrable following local injury. Whether these 2 facets of tissue repair are merely associated or interdependent is uncertain. The metabolic demands of proliferating, regenerating tissue are probably high, entraining rises in local perfusion. The proliferation of new microvessels in our preparation appears to be orchestrated with mast cell accumulation, axonal sprouting and gross nerve trunk enlargement. Enlargement of outgrowth from proximal nerve stump results in the eventual formation of a classic neuroma.

The apparent time relationship between angiogenesis, mast cell proliferation and axonal sprouting may suggest that these events share trophic signals. One shared trophin candidate might be nerve growth factor (NGF), generated locally from infiltrates of macrophages that stimulate nonneuronal NGF production by interleukin-1β (Lindholm et al. 1987). One difficulty is knowing whether aberrant axonal sprouting in a neuroma is NGF dependent. NGF supports collateral sprouting of intact sensory axons but apparently not regenerative sprouting (Diamond et al. 1992*a*, *b*). NGF does promote mast cell proliferation (Aloe, 1988).

A more plausible approach would involve the activity of multiple trophins with overlapping growth promoting actions. There is an extensive list of nonneurotrophin candidates including bFGF (Walicke, 1986; Finklestein et al. 1988), EGF (Morrison et al. 1987), TGF alpha (Chalazonitis et al. 1992) and TGF beta (Flanders et al. 1990) that might

Table. *Microvessel luminal areas and nerve transverse areas**

Tissue (n)	Mean microvessel area ($mm^2 \times 10^{-4}$)	Total microvessel area ($mm^2 \times 10^{-2}$)	Nerve area (mm^2)
24 hour			
Contralateral control (4)	1.9±0.4	1.9±0.7	1.0±0.2
Stump (4)	2.7±0.8	2.1±0.8	1.3±0.3
Sham-exposed control (3)	2.6±1.2	2.6±1.9	0.8±0.1
48 hour			
Contralateral control (5)	1.8±0.2	1.4±0.3	0.9±0.1
Stump (5)	3.0±1.0	3.0±1.6	1.4±0.3[c]
Sham-exposed control (3)	1.4±0.2	1.0±0.3	1.1±0.1
7 day			
Contralateral control (6)	2.1±0.5	2.1±0.6	1.0±0.1
Stump (6)	1.3±0.3[a]	3.5±0.7	1.6±0.3[d]
Sham-exposed control (3)	1.6±0.2	4.4±1.3	1.4±0.1
14 day			
Contralateral control (4)	2.0±0.7	1.2±0.2	0.8±0.1
Stump (4)	3.1±1.8	6.9±2.7[b]	1.6±0.1[e]
Sham-exposed control (3)	2.4±0.6	2.3±0.2	0.9±0.1

* Results are means±S.E.M. The mean microvessel area refers to the average perfused area of a single vessel profile. The total microvessel area is the summed total of areas from all perfused vessels. The nerve area is the total tissue area of the transverse section studied.

[a] stump vs contralateral control $P = 0.06$ (1-tailed); [b] stump vs contralateral control $P = 0.03$ (1-tailed); [c] stump vs contralateral control $P = 0.01$ (1-tailed); [d] stump vs contralateral control $P = 0.01$ (1-tailed); [e] stump vs contralateral control $P = 0.001$ (1-tailed); stump vs sham $P = 0.003$ (1-tailed).

accumulate in injured nerve trunks. Each of the listed candidates is a known angiogenic factor with additional demonstrated neurotrophin-like activity. None of them, however, has been evaluated in experimental circumstances that would allow direct comparison between their actions on sprouting peripheral axons, mast cells and endothelial cells. Further angiogenic signals that may be important are TNF alpha, VEGF, histamine and heparin (Scholl & Morrow, 1993; Cockerill et al. 1995; Norrby, 1995). The inherent complexity of this interplay should not detract us from evaluating trophin action, however, because multiple and sequential neurotrophin action itself seems crucial in several scenarios, especially during neurobiological development. Growth promoting redundancy is probably an important feature of general wound, and in particular, peripheral nerve repair.

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