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Original Article

Origins of the neurovascular bundle: interactions between developing nerves and blood vessels in embryonic chick skin

PAUL MARTIN and JULIAN LEWIS*

ICRF Developmental Biology Unit, Zoology Department, Oxford University, Oxford, U.K.

ABSTRACT Growth cones of nerves and endothelial cells of blood vessels are closely analogous in their migratory behavior, and they are both set a similar task during the early development of a limb. Both must invade the mesenchyme to form ramifying networks of large nerves and vessels. Both systems must densely pervade certain regions of the developing limb, such as muscle rudiments, and both form dense cutaneous plexuses at precisely the same depth beneath the epidermis. Moreover, adult tissues show many examples of neurovascular bundles in which nerves and blood vessels run closely parallel and branch in a correlated fashion, suggesting some interdependence during development. We have examined the interrelationship between developing nerves and blood vessels in chick wing skin because it allows a particularly convenient two-dimensional analysis of the two systems which can be revealed simultaneously in the same preparation by injection of Indian ink combined with silver-staining. We show that nerves do not use blood vessels as pathways along which to crawl, but that there are two other ways in which neurovascular associations arise: in some situations nerves and blood vessels follow the same route because they are responding independently to the same mesenchymal cues; and in some situations nerves induce blood vessels to remodel around them.

KEY WORDS: *nerves, blood vessels, chick limb, skin*

Introduction

Developing limbs are invaded at early stages by endothelial cells, which will form the vasculature, and a little later by the axons of peripheral neurons, which will form the nerves. The mechanisms of invasion are similar for the two classes of cells: endothelial cells and axonal growth cones appear both to move by throwing out filopodia, while dissolving obstructions ahead of themselves by secreting proteases (Clark, 1918; Speidel, 1933; Krystosek and Seeds, 1981; Kalebic *et al.*, 1983; Pittman, 1985; Bray and Hollenbeck, 1988). The vascular and nervous systems go on to develop in parallel and are therefore confronted by the same mesenchymal environment as they extend and ramify in the limb. Both developing systems may be guided by differential adhesivity cues provided by the extracellular matrix (ECM) of the environment that they invade, and both may also respond in a chemotactic or a trophic manner to cues provided by diffusible factors (Davies, 1987; Folkman and Klagsbrun, 1987). Probably the patterns of invasion depend on a complex combination of different cues (Martin *et al.*, 1989), some of which may be the same for the two cell types. Indeed, the two systems develop similar patterns in the limb, with large trunks that progressively branch to form blood vessels and nerves of smaller and smaller diameter. Major branches of both systems ramify extensively within the same regions, such as the developing muscles and the skin (Fig. 1). Furthermore, both nerves and blood vessels appear to be inhibited from invading the same particular regions, notably chondrogenic mesenchyme (Tosney and Landmesser, 1985; Wilson, 1986) and a mesenchymal zone immediately beneath the epidermis and including the presumptive dermis (Feinberg *et al.*,1983; Verna, 1985; Martin *et al.*, 1989). A still closer correlation be-

Abbreviations used in this paper: ECM, extracellular matrix; HA, hyaluronic acid.

***Address for reprints:** ICRF Developmental Biology Unit, Zoology Department, Oxford University, South Parks Road, Oxford, OX1 3PS, U.K.

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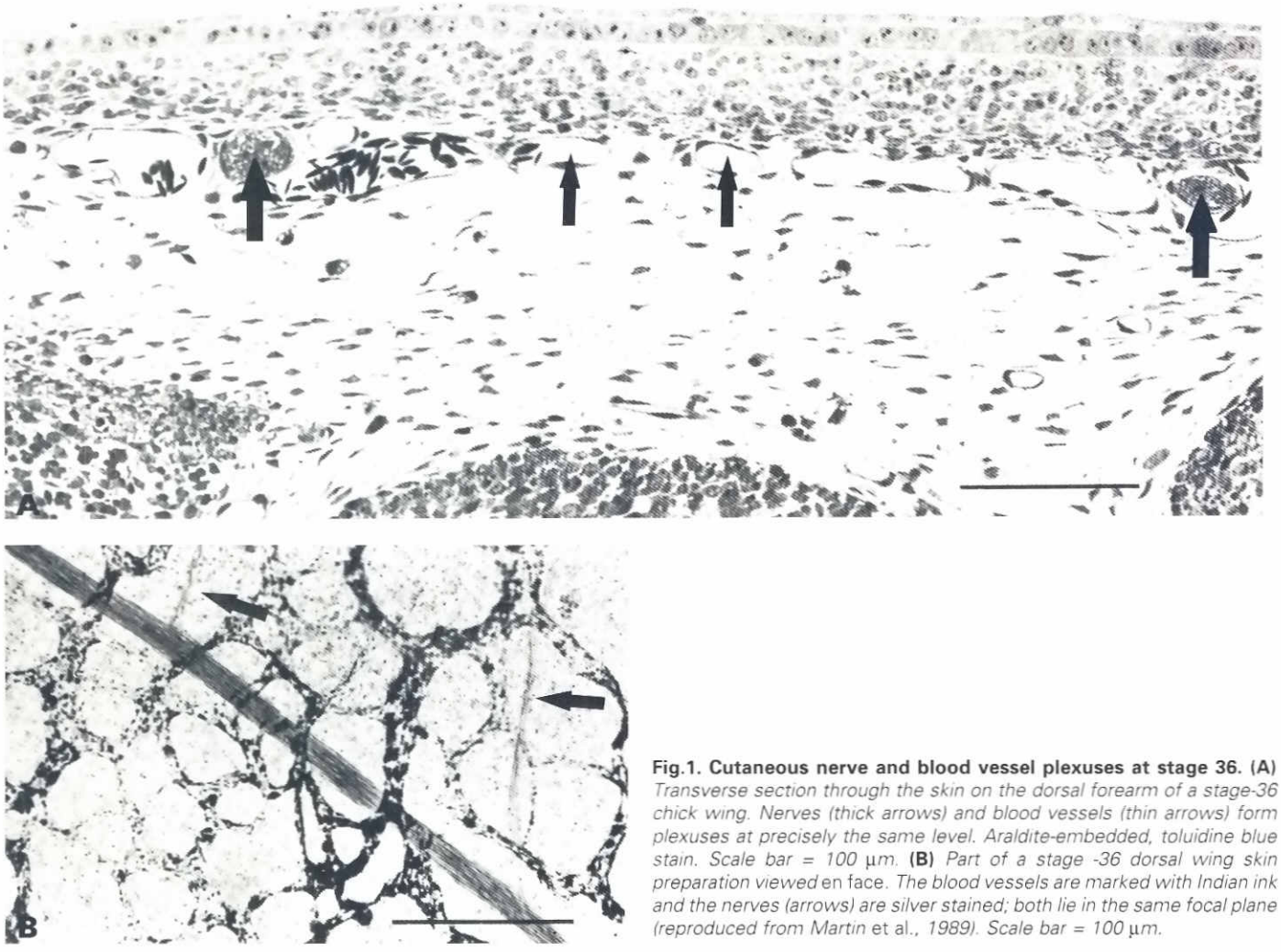

Fig.1. Cutaneous nerve and blood vessel plexuses at stage 36. (A) *Transverse section through the skin on the dorsal forearm of a stage-36 chick wing. Nerves (thick arrows) and blood vessels (thin arrows) form plexuses at precisely the same level. Araldite-embedded, toluidine blue stain. Scale bar = 100* μm. **(B)** *Part of a stage -36 dorsal wing skin preparation viewed* en face. *The blood vessels are marked with Indian ink and the nerves (arrows) are silver stained; both lie in the same focal plane (reproduced from Martin* et al., *1989). Scale bar = 100* μm.

tween nerves and blood vessels is seen in the neurovascular bundles of the adult body, especially in the limbs (Lucas and Stettenheim, 1972).

Such parallelism could arise in two ways: the nerves and blood vessels could be dependent upon one another during their development (in the sense that one provides guidance for the other), or they might independently follow common pathways during development (Hamburger, 1929). It has been reported that nerves sometimes appear to follow blood vessels in circumstances where other favorable tracks have been experimentally denied them, for example, as a result of ablation of the neural crest early in development (Carpenter and Hollyday, 1986), or when sensory nerves are deprived of their normal interactions with motoneurons by early removal of the ventral neural tube from which the motor neurones are derived (Landmesser and Honig, 1986). However, serial reconstructions of histological sections of the embryonic chick wing show no close relationship between blood vessels and established major deep nerves (Tosney and Landmesser, 1985), and the weight of current opinion is that blood vessels do not normally provide guidance for nerves.

In view of the current interest in mechanisms of axonal guidance and in the control of angiogenesis, it is worth considering in more detail whether there is any evidence either for nerves using blood vessels as pathways along which to crawl, or for blood vessels remodeling around nerves. However, the relationship between developing nerves and blood vessels deep within the mesenchyme is difficult to survey because the structures are complex and 3-dimensional systems (Bennett *et al.*,1980). Most conveniently, in wing skin, developing nerves and blood vessels both form plexuses in the same plane just beneath the dermis (Martin *et al.*, 1989), making feasible a more simple 2-dimensional analysis of their relationship. Another simplifying feature of the cutaneous system is the absence of restricting obstacles such as cartilage elements (although dermal condensations, which will eventually give rise to the feather papillae, do arise at the later stages and begin to cause complications).

We have used a limb skin preparation from the dorsal face of the wing with both cutaneous nerves and blood vessels marked. This system is easily accessible to analysis until fairly late stages of development, and by depriving the limb of its source of innervation, we can test for certain types of developmental interactions between nerves and blood vessels. The region of skin that we have chosen to examine (Figs. 2 and 3) is innervated by ramifications from two reproducibly positioned cutaneous nerve branches, DC AI and DC Int (nomenclature as in Martin *et al.*, 1989; and see Fig. 3A), which diverge from the main dorsal mixed nerve trunk of the wing as it traverses the humerus. These two nerve branches can be identified both shortly after they have begun to invade the skin, at stage 29/30 (6-7 days of incubation) and 3-4 days later, at stage 35/36 (9-10 days) when each nerve branch has extensive ramifications. Our observations suggest that nerves and blood vessels develop independently of one another, for the most part. At no stage do we see nerves using blood vessels as pathways along which to crawl. At later stages, however, some nerves and blood vessels do appear intimately related. We show that some of these neurovascular associations are accounted for simply by nerves and blood vessels responding independently to the same mesenchymal cues; but a second category of neurovascular relationship must be the result of vascular remodeling around large nerves, implying that the nerves emit some weak angiogenic signal.

Results

Eleven normal embryos at stages 35 and 36 were successfully perfused with ink and silver-stained. Eight wings from 4 of these embryos were dissected to give dorsal wing skin preparations. The remaining wings were examined as whole-mounts, allowing nerve and blood vessel patterns of the alar web to be recorded with the aid of a drawing tube. Two of these wings were later embedded for routine histology to examine the distribution of blood vessels and nerves in the transverse plane.

Six normal stage 29/30 embryos were successfully perfused with ink and processed as above, and of these, 4 wings from 2 embryos were dissected to give dorsal wing skin preparations. The remaining stage 29/30 wings were analyzed as whole-mounts.

A further set of 8 experimental embryos with partially or completely nerveless wings were analyzed at stage 35/36 in a similar way (see below).

Nerves ramify to form a well-defined cutaneous plexus

Nerves begin migrating into the wing bud at about stage 24/25 (E4) (Swanson and Lewis, 1982) and by stage 29/30 (E6) the nerve branches that will innervate muscles and skin at the forearm level have diverged from the dorsal mixed nerve trunk (Fig. 2A). On reaching a specific level beneath the epidermis, the four cutaneous nerve branches serving the dorsal forearm (DC AI, DC Int, DC Uln and DC Elb) then ramify to form a plexus of fine nerve twigs, such that the four nerve branch territories will occupy the whole dorsum of the limb by stage 35/36 (E10) (Fig. 3A). As each cutaneous nerve branch ramifies, some of its twigs encounter those of adjacent branches and fasciculate with them, weaving a plexus in which nerve fibers both diverge into separate fascicles and converge into composite fascicles. The meshes of this plexus are typically lozenge-shaped, measuring about 1-2 mm by 250-500 μm at stage 36, with their long axis parallel to the long axis of the limb. Most strikingly, the plexus is confined to a very narrow stratum, only about 20 μm thick, at a depth of 70 μm beneath the epidermis on the dorsal side of the wing, or about 50 μm on the ventral side (Martin *et al.*, 1989).

The vasculature in developing wing skin consists largely of a polygonal network of capillaries

It is clear both from sections (Fig. 1A) and from whole-mounts (Fig. 1B) that the cutaneous vascular plexus forms in precisely the same stratum beneath the epidermis as the cutaneous nerve plexus. The time course of its development is somewhat different, however. Blood vessels are present in the limb bud from a very early stage, and a cutaneous vascular plexus can be seen already at stage 20 (Caplan and Koutroupas, 1973). By stage 29/30, the cutaneous capillary vessels form a network of randomly interconnected polygons, about 75 μm in diameter and roughly as long as they are wide. The meshes of this network are roughly uniform in size over the dorsal surface of the limb. The capillaries have a diameter of about 10 μm.

At stage 35/36, the cutaneous vascular plexus still appears as a polygonal network of capillaries, with the vessels and the polygons in most regions both having similar dimensions to their counterparts at stage 29/30. This does not mean that the system of vessels has remained static — quite the contrary. The wing as a whole has roughly doubled in length and width in the period from stage 29/30 to 36, so that if the initial polygonal network had undergone no active growth or remodeling its polygons would have been stretched to double their previous diameter. If they still have the same average size as before, it must be because new capillaries have formed, roughly quadrupling the total length of cutaneous vasculature so as to maintain a constant density of vessels per unit area as the limb grows. The plasticity of the vascular network over this period is emphasised also by the development of some departures from the initially uniform basic pattern of polygons. Thus, the proximal area of our preparations (corresponding to the region of skin overlying the mid-

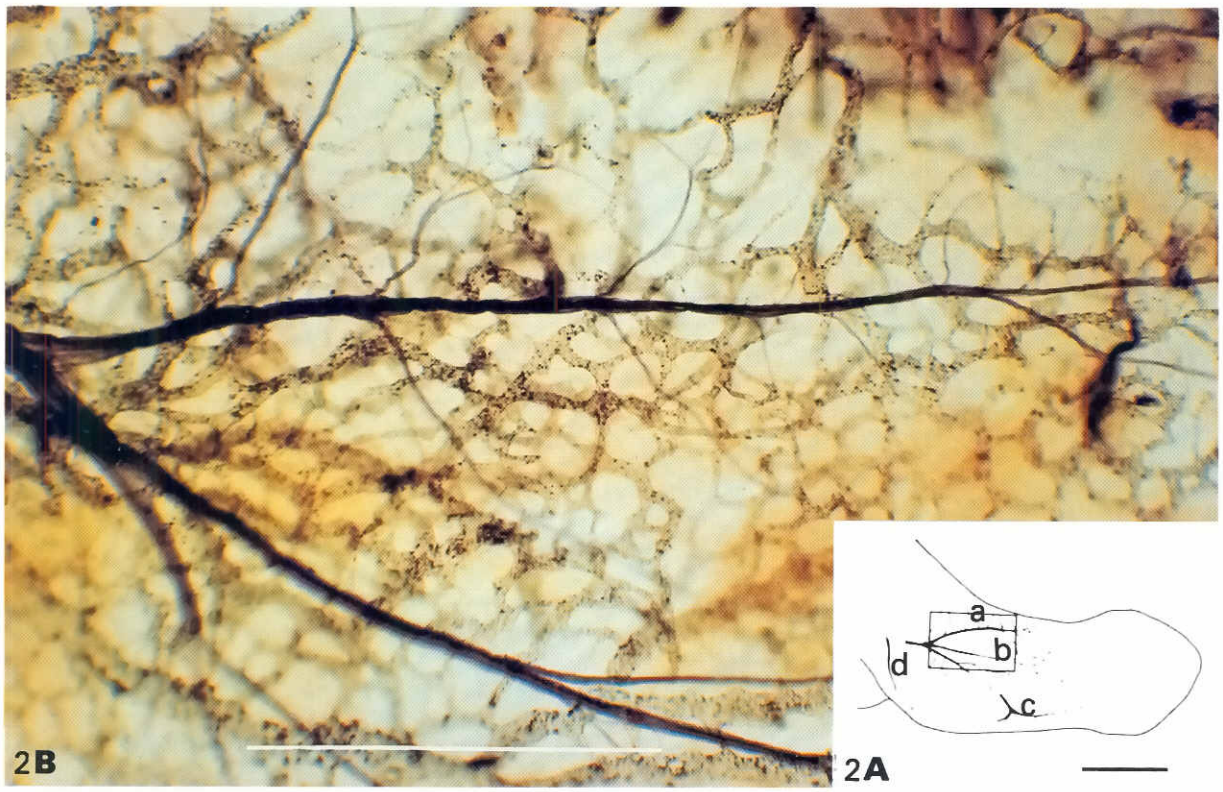

Fig. 2. Cutaneous nerve and blood vessel at plexuses at stage 30. (A) Camera lucida *drawing of the pattern of cutaneous innervation of the dorsal forearm of a stage -30 chick wing, as seen by silver-staining. Cutaneous nerve branches are shown as solid lines (A = DC Al; B = DC Int; C = DC Uln; D = DC Elb) and the proximal part of the dorsal mixed nerve trunk is shown with broken lines). Scale bar = 1 mm.* **(B)** *View at higher magnification of dissected skin preparation from another stage -30 specimen, corresponding approximately to the boxed region in (A). Nerves are silver-stained; blood vessels are marked by Indian ink. Neither the main nerve trunks nor the fine nerve twigs run parallel to blood vessels. Scale bar = 500 μm.*

humerus) shows smaller numbers of larger polygons (up to 250 μm in diameter), implying that no new vessels have formed or even that there has been a net regression of vessels, while the posterior edge shows a slightly increased density of smaller-diameter polygons (about 25-50 μm in diameter), implying that the vessels here have proliferated faster than required to keep pace with the growth of the limb.

One other major feature stands out from the polygonal capillary network pattern. This is a vessel of diameter up to 100 μm coursing in a proximo-distal direction across the alar web. It was visible in half (11/22) of the stage 35/36 specimens examined, always following the same route; and in all cases where it was present it ran parallel with, and close to, the cutaneous nerve branch DC Al. We shall discuss this association in greater detail below.

Initially, the neural and vascular plexuses, though they form at the same depth, are otherwise uncorrelated

The patterns of cutaneous nerves and blood vessels can be compared in whole-mount preparations of dorsal wing skin: since both plexuses are confined to a thin stratum at exactly the same depth beneath the epidermis, the relationships between them within that plane are easy to see. At stage 29/30, the nerves and vessels follow uncorrelated paths, criss-crossing at random (Fig. 2B). Extending nerve twigs rarely travel as neighbors to blood vessels — no more often than would be expected by chance; they are often seen traversing regions as far from the nearest blood vessel as is possible in their area (i.e. half the local diameter of the vascular polygons). Evidently, intimate association with a blood vessel is not required for nerve extension, and blood vessels are not likely to be defining nerve pathways. Conversely, the formation of blood vessels at and before this stage does not depend on guidance from nerves — indeed, a vascular plexus has already developed before nerves appear on the scene.

Within 3-4 days after invading the skin, some main nerve branches have become closely associated with blood vessels

Between stages 29 (E6) and 36 (E10), adjustments take place in the spatial relationships between blood vessels and the major cutaneous nerves. Stage 36 dor-

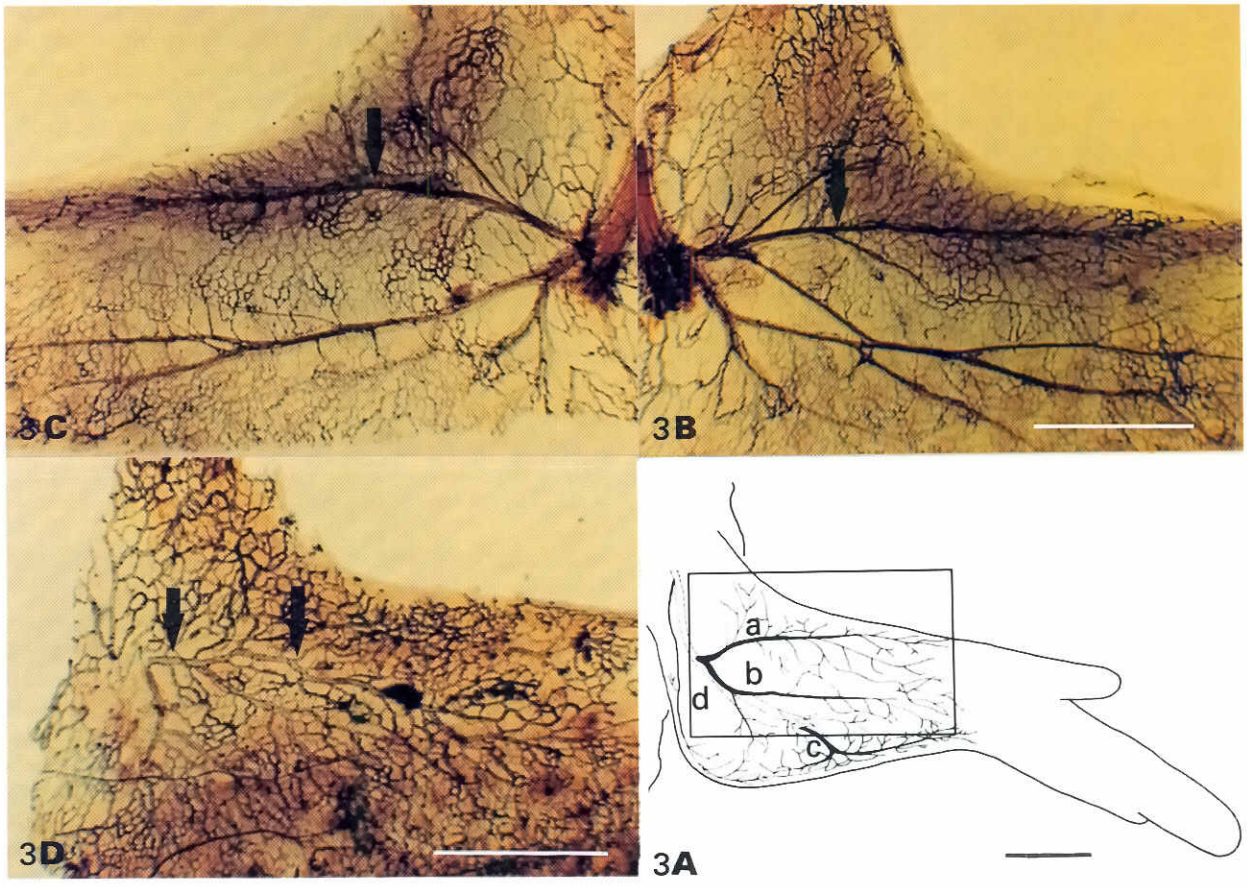

Fig. 3. Dorsal wing skin preparations from normal and nerveless wings at stage 36. (A) Camera lucida *drawing of the pattern of cutaneous innervation of the dorsal forearm of a stage -36 chick wing, as seen by silver-staining. Labeling conventions as for Fig. 2A. Scale bar = 1 mm.* **(B, C)** *View of dissected skin preparations from right and left wings of another stage-35/36 specimen, corresponding approximately to the boxed region in (A). Nerves are silver-stained (brown); blood vessels are marked by Indian ink (black). Large blood vessels (arrows) accompany nerve branch DC AI. Scale bar = 1 mm.* **(D)** *Dissected skin preparation corresponding to that shown in (B), but from a wing almost entirely deprived of innervation. A large blood vessel (arrows) can be seen following roughly the normal route along the alar web. Scale bar = 1 mm.*

sal wing skin preparations show that some regions of major cutaneous nerve branches, which at stage 26 had no correlation with blood vessels, are now closely associated with blood vessels, although there is still no neurovascular relationship apparent at the level of the smaller branches and growing twigs (Figs. 1B, 3 and 4). Two major cutaneous nerves were regularly included in our whole-mount skin preparations, DC AI and DC Int, and both – to varying degrees in different specimens — acquired close companion blood vessels for a good proportion of their length (more than half their length, in 50% (DC AI) and 40% (DC Int) of the specimens assessable). These neurovascular associations are, however, of two rather different sorts:

I) Of the 22 normal wings examined, as mentioned above, 11 showed a large-diameter blood vessel which in all 11 cases appeared to weave a pathway along the cutaneous nerve DC AI. The vessel had a diameter of 50-100 μm, that is, up to three times the diameter of the nerve it was accompanying; the exact pathway was not identical from limb to limb, occasionally crossing the nerve a number of times but at no point, in any specimen, diverging more than 10-20 μm (edge to edge) from DC AI (Figs. 3B, C and 4A, B). In the other 11 wings no large blood vessel was closely associated with DC AI.

II) A much less consistent relationship was seen between DC Int (and some of the secondary nerve branches) and the local vasculature. The impression was of a more intimate relationship, though for shorter distances than described in I) above. Either (a) smaller vessels, usually 10 μm capillaries, form a sheath around the nerve, with vessels above, below and on both sides of the nerve or, (b) a single vessel (capillary or slightly larger) follows a route precisely adjacent and parallel to the nerve (Figs. 3B, C and 4A, C); examples of both of these types of intimate association could be seen in all

limbs examined, although the locations varied from limb to limb.

Another striking but again rather inconsistent relationship between nerves and blood vessels, seen in a number of specimens, was the parallel branching of nerves and blood vessels. The relationship, where present, appeared very precise with the branching of one system exactly matched by that of the other. This precise matching of the locations of branching (Fig. 4C) argued that it was not a random coincidence.

Remodeling of the vasculature between stage 29/30 and stage 35/36 must account for the neurovascular relationships seen at the later stage

The major dorsal cutaneous nerves DC AI and DC Int examined at stage 29/30 show no relationship with the surrounding vasculature, and yet 3-4 days later both nerve branches often show clear associations with blood vessels. Nerve fibers grow from their tips, and once a nerve has formed it does not easily shift during development; it has no locomotor apparatus to pull it sideways. The vasculature, by contrast, is continually changing as new vessels form and old ones regress; so if an association arises sometime after both systems have invaded beneath the dermis, then the blood vessels must be the adaptive party. In principle the blood vessels might be either adjusting according to an influence from the nerves, or independently responding to the same (or similarly located) non-neural cues that served earlier to guide the nerves. The following observations suggest that both of these mechanisms may play a part in the spatial rearrangement of the cutaneous capillary bed.

Nerveless limbs have a practically normal blood vessel pattern

By destroying a large part of the neural tube with UV irradiation at two days of incubation, we were able to make nerveless wings (Lewis, 1980) and examine their cutaneous vasculature. Of the 15 embryos surviving irradiation at 2 days of incubation, 8 were successfully perfused with ink and silver stained at stage 35/36, to yield 13 wings with little or no innervation to the dorsal limb skin (in particular, no recognizable DC AI or DC Int nerve branches); 3 of the embryos had one nerveless wing and one almost normal wing. Two of the nerveless wings were dissected so as to make dorsal wing skin preparations; the other 11 nerveless wings were examined as whole-mounts. The polygonal capillary network of cutaneous blood vessels in these nerveless wings was indistinguishable from that in normal wings (compare Figs. 3B and D). Both the dimensions of the polygons and their variation from one limb region to another appeared normal. Moreover, in 4 out of the 13 nerveless wings (1 dissected skin preparation and 3 intact whole-wing preparations), a large blood vessel could be seen coursing in a proximo-distal direction across the alar web, along a route similar to the pathway that the nerve DC AI and its companion blood vessel would have taken if DC AI had been present (Fig. 3D). This indicates that there is a strong tendency for a large blood vessel to form along this route independently of the nerve, and that guidance for the vasculature is provided by some non-neural cue or cues that just happen to align the vessel with DC AI in a normal limb. Such cues, acting on nerves and blood vessels alike, might be mechanical, depending, say, on the oriented elongation of the limb, or chemical, marking a specific pathway in the extracellular matrix, for example.

By contrast with the companion vessel to DC AI, the smaller vessels that swathe DC Int in a normal wing have no identifiable counterpart in the nerveless limbs. These vessels are indeed normally so closely aligned with or wrapped around the nerve that it seems that the nerve must have guided their development. As this type of neurovascular association is variable in location from wing to wing, it is hard to confirm the neural dependency rigorously by examining the vascular patterns in nerveless wings. However, this very variability supports our interpretation of events and can be seen as a reflection of the way in which the relationship normally arises. It seems likely that nerve and blood-vessel patterns are laid down with little regard to one another and with some randomness in the details of each, only the major nerve branches (and occasionally large blood vessels) being defined precisely. This would result in a largely random relationship between the two systems. Nerve-dependent remodeling of the vasculature would then occur in the vicinity of the larger nerves in those cases where a blood vessel is traveling close enough to be 'captured' by short-range angiogenic factors released by the nerve. These sites of remodeling would be variable from limb to limb because of the initially random relationship between nerve and blood vessel pattern.

Growing nerves and growing blood vessels may be responding to the same inhibitory factor from the ectoderm

The initial lack of correlation between nerves and blood vessels in the plane of the skin contrasts strikingly with the exact correlation between the two plexuses with regard to the depth at which they form. This

Fig. 4. Neurovascular associations at stage 36. (A) *View of dissected skin preparation from a stage-36 wing corresponding approximately to the region boxed in Fig. 3A. Scale bar = 500 μm.* **(B)** *Higher magnification of upper boxed region of (A), showing a single large blood vessel closely accompanying the nerve DC AI. Asterisks indicate the lumen of the blood vessel. Scale bar = 200 μm.* **(C)** *Higher magnification of lower boxed region of (A), showing a number of blood vessels – some large, some small – running parallel to and ensheathing the nerve DC Int. Note also that the branching of the nerve proximally (arrow) is precisely matched by blood vessel branching. Scale bar = 200 μm.*


A
B
C

reinforces a tentative conclusion of a previous paper (Martin *et al.*, 1989): the plexuses form at the same level not because the neural growth cones are guided by the vascular endothelial cells, or vice-versa, but because both are responding independently to the same depth cues. In particular, it is tempting to hypothesize that the same mechanism inhibits both of them from sprouting too close to the ectoderm.

What might this mechanism be? Feinberg and Beebe (1983) have argued that the "avoidance factor" keeping blood vessels away from the epidermis may be hyaluronic acid (HA): the avascular zone beneath the epidermis is correlated with high levels of HA, and a slow-release compound (Elvax) impregnated with HA creates an avascular zone around itself when implanted into the embryonic limb. Analogous findings have been reported in relation to nerve outgrowth. *In vitro* experiments show that embryonic epidermis, but not dermis, appears to secrete a "neural avoidance factor" so that axons from dorsal root ganglia refrain from traveling closer than about 40 μm to a co-cultured epidermal explant (Verna, 1985; Verna *et al.*, 1986). Moreover, nerve outgrowth in culture has been shown to be inhibited both by HA and by two other positively charged glycosaminoglycans that are normally plentiful beneath the epidermis — dermatan sulphate and chondroitin-6-sulphate (Carbonetto *et al.*, 1983; Verna *et al.*, 1989).

It is tempting to try to marry all these results by supposing that HA is the "neural avoidance factor" and is preventing neural as well as vascular outgrowth into the subectodermal region *in vivo*. Verna (1987) has, however, observed the neural avoidance phenomenon even in the presence of hyaluronidase (from Streptomyces), suggesting that HA is not necessary (though it might still be sufficient). Perhaps dermatan sulphate and/or chondroitin-6-sulphate may be sufficient to account for the effect; or perhaps it may be mediated by some other type of molecule — a glycoprotein, for example (Verna, 1987; Fichard *et al.*, 1989).

Discussion

To a first approximation, it appears that nerves and blood vessels are independently guided: both respond to cues provided by the epidermis and the connective tissues of the limb, and the gross similarities in the neural and vascular patterns — their depth beneath the epidermis, the parallelism of the nerve DC AI and its companion vessel — reflect similar responses to those cues by migrating endothelial cells and by neural growth cones. This emphasizes once again the primacy of the connective tissues and epidermis in regulating the assembly of the limb (Lewis *et al.*, 1983; Bryant *et al.*, 1987; Martin and Lewis, in preparation): they provide the master template that defines the patterning of all other limb components.

The gross pattern of cutaneous nerves and blood vessels is secondarily refined, to a small extent, through local remodeling of the vasculature under the influence of short-range signals from the larger cutaneous nerve branches. It seems that neurovascular bundles in the skin often arise in this way.

The present type of analysis of the rules of behavior of the developing nerves and blood vessels — that is, of the neural growth cones and the endothelial cells — provides the necessary background for the next level of investigation — the search for the specific chemical and physical factors through which the rules are implemented.

Materials and Methods

Fertilized chicken eggs (White Leghorn x Rhode Island Red, from Park Farm, Oxford) were incubated at 38±1°C and windowed at approximately stage 18 (Hamburger and Hamilton, 1951). They were then returned to the incubator and left for a further 3-7 days, until either stage 29/30 or 35/36, when they were injected with approximately 10-50μl (according to stage) of Indian ink (Pelikan, No. 17 mixed 2:1 with phosphate-buffered saline and sonicated briefly before use). The injection was made directly into one of the major veins overlying the yolk sac. This procedure clearly marks even the tiniest capillary (Caplan and Koutroupas, 1973). Embryos were then immediately transferred from their eggs to a dish of saline where they were eviscerated. After this they were fixed overnight in Bodian's fixative and processed by an adapted Bodian's silver method to reveal the nerves (Lewis *et al.*, 1981). Finally, they were dehydrated through alcohols and cleared in methyl salicylate. To obtain a preparation of dorsal limb skin for 2-dimensional analysis of blood vessel and innervation pattern, we carefully dissected away the obscuring soft tissues and cartilage, working with the ventral surface uppermost in the dish, and cutting major nerve and blood vessel branches as they diverged towards the skin. The shape of the preparation, together with the pattern of cutaneous nerves, allowed us to retain a knowledge of its relative orientation on the intact limb and therefore to compare preparations from different wings. The combination of ink injection and silver staining made undissected whole-mount specimens too dark to photograph but we were able to make *camera lucida* drawings of cutaneous nerve and blood vessel patterns of the whole dorsal surface of stage 29/30 specimens and of the dorsal alar web region of stage 35/36 specimens.

Nerveless wings were created as previously reported (Lewis, 1980), using long-wave UV irradiation at two days of incubation to ablate the region of the neural tube between somites 12 and 20 of a stage 12/13 embryo, which includes the region contributing neural tissue to the wing. Embryos with neural tube ablations were allowed to grow until stage 36 and then processed in the same way as normal embryos. Dorsal wing skin preparations were dissected from the nerveless wings, and the alar web region was also examined in undissected whole-mount specimens, as before.

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