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Original Article

Developing blood vessels and associated extracellular matrix as substrates for neural crest migration in Japanese quail, *Coturnix coturnix japonica*

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ABSTRACT Japanese quail embryos were used to examine paths of neural crest cell (NC) migration in relationship to the embryonic vasculature. Immunolabeling for NC, angioblasts and the extracellular matrix (ECM) glycoproteins fibronectin (FN), laminin (LN) and tenascin (TN) revealed several instances where spatiotemporal patterns of NC migration coincide with the embryonic vascular pattern and its associated ECM. An *in vitro* model for angiogenesis was modified to include NC, and associations with "capillary-like" endothelial cell structures were demonstrated. A working hypothesis is that the embryonic vasculature may, in specific instances, be used as a substratum for directed NC migration and that these interactions are mediated primarily through the adhesive interactions of FN. Some members of the TN family of glycoproteins, through their relatively non-adhesive properties, may act to help guide neural crest cells to the FN-rich blood vessel surface.

KEY WORDS: *neural crest, blood vessels, HNK-1, QH-1, cell migration, angiogenesis, fibronectin, laminin, tenascin*

Introduction

Two pathways of trunk neural crest cell (NC) migration contribute to the formation of the sympathetic ganglia and the aortic plexus (Thiery *et al.*, 1982; Duband *et al.*, 1985; Lallier and Bronner-Fraser, 1988). The first pathway is between two consecutive somites, where NC cells migrate in a ventral direction toward the dorsal aorta (Thiery *et al.*, 1982; Erickson, 1985a; Loring and Erickson, 1987). The second more predominant pathway is within the rostral part of the sclerotome, under the dermamyotome, where NC cells migrate in a ventrolateral direction to the posterior cardinal vein and the dorsal aorta (Rickmann *et al.*, 1985; Bronner-Fraser, 1986a; Lallier and Bronner-Fraser, 1988).

Some reports claim the intersomitic stream is minimal (Rickmann *et al.*, 1985; Bronner-Fraser, 1986a; Lallier and Bronner-Fraser, 1988), while others maintain that a significant number of cells follow this route (Loring and Erickson, 1987; Teillet *et al.*, 1987). Loring and Erickson (1987), using whole-mount preparations, noted a number of NC cells intersomitically and proposed that these cells represented the initial path of NC cell migration. They further suggest that NC cells which migrate in the intersomitic space are closely associated with the intersomitic blood vessels (Erickson 1985a; Loring and Erickson, 1987). Other studies have also implicated developing blood vessels and their associated extracellular matrix (ECM) in the migration and aggregation of trunk NC cells into ganglia (Le Douarin, 1980; Newgreen and Thiery 1980; Newgreen and Erickson, 1986; Thiery and Duband, 1986).

Based on their spatiotemporal distribution *in vivo*, the ECM glycoproteins fibronectin (FN), laminin (LN) and tenascin (TN) have been frequently correlated with NC migration (Timpl *et al.*, 1979; Newgreen and Thiery, 1980; Duband and Thiery, 1982; Thiery *et al.*, 1982; Newgreen, 1984; Loring and Erickson, 1987; Tan *et al.*, 1987; Mackie *et al.*, 1988). FN and LN have also been shown to support NC adhesion and migration *in vitro* (Rovasio *et al.*, 1983; Bilozur and Hay, 1988). Conversely, similar studies indicate that TN may act as an anti-adhesive matrix glycoprotein that promotes NC cell motility (Chiquet-Ehrismann *et al.*, 1988; Halfter *et al.*, 1989). NC cells have also been shown to produce TN (Tucker and McKay, 1991). Finally, studies involving microinjection of antibodies or synthetic peptides which disrupt cell-matrix interactions have been used to test the roles of these molecules, *in situ*. Such studies have established that FN, a LN-heparin sulfate proteoglycan complex, TN and the integrin receptor are all involved in some aspect of cranial NC migration (Boucaut *et al.*, 1985; Bronner-Fraser, 1985, 1986b, 1989; Bronner-Fraser and Lallier, 1988).

Abbreviations used in this paper: NC, neural crest; FN, fibronectin; LN, laminin; TN, tenascin.

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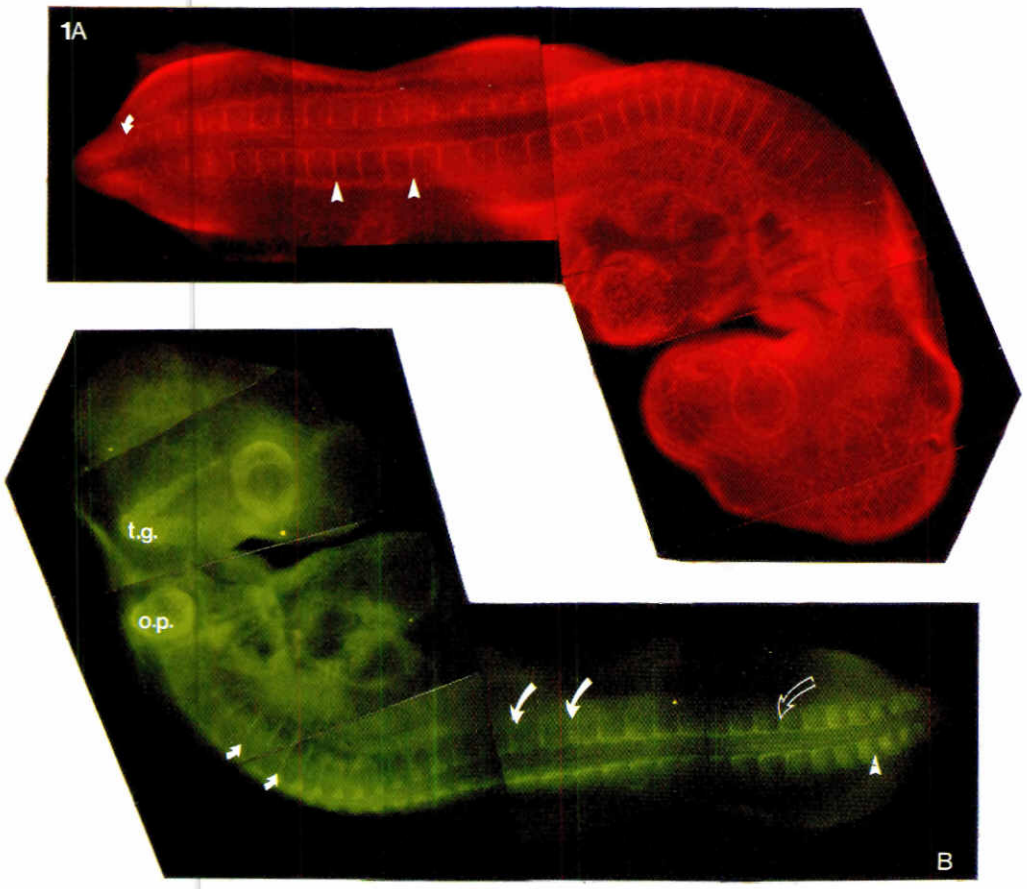


Fig. 1. Whole-mount preparation of a 32-somite quail embryo double immunolabeled for QH-1 (A) and HNK-1 (B). (A) *QH-1: Intersomitic blood vessels (arrowheads) branch from the dorsal aorta between somites. Intersomitic vessels (small curved arrow) are evident in the intersomitic cleft at the level of the segmental plate and the last formed somite.* **(B)** *HNK-1: Neural crest cells are evident in the dorsal aspect of the intersomitic space 3-4 somites anterior to the segmental plate (arrowhead). Crest cells are also evident in the rostral part of the sclerotome, 5-7 somites anterior to the segmental plate (open curved arrow represents the 7th to last somite). At more cranial levels (large solid curved arrows) crest migration in the rostral part of the somite is more pronounced. In vagal regions crest cells and ventral root axons are immunolabeled with HNK-1 (small solid curved arrows). Note the endogenous HNK-1 staining present within the rostral part of the last 3-4 somites. o.p., otic vesicle; t.g., trigeminal ganglion*

The purpose of this study was to examine spatiotemporal patterns of NC cell-blood vessel interactions *in situ* and *in vitro*. By immunolabeling for NC (HNK-1, Tucker and Erickson, 1984), angioblasts (QH-1, Lebastie *et al.*, 1986; Pardanaud *et al.*, 1987; Coffin and Poole, 1988) and the ECM glycoproteins FN, LN and TN, in whole-mount and sectioned embryos, a detailed description of NC-blood vessel-ECM interactions was obtained. To test the hypothesis that migrating neural crest cells may use the surface and/or ECM of developing blood vessels as a substratum, an *in vitro* model for angiogenesis (Kubota *et al.*, 1988) was altered to include neural crest cells and associations were noted.

Results

In the trunk of the avian embryo the development of the intersomitic blood vessels proceeds in a cranial to caudal direction (Coffin and Poole, 1988). NC cell migration also proceeds in a cranial to caudal direction so that several stages of migration can be examined within one embryo. For example, initial stages of migration are evident in caudal regions of the embryo, whereas more advanced stages of migration can be examined in more cranial regions (Newgreen and Erickson, 1986). Although inter-regional differences in paths of NC migration exist, i.e. head vs trunk, intra-regional differences in paths of trunk NC cell migration are minimal (Le Douarin, 1982; Loring and Erickson, 1987). Therefore, this study is limited to the trunk region of embryonic day 3 embryos (HH stage 17-18; Hamburger and Hamilton, 1951).

Blood vessel development precedes neural crest migration in the intersomitic space

In whole-mount preparations of embryonic day 3 quail embryos, blood vessels are evident in the intersomitic cleft throughout the craniocaudal axis of the embryo. In Fig. 1A, intersomitic blood vessels are evident between the segmental plate and the last formed somite. NC cells, on the other hand, initiate migration away from the neural tube two somites cranial to the segmental plate. These crest cells appear as a thin wedge of cells extending no further ventrally than the interface between the neural tube and the somite.

At more advanced stages of migration, more NC cells emigrate from the neural tube and conform to the dorsal boundaries of the intersomitic space (Fig. 1B). This stage of migration is evident at the level of the intersomitic cleft between the second and third most caudal somites in a 32-somite embryo. Three to four somites cranial to the segmental plate, NC cells are present in more ventral regions of the intersomitic cleft (Fig. 2B).

Careful analysis of each intersomitic cleft (see semi-serial cross sections Fig. 3) reveals a repetitive pattern in which the intersomitic artery is rostral to the intersomitic vein. This vein is closely apposed to the rostral wall of the adjacent somite. In the rostral part of the cleft, these blood vessels overlap considerably. In the caudal part of the cleft, which contains only the intersomitic vein, there is more cell-free space as compared to the rostral part of the cleft. Distinctions between arteries and veins could only be made in cross sections and were based primarily on whether an anastomosis was made with the dorsal aorta or the posterior cardinal vein.

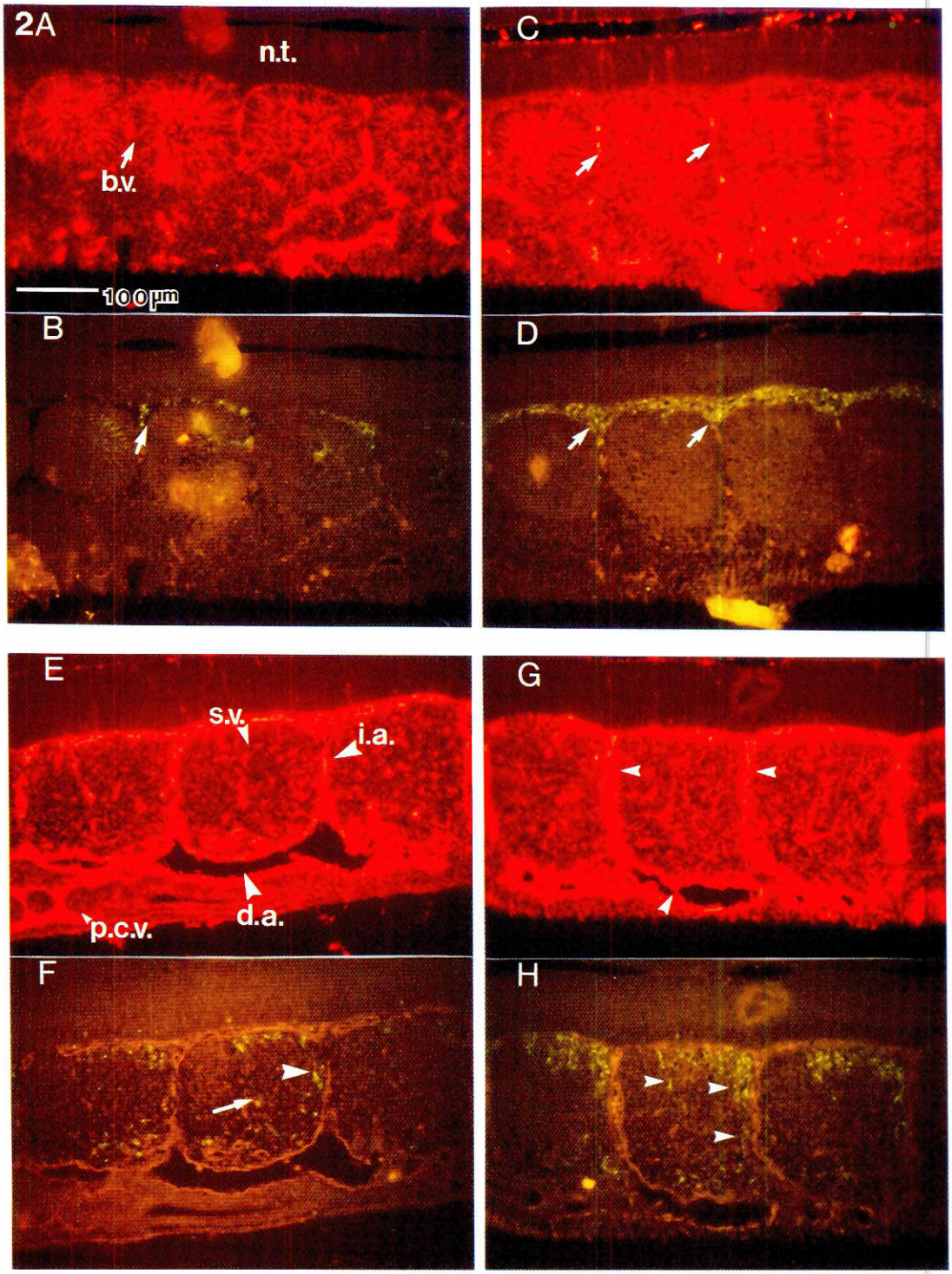


Fig. 2. Sagittal sections through the trunk of a 32-somite embryo. QH-1 (A,C,E,G). HNK-1 (B,D,F,H). (A,B) *Three somites cranial to the segmental plate, blood vessels (b.v., blood vessel, arrow in* **A***) are evident in the intersomitic cleft. Neural crest cells (arrow in* **B***) are also evident in the same space. Note the hemisegmental distribution of HNK-1 staining endogenous to the rostral half of each newly formed somite. This staining appears to be limited to the internal part of these somites.* **(C,D)** *Five somites cranial to the segmental plate, a stream of neural crest cells* **(D)** *enters the intersomitic space adjacent to blood vessels* **(C)**. **(E,F)** *Ten somites cranial to the segmental plate and slightly more ventral than preceding sections, neural crest cells (arrowhead in* **F***) are closely apposed to the intersomitic artery (i.a. in* **E***) in the rostral part of the somite. These cells represent the initial edge of crest migration within the rostral part of each somite. In addition, some crest cells (arrows,* **F***) are within Von Ebner's fissure, in close proximity to the somitic vein (s.v. in* **E***, arrows-neural crest; d.a., dorsal aorta; p.c.v., posterior cardinal vein).* **(G,H)** *Twelve to fifteen somites anterior to the segmental plate, crest cells (arrow heads in* **H***) are evident in the rostral part of the somite extending to the dorsal aorta. Note, the leading edge of this mass of cells is adjacent to the intersomitic artery (arrowhead in* **G***) and the midsomite region, within Von Ebner's Fissure.*

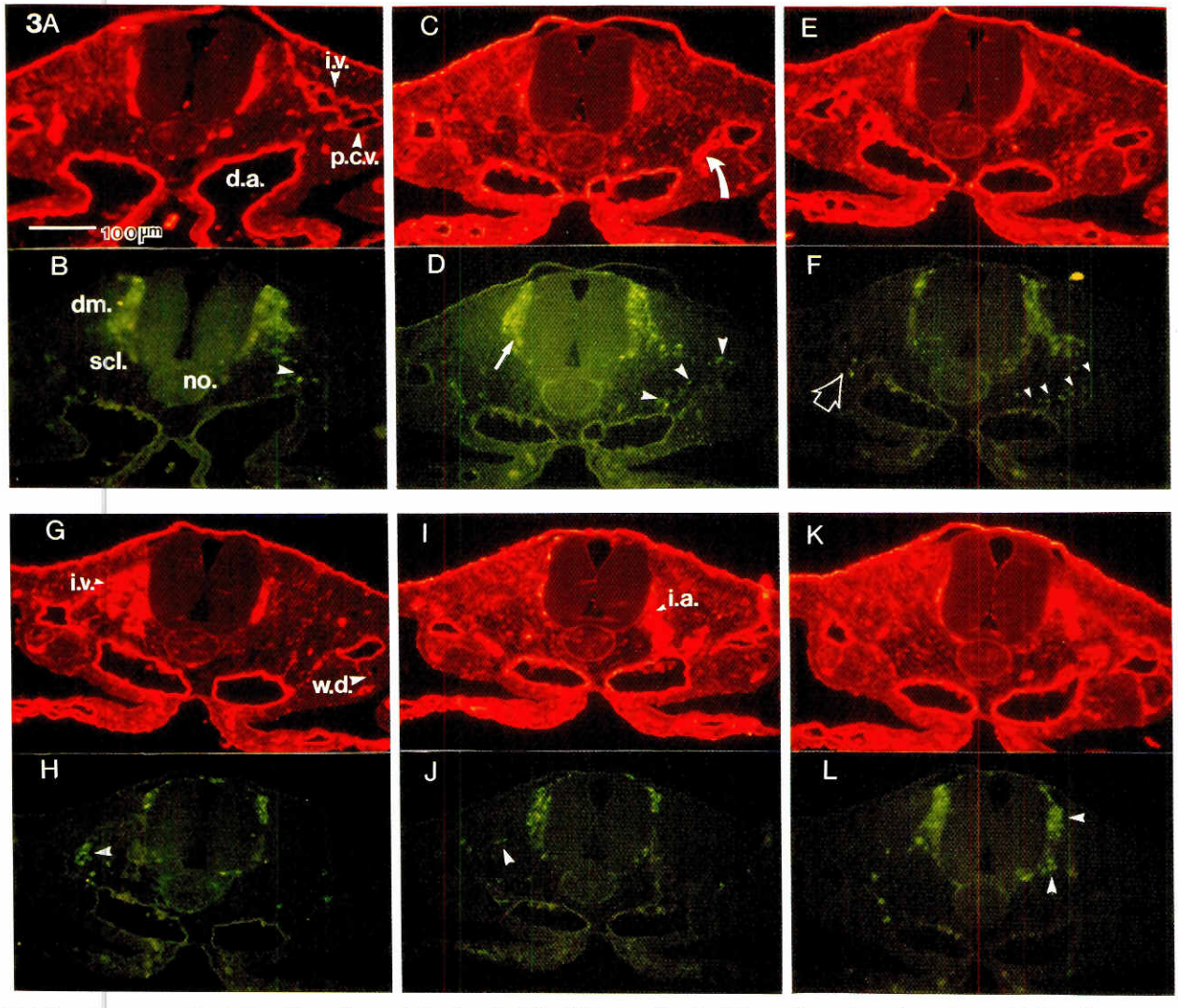


Fig. 3. Slightly oblique semiserial sections through the level of the 17th somite of a 32-somite embryo (craniocaudal direction, respectively) double immunolabeled with QH-1 [RITC] (A,C,E,G,I,K) and HNK-1 [FITC] (B,D,F,H,J,L). (A,B) *On the left, the rostral aspect of the left somite. On the right, the intersomitic space between the 16th and 17th somite, the intersomitic vein (i.v. in* **A***) branching from the posterior cardinal vein (p.c.v.). On the right, crest cells are evident beneath the intersomitic vein and adjacent to the posterior cardinal vein, p.c.v. and the dorsal aorta, d.a. (dermamyotome, d.m.; no, notochord; sclerotome, scl).* **(C,D)** *On the left, the midsomite region of the 17th somite. In the caudal part of the left somite, crest cells (arrow in* **D***) are evident between the developing sclerotome and the neural tube. On the right, in the rostral part of the 17th somite, crest cells (arrowheads) migrate under the dermamyotome to the p.c.v. and then adjacent to an anastomosing blood vessel (curved arrow in* **C***) to the dorsal aorta.* **(E,F)** *On the left, the posterior part of the 17th somite, adjacent to the intersomitic space. Crest cells are evident between the sclerotome and the neural tube; some cells are also evident between the p.c.v. and d.a. (open arrow). On the right, the rostral part of the 17th somite. Note the crest cells (arrowheads) adjacent to the anastomosing blood vessel (curved arrow in* **C** *not labeled in* **E***).* **(G,H)** *On the left, the intersomitic space between the 17th and 18th somites, crest cells are interposed between the p.c.v. and the d.a. (small arrowhead in* **H***). On the right, the posterior aspect of the 17th somite; wolfian duct (wd).* **(I,J)** *On the left, the rostral part of the 18th somite with crest cells in the sclerotome (arrowhead). On the right, the rostral part of the intersomitic space between the 17th and 18th somites, individual crest cells located next to the p.c.v. and dorsal aorta.* **(K,L)** *On the left, the rostral part of the 18th somite. On the right, crest cells adjacent to the intersomitic artery. Note, the aggregates of crest cells dorsal and ventral to the intersomitic artery, adjacent to the neural tube. Some of the HNK-1 positive cells located ventral to the intersomitic artery may be satellite cells derived from the ventral portion of the neural tube.*

At later stages of migration, some NC cells are present in the same section as those containing intersomitic blood vessels (see Fig. 3). However, by this stage of migration most of these cells are rarely adjacent to both blood vessels and are usually most concentrated in the caudal aspect of the intersomitic cleft in close association with the intersomitic vein (see Fig. 3A,B). The presence of the intersomitic vein in this section confirms the fact that these NC cells are in the intersomitic cleft itself rather than in the rostral part of the adjacent somite.

Neural crest migration in the rostral aspect of the sclerotome

NC cells enter the rostral part of the sclerotome approximately 4-5 somites cranial to the segmental plate, where the somite develops into the dermamyotome and the sclerotome. The leading edge of this mass of NC cells is contained within the most rostral part of the sclerotome, in those areas directly apposed to the intersomitic blood vessels (Fig. 2E,F,G and H). These cells will eventually encounter NC cells adjacent the posterior cardinal vein which arrived earlier, through the intersomitic pathway (also see

Loring and Erickson, 1987). A less prominent mass of NC cells is also found in the region of von Ebner's fissure (Fig. 2E,F,G, and H), a small shallow trough present in the middle of the somite that contains a blood vessel we have designated the somitic vein (Fig. 2E,F). This blood vessel branches from the posterior cardinal vein. Thus, NC cell migration in the rostral part of the sclerotome is initiated in the mesenchyme of the cranial wall of the sclerotome and followed soon after by movement in the middle of the sclerotome, within von Ebner's fissure. Crest cells traversing this space are noted adjacent to the dorsal aorta and the posterior cardinal vein by the level 7-8 somites cranial to the segmental plate.

Neural crest migration in the vicinity of an anastomosing vessel which connects the posterior cardinal vein with the dorsal aorta

In the most advanced stages of NC migration, cells traversing the rostral part of the sclerotome reach the ventral aspect of the embryo, adjacent to the posterior cardinal vein. This is indicated in serial sections taken through the level of the 17th to 18th somites of a 32-somite embryo (Fig. 3). In these micrographs, NC cells are evident in the rostral part of the sclerotome, just under the dermamyotome extending to the posterior cardinal vein. It is not known how these cells make their way from the posterior cardinal vein to the surface of the dorsal aorta, a distance of approximately 75-100 μm.

Careful examination suggests that these cells traverse the most lateral edges of the sclerotome to the posterior cardinal vein and migrate in close proximity to an anastomosing vessel which joins the dorsal aorta (Fig 3). The anastomosing vessel appears to be most prominent in the rostral part of each somite. Furthermore, it appears to be a transient structure since it is not always present at more cranial levels. As NC cells reach the posterior cardinal vein and the dorsal aorta they redistribute from a segmented to nonsegmented pattern along the surface of these two vessels (see Fig. 4).

Fibronectin

FN appears to be ubiquitously distributed throughout the trunk of the 32-somite embryo (Fig. 4). FN immunoreactivity is most heavily concentrated around developing blood vessels. For example, newly formed blood vessels in the caudal eminence, the intersomitic blood vessels (Figs. 4, 7A', see also Fig. 8A'), the vertebral artery (Fig. 7A'), and the dorsal aorta (Fig. 4 see also Fig. 8A' and B') all stain intensely for FN. Approximately 3-4 somites cranial to the segmental plate (Fig. 4C), NC cells preferentially enter the intersomitic space. A cross section through this area (Fig. 8A-A") reveals that the developing intersomitic blood vessels and the dorsal aorta possess an ECM rich in FN.

As NC cells enter the rostral part of the sclerotome and migrate ventrally to the posterior cardinal vein, FN appears to be uniformly distributed within the sclerotome (Fig. 7A-A"). When viewed in cross section (Fig. 8B-B"), FN is most concentrated in the basal lamina under the dermamyotome and within the ventrolateral aspect of the sclerotome in areas containing many crest cells. As NC redistribute along the dorsal aorta, FN is evident along the rostrocaudal length of the dorsal aorta (Fig. 4). FN is also concentrated between the ectoderm and the dermamyotome in a region traversed by migrating melanocyte precursors (Fig. 8B-B"). Conversely, FN immunofluorescence is also evident in areas where NC cells will not migrate, such as the basal lamina surrounding the notochord and the mesonephros. Very little immunoreactivity for FN is apparent in the dermamyotome, the neural tube, the notochord or the urogenital ridge.

Laminin

Laminin (LN) appears to have a more restricted distribution than FN in the trunk of the day-3 embryo (Fig. 5B). LN is not associated with newly formed blood vessels. In newly formed somites, about 3-4 somites cranial to the segmental plate, LN immunoreactivity is occasionally observed in the caudal part of the somite in a pattern that complements HNK-1 staining endogenous to the rostral part of the somite. HNK-1 labeling of the rostral somite cells has been well documented by Newgreen *et al.* (1990) and diminishes as these somites mature. As dermamyotome and sclerotome develop, about 5-6 somites cranial to the segmental plate, NC cells initiate their migration through the rostral part of the sclerotome and LN is uniformly distributed throughout the somite (see open arrow Fig. 5B).

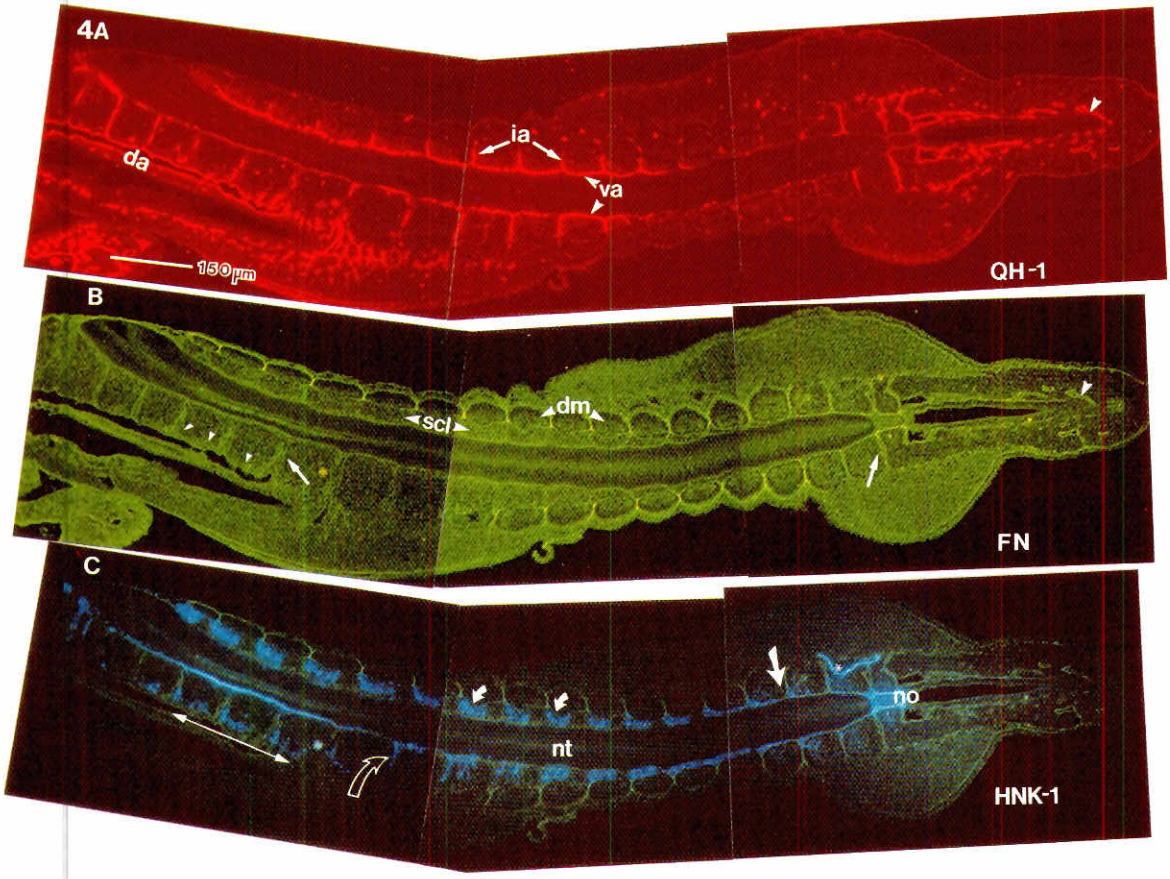
Eight to ten somites cranial to the segmental plate (see Figs. 5 and 7B-B") LN, although present along the entire rostrocaudal length of the somite, is most predominant in the rostral part of the sclerotome and codistributes with migrating NC cells and emerging ventral root fibers. Between the posterior cardinal vein and the dorsal aorta in the rostral part of the sclerotome, there is punctate labeling for LN which is superimposable on individual NC cells (Fig. 8C'-C"). Dorsally, where NC cells come in initial contact with the basement membrane of the dermamyotome (Fig. 8C-C"), LN labels the rostrocaudal length of the basal lamina of the dermamyotome (Figs. 5B, 7B and 8C'). Ventrally, LN immunofluorescence associated with the basement membrane of the myotome is most predominant in the rostral part of the somite as compared to the caudal part of the somite (Figs. 5B and 7B'). As these somites mature, LN becomes evenly distributed throughout the rostrocaudal length of the basement membrane of the dermamyotome. At this stage of development LN's only association with blood vessels is between the paired dorsal aortae and within the basal lamina of the endoderm (Fig. 8C-C").

In more cranial regions of the 32-somite embryo, the maturing dorsal aorta becomes more LN immunoreactive (Fig. 5); however, LN is not associated with the intersomitic blood vessels. At these levels, crest cells are observed adjacent to the dorsal aorta. Within the rostral part of the somite, LN immunoreactivity becomes more pronounced and has a "fan-like" appearance (Fig. 5B), emerging from the neural tube and expanding into the ventral sclerotome. As with FN, LN is also associated with structures where NC cells will not migrate, such as the notochord and the basal lamina surrounding the mesonephric (Wolffian) duct. A line of LN immunofluorescence appears to surround the neural tube (Fig. 8C').

Tenascin

TN immunoreactivity was tested with two polyclonal antibodies (rabbit and guinea pig). Similar staining patterns were observed regardless of the antibody used.

Newly formed somites appear to be surrounded by TN immunoreactivity (Fig. 6). TN labeling is also associated with the intersomitic cleft and is most intense in the basement membrane surrounding the caudal aspect of the somite (Fig. 6). In these areas, TN appears to be more closely associated with the intersomitic artery than with the intersomitic vein. As noted earlier, NC cells are usually more numerous in the caudal aspect of the intersomitic cleft in close association with the intersomitic vein. In a cross section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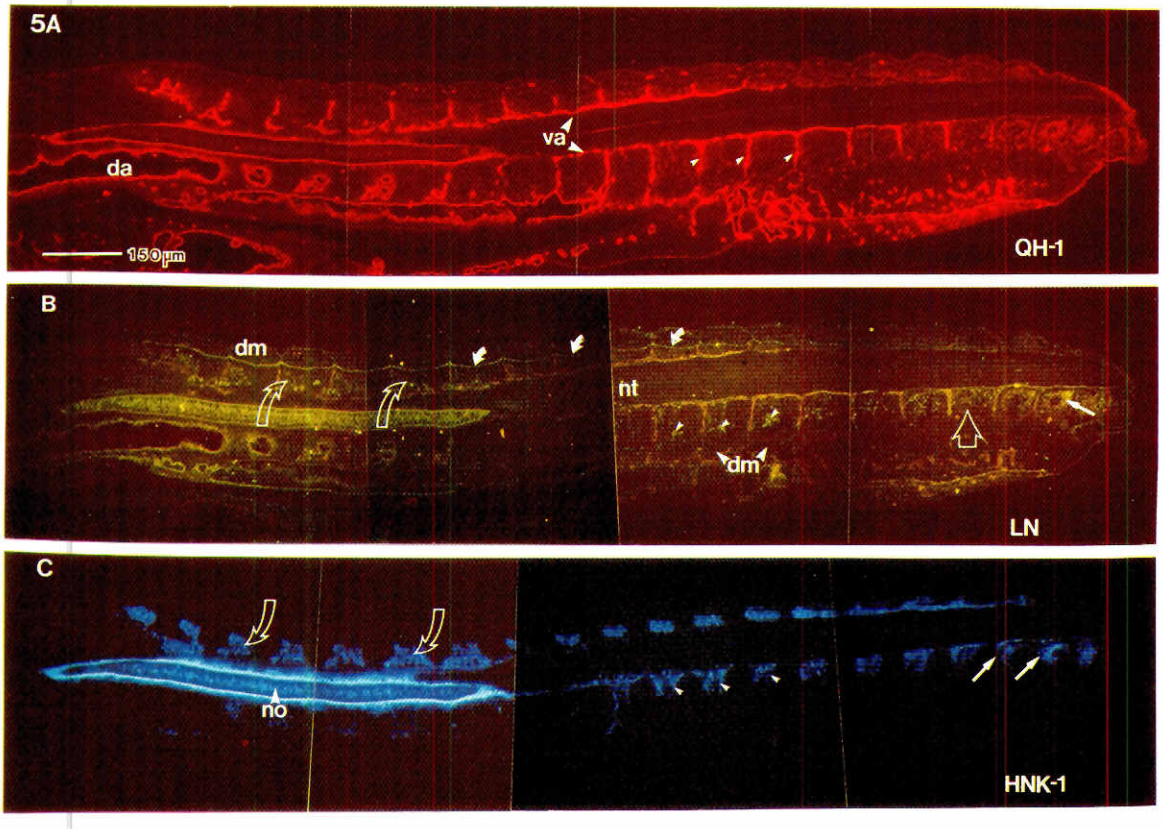
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through the rostral part of the intersomitic cleft, which contains the intersomitic artery (Fig. 8D-D"), TN immunoreactivity is not superimposable around this vessel. Most TN immunoreactivity appears to be in the somitic mesenchyme slightly rostral to the intersomitic artery, a few cell widths away. NC cells observed in this space are localized in a small channel between the TN immunoreactive somitic mesenchyme, the neural tube and the intersomitic blood vessels (see Fig. 8D"). TN is also evident surrounding the outlines of the dorsal aorta and, to a lesser extent, adjacent to the posterior cardinal vein, from which the intersomitic vein arises. As crest cells enter the rostral somite, TN immunoreactivity is evident within the mesenchyme of the caudal part of the developing sclerotome (see Fig. 7C-C"). Ten to fifteen somites cranial to the segmental plate, TN is evenly distributed along the rostrocaudal axis of the ventral sclerotome in the angle between the dermamyotome, posterior cardinal vein and the dorsal aorta. These areas are not immediately accessible to migrating NC cells.

Within the vagal region, TN immunoreactivity eventually appears in the rostral part of the sclerotome. The labeled area corresponds to slightly more than half of the sclerotome and colocalizes with migrating crest cells (data not shown). Unlike the organized fibrillar presence of tenascin in the caudal aspect of younger somites, TN immunoreactivity associated with migrating crest cells is punctate and appears sparse. TN immunoreactivity is also associated with the basement membranes of the neural tube, the notochord and as a diffuse deposit along the ectodermal surface of the dermamyotome.

NC cells will associate with "capillary-like" endothelial cell structures in vitro

Five to six days after explanting aortic segments onto 3-D basement membrane gels, "capillary-like" endothelial cell sprouts appeared from all ends of the explant (Fig. 9). Immunocytochemical staining for QH-1 indicates that the "capillary-like" structures were composed of endothelial cells and not fibroblasts (data not shown).

To examine NC cell-endothelial cell interactions, NC cells were introduced into aortic explant cultures that exhibited significant "capillary-like" endothelial cell differentiation. After two days, these cultures were fixed and double immunolabeled for QH-1 and HNK-1. Of the 8 aortic explant cultures inoculated with NC cells, 7 exhibited close associations between the "capillary-like" structures and NC cells. One culture could not be viewed because the gel had lifted off the bottom of the culture well. NC cells selectively associate with the surface of "capillary-like" structures rather than adhering to the gel (Fig. 9). Crest cells associated with the "capillary-like" endothelial cell structures assumed a rounded morphology (Fig. 9).

Discussion

NC migration in the intersomitic cleft

Our results indicate that crest cells initially migrate through the intersomitic cleft. The formation of the intersomitic blood vessels preceded NC cell migration within the intersomitic cleft by 2-3 somites (approximately 3 h of developmental time, assuming that a somite is formed every 70 min; see Newgreen *et al.*, 1990). During the initial stages of crest migration through the intersomitic space, NC cells migrate in close apposition to the intersomitic blood vessels. Although NC cells were noted adjacent to the medial surface of the intersomitic artery, it appears that the majority of crest cells traversing this space prefer to associate with the intersomitic vein, in the caudal part of the cleft. NC cell migration may be excluded within the rostral part of the cleft due to the absence of cell-free space caused by the overlap of these vessels in this area. Three to five somites cranial to the axial level where migration in the intersomitic space is initiated, this pathway is relatively obscured by NC cells migrating through the rostral part of the somite. These results reflect the transient nature of the intersomitic pathway and may explain why there has been some controversy as to its existence.

NC migration within the somite

The majority of NC cells in the rostral part of the somite appeared to be localized to those areas directly apposed to the intersomitic cleft. In the midsomite region, within von Ebner's fissure, a smaller mass of cells was also observed. Both of these areas lie in close relation to or contain blood vessels.

As NC cells become localized along the dorsal aorta they redistribute from the segmented pattern present within the rostral part of the somite to a nonsegmented continuous pattern along the surface of this vessel. Using quail/chick chimeric embryos Yip (1986) was able to determine that crest cells migrated, on the average, 2 segments cranially and 3 segments caudally once they

Fig. 4. Frontal section of a 32-somite embryo, triple labeled with QH-1 (A); anti-FN (B); and HNK-1 (C). Cranial regions of the embryo are oriented towards the viewer's left while the caudal regions are on the viewer's right. (A) *QH-1: all of the major embryonic vessel primordia is present: ia, intersomitic arteries; va, the vertebral arteries; da, dorsal aorta. Newly formed blood vessels in the caudal eminence (arrow head).* **(B)** *FN: small arrows, FN-rich areas around the intersomitic blood vessels. Small arrowheads, FN-rich matrix surrounding the dorsal aorta. In caudal eminence, FN surrounds newly formed blood vessels (arrowhead) dermamyotome (dm) sclerotomes (scl).* **(C)** *HNK-1: in caudal regions crest cells are evident within the intersomitic space 3-4 somites cranial to the segmental plate (large solid curved arrow). More cranial regions, crest cells are evident in the rostral part of each sclerotome, just under the dermamyotome (small solid curved arrows). Crest cells (open curved arrow) in the rostral sclerotome adjacent to the intersomitic cleft. Redistribution of crest cells adjacent to the dorsal aorta (doubleheaded arrow). *, a piece of lint caught under the coverslip; no, notochord; nt, neural tube.*

Fig. 5. Frontal section of a 32-somite embryo, triple-immunolabeled for: QH-1 (A); anti-LN (B); and HNK-1 (C). Orientation as in Fig 4. (A) *QH-1: dorsal aorta (da), vertebral arteries (va) and the intersomitic blood vessel (arrowheads).* **(B)** *LN: initially localized in the caudal aspect of the immature nascent somite (solid small arrow). As the somite develops into the dermamyotome/sclerotome, LN is evident throughout the sclerotome (open large arrow); LN becomes progressively localized to the rostral part of the sclerotome and the ventral part of the basal lamina of the dermamyotome (small arrowheads). Dorsally, LN immunoreactivity is evident along the entire length of the basal lamina of the dermamyotome (small curved arrows). In more cranial regions, LN labeling in the rostral sclerotome is superimposable with neural crest cells and ventral root fibers (open curved large arrows). LN is also associated with the developing heart and the dorsal aorta.* **(C)** *HNK-1: in caudal regions of the embryo, HNK-1 immunoreactivity predominates in the rostral half of the nascent somite (small arrows). At more advanced stages of migration, crest cells enter the rostral part of the sclerotome (small arrowheads). HNK-1 positive ventral root fibers (large open curved arrows) overlap with the pattern of laminin expression. The notochord (no) is also HNK-1 positive.*

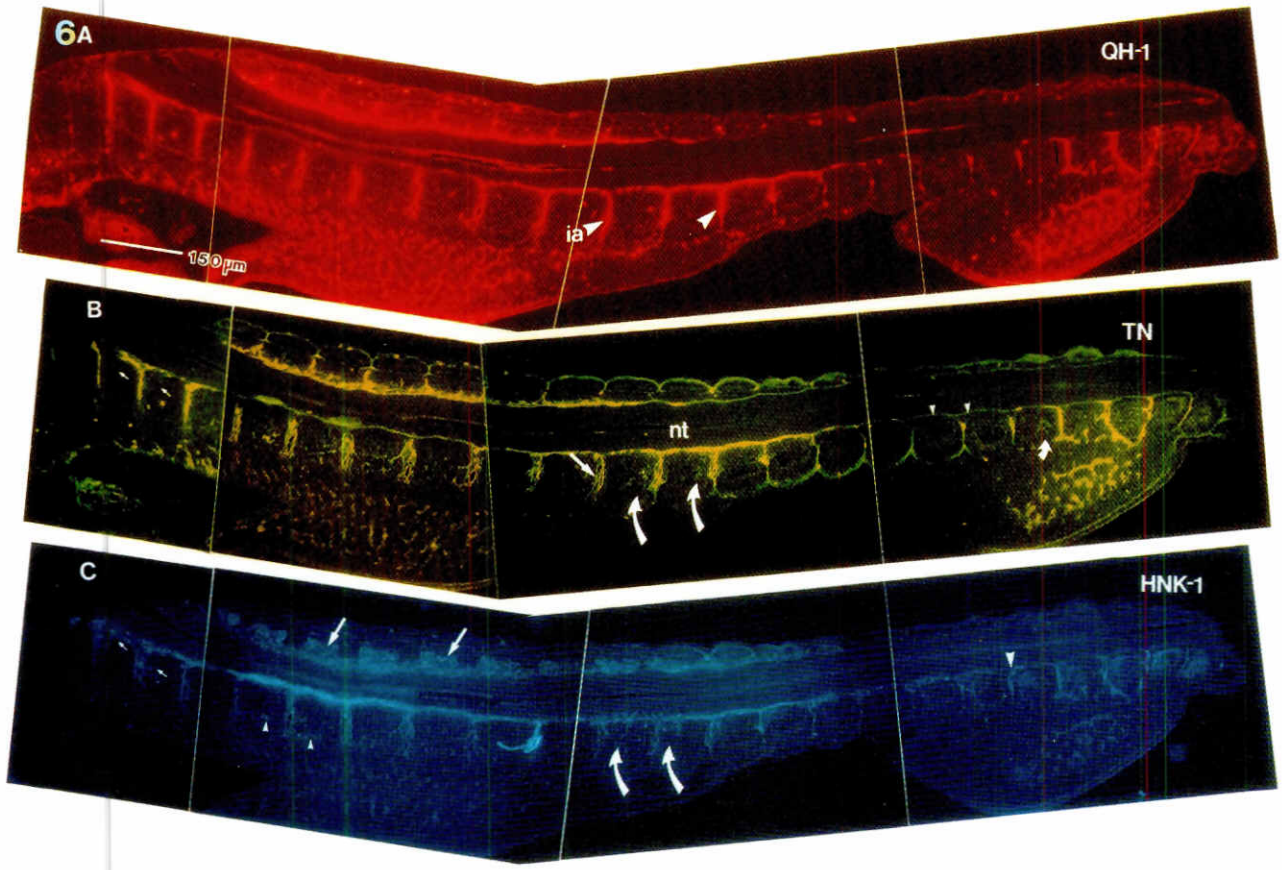


Fig. 6. Frontal section of a 32-somite embryo triple immunolabeled for QH-1 (A), anti-TN (B), and HNK-1 (C). Orientation as in Figs. 4 and 5. (A) *QH-1: i.a. the intersomitic blood vessels. Note the vertebral blood vessels present on either side of the neural tube.* **(B)** *TN: immunoreactivity surrounds each somite and is also localized to the caudal part of the sclerotome (small curved arrow) prior to neural crest migration in the rostral part of the sclerotome. TN surrounds the neural tube and the vertebral blood vessels (small arrowheads). As neural crest cells enter the rostral part of the sclerotome (see* **C** *large curved arrows), TN predominates in the caudal half (large curved arrows in* **B**). *TN associated with the intersomitic cleft is most predominant in the basement membrane of the posterior aspect of the next somite rostrally (see, small straight arrow in* **B**). *Over time TN becomes more localized to the rostral part of the somite (very small arrows in* **B**), *this labeling obscured at these magnifications.* **(C)** *HNK-1: initial stages of neural crest migration are first observed in the intersomitic cleft 3-5 somites anterior to the segmental plate (small arrowhead). In more advanced stages of migration, neural crest cells enter the rostral part of the sclerotome (large curved arrows). Crest cells in the ventrolateral aspect of the embryo medial to the posterior cardinal vein (very small arrowhead). Developing dorsal root ganglion (straight arrows), neural crest cells and emerging ventral root fibers in the rostral part of the sclerotome (very small arrows, cranially).*

reached the surface of the dorsal aorta. Such results implicate the surface and extracellular matrix of the dorsal aorta as a substrate for NC cell migration. Similar studies by Teillet *et al.* (1987) have demonstrated that NC cells located between the neural tube and the caudal sclerotome migrate for a short distance either cranially or caudally along the neural axis. NC cells in the caudal sclerotome were never observed further ventrally than the vertebral blood vessels. Perhaps crest cells in these areas are utilizing the vertebral blood vessel to migrate in the cranial-caudal plane.

Extracellular matrix

Based on immunolabeling studies, a model (Fig. 10) has been constructed which illustrates the distribution of FN and TN with regard to NC cell-blood vessel interactions. The distribution of LN has been omitted since it is not found in areas of initial NC cell-blood vessel interactions. In this model FN, a very adhesive ECM molecule (Newgreen, 1982; Rovasio *et al.*, 1983; Tucker and Erickson, 1984), is most concentrated on the blood vessel surface. TN, a relatively non-adhesive ECM molecule (Tan *et al.*, 1987; Mackie *et al.*, 1988), is most concentrated a short distance from the blood vessel surface. In this scenario TN may channel NC cells to the FN rich blood vessel surface. FN then mediates adhesion and migration of the NC cells along the blood vessel surface. This model suggests that directed cell migration can be accomplished by the exclusion of cell migration from areas containing a nonadhesive substratum to areas which contain an adhesive substratum. Similar functions have been proposed for molecules bearing a moiety that binds peanut lectin which have been identified in the caudal half of the somite prior to and during NC migration in the rostral part of the somite (Stern *et al.*, 1986).

The presence of the ECM glycoprotein FN, *in vivo*, has been spatiotemporally correlated with NC migration (Thiery *et al.*, 1985; Thiery and Duband, 1986). In addition, the cessation of movement of NC cells in some cases coincides with the local disappearance of FN (Thiery *et al.*, 1982). *In vitro* studies have shown FN to be the most suitable substrate for NC cell adhesion and motility (Newgreen,

1982; Rovasio *et al.,* 1983; Tucker and Erickson, 1984). Although ubiquitously distributed in the quail embryo, FN is most concentrated around developing blood vessels. Within the intersomitic cleft, FN is superimposable with the developing intersomitic blood vessels, and NC cells which traverse this space do so in close apposition to these vessels (Erickson, 1985a,b; Loring and Erickson, 1987). Once NC cells reach the dorsal aorta, they redistribute along the surface of this vessel for several segments (Yip, 1986). Interestingly, the dorsal aorta is the most FN-rich area in the developing embryo and this immunoreactivity persists after migration is complete (Duband *et al.,* 1985). FN has also been proposed to promote the adrenergic differentiation of crest cells, a property of sympathetic neurons located adjacent to the dorsal aorta (Sieber-Blum *et al.,* 1981). Perhaps the FN-rich ECM of the dorsal aorta is responsible for the redistribution of crest cells adjacent to this vessel and the differentiation of these cells into sympathetic neurons.

Although TN colocalizes with trunk NC cells at advanced stages of NC dispersion through the rostral part of the sclerotome (Tan *et al.,* 1987; Mackie *et al.,* 1988; Bronner-Fraser, 1989; Stern *et al.,* 1989), the purified molecule itself appears to be a poor substrate for NC cell attachment and migration *in vitro* (Tan *et al.,* 1987; Mackie *et al.,* 1988). With regard to blood vessel development within the intersomitic cleft, TN expression does codistribute with blood vessels in these areas; however, it appears to be more closely associated with the intersomitic artery than the intersomitic vein. When crest cells traverse the intersomitic cleft they are found predominantly within the caudal part of the cleft, which contains the intersomitic vein and significantly less TN immunoreactivity than the rostral part of the intersomitic cleft. From these results it appears that migrating NC cells initially favor those areas which contain significantly less TN immunoreactivity.

TN, when isolated from chicken cell cultures, shows a characteristic pattern of a high molecular weight form and two lower molecular weight forms (220 kD, 200 kD, and 190 kD, respectively; Jones *et al.,* 1989). By ablating NC cells, Stern *et al.* (1989) were able to suppress the expression of the higher molecular weight form of TN. Recently, it has been demonstrated that NC cells are capable of synthesizing TN (Tucker and McKay, 1991), probably the high molecular weight form. While this form of TN is not thought to direct migration it may generate a local environment which promotes motility (Tucker and McKay, 1991). This phenomenon can be explained by assuming that the role of the extracellular matrix in cell migration is twofold: a certain amount of adhesion is required to generate traction within the environment at the front part of the cell, and the cells must be able to detach their trailing edge from the substrate when migrating from one place to another. Increased cell migration in a nonadhesive matrix has been obtained for NC cells cultured on collagen gels containing chondroitin sulfate or chondroitin sulfate proteoglycans, both nonadhesive ECM constituents (Tucker and Erickson, 1984). TN has also been shown to interfere with cell to FN adhesive interactions (Chiquet-Ehrismann *et al.,* 1988; Riou *et al.,* 1990). During embryonic development the high molecular weight form of TN is expressed in regions of dynamic tissue remodeling and cell motility, whereas the low molecular weight forms are associated with cell proliferation and differentiation (Kaplony *et al.,* 1991; Tucker *et al.,* 1993). TN associated developing blood vessels and in the caudal part of the somite is probably of the low molecular weight form and this form of TN may be inhibitory to NC cell migration at high concentrations.

LN has a more restricted distribution in the developing avian embryo than FN (Timpl, 1979). LN was never associated with the intersomitic blood vessels during these stages of development and was only associated with the ECM of larger and more mature blood vessels such as the dorsal aorta. The fact that no LN is associated with the intersomitic space at this time in development precludes it from playing a role in the migration of NC cells within this area. However, since LN is associated with the dorsal aorta, it may play a role in the redistribution of NC cells along this vessel.

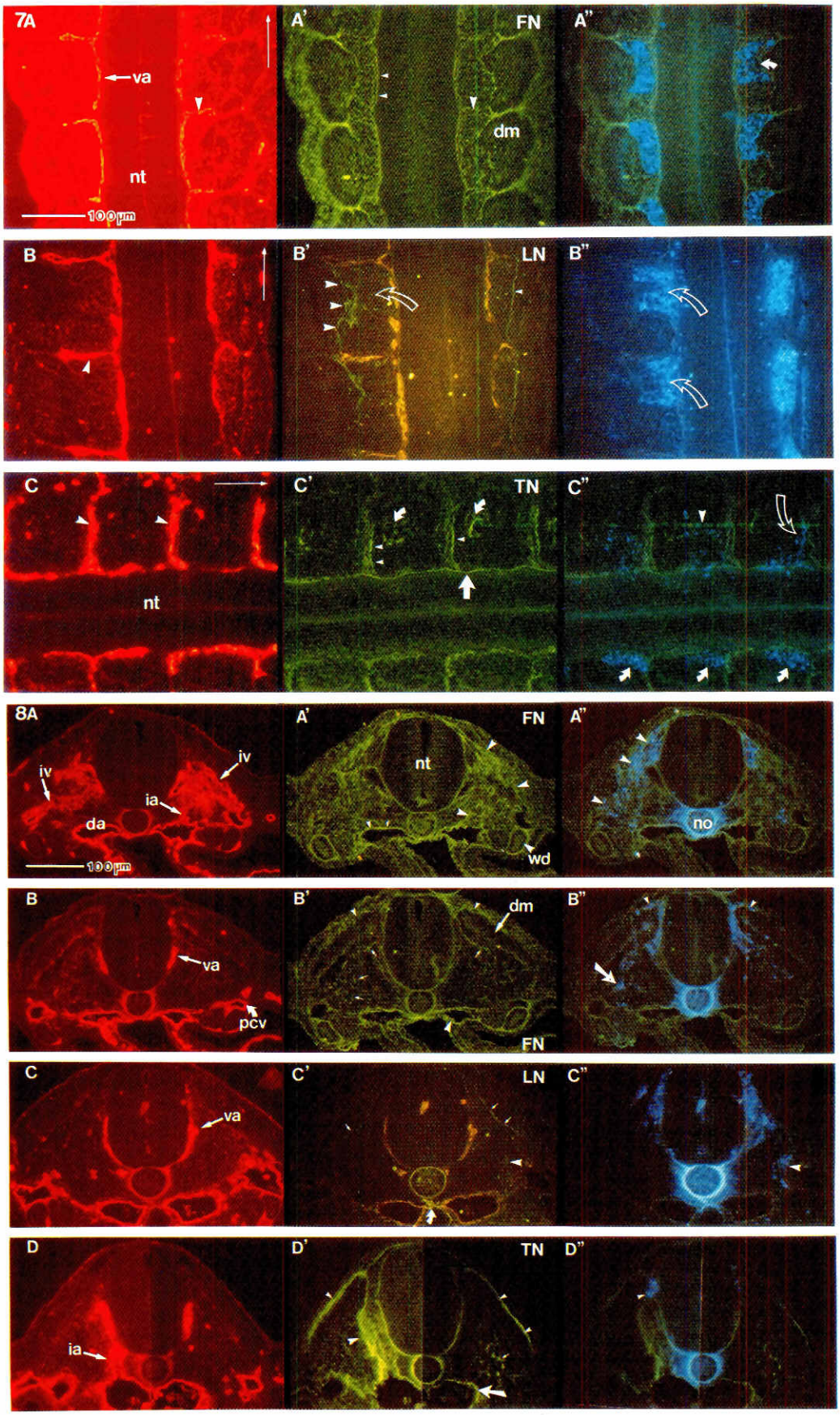
It is interesting to note that these blood vessels form by two different morphological mechanisms. The dorsal aorta forms by vasculogenesis, a process where blood vessels develop in place by the aggregation of angioblasts (Coffin and Poole, 1988; Poole and Coffin, 1989, 1991). The intersomitic blood vessels form by angiogenesis, where blood vessels form by sprouting from preexisting blood vessels such as the dorsal aorta (Coffin and Poole, 1988; Poole and Coffin, 1989, 1991). The differential distribution of LN could reflect a difference between these two mechanisms of development or differences in maturity of these vessel rudiments at these stages. Recently, LN has been shown to be a potent promoter of tube formation of cultured endothelial cells (Kubota *et al.,* 1988). Perhaps the presence of LN around these vessels signifies a change in morphology from nonfunctional, coalesced, endothelial cell sprouts to functional tube-like capillaries. The intense staining observed between the paired dorsal aortae suggests that LN may also play a role in the eventual fusion of these vessels.

LN is initially most concentrated in the basement membrane of the dermamyotome in the antero-dorsal part of the somite, paralleling myotome development (Kaehn *et al.,* 1988). As the somite matures, LN becomes more concentrated in the rostral part of the sclerotome. The "fan-like" distribution of laminin immunoreactivity observed in rostral somite at advanced stages of development suggests that LN immunoreactivity is closely associated with ventral root fibers emerging from the neural tube (Rogers *et al.,* 1986). Like others (Rogers *et al.,* 1986; Duband and Thiery, 1987; Newgreen *et al.,* 1990), these results indicate that the distribution of LN overlapped with, but did not correspond precisely to the spatiotemporal distribution of early migrating NC cells. Therefore, LN alone could not be responsible for the initial segmented distribution of NC cells into the rostral part of the sclerotome.

NC associations with "capillary-like" structures in vitro

Models of angiogenesis similar to those utilized here have been characterized and studied extensively (Folkman and Haudenschild, 1980; Kubota *et al.,* 1988). These models have also been used to obtain purified cultures of endothelial cells from several different species of animals. The purity of the cultures derived from these methods has been confirmed by the uptake of fluorescent acetylated low density lipoprotein (Di-I-Ac-LDL) and immunostaining with antibodies for von Willebrand Factor (vWF), both of which selectively label endothelial cells (Voyta *et al.,* 1980; Wagner *et al.,* 1982). We examined the cellular composition of the "capillary-like" structures in our cultures by immunolabeling with QH-1 and confirmed that these sprouts are composed of endothelial cells.

The fact that NC cells preferentially associate with the surface of the "capillary-like" structures, rather than the substance of the gel itself, suggests several hypotheses explaining these interactions. First, the "capillary-like" structures may be a more adhesive substrate than the surrounding gel. These structures are immunocytochemically positive for laminin, fibronectin and collagen IV (Timpl *et al.,* 1979; Newgreen and Thiery, 1980; Thiery *et al.,* 1982; Newgreen, 1984; Duband and Thiery, 1987; Loring and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Erickson, 1987), all of which support NC cell migration. The second hypothesis is that the endothelial cell structures are producing some substance or growth factor which acts as a chemo-attractant and also stimulates NC cell growth and or survival. NC cells not associated with these structures may die due to lack of trophic support. In this regard laminin, associated with the "capillary-like" structures, has neurotrophic properties as well as adhesive properties (Edgar *et al.*, 1984; Lander *et al.*, 1985). Laminin is also a potent promoter of the "capillary-like" differentiation of endothelial cells *in vitro* (Kubota *et al.*, 1988). The third possibility would be some combination of the first and second. In this case, NC cells would be initially attracted to the surface of the "capillary-like" structures by a diffusible chemotactic agent secreted by the endothelial cells and, once contact is established, preferentially adhere to the ECM surrounding these structures.

The results presented here suggest that the embryonic vascular pattern may play a role in the patterned migration of NC cells in certain locations. Developing blood vessels may be a more adhesive substratum than the surrounding mesenchyme and once NC cells become attached they may be unlikely to move away. Further investigation will be required to determine the molecular basis for the oriented migration described in this paper.

Materials and Methods

Preparation and immunolabeling of whole-mount specimens

Fertilized Japanese quail eggs were obtained from Cornell University Hatchery and incubated in a forced draft, humidified incubator at 38.5°C. On embryonic day 3, eggs were opened, embryos removed, staged by counting the number of somite pairs and fixed in 10% buffered formalin for 24 h. Following fixation embryos were: washed in phosphate buffered saline (PBS, pH 7.4, 3 changes 10 min each), immersed in ice cold 100% methanol (3 changes, 30 min each, 4°C), and placed on a rotary shaker at -4°C for 24 h. Specimens were rehydrated in a graded series of ethanol (100%, 90%, 70%, 50%, 30%, dH20 to PBS, 2 changes each for 10 min), immersed in a 3% solution of bovine serum albumin (BSA) in PBS and placed on a rotary shaker at ~4°C for 24 h. All immunolabeling and subsequent washes were performed at 4°C on a rotary shaker. Embryos were washed in PBS (3x5 min), incubated in a mixture of QH-1 and HNK-1, (both at concentrations of 1:400 for 24 h), washed in PBS for 6 h and incubated with goat antimouse FITC Fab'2 IgM, μ chain specific 1:400 (Accurate Chemical, for HNK-1) and goat anti-mouse RITC Fab'2 IgG 1:400 (Accurate Chemical, for QH-1) for 24 h. Embryos were washed in PBS for 4 h, dehydrated in a graded series of ethanol, cleared in toluene, mounted on slides and coverslipped with Entellan (VWR Scientific) for observation and photography. Specimens were photographed with Ektachrome 160 tungsten slide film (Kodak) or Ektachrome 200 daylight slide film (Kodak). Color prints were made directly from slides using Cibachrome color paper (C-Max Custom Color Photography Inc., Syracuse, NY, USA).

Paraffin embedding/immunolabeling

Embryos were oriented in a culture dish and a small amount of Omnifix was applied with a transfer pipette. When the embryos turned white they were transferred to a scintillation vial, immersed in Omnifix for 1-2 h, transferred to 100% ETOH, 100% xylene (each: 3 changes, 7 min) and embedded in Paraplast (Fisher) starting with: 50% Paraplast/50% xylene for 5 min, followed by 3 changes of 100% Paraplast (56°C). The embryos were oriented and sectioned on a microtome at 7 μm, mounted on

Fig. 7. Three frontal sections (A-C), taken from 33 somite embryos, triple stained with QH-1; ECM antibodies to: FN, LN, and TN; and HNK-1 photographed at 25x. The thin white arrow present in each set of photos indicates cranial direction. (A-A'') *Eight to ten somites cranial to the segmental plate triple immunolabeled with QH-1* **(A)**; *anti-FN* **(A')** *and HNK-1* **(A'')**. *In* **A**, *the vertebral artery (va), neural tube (nt), intersomitic blood vessels (arrowhead). In* **7A'**, *FN, although ubiquitously distributed, is most concentrated between each dermamyotome (dm), around the intersomitic blood vessels (arrowhead) and along the vertebral artery (small arrowheads). In* **A''**, *crest cells are within the rostral part of the sclerotome (curved arrow).* **(B-B'')** *Eight to ten somites cranial to the segmental plate triple labeled with QH-1* **(B)**; *anti-LN* **(B')**; *and HNK-1* **(B'')**. *This section is more ventral than those in A-A''. In* **B**, *intersomitic vessels (arrowhead). In* **B'**, *LN is most concentrated in the rostral part of the sclerotome (curved open arrow) and in the basal lamina of the dermamyotome (arrowheads). In dorsal regions (small arrowhead), laminin is evident in the basement membrane of the dermamyotome. In* **B''**, *crest cell in the rostral part of the sclerotome are superimposable with LN (see* **B'**). **(C-C'')** *Six to eight somites cranial to the segmental plate triple labeled with QH-1* **(C)**; *anti-TN* **(C')**; *and HNK-1* **(C'')**. *Cranial regions oriented towards the viewer's left. In* **C**, *the intersomitic blood vessels (arrowheads) and the vertebral arteries are evident. In* **C'**, *TN surrounds the neural tube and is associated with the vertebral arteries (large solid arrow). TN also appears to be associated with the intersomitic blood vessels (small arrowheads). Note that TN is most evident in the rostral part of the intersomitic space (associated with the basement membrane surrounding the caudal part of each somite). TN is also evident in the mesenchyme of the caudal part of the sclerotome (see curved small arrows). In* **C''**, *crest cells in the rostral part of each somite (see curved arrows and arrowhead).*

Fig. 8. Four cross sections (A-D) through the trunks of 32-33 somite embryos triple immunolabeled with QH-1 (A,B,C,D); various ECM glycoproteins (A', FN; B', FN, C', LN; D', TN) and HNK-1 (A'',B'',C'', D''). (A-A''.) A, *Rostral part of the intersomitic space (the viewer's right) 4-5 somites cranial to the segmental plate, intersomitic artery (ia) and the intersomitic vein (iv). On the left, the caudal part of the intersomitic space which contains the intersomitic vein branching from the posterior cardinal vein. In* **A'**, *FN, although ubiquitously distributed, is most concentrated in the ECM surrounding the intersomitic blood vessels (large arrowheads) and the dorsal aorta (small arrowheads). FN is also associated with the wolfian duct (wd, mesonephros). In* **A''**, *crest cells (arrowheads) in the caudal part of the intersomitic space on the left. On the right, large mass of the overlapping blood vessels, some crest cell are located ventrally, adjacent to wolfian duct.* **(B-B'')** *Five somites cranial to the segmental plate; the vertebral artery (va), the posterior cardinal vein (pcv, see* **B**) *and dermamyotome (dm). FN* **(B')**, *associated with the dorsal aorta (large arrowhead) and the wolfian duct. Fibrillar matrix of FN (small arrows) lines the perspective path of neural crest migration (see* **B''**, *curved arrow) through the rostral sclerotome. Premelanocyte cells are evident under the ectoderm (see arrowheads in* **B''**) *in a FN-rich matrix (in* **B'**). **(C-C'')** *Ten somites cranial to the segmental plate, all of the major blood vessel primordia* **(C)** *is evident. In* **C'**, *LN is evident in the basal lamina of the dermamyotome (small arrows) and as punctate staining (arrowhead) within the most ventrolateral aspect of the sclerotome just medial to the posterior cardinal vein. In addition, some LN is evident the most dorsal region of the sclerotome, between the dermamyotome and the neural tube just dorsal to the vertebral artery. In* **C''**, *neural crest cells in the sclerotome, superimposable with LN (compare arrowheads in* **C'** and **C''**). *Intense laminin staining is also observed between the dorsal aortae (curved arrow in* **C'**). **(D-D'')** *Two adjacent sections spliced together. On the left, the intersomitic space 15-14 somites cranial to the segmental plate; on the right, the caudal aspect of the 15th somite cranial to the segmental plate. On the right (in* **D**), *the intersomitic artery branches from the dorsal aorta. TN, as shown in* **D'**, *is evident in the intersomitic space (large arrowhead in* **D'**); *however, unlike FN it is not superimposable with the intersomitic blood vessels. Crest cells, in this area (arrowhead in* **D''**), *are evident in channels lined by TN. TN is also associated with the dorsal aorta (arrow in* **D'**). *Within the sclerotome, TN is most concentrated caudo-ventrally (small arrow on the right in* **D'**) *in the angle between the dermamyotome and the dorsal aorta, an area not accessible to migrating neural crest cells. TN is also evident on the ectodermal surface of the dermamyotome (small arrowheads in* **D'**), *around the neural tube and the notochord.*

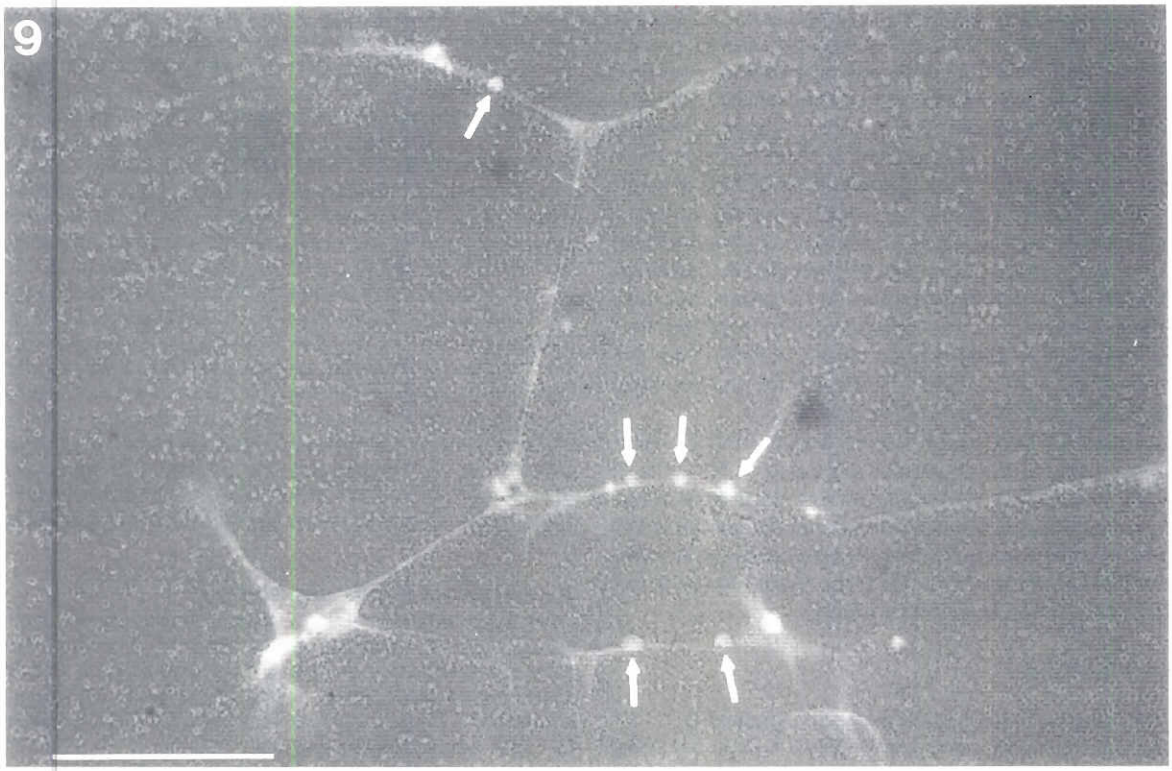

Fig. 9. Combination phase contrast and HNK-1 epifluorescence indicating HNK-1 positive neural crest cells (arrows) associated with "capillary-like" endothelial cell structures. *Scale bar, 100 μm.*

albuminized slides, dewaxed and rehydrated in the following series of solutions: 100% xylene (2 changes, 2 min), 100% ETOH (2 changes, 2 min), 70% ETOH (2 min), 50% ETOH (2 min), 30% ETOH (2 min) to PBS (pH. 7.4). Primary antibodies were then applied. The following antibodies were used: QH-1 (Developmental Studies Hybridoma Bank), a monoclonal IgG made in mouse which labels quail endothelial cells (Lebastie *et al.*, 1986; Pardanaud *et al.*, 1987; Coffin and Poole, 1988); HNK-1 (Art Tatum, SUNYHSC at Syracuse), a monoclonal IgM made in mouse which labels migrating NC cells (Tucker *et al.*, 1984); Rabbit anti-Human Fibronectin, polyclonal (Calbiochemical); Rabbit anti-Laminin, polyclonal (Sigma Chemical); Rabbit anti-Tenascin, polyclonal (from Douglas Fambrough PhD.); and Guinea Pig anti-Tenascin, polyclonal (from Eleanor Mackie Ph.D., also see Chiquet-Ehrismann *et al.*, 1986).

We used the following combination of primary antibodies for triple labeling:

QH-1	QH-1	QH-1
HNK-1	HNK-1	HNK-1
anti-FN (rabbit)	anti-LN (rabbit)	anti-TN (rabbit or guinea pig)

Because of the possible cross-reactivity between primary and secondary antibodies, it was necessary to select secondary antibodies which were specific for unique domains of our primary antibodies. Antibodies were also selected which had cross reactivity with other species of animals removed; these antibodies were made for multiple labeling procedures (Jackson Immunoresearch). We used the following secondary $F(ab')_2$ fragments of affinity purified and affinity absorbed antibodies: RITC-conjugated Goat anti-Mouse IgG (specific for the Fc region) cross-reactivity removed for human, bovine, horse and serum protein (Jackson Immunoresearch), to visualize QH-1; AMCA-conjugated Goat anti-Mouse IgM (μ chain specific) to visualize HNK-1 (Jackson Immunoresearch); DTAF-conjugated Goat anti-Rabbit IgG (h and l chain specific) minimal cross reactivity with human serum protein. (Jackson Immunoresearch); and FITC-conjugated Goat-anti-Guinea Pig (h and l chain specific) (Jackson Immunoresearch).

Sections were incubated for 1 h with 3% BSA (bovine serum albumin, Sigma) in PBS (pH. 7.4) to block any nonspecific binding sites. One of the three combinations of antibodies shown above was applied to tissue sections and incubated at 37.5°C. for 1 h. The antibody concentrations were as follows: QH-1, 1:1000; HNK-1, 1:500; anti-Fibronectin, 1:100-1:200; anti-Laminin, 1:50-1:100; anti-Tenascin (rabbit), 1:50-1:100; anti-Tenascin (guinea pig), 1:50-1:100 (all diluted in 3% BSA in PBS). Following incubation, sections were washed in PBS (3 changes x10 min), secondary antibodies (also diluted in 3% BSA in PBS) applied and incubated in a humidified chamber for 1 h. Sections were washed in PBS (3 changes x10 min) and coverslipped with Citifluor PBS/Glycerol solution (Ted Pella Inc.). The coverslips were sealed with clear nail polish and sections viewed for immunofluorescence. Sections were photographed using Ektachrome 400 (Kodak) color slide film.

Primary antibody cross-reactivity with the secondary antibodies generated against other isotypes and species was tested. For example, some sections were labeled with QH-1 and processed (as noted above) with goat anti-rabbit IgG (H&L), goat anti-mouse IgM (μ), or goat anti-guinea pig secondary antibodies. No cross-reactivity was observed. Furthermore, each primary antibody was also used alone, labeled with the appropriate secondary and viewed. The same staining pattern was observed regardless of the number of antibodies used.

Preparation of thoracic aorta explant cultures

Frozen stock basement membrane gel, derived from mouse EHS tumor cells (obtained from Dr. Roy Ogle University of Virginia School of Medicine; also see Kleinman *et al.*, 1986) was diluted 1:4 with Dulbecco's Modified Eagles Medium (DMEM, Sigma) at 4°C to prevent gelling. Two chamber tissue culture slides (Nunc, from VWR Scientific) were coated with 0.4-0.5 ml of diluted basement membrane solution. Gelation was induced by

incubation at 37°C for 30-60 min in a humidified incubator containing 90% air and 10% CO_2. Gels were equilibrated with complete medium [DMEM, 1% Penicillin/streptomycin, glutamine (Irvine Scientific, Irvine, CA, USA) and 7% fetal bovine serum (FBS, Sigma)] for 1-2 h at 37°C. The medium was removed prior to use.

Adult Japanese quail were anesthetized by etherization followed by decapitation and thoracotomy. The vessel for explant was aseptically removed, rinsed in DMEM and gently cleaned of periadventitial fat. The thoracic aortae were split longitudinally, cut into flat segments (approximately 1-2 mm squares) and transferred to the desired substratum endothelial side down with enough medium to keep moist. Approximately 2-3 explants were placed in each chamber. Explants were incubated 24-48 h in a humidified incubator at 37°C (90% air/10% CO_2), after which complete medium (1 ml/chamber) was added to each culture. The initial incubation period with very little medium was important to ensure that the explants adhered to the substratum. Once the explant adhered, medium was replenished every 2-3 days. After 5-7 days in culture, a "capillary-like" endothelial cell structure sprouted from the explants, and NC cells (see preparation below) were added to the aortic cultures.

Preparation of neural crest cells

Japanese quail eggs were incubated at 38°C for approximately 50 h to reach stage 10-13 of Zacchei (1961), equivalent to approximately 15 pairs of somites. The embryos were aseptically removed from the eggs and the caudal 5-7 somite regions were excised with microsurgery scissors. The trunk fragments were incubated for 15 min at room temperature with 200 μg/ml dispase (Sigma) in Hanks Balanced Salt Solution (Ca^{+2}, Mg^{+2} free; Sigma) in 35-mm Petri dishes (Falcon Labware). The neural tube segments corresponding to only the lumbar levels were freed from the ectoderm, the somites, the endoderm and from the notochord with tungsten needles under a dissecting microscope. The neural tubes were transferred to a 35 mm Petri dish containing culture medium (with 10% FBS), incubated at 37°C in a humidified 90% air/10% CO_2 incubator for 1 h to recover, and transferred to multiwell slide chambers which had been coated with fibronectin (FN, 10 μg/ml, Sigma). Neural tubes were left in position for 24 h and removed. This was a sufficient amount of time for the NC cells to migrate onto the substratum. NC cells which migrated from the neural tube onto the FN-treated substrate were collected by gentle pipetting after a brief exposure to trypsin (Halfter *et al.*, 1989). The dissociated NC cells were resuspended in complete medium, containing 10% FBS (to inhibit enzyme), and transferred to the aortic cultures.

After co-culturing NC cells with the aortic explants for 2-3 days, the cultures were washed with PBS, fixed with 4% paraformaldehyde in PBS (pH 7.4) for 8 h, washed with PBS and labeled with primary antibodies at 4°C for 24 h. Some preparations were double labeled with HNK-1 and QH-1 (1:400 and 1:1000, respectively). Other preparations were only labeled with HNK-1. The cultures were then extensively washed in PBS for 6 h and incubated with goat anti-mouse FITC Fab'2 IgM (μ chain specific, Jackson Immunoresearch) 1:400 in PBS and goat antimouse RITC Fab'2 IgG (Jackson Immunoresearch)1:1000 in PBS for 24 h at 4°C. Cultures were washed in PBS for 4-6 h, examined with an inverted microscope (Nikon) and photographed with Ektachrome 400 tungsten slide film (Kodak) or TriX400 (Kodak). All prints made using Cibachrome color paper (C-Max Custom Color Lab, Syracuse, NY, USA).

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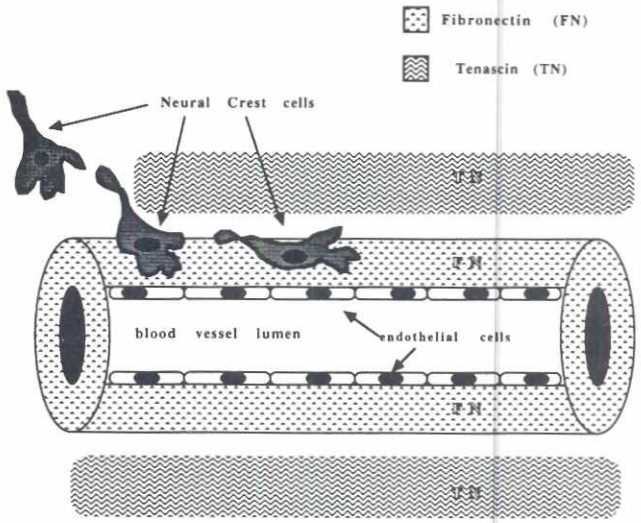

Fig. 10. A model illustrating the distribution of FN and TN with regard to NC-blood vessel associations. *FN is most concentrated on the blood vessel surface. TN is most concentrated a short distance from the blood vessel surface. TN, through its relatively non-adhesive properties, helps channel neural crest cells to the FN-rich surface of the intersomitic blood vessel. FN then facilitates the adhesion and migration of NC cells along the vessel surface.*

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