Differential deposition of basement membrane components during formation of the caudal neural tube in the mouse embryo

K. SUE O'SHEA

Department of Anatomy and Cell Biology, University of Michigan Medical School, Ann Arbor, MI 48109, USA

Summary

The distribution of basement membrane and extracellular matrix components laminin, fibronectin, type IV collagen and heparan sulphate proteoglycan was examined during posterior neuropore closure and secondary neurulation in the mouse embryo. During posterior neuropore closure, these components were densely deposited in basement membranes of neuroepithelium, blood vessels, gut and notochord; although deposition was sparse in the midline of the regressing primitive streak. During secondary neurulation, mesenchymal cells formed an initial aggregate near the dorsal surface, which canalized and merged with the anterior neuroepithelium. With aggregation, fibronectin and heparan sulphate proteoglycan were first detected at the base of a 3- to 4-layer zone of radially organized cells. With formation of a lumen within the aggregate, laminin and type IV collagen were also deposited in the forming basement membrane. During both posterior neuropore closure and secondary neurulation, fibronectin and heparan sulphate proteoglycan were associated with the most caudal portion of the neuroepithelium, the region where newly formed epithelium merges with the consolidated neuroepithelium. In regions of neural crest migration, the deposition of basement membrane components was altered, lacking laminin and type IV collagen, with increased deposition of fibronectin and heparan sulphate proteoglycan.

Key words: basement membrane, extracellular matrix, fibronectin, collagen, heparan sulphate proteoglycan, laminin, neurulation, neural crest, mouse embryo.

Introduction

Reciprocal cell–cell interactions appear to play an important role in determining the nature and timing of coordinated cell divisions, migrations and other events critical to the early development of the vertebrate embryo. Recently, considerable attention has focused on epithelial–mesenchymal interactions, transitions and the role of the extracellular environment in these processes.

In the morphogenesis of a number of organs, spaces between mesenchymal cells collapse, cells aggregate, secrete a basement membrane and form a lumen, taking on an epithelial configuration. Similarly, with the loss of the basement membrane (or components) cells may escape from an epithelium, 'becoming' mesenchymal. The occurrence of these transitions is well established; however, the mechanisms involved, particularly the roles of the cells themselves in producing the local alterations that permit/participate in the transitions remain to be determined.

The current investigation has examined one such system characterized by mesenchymal–epithelial conversion, the development of the caudal portion of the neural tube. Unlike the cephalic neural tube where neurulation movements are produced by neuroepithelial cell shaping changes (cf. Karfunkel, 1974), and support from the underlying mesenchyme (Morris & Solursh, 1978), formation of the caudal portion of the neuraxis is complicated by a second series of events in which mesenchymal cells aggregate, epithelialize and merge with the anterior neuroepithelium; a process termed 'secondary neurulation' (Schoenwolf, 1984). In the current investigation, the time of appearance and pattern of localization of extracellular matrix and basement membrane components: fibronectin (FN), laminin (LN), heparan sulphate proteoglycan (HSPG), and type IV collagen (IV) are correlated...
with cell shaping and topographical alterations involved in posterior neuropore closure and secondary neurulation in the mouse embryo.

Materials and methods

Tissue

Embryos were obtained from matings of CD-1 (Charles River Laboratories) mice; the morning of finding a vaginal plug was considered to be the first day of gestation. At intervals on the 10th, 11th and 12th days, decidual swellings were removed from the uterus, and embryos dissected from decidua, chorion and amnion. Over 350 embryos from 33 litters were examined in the course of this study. Embryos were staged and caudal portions (caudal to the hindlimb buds) were excised and immediately frozen in OCT-embedding compound (Miles) in hexane cooled over an acetone/dry ice slurry.

Sections to be examined using immunocytochemistry (ICC) were air dried, exposed to 5% normal goat serum followed by the primary antibody for 2 h at room temperature in a moist chamber. They were then washed in PBS and exposed to the second antibody (goat anti-rabbit IgG) conjugated to FITC (1:50, Cappel) for 30 min at room temperature. Sections were washed extensively in PBS, coverslipped with glycerol containing 0.1% phenylenediamine and sealed with Kronig wax. Controls were incubated in preimmune serum or PBS in place of the primary antibody.

Because of the extensive codistribution and numerous interactions of these components (cf. Ruoslahti & Engvall, 1980), a number of enzyme digestions were applied in an attempt to augment the staining or change the pattern of localization (Holund & Clemmensen, 1982). Collagenase (275 i.u. ml⁻¹; pH 7.4, Gibco), Streptomyces hyaluronidase (100 Tru ml⁻¹; Miles), testicular hyaluronidase (400 i.u. ml⁻¹; Cappel) were applied to the sections for 1 h at 37°C prior to exposure to the primary antibody. Exposure to testicular hyaluronidase significantly improved the intensity of fibronectin immunoreactivity and was employed throughout these studies. No other treatment was effective and none altered the pattern of localization.

Immunocytochemistry

Sections to be examined using immunocytochemistry (ICC) were air dried, exposed to 5% normal goat serum followed by the primary antibody for 2 h at room temperature in a moist chamber. They were then washed in PBS and exposed to the second antibody (goat anti-rabbit IgG) conjugated to FITC (1:50, Cappel) for 30 min at room temperature. Sections were washed extensively in PBS, coverslipped with glycerol containing 0.1% phenylenediamine and sealed with Kronig wax. Controls were incubated in preimmune serum or PBS in place of the primary antibody.

Because of the extensive codistribution and numerous interactions of these components (cf. Ruoslahti & Engvall, 1980), a number of enzyme digestions were applied in an attempt to augment the staining or change the pattern of localization (Holund & Clemmensen, 1982). Collagenase (275 i.u. ml⁻¹; pH 7.4, Gibco), Streptomyces hyaluronidase (100 Tru ml⁻¹; Miles), testicular hyaluronidase (400 i.u. ml⁻¹; Cappel) were applied to the sections for 1 h at 37°C prior to exposure to the primary antibody. Exposure to testicular hyaluronidase significantly improved the intensity of fibronectin immunoreactivity and was employed throughout these studies. No other treatment was effective and none altered the pattern of localization.

Antibodies

Antibodies to laminin were obtained from Dr J. P. McCoy, Department of Pathology, University of Michigan; antibodies to type IV collagen from Dr H. Furthmayr, Department of Pathology, Yale University; and antibodies to heparan sulphate proteoglycan from Dr J. Hassell, National Institute of Dental Research. All three antigens were isolated from EHS sarcoma. Antibodies to human serum fibronectin were purchased from Cappel laboratories. All were raised in rabbits, were affinity purified and exhibited no cross reactivity by ELISA. Anti-laminin was used at a concentration of 1:50, anti-fibronectin 1:10, anti-type IV collagen 1:100, and anti-heparan sulphate proteoglycan 1:20.

After staining, slides were viewed and photographed in a Leitz Dialux orthoplan photomicroscope using transmitted or u.v. light. Toluidine blue-stained sections were photographed using Technical Pan 2415 film; immunofluorescence was photographed using Kodak 2475 recording film.

Results

Regional development

Cellular alterations involved in posterior neuropore closure are essentially as described previously (Waterman, 1976; Wilson & Finta, 1980). The timing and pattern of events involved in secondary neurulation varies only slightly from that described by Schoenwolf (1984) in C57BL/6 mouse embryos. These events will be outlined briefly to provide a basis for evaluating the immunocytochemical results.

At the earliest stage of development examined (day 10.5, 25- to 28-somite stage), the posterior neuropore was a trough-shaped extension of the neural tube (Fig. 1A). At the caudal portion of the posterior neuropore was a bulbous end region, the most caudal extent of the primitive streak. Mesenchymal cells in this region were stellate and loosely packed, the regressing primitive streak was present as a plate-like epithelial thickening. Cephalad, the elevating neural folds were present on the dorsal surface of the embryo. Neuroepithelial cells were tightly organized around the forming lumen while the basal surface was still somewhat uneven. With neural tube closure, the neuroepithelium consolidated and detached from the surface ectoderm. The hindgut was typically well organized at this level and blood vessels coursed near it.

During secondary neurulation (day 11, 29- to 32-somite stage), the posterior neuropore had closed and the primitive streak regressed forming the tailbud of the embryo (Fig. 1B). At the tip of the tailbud was a region of loosely packed, stellate mesenchymal cells, bounded by surface ectoderm. Rostrally, mesenchymal cells aggregated; a clear boundary separated the three to four layers of radially oriented cells from the
Fig. 1. (A) Sagittal section through the closing posterior neuropore region of a day 10½ embryo. The neural tube has nearly closed in the dorsal midline, the notochord (N) is well established between neuroepithelium (NE) and hindgut (G). The regressing primitive streak (PS) can be seen near the caudal extent of the embryo. (B) Sagittal section through the tailbud region on the 11th day. The neural tube is closed throughout its extent and surface ectoderm covers the entire region. Notochord and hindgut (G) are well formed. NE, neuroepithelium. Arrowhead a, level of mesenchymal aggregation; arrowhead b, level of cavitation. Scale bars, 100 μm.
adjacent mesenchyme. At more cephalic levels, a horizontal lumen formed between aggregated cells; a thick plate of cells was found at its dorsal surface. There was an obvious demarcation between its uneven ventral border and the surrounding mesenchyme.

As the lumen of the forming tube consolidated, the neuroepithelial cells became more organized, particularly at the dorsal surface where there was little intercellular space, unlike ventral and lateral areas. The notochord and gut had consolidated and there were numerous blood vessels in the region. Secondary neurulation continued into the 12th day extending the length of the tailbud, but the basic pattern of events was similar to that seen on the 11th day of development.

Immunocytochemistry

**Posterior neuropore closure**

At the distal tip of the tailbud, in the region of unaggregated mesenchyme, there was scattered FN and HSPG staining, very little IV or LN was present in this region. Cephalad, at the base of the primitive streak, all components were present, but were typically less-densely deposited near the midline, where cells from the streak migrate into the mesenchyme (Fig. 2A). As the neural folds began to elevate, the deposition of these components became more linear at the base of the neuroepithelium. Type IV collagen (Fig. 2B) and LN were densely deposited in the lateral and medial regions of the neuroepithelial basement membrane, while in the dorsolateral margin, the region of active neural crest cell migration, there was little LN or IV. FN (Fig. 2C) and HSPG were often less-densely deposited near the midline. Basement membranes of blood vessels and of the hindgut stained intensely with all components.

With neural tube closure, HSPG and FN formed a continuous boundary around the neuroepithelium and were densely deposited in the mesenchyme dorsolateral to the neural tube. LN and IV similarly surrounded the neuroepithelium but were depleted in the dorsolateral areas of neural crest emigration (Fig. 2D). Basement membranes of blood vessels, hindgut and the notochord stained intensely. Unlike FN and HSPG which were also components of the mesenchyme, IV and LN were restricted to basement membranes.

At the caudal extent of the neuroepithelium, the region where newly formed epithelium merges with neuroepithelium of the posterior neuropore, FN and HSPG (Fig. 3A) were densely deposited, while LN and IV (Fig. 3B) were absent from this zone.

**Secondary neurulation**

As during posterior neuropore closure, FN and HSPG were found in the unorganized mesenchyme at the tip of the tailbud, LN and IV were not deposited in this region. Cephalad, with the formation of the mesenchymal aggregate near the dorsal surface, punctate deposits of HSPG and FN were present at the base of the aggregated cells. Both were excluded from between the rosette of cells but were general mesenchymal components (Fig. 4A,C). As the aggregate consolidated and a central lumen began to form, FN was densely deposited at the base of these cells (Fig. 4B). HSPG formed a delicate border at the base of the forming neuroepithelial cells and, like FN, was a general mesenchymal component (Fig. 4D).

The pattern of deposition of LN and IV was considerably different to that of FN and HSPG during secondary neurulation. With aggregation, LN and IV were also excluded from between the cells of the rosette. Slight punctate IV staining was present with aggregation (Fig. 5A); however, laminin was not present at this stage (Fig. 5C). With cavitation of the aggregate, LN and IV were found in the neuroepithelial basement membrane especially above the notochord, along its lateral border, but were absent at its dorsolateral margin (Fig. 5B,D).

Cephalad, at the level of the consolidated neural tube, deposition was similar to that seen during posterior neuropore closure, FN and HSPG completely surrounded the neural tube and were enriched at its dorsolateral border. IV and LN were most-densely deposited medially, slightly less densely in the lateral region of the neuroepithelial basement membrane. Very little LN or IV were present at the dorsolateral border in the neural crest migration zone. All four components were associated with basement membranes of forming blood vessels in the region.

In sagittal section, HSPG and particularly FN were densely deposited at the caudal portion of the consolidated neuropore (Fig. 3C), the zone where epithelium formed via secondary neurulation fuses with the more anterior neuroepithelium. This zone continued to be enriched in these components when examined at later phases of secondary neurulation on the 12th day of development. IV (Fig. 3D) and LN were not present in the merging zone at any stage of development.

**Discussion**

**Posterior neuropore closure**

This study demonstrates the presence of fibronectin, heparan sulphate proteoglycan, type IV collagen and laminin in the neuroepithelial basement membrane.
during closure of the posterior neuropore. Deposition of these components was especially dense in the lower two-thirds of the neural tube, while at its dorsolateral surface, type IV collagen and laminin were typically absent, and the deposition of FN and of HSPG was increased.

In addition to providing general 'structural stability' to the neuroepithelium, the roles of individual components of the neuroepithelial basement membrane remain to be determined. It seems likely that as in other systems (Singer, 1979; Woods, Hőök, Kjellén, Smith & Rees, 1984), they may interact with the

Fig. 2. (A) Cross section through the primitive streak in a day 10.5 embryo, illustrating heparan sulphate proteoglycan deposition in basement membranes and lateral but not medial regions of the base of the primitive streak. (B) Cross section at a more rostral level illustrating the more linear deposition of type IV collagen along the neuroepithelial basement membrane (arrowed) as the neural folds begin to elevate. It is also a particularly prominent component of the hindgut (G) basement membrane. (C) Section through the elevating neural folds of the posterior neuropore illustrating the considerable staining of the basement membrane of the neuroepithelium (NE), with less staining medially. Fibronectin is also associated with basement membranes of blood vessels, hindgut (G), and is deposited in the mesenchyme. (D) More rostral section illustrating laminin deposition in basement membranes of the region. Note its absence with neural crest migration (arrowed). Scale bars, 100 μm.
microfilaments located in the base of the neuroepithelial cells (Sadler, Greenberg, Coughlin & Lessard, 1980), to provide anchorage and thus functional polarization to these cells. Abnormalities of the neuroepithelial basement membrane result in a loss of this polarity, with the normally radially oriented neuroepithelial cells making transverse progress across the neuroepithelium (Morris & O'Shea, 1983).

Fig. 3. (A) Sagittal section illustrating anti-heparan sulphate proteoglycan staining in the posterior neuropore region on day 10i. Basement membranes of notochord (N), neuroepithelium (NE), hindgut (G), and blood vessels are densely stained as is the mesenchyme. The caudal extent of the neuroepithelium (arrowed) was also stained. (B) Sagittal section through the posterior neuropore region on day 10i illustrating the distribution of type IV collagen immunoreactivity primarily associated with basement membranes. Interestingly, it is densely deposited at the base of the neuroepithelium (NE) until the primitive streak zone when it is absent. N, notochord; G, hindgut. (C) Sagittal section through the tailbud region on the 11th day illustrating the pattern of anti-fibronectin staining. Basement membranes of notochord (N), hindgut (G) and neuroepithelium (NE) are stained. There is also mesenchymal staining and the caudal extent of the neuroepithelium (arrowed) contains considerable fibronectin. (D) Pattern of type IV collagen staining in the tailbud on the 11th day. Basement membranes of hindgut (G), blood vessels, notochord and neuroepithelium (NE) are intensely stained. Unlike fibronectin and heparan sulphate proteoglycan, there is no staining associated with the most caudal extent of the neuroepithelium. Scale bars, 100 μm.
Ultimately, small changes in basement membrane composition may be responsible for highly localized events that occur within the neuroepithelium: regional patterns of proliferation (e.g. neumeres), localized medial bending of the neural folds (Schoenwolf & Franks, 1984), and neural crest rearrangements and shape changes prior to emigration from the neuroepithelium (Newgreen & Gibbins, 1982), via the cytoskeleton.

With neural fold elevation, there was little type IV collagen or laminin at the dorsolateral border of the neuroepithelial basement membrane in the region of imminent neural crest migration. Alterations in the integrity of the neuroepithelial basement membrane

---

**Fig. 4.** (A) Cross section through the region of mesenchymal cell aggregation in an 11 day embryo (arrowhead a, Fig. 1B) illustrating anti-fibronectin staining. Fibronectin is a general component of the mesenchyme and is present at the basal region of the radially organized cells (arrowed), but not between them. (B) Section through the region of lumen formation (arrowhead b, Fig. 1B) illustrating fibronectin staining associated with basement membranes of neural tube, notochord (N), hindgut (G) and blood vessels. (C) Section through the area of initial aggregation illustrating the deposition of heparan sulphate proteoglycan at the lower border of the radially aligned cells (arrowed). (D) Zone of cavitation illustrating the lack of heparan sulphate proteoglycan within the epithelium but dense deposition at its lower border (arrowed), in the mesenchyme and in the basement membrane of the hindgut (G). Scale bars, 100 μm.
during neural crest migration have previously been described in the cephalic (Nichols, 1986; Tosney, 1982), spinal (Erickson & Weston, 1983), and in the secondary neuroepithelium (Schoenwolf, 1984). Steinberg & Kimber (1986) and Tuckett & Morriss-Kay (1986) have recently illustrated alterations in the deposition of laminin and fibronectin in regions of active neural crest migration in the cephalic and trunk neural folds of rodent embryos.

The reduced deposition of type IV collagen in the region of neural crest migration is consistent with its structural function in basement membranes (cf. Timpl, Oberbaumer, Furthmayr & Kuhn, 1982); however, the absence of laminin in this region is

Fig. 5. (A) Sparse anti-type IV collagen immunoreactivity at the base of the aggregated cells (arrowed) on the 11th day. Section corresponds to arrowhead a, Fig. 1B. The basement membrane of blood vessels also stains intensely. (B) More rostral section taken at the level of lumen formation illustrating the deposition of type IV collagen in basement membranes. Note its patchy deposition in the neuroepithelial basement membrane and absence from the dorsolateral boundary. Section from level indicated by arrowhead b, Fig. 1B. N, notochord; G, hindgut. (C) Section through the level of aggregation illustrating scattered laminin immunoreactivity in the region of the forming hindgut. No laminin is associated with the base of the aggregate. (D) More rostral level at the region of cavitation. Laminin is found along basement membranes and is absent from the dorsolateral border of the neuroepithelium (arrowed) and from the adjacent mesenchyme. N, notochord; G, hindgut. Scale bars, 100 μm.
somewhat unexpected, as laminin has been shown to stimulate epithelial spreading and neurite outgrowth (Terranova, Rohrbach & Martin, 1980; but see Newgreen, 1984). Martin, Kleinman, Terranova, Ledbetter & Hassell (1984) have suggested that there is a reciprocal expression of cell surface receptors for LN and FN and that binding to one receptor may suppress the receptors for the other, thus dictating cell phenotype. Perhaps neural crest even within the neuroepithelium must already be considered ‘mesenchymal’ rather than ‘epithelial’ and LN disappears from their subjacent basement membrane. Alternatively, since as neural crest cells prepare to migrate they must change their attachments, both cell–cell and cell–BM, absence of LN from this region may be interpreted in light of its suggested role in linking cells to basement membranes (Laurie, Leblond & Martin, 1982). Whether the observed alterations in basement membrane composition result from active degradation, lack of extrusion, or cessation of production of these components remains to be determined.

Secondary neurulation
During secondary neurulation, FN and HSPG were general components of the loose mesenchyme at the tip of the tailbud; with aggregation they were lost between cells and formed a distinct boundary at their base. With consolidation of the lumen, type IV collagen and LN were also components of the forming basement membrane. Presence of FN in the undifferentiated tailbud mesenchyme is consistent with its role in maintaining space for and promoting cell migrations (Ali & Hynes, 1978; Duband & Thiery, 1982; Katow, Yamada & Solursh, 1982; Darribere, Boulekosche, Shi & Boucaut, 1985). FN is lost with mesenchymal condensation in the development of the nephron (Ekblom, 1981), odontoblasts (Thesleff, Sterman, Vaheri & Timpl, 1979) and the Müllerian duct (Ikawa, Trelstada, Hutson, Manganaro & Dornahoe, 1984). Thus, formation of the aggregate may be related to alterations in the deposition of FN and perhaps of hyaluronate, which plays a role in creating and maintaining extracellular space within the embryo (cf. Toole, Goldberg, Chi-Rosso, Underhill & Orkin, 1984).

Both FN and HSPG were densely deposited at the most caudal extent of the neuroepithelium, the region where the secondary epithelium merges with the consolidated neuroepithelium. This extracellular matrix material may be involved in providing guidance cues to the newly formed epithelium or in maintaining a favourable substrate for the merging of these two populations.

Rather than the sequential appearance of BM components reported here, more typically, BM components (LN, IV, HSPG) have been reported to appear concurrently, as in nephron (Ekblom, 1981), lung (Jaskoll & Slavkin, 1984) and retinal (Turk, Aubin, Sodek & Kalnins, 1985) development, although Leivo (1983) has noted that the presence of BM components may be detected prior to the formation of an ultrastructurally complete basal lamina. The early deposition of FN and HSPG at the base of the aggregate may be important in the reorganization of the cytoskeleton which presumably occurs with aggregation. The suggested role of HSPGs in the linking of cells to basement membranes and in initiating BM assembly via interaction with interstitial collagens (Koda & Bernfield, 1984) is also consistent with the early localization of the matrix HSPG at the base of the mesenchymal aggregate. This compositional and structural immaturity of BM of developing epithelia may provide flexibility to these tissues; with increasing age and structural completion of the BMs, the rigid scaffolding characteristic of adult BMs is formed and developmental options reduced (cf. Bernfield, Banerjee, Koda & Rapraeger, 1984).

When is an aggregate an epithelium?
A series of steps may be involved in the formation of an epithelium from mesenchyme: aggregation, cellular polarization, membrane polarization and junction formation, basement membrane formation. Studies of isolated cells have indicated that attachment to basement membrane or extracellular matrix material can initiate an epithelialization cascade (Inger, Madri & Jamieson, 1986), and polarization of retinal pigment epithelium (Turiksen et al. 1988), nephric tubule epithelium (Ekblom, 1981) and odontoblasts (Thesleff et al. 1979), correlates with the attachment of these cells to a basement membrane. The precise hierarchical relationship of these events is somewhat difficult to determine in the current example, as with aggregation cells polarized and FN and HSPG were observed at their base. Deposition of additional basement membrane components LN and type IV collagen was secondary to initial aggregation, appearing with the organization and stabilization of the epithelium. In addition to LN and IV, it will be of interest to examine the expression of other tissuespecific products (cf. Jackson, Grund, Winter, Franke & Illmensee, 1981).

Because of their role in cytoskeletal polymerization and thus cell migration, division, cell shape and gene expression (cf. Bissell, Hall & Parry, 1982), sequential alterations in the presence or relative proportion of extracellular components or in the ability of cells to respond to these components may ultimately direct
the events involved in early formation of the nervous system.

The author is grateful to Ms L.-H. J. Liu and Ms J. S. T. Rheinheimer for excellent technical assistance. Supported by NIH grant NS-21108 and grants from the Spina Bifida Association of America.

References


**Basement membrane components in the caudal neural tube**


(Accepted 9 December 1986)