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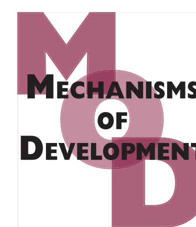

An organizing function of basement membranes in the developing nervous system

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ABSTRACT

The basement membranes (BMs) of the nervous system include (a) the pial BM that surrounds the entire CNS, (b) the BMs that outline the vascular system of the CNS and PNS and (c) the BMs that are associated with Schwann cells. We previously found that isolated BMs are bi-functionally organized, whereby the two surfaces have different compositional, biomechanical and cell adhesion properties. To find out whether the bi-functional nature of BMs has an instructive function in organizing the tissue architecture of the developing nervous system, segments of human BMs were inserted into (a) the parasomitic mesoderm of chick embryos, intersecting with the pathways of axons and neural crest cells, or (b) into the midline of the embryonic chick spinal cord. The implanted BMs integrated into the embryonic tissues within 24 h and were impenetrable to growing axons and migrating neural crests cells. Host axons and neural crest cells contacted the epithelial side but avoided the stromal side of the implanted BM. When the BMs were inserted into the spinal cord, neurons, glia cells and axons assembled at the epithelial side of the implanted BMs, while a connective tissue layer formed at the stromal side, resembling the tissue architecture of the spinal cord at the pial surface. Since the spinal cord is a-vascular at the time of BM implantation, we propose that the bi-functional nature of BMs has the function of segregating epithelial and connective cells into two adjacent compartments and participates in establishing the tissue architecture at the pial surface of the CNS.

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1. Introduction

Basement membranes (BMs) are thin sheets of extracellular matrix (ECM) that are located at the basal side of every epithelium, that outline muscle fibers and that are present at the basal surface of the vascular endothelial cells (Yurchenco and Patton, 2009; Halfter et al., 2013a). BMs are comprised of multi-domain extracellular matrix (ECM) proteins that either polymerize or bind to other ECM proteins (Timpl and Brown, 1996; Erickson and Couchman, 2000). Essential in the assembly of BMs are cellular receptors, such as integrin family members (Stephens et al., 1995; Fassler and Meyer, 1995) and dystroglycan (Henry and Campbell, 1998). BMs are evolutionary conserved in all metazoans, and mutations of BMs are either embryonic lethal or lead to muscular dystrophy, vasculature ruptures, skin blistering or CNS malformations (Gautam et al., 1996; Arikawa-Hirasawa et al., 1999; Smyth et al., 1999; Costell et al., 1999; Willem et al., 2001; Fukai et al., 2002; Halfter et al., 2002; Pöschl et al., 2004; Bader et al., 2005; Gould et al., 2005; Lee and Gross, 2007).

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BMs are very thin, and direct experimental testing of isolated BMs has been complicated by the difficulty of handling the delicate and barely visible ECM sheets. The functions of BMs have, therefore, mainly been indirectly deduced from the phenotype analysis of humans and mice with mutations of BMs proteins. The current data indicate that the underlying cause for BM-related pathologies, such as muscular dystrophy, vascular instability, skin blistering diseases and CNS dysplasias, are due to (a) the rupture of BMs, (b) the failure to provide a stable border for epithelial/endothelial tissues or muscle fibers or (c) the separation of epithelia from the underlying connective tissue layers.

The problem of direct testing for BM properties has been recently overcome by using adult human BMs for experimentation: adult human BMs are thicker than BMs from short-lived laboratory animals and can be isolated, handled, mounted on glass slides and tested for their composition, their biomechanical properties and their cell adhesion activities (Halfter et al., 2013b). These studies have shown that human as well as non-human BMs are bi-functionally organized with an epithelial side that is characterized by a high abundance of laminin, a high stiffness and a strong cell adhesion-promoting activity. The connective tissue side of BMs is softer, has a different protein composition and is anti-adhesive for epithelial cells and neurons. In the present study, we examined whether the bi-functional nature of BMs has organizing functions in embryogenesis by isolating human BMs and transplanting them into developing chick embryos.

2. Methods

2.1. Basement membranes isolation and flat mounting

Human cadaver eyes ($n = 6$) ranging from 60 to 72 years of age were obtained from CORE, the Center of Organ Recovery and Education. A list of the donor characteristics has been provided previously (Halfter et al., 2013b). The eyes were harvested within 4–24 h of death and delivered to the laboratory in less than two days. The use of the human eyes for this project was approved by the internal review board of the University of Pittsburgh under the IRB protocol number #0312072. The inner limiting membrane (ILM) was obtained by incubating segments of retinas in 2% Triton-X-100 in distilled water overnight, and transferring the ILMs into new detergent that also included 2% deoxycholate (Candiello et al., 2010). The Descemet's membrane (DM) was obtained from corneas that were incubated in 2% Triton-X-100 for 3 h followed by micro-dissection of the DM from the corneas. Lens capsules were obtained by micro-dissection. Flat mounting of the BMs was achieved by suspending segments of DM or ILM in a droplet of PBS on poly-lysine-coated slides, draining the liquid and firmly immobilizing the BMs by centrifugation at 1200 rpm for 5 min (Halfter et al., 2013b).

2.2. Implantation of human BMs into chick embryos

Segments of lens capsule (LC) and Descemet's membrane (DM) from 60 to 72-year old human eyes were stained for 1 min in 0.5% α-naphthol blue (Sigma), washed multiple times in PBS and inserted into embryonic day (E) 2.2/HH stage 14–15 chick embryos. The pre-staining of the BMs was necessary to render the otherwise transparent and very thin BMs visible and facilitate the positioning and insertion of the BMs. BMs were transplanted into the parasomitic mesoderm ($n = 28$) or into the midline of the neural tube ($n = 32$). The implants were inserted lateral to the neural tube, at the level of the segmental plate below the last formed somite (somite 27). As shown previously, neural crest cell outgrowth has not occurred at that level at that stage (Yip, 1987). For controls, segments of saran wrap (PGC Scientific #12901173; $n = 5$) or thin sheets of nitrocellulose ($n = 15$) were also transplanted. The nitrocellulose sheets were prepared by drying a drop of nitrocellulose dissolved the methanol (Lagenaur and Lemmon, 1987) onto a glass slide. The nitrocellulose sheets were lifted off the glass slides after submerging in PBS.

2.3. Immunocytochemistry

Flat mounted BMs were incubated with primary antibody for 3 h to overnight, washed, and incubated with Cy3 or Cy2-labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 2 h. For staining of spinal cord cross sections, embryos were fixed in 4% paraformaldehyde, cryoprotected in 25% sucrose, embedded in OCT compound and sectioned at 25 μm. The sections were mounted on poly-lysine-coated Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Images were taken with an Olympus FlowView confocal microscope. A polyclonal antibody to mouse laminin-111 and a monoclonal antibody to human laminin α5 (Sigma, St. Louis, MO; Invitrogen) were used to detect laminin in the human BMs. A mouse monoclonal antibody to the 7S domain of human collagen IV α3 (Mab J3-2; provided by Dr. Nirmal SundarRaj (SundarRaj and Wilson, 1982) was used to detect the human BM implants in the host chick embryos. This antibody also served as a marker for the stromal side of implanted BMs. An IgG monoclonal antibody to NCAM 180 (Mab 9H2; Halfter et al., 1997) and an IgM monoclonal to neurofilament (Mab EC/8; Developmental studies Hybridoma Bank) were used to trace axons in the chick embryos. Migrating neural crest cells were detected with a monoclonal antibody to the HNK-1 epitope (Mab 1C10; Developmental Studies Hybridoma Bank). Chick-specific monoclonal antibodies were used to detect host ECM proteins. These included antibodies to collagen II (3F1; Halfter et al., 2006), laminin (Mab 3H11; Halfter, 1993; Dev Studies Hyb Bank), perlecan (Mab 5C9; Balasubramani et al., 2004; Dev Studies Hyb Bank), fibronectin (Mab 5F3), tenascin-C (Mab M1; Dev Studies Hyb Bank) and CSPG (Mab 9BA12; Ring et al., 1995; Dev Studies Hyb Bank). A monoclonal antibody (Mab 1A3) was used to detect vascular endothelial cells. For TEM, the chick embryos were fixed in 2.5% glutaraldehyde and 0.05% tannic acid overnight. The fixed samples were osmicated and embedded in EPON according to standard protocols.

2.4. Cell adhesion and neurite outgrowth assays

Embryonic chick retinal cells were suspended at 200,000 cells/ml in DMEM/2% ovalbumin and plated onto the flat mounted BMs. After incubation for 15 min, the

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