Neural tissue co-culture with mesenchyme to investigate patterning of peripheral nerve during murine embryonic limb development

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Abstract

Lateral plate mesoderm is native to the developing limb while other cells such as neurons extend migratory axonal processes from the neural tube. Questions regarding how axons migrate to their proper location in the developing limb remain unanswered. Extracellular matrix molecules expressed in developing limb cartilages, such as the versican proteoglycan, may function as inhibitory cues to nerve migration, thus facilitating its proper patterning. In the present study, a method is described for co-culture of neural tissue with high density micromass preparations of mouse limb mesenchyme in order to investigate neurite patterning during limb chondrogenesis *in vitro*. Comparison of *hdf* (heart defect) mouse limb mesenchyme, which bears an insertional mutation in the versican proteoglycan core protein, with wild type demonstrated that the described technique provides a useful method for transgenic analysis in studies of chondrogenic regulation of neurite patterning. Differentiating wild type limb mesenchyme expressed cartilage characteristic Type II collagen and versican at 1 day and exhibited numerous well defined cartilage foci by 3 days. Wild type neurites extended into central regions of host cultures between 3 and 6 days and consistently avoided versican positive chondrogenic aggregates. Wild type neural tubes cultured with *hdf* limb mesenchyme, which does not undergo cartilage differentiation in a wild type pattern, showed that axons exhibited no avoidance characteristics within the host culture. Results suggest that differentiating limb cartilages may limit migration of axons thus aiding in the ultimate patterning of peripheral nerve in the developing limb.

Introduction

In the developing embryo, limb morphogenesis provides a useful model to study developmental processes such as pattern formation. While much has been learned about many transcription and paracrine signaling factors that regulate formation of the cartilaginous skeletal template, less is

understood regarding the role of the cartilage model on the patterning of other cell types in the developing limb.

The formation of limb cartilage begins with an increase in mesenchymal cell condensation (Mariani and Martin 2003). Extracellular matrix (ECM) and cell adhesion molecules suggested to play a role in the process of precartilage aggrega-

tion and chondrogenesis include hyaluronan, tenascin, N-CAM and N-cadherin (reviewed in Hall and Miyake 2000). Shortly thereafter, other ECM molecules such as type II collagen and aggrecan proteoglycan are expressed in differentiating cartilages.

The large chondroitin sulfate proteoglycan, versican, is also localized in the precartilaginous and early chondrogenic limb ECM (Kimata et al. 1986; Shinomura et al. 1990, 1995; Snow et al. 2005). Versican interacts with numerous matrix and cell surface proteins including CD44, hyaluronan, tenascin, fibulins, fibrillin, fibronectin, selectins, and EGF receptors (Yamagata et al. 1986; LeBaron et al. 1992; Aspberg et al. 1995, 1997; Zhang et al. 1998; Kawashima et al. 2000; Olin et al. 2001; Isogai et al. 2002). Its function has been linked to cartilage differentiation (Kimata et al. 1986; Shinomura et al. 1990), cell proliferation (Zhang et al. 1999; Yang et al. 2003), and tissue patterning (Chalepakidis et al. 1993; Harris and Jahoda, 2001). The recent availability of the heart defect (*hdf*) mutant mouse (Zhang et al. 1994), which bears an embryonic homozygous lethal mutation of *Cspg2* encoding the core protein of versican (Mjaatvedt et al. 1998), has shown that expression of the mature versican proteoglycan is pivotal to limb chondrogenesis (Williams et al. 2005).

Motor axons migrate from the ventral root of the developing spinal cord to innervate the embryonic limb led by a growth cone that serves both a locomotor and sensory function (Letourneau 1979). The ECM has been implicated in creation of pathways that growth cones and axons will follow (Akers et al. 1981; Gundersen 1987) and versican is expressed in regions avoided by both motor and sensory axons, suggesting that versican may serve a guidance function in direction of outgrowing axons to their target sites (Landolt et al. 1995; Harris and Jahoda 2001; Snow et al. 2005). Enzymatic hydrolysis of a versican-like proteoglycan *in vitro* reduced inhibitory activity on axonal migration, further suggesting that differentiating cartilage-associated ECM molecules such as versican may be a functional component of barrier activity (Fidler et al. 1999). Though these studies demonstrated a putative function for versican in regulation of peripheral nerve outgrowth, to date no investigations of axonal patterning have been attempted

with a model lacking the mature versican molecule in which limb chondrogenesis is disrupted. Because the *hdf* mutation is embryonic lethal in homozygous mice prior to formation of limb cartilages due to severe cardiac malformations, the present study was undertaken in order to develop an *in vitro* co-culture model to assess effects of wild type and *hdf* limb mesenchyme on neural patterning.

Materials and methods

Genotyping

All animal procedures were approved by the East Carolina University Institutional Animal Care and Use Committee (IACUC). Genomic DNA extraction was a modification of Laird et al. (1991). Briefly, embryonic tissue or 1 cm tail clips were placed in 500 μ l lysis buffer (0.1 M Tris HCL, pH 8.5, 5.0 mM EDTA, 0.2% SDS, 0.2 M NaCl, 100 μ g ml⁻¹ proteinase K (Invitrogen 25530-015) and incubated at 56 °C for 24 h. Following proteinase K treatment, extracts were clarified 5 min at 12,000 $\times$ g and supernatant mixed with an equal volume of isopropanol. Following gentle mixing, DNA precipitate was removed using a sterile pipet tip and dissolved in 50 μ l 10 mM Tris Cl, pH 8.0. Three to five hundred ng genomic DNA, 10 mM dNTPs (Invitrogen 18427-013), 2.5 units *Taq* polymerase (Invitrogen 10342-020), and 13 mM forward/reverse *lacZ* primers (5'-CGGCCAG-GACAGTCGTTTGCCGTCTG-3' and 5'-CC TGACCATGCAGAGGATGATGCTCG-3'; Yamamura et al. 1997) were combined for PCR amplification (MJ Research PTC-0150) of the *lacZ* portion of the *hdf* transgene. Template DNA was amplified by 36 cycles of denaturing (95 °C for 30 s), annealing (58 °C for 30 s), extension (72 °C for 30 s), and a final extension (72 °C for 10 min.). PCR products were analyzed on 1% agarose gels.

Trunk explant culture

Mice at 10.5–11 days post coitum (dpc) were harvested from timed pregnant females following IACUC-approved procedures and placed in ice cold Hank's Balanced Salt Solution (Gibco 14065-056). Homozygous *hdf* embryos could be easily

identified by distinct morphology including enlarged primitive ventricle and overall reduction in size relative to littermates (Yamamura et al. 1997). In order to isolate sufficient numbers of mesenchyme from smaller *hdf* mutant limb buds for high density micromass culture (Owens and Solursh 1981), a modification of Niswander and Martin (1992) was utilized for trunk explant culture of both wild type and mutant specimens. Briefly, trunks with attached forelimbs were dissected from embryos in phosphate buffered saline (PBS; 1 mM KH_2PO_4 , 2.6 mM KCl, 150 mM NaCl, 6 mM Na_2HPO_4) containing 10% fetal calf serum (FCS; Gibco 16170-086). Truncated embryos were placed in 14 ml polypropylene round-bottom culture tubes (Falcon 352059) containing 3 ml (Dulbecco's Modified Eagle Medium (DMEM; Gibco 12800-017), 10% fetal bovine serum (FBS; Gibco 26140-087), 0.1% penicillin-streptomycin (Gibco 15140-048) and 50 ng ml^{-1} FGF-2 (Sigma F 0291). Culture tubes were placed on a Wheaton roller apparatus (Wheaton Scientific Products 348921A) and incubated at 30 revolutions per minute at a 15° elevation for 2 days in a humidified 5% CO_2 incubator at 37 ° C. Half of the medium was replaced every 24 h with fresh DMEM–10% FBS containing 50 ng ml^{-1} FGF-2 as above.

Micromass culture of limb mesenchyme

Following 2 day roller bottle incubation, trunk explants were removed and placed in PBS-10% FCS. High density culture of limb mesenchyme from individual embryos was a modification of Gillotte et al. (2003). Limbs were removed at the shoulder girdle using tungsten needles (Fine Science Tools 10130-05), washed twice in 0.5 ml PBS, and tissues from each embryo incubated individually for 5 min in 0.25 ml 0.1% trypsin in PBS (Invitrogen 15090-046) at 37 °C. Trypsin hydrolysis was quenched with addition of 0.75 ml DMEM–10% FBS, limb cell suspensions centrifuged 400×*g* for 7 min, supernatant removed, and cell pellet resuspended in 0.5 ml DMEM–10%FBS with antibiotics. Cells were counted by hemocytometer and viability (consistently >95%) estimated by trypan blue exclusion (0.25% in PBS; Sigma T 6146). Cell density was adjusted to 1×10^7 cells ml^{-1} and 5 μl was spotted onto

35 mm culture dishes (Corning 430165) and allowed to attach for 2 h at 37 °C in a humidified 5% CO_2 incubator. Following cell attachment, plates were supplemented with gentle addition of 2 ml prewarmed DMEM–10% FBS and returned to the incubator. Half of the medium (1 ml) was replaced every day.

Neural tube explant co-culture

For study of neural outgrowth interactions with differentiating limb mesenchyme, 1 ml of medium was removed from limb cell cultures after 24 h of incubation. Neural tubes from 11 to 13 dpc embryos were removed using tungsten needles, placed in PBS, cleaned carefully of adherent tissue, and portions of the cervical and upper thoracic regions sectioned sagittally. Neural tube sections (approximately 1 mm) were placed cut edge down on the outer perimeter of the limb cell culture and allowed to attach for 8 h at 37 °C in a 5% CO_2 incubator before the plates were supplemented with an additional 1 ml DMEM–10% FBS and antibiotics. In some experiments, prior to establishment of micromass cultures, marked 10 μl spots on dishes were pretreated with 20 $\mu\text{g ml}^{-1}$ human plasma fibronectin (Sigma F2006) overnight and washed with PBS. Micromass cultures were established adjacent to fibronectin treated spots as described and after 1 day, neural tube explants were set on fibronectin-coated spots as outlined above.

Immunohistochemistry

After 1, 3, and 6 days, cultures were washed twice with PBS and fixed in Dent's fixative (80% methanol/20% dimethyl sulfoxide; Dent and Kymkowsky 1987). Fixed cultures were sealed with parafilm and stored at 4 °C until processed for immunohistochemical staining. Using primary antibodies specific for each tissue type, samples were whole mount immunostained in the 35 mm tissue culture plates. Mouse anti-type II collagen (Developmental Studies Hybridoma Bank II-II6B3 hybridoma supernatant, 1:5 dilution) and rabbit anti-mouse versican GAG- β (anti-chondroitin sulfate glycoaminoglycan attachment domain beta, 2 $\mu\text{g ml}^{-1}$; Snow et al. 2005) were

utilized to mark developing cartilage tissues. Neurite outgrowth was assessed by mouse anti-neurofilament associated antigen (Developmental Studies Hybridoma Bank 2H3 hybridoma supernatant, 1:5 dilution). In selected experiments, skeletal muscle myoblasts were detected using mouse anti-sarcomeric myosin (Developmental Studies Hybridoma Bank MF 20 hybridoma supernatant, 1:3 dilution).

Briefly, fixative was removed and plates washed three times with PBS, blocked 1 hr in 3% bovine serum albumin (Sigma A7906), 1% goat serum (Sigma G 9023) in PBS, and incubated overnight with primary antibodies at 4 °C. The following day, specimens were washed four times with PBS, incubated with 1:200 dilution of goat anti-rabbit rhodamine (ICN/Cappel 55673) or goat anti-mouse fluorescein isothiocyanate (ICN/Cappel 55514) in blocking buffer for 2 h at room temperature, washed five times with PBS and postfixed in 85 and 50% ethanols. Samples were mounted in 100 mg ml⁻¹ 1,4-diazabicyclo (2,2,2) octane (Johnson et al. 1982; Sigma D2522) in 90% glycerol, 10% PBS to retard fading of fluorescent signal. In order to more precisely localize neurite outgrowth relative to cartilage matrix, samples were routinely double-labeled using anti-versican and 2H3 or II-II6B3 antibodies. Controls utilized included use of irrelevant primary antibodies of the same isotype and omission of primary antibodies. Samples were visualized using an Olympus BX-40 photomicroscope quipped with epifluorescence optics and images captured using a SPOT RT digital camera and software (Diagnostic Instruments).

Data analysis

In double-immunolabeled preparations, 2H3-positive neurites were scored according to their relative position to versican-positive cartilage forming regions. Measurement of neurite outgrowth in captured images was performed with SPOT RT software. The total number of neurites from each explant was recorded; those not extending into the limb mesenchyme culture were disregarded. A minimum of 4–5 separate neurofilament-immunostained samples derived from separate experiments at 3 and 6 days of micromass culture was used for analysis.

Results and discussion

While previous reports have examined either axonal growth or chondrogenesis *in vitro*, to our knowledge the present study is the first to describe a readily usable method for neural explant co-culture with high density chondrogenic preparations of mouse limb mesenchyme to assess the role played by differentiating limb cartilages on patterning of peripheral nerve. Versican and other extracellular molecules have been previously implicated as key to the chondrogenic process, however, there have been few inquiries regarding potential regulation by differentiating cartilage of other cells that migrate into the developing limb. Evidence from other studies suggests that versican or other ECM molecules might serve as a barrier to axonal outgrowth (Oakley and Tosney 1991; Oakley et al. 1994; Landolt et al. 1995; Harris and Jahoda 2001; Snow et al. 2005). The technique described in the present study provides a simple experimental model for study of the role of cartilage-derived ECM molecules on peripheral nerve patterning in limb and can be easily used to investigate the effect of specific transgenic mutations of ECM molecules on this process.

In the present study, *in vitro* cartilage differentiation of wild type mouse limb mesenchyme followed a time course similar to that previously described (Owens and Solorsh 1981; Gillotte et al. 2003). Cartilage differentiation *in vitro* is density dependent (Solorsh et al. 1978) and at one day, versican and Type II collagen-positive cellular aggregates were noted primarily in the more densely populated central region of wild-type cell layers, indicating formation of incipient cartilage foci (Figure 1a–c). Between 3 and 6 days, established cartilaginous areas increased in size and there was increase in number of additional small aggregates (Figure 1d–f, Figure 2a). Control incubations with irrelevant primary antibodies or with omission of primary antibody showed that staining of aggregate structures for cartilage characteristic type II collagen and versican was specific and not the result of spurious trapping of immunoreagents (Figure 2).

Interestingly, by 3 days MF-20 positive skeletal myoblasts, which typically migrate over the surface of high density whole limb mesenchyme cultures (Owens and Solorsh 1981), were observed to

Figure 1. Cartilage differentiation in wild type mouse limb mesenchyme. At day 1 (a–c), double labeling with anti-Type II collagen (b) and versican (c) show overlapping localization in aggregate structures (a). At day 3 (d–f), Type II collagen (e) and versican (f) double labeling show increased accumulation of cartilage matrix in the chondrogenic nodule (a). Arrowheads mark position of representative cartilage nodules. Scale bar = 50 μ m for all panels.

largely avoid versican positive wild type chondrogenic regions (Figure 3). In an effort to quantify whether chondrogenic regions *in vitro* may also potentially function in patterning of migratory skeletal muscle precursors, myoblasts were scored by their position relative to cartilage-forming areas, i.e., regions of strong versican expression. Chi square analysis of the observed vs. expected distribution of murine myoblasts in cultures at day 3 indicated a significant difference in numbers of myoblasts between versican positive and versican negative regions ($p \leq 0.0005$). As suggested in previous studies (Schramm and Solursh 1990; Snow et al. 2005), developing cartilage

regions within the limb may exclude localization of skeletal myoblasts, aiding in their organization into premuscle masses around the developing hyaline cartilage model. One previous study using transfilter culture of chick limb mesenchyme showed that exclusion of myoblasts from cartilage-forming areas may be due to myoblast interactions with cartilage matrix molecules or the chondrocyte cell surface rather than through a soluble paracrine signaling mechanism (Schramm et al. 1994). Further study will be required to determine whether matrix versican is a key regulator of this chondrogenic influence on myoblast migratory behavior.

Figure 2. Representative immunohistochemical control day 3 wild type micromass culture. (a) Numerous chondrogenic aggregates (asterisks) are noted at this time. (b) Note lack of non-specific immunoreactivity with rhodamine-conjugated secondary antibody alone. Scale bar = 50 μ m for both panels.

Figure 3. Myoblasts largely avoid versican positive chondrogenic aggregates at day 3. (a) Phase contrast image of panels b and c. Asterisks identify corresponding aggregate structures in panels b and c. (b) Anti-sarcomeric myosin (MF-20) immunostaining shows myoblast localization outside of developing wild type cartilage structures. (c) Double labeling shows strong versican immunoreactivity in developing cartilages. Scale bar = 50 μ m for all panels.

Preliminary neural co-culture experiments with wild type mesenchyme showed that similar and consistent neurite outgrowth could be achieved using wild type neural tube explants from 11 to 13 dpc embryos and in the present study, all data were gathered using wild type 11 dpc neural tissue. During dissection of the neural tube, careful removal of extra-neural tissue (epidermis and connective tissue) with tungsten needles was also found important to minimize the growth of contaminating tissue which also expressed versican. Neural tube explants were added one day after initial limb culture preparation in order to permit firm attachment and initial development of the mesenchymal cell layer. As such, all time points mentioned in this report are in reference to the initial establishment of the limb mesenchyme culture.

Initial experiments also utilized fibronectin coating to provide a suitable substrate for attachment of the neural explant, which otherwise did not adhere well to the plastic surface of the tissue culture dishes. Fibronectin-coated spots were always placed just peripheral to the area where limb micromass cultures were to be established as fibronectin coating has been documented to inhibit chondroblast differentiation (West et al. 1979). As

reported in other studies of axon migration (Krull and Kulesa 1998), precoating with fibronectin worked well for explant attachment. However, in our hands, further experiments determined that the ECM associated with the lower cell density, non-chondrogenic peripheral edge of the micromass culture at one day of culture also provided a suitable substrate for neural explant attachment. This modification provided the added benefit of reducing the overall time required for neurite extension into the higher cell density central chondrogenic region of the limb micromass culture so that fibronectin coating of culture plates was no longer a requisite preliminary step.

Neurite outgrowth away from the explant and into wild type high density limb micromass culture is summarized in Table 1 and illustrated by a representative example in Figure 4. At 3 days, neurites were too thin to be scored accurately by direct morphological observation; therefore neurofilament (2H3) immunostaining was employed. Stained axons appeared as a network of processes around the explant and were easily differentiated from each other facilitating accurate quantification. At this time point, neurites had begun extension across the limb culture surface, but still had not yet reached the central region of wild type cell layers where the number of developing chondrogenic foci was greater. By 6 days, neurites on average had extended 3-fold further from the explant, many reaching the center of wild type limb cultures (Figure 4c & d). Continued neurite growth over 6 days demonstrated that the neural explant remained viable throughout the culture period.

In wild type limb culture at 6 days (Figure 5a–c) numerous 2H3-positive neurites were observed

Table 1. Murine neurite outgrowth from 11 day post coitum cervical/upper thoracic neural tube explants across high density micromass cultures of wild type mouse limb mesenchyme.

Explant age (days)	Neurite outgrowth (μ m)
3	230.8 $\pm$ 80.6
6	750.4 $\pm$ 127.8

Measurements represent mean $\pm$ SEM of 4–5 separate neurofilament (2H3)-immunostained samples from 3 and 6 days of co-culture.

Figure 4. Co-culture of neural tube explants at day 11 post coitum with wild type high density micromass preparations of mouse limb mesenchyme show extensive neurite growth at days 3 (a & b) and 6 (c & d). (a) Phase contrast image of panel b. Neural tube explant is marked by asterisk. (b) Anti-neurofilament (2H3) immunolocalization shows many neurites beginning to extend into the mesenchymal cell layer. (c) Phase contrast image of panel d shows neural tube explant (asterisk). (d) Anti-neurofilament staining shows increased growth of neurites into the limb mesenchyme co-culture. Scale bar = 200 μ m for all panels.

Figure 5. Co-culture of wild type neural tube explants at day 11 post coitum with day 6 wild type (a–c) and *hdf* mutant (d–f) high density micromass preparations of mouse limb mesenchyme shows exclusion of neurites from developing cartilages. (a) Phase contrast image of panels b and c. Arrowhead marks position of cartilage. (b) Anti-neurofilament (2H3) reactivity illustrates multiple neurites outside of the developing cartilage. (c) Double labeling shows strong versican localization in incipient cartilage. (d) Phase contrast image of panels e and f. Note lack of refractile hyaline cartilage foci in the *hdf* mesenchymal cell layer. (e) Anti-neurofilament staining lacks discrete preferential localization of neurites in the *hdf* mutant mesenchymal cell layer. (f) Double labeling shows lack of anti-versican reactivity in the *hdf* cell layer. Scale bar = 50 μ m for all panels.

within the cell layer. In all samples examined, neurites always localized in areas outside of developing versican-positive cartilages, giving the impression of having avoided these regions by migration around them. Previous observations *in vivo* showed versican localization in the chick pelvic girdle, a barrier tissue avoided by axons migrating into the leg bud (Landolt et al. 1995) and an inverse correlation between versican localization and sensory innervation of rat vibrissa (Harris and Jahoda 2001). Most recently, Snow et al. (2005) found that in developing mouse limbs, branches of the brachial plexus avoided versican rich chondrogenic areas, supporting observations of neural avoidance of versican-positive chondrogenic foci *in vitro* in the present study. There are several possibilities with regard to versican function as a barrier molecule to axonal growth that could facilitate neuronal patterning in the developing limb. The lectin domain of versican binds tenascin (Aspberg et al. 1995), increasing tenascin's anti-adhesive properties (Probstmeier et al. 2000). Likewise, versican binding to other ECM molecules may block neurite adhesion to other permissive matrix substrates such as fibronectin and collagen (Yamagata et al. 1989), which, along with versican, are all expressed in incipient limb cartilage tissues (Shinomura et al. 1990).

As reported previously (Williams et al. 2005), in limb cell cultures derived from *hdf* homozygous mutant embryos, at either 3 or 6 days Type II collagen or versican-positive cartilage foci were not observed (Figure 5f). Cartilage nodules do not differentiate in the versican-deficient *hdf* mutant in spite of expression of other molecules implicated in chondrogenesis, including fibronectin and cadherins (Williams et al. 2005). The defect in the chondrogenic pathway appears to reside in lack of mesenchymal aggregation along a wild type time course, suggesting an interactive role between mature versican proteoglycan and other molecules in this process. Unlike complete neurite avoidance of chondrogenic aggregates as noted in wild type preparations, wild type axons co-cultured with *hdf* derived mesenchyme exhibited no preferential localization at 6 days (Figure 5e). It is important to note that while individual neurites displayed no preferential directional orientation, they tended to cluster with other axons more so than observed in control co-cultures, often forming fascicle-like structures. A similar effect on axonal fasciculation

was reported in sonic hedgehog (Shh)-deficient chick embryos (Fedtsova et al. 2003). In that report, addition of Shh caused an increase in versican expression in a zone that excluded migrating trigeminal nerve precursors, distorting morphology of the trigeminal ganglion. It is interesting to speculate that perhaps in addition to service as a barrier molecule reserving space for the limb skeletal template, wild type versican downstream of limb Shh expression may ensure separation of individual axons to provide complete rather than partial innervation of target tissues.

Although results are entirely consistent with a role for versican in patterning of nerve in limb tissues, it may also be possible that other cartilage-derived molecules function co-operatively or are responsible for exclusion of nerve and that lack of chondrogenesis in the versican-deficient *hdf* mutant prohibits synthesis of such a factor(s). Future experiments utilizing the described co-culture method will explore further the potential role of other cartilage-derived factors on migratory cell patterning in the developing limb. In summary, the present study has provided a ready protocol for neural/limb mesenchyme co-culture that can be easily used to study patterning events during embryonic limb morphogenesis and may be extended to include investigations into the effects of transgenic mutations on this important developmental process.

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