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The differential influence of colocalized and segregated dual protein signals on neurite outgrowth on surfaces

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Abstract

We present an in vitro micropatterning approach in which the density and spatial presentation of two separate protein layers can be independently controlled to form cell stripe assays through (1) the simultaneous application of microcontact printing (μ CP) and microfluidic network patterning (μ FN) to generate alternating stripes of pure single protein layers or (2) through μ CP onto a pre-adsorbed homogeneous protein layer to generate alternating single and dual protein stripes. This approach enabled the creation of choice boundaries in which protein-protein interactions were limited and the effects of spatially segregated or colocalized dual protein signals on model primary neuronal behavior could be readily interrogated and compared on both glass and tissue culture polystyrene substrates. Dorsal root ganglion (DRG) cell body attachment was dictated largely by non-specific cell adhesion interactions and interactions between the guidance molecules laminin and aggrecan were insufficient to explain aggrecan inhibition on neurite outgrowth. The presentation of a specific laminin epitope stabilized by interactions with aggrecan and destabilized by μ CP was a strong predictor of neurite promoting activity. These observations provide evidence that aggrecan is intrinsically inhibitory and that laminin-aggrecan interactions do not diminish laminin growth promoting properties.

1. Introduction

Several in vitro cell choice assay experiments have demonstrated the inhibitory effects of proteoglycans (PG) in the presence of laminins [1–4] and have provided evidence that neurons integrate mixed growth promoting and growth inhibitory signals and discriminate the level of inhibition according to the mass ratio of PG to laminin (LN) [5–7]. Neuronal guidance depends on the integration of multiple signals [8–11], but it is unclear if colocalized PG and LN signals are simply additive since a variety of interactions between these molecules have been elucidated including direct PG masking of LN integrins important to neuron growth promoting activity [12–14]. In other examples, neuronal adhesion to fibronectin was inhibited by versican and decorin but was not affected by the same molecules in the presence of laminins [15]; NG2 overrode the growth promoting properties of laminin even after chondroitin sulfate (CS) chain digestion but did not affect outgrowth on L1 surfaces [16]. Additionally, CS sugars and keratin sulfate inhibited DRG outgrowth on laminin but not on L1 [17].

In light of past findings, it has been postulated that certain PGs are not intrinsically inhibitory but act by blocking the growth promoting properties of other proteins through specific protein-protein interactions. Consequently, where such interactions alter the intrinsic signal of either

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of the participating proteins, any variation in vitro deposition technique or minute substrate difference that affects the magnitude of these interactions will also affect resulting cell behavior.

We postulate therefore that in cases where protein-protein interactions ultimately dictate neuronal behavior that their effect may be discovered by observing neuronal attachment and neurite outgrowth on substrata where protein-protein interactions have been greatly diminished or abolished. To test this hypothesis, we used a set of cell choice stripe assays aimed at separating possible PG-laminin interactions from intrinsic effects, which PGs may exert on neuronal cell attachment and neurite outgrowth. We employed soft lithography techniques combining microcontact printing (μ CP) and microfluidic networks (μ FN), or μ CP and solution adsorption to produce patterns with sharp stripe boundaries, precise stripe periodicity, and controlled LN and PG densities. In case (1) alternating lanes of pure LN and the proteoglycan aggrecan (AG) were produced by the simultaneous application of μ CP and μ FN, and in case (2) alternating pure LN lanes and colocalized LN and AG lanes were produced by stamping an AG stripe pattern onto a homogeneous pre-adsorbed LN layer. The resulting patterns were extensively characterized by quantitative fluorescence imaging, scanning force microscopy (SFM), and antibody staining for the identification of specific LN epitopes.

We further investigated the role that deposition method played on protein activity. Retention of aggrecan activity after μ CP was confirmed through a CS digestion and neurite outgrowth assay, while laminin activity was determined by elucidating the presentation of a specific epitope in the laminin C-terminus domain critical to $\alpha_6\beta_1$ integrin receptor attachment and neurite outgrowth [18,19]. By correlating epitope presentation with neurite promoting activity, as measured by neuronal outgrowth on the various laminin layers, we were able to confirm that epitope presentation is in fact an indicator of growth promoting activity.

2. Materials and Methods

2.1. Microfluidic/microcontact printing device fabrication

The University of Utah Microfabrication Laboratory was used to design, model, and fabricate the micropatterning devices used in this study. Various pattern designs were modeled in Autocad R14 (Autodesk, San Rafael, CA) software and translated onto a photolithographic mask (Nanofilm, Valley View, OH) by use of a pattern generator (Electromask). Masks were developed and applied to UV patterned thin films of SU-8 2 (Microchem, Newton, MA) photoresist coated on silicon wafers. SU-8 films were subsequently cured then etched to yield a mold for micropatterning device fabrication. All μ FN/ μ CP devices were produced by curing polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI) against SU-8 Si molds. Cured devices were soaked overnight in hexane (Aldrich, St. Louis, MO) to remove polymerization reaction byproducts, then dried by heating before use.

2.2. Protein preparation and dye conjugation

Alexa Fluor (AF) dyes AF488 and AF594 were purchased from Invitrogen (Carlsbad, CA) and fluorescein (FITC) from Sigma (St. Louis, MO). Englebreth-Holm-Swarm mouse tumor laminin (LN) was purchased in solution form from Invitrogen, lyophilized bovine articular cartilage aggrecan (AG) from Sigma, and lyophilized fraction V fatty acid free human serum albumin (HSA) from MP Biomedicals (Irvine, CA). Laminin and AG were labeled with AF488 and HSA was labeled with FITC using protocols designed by Invitrogen. Alternatively, AF594 was mixed with AG in a 10:1 AF594:AG molar ratio to produce a physically labeled AG species. All covalently labeled species were isolated through gel permeation chromatography using Sephadex G-25 columns (GE Healthcare, Piscataway, NJ). Thin layer chromatography was used to verify free dye removal from the labeled proteins. Final protein solution

concentrations and dye to protein molar ratios were determined through absorbance spectroscopy and the Bradford method using a UV/Vis spectrophotometer and plate reader respectively.

2.3. Surface patterning

Glass coverslips were cleaned prior to surface modification in a 1 H₂O₂: 2 H₂SO₄ mixture, rinsed 4x with double distilled deionized (DD) water, and then dried under a nitrogen stream. PDMS devices were cleaned with ethanol then dried prior to protein adsorption. Laminin (900 µg/ml) was adsorbed on glass coverslips for 1 hour and AG (1 mg/ml), HSA (4.5 mg/ml), or LN (900 µg/ml) were applied to PDMS devices for 20 minutes before stamping. In colocalized patterned protein layers, LN was first homogeneously adsorbed to dry glass coverslips, which were then rinsed and dried. A second protein layer was then patterned onto the LN layer through µCP using an AG or HSA coated PDMS device that had been rinsed with DD water and briefly dried after the adsorption step.

Segregated patterned protein layers were produced by stamping AG or HSA onto a clean glass coverslip, and before removing the PDMS device from the glass surface, simultaneously adsorbing LN from solution placed in the PDMS microfluidic networks. Briefly, after the PDMS device was brought into contact with the glass coverslip, microchannels were filled with phosphate buffered saline (PBS) then LN solution was allowed to exchange into the microchannels through a process of diffusion and fluid movement created by PBS evaporation from the channel exits. After the adsorptive process, channels were rinsed with PBS and allowed to dry before PDMS device removal. All samples were rinsed with running DD water and dried by a nitrogen stream after the patterning process. Figures 1A and 1B contain a representative flow chart of the patterning process used to produce colocalized and segregated protein patterns.

Sequence-adsorbed AG-LN layers were prepared by adsorbing AG (1 mg/ml) for 1 hour to clean glass then, after rinsing and drying, adsorbing LN (900 µg/ml) for 1 hour onto the AG layer. Co-adsorbed AG and LN layers were produced by co-adsorbing LN and AG in a 1:1 mixture of the above AG and LN solutions (500 µg/ml AG and 450 µg/ml final concentrations) for 1 hour.

2.4. Protein pattern characterization

Protein layer densities were determined by preparing dual protein patterns with one labeled and one non-labeled protein species. A fluorescence microscopy imaging technique described in previous work [20] was utilized to determine absolute protein film densities for the various patterns produced. Briefly, a Nikon Eclipse E600 Microscope (Melville, NY) equipped with a Nikon Plan Fluor 100x, 1.30 N.A. objective and Micromax Peltier-cooled CCD camera (Princeton Instruments, Trenton, NJ) was used to image the various patterned protein layers on glass and corresponding protein solutions in a thin liquid film above the layers. Fluorescence intensities of the patterned protein layers were equated to absolute densities by finding the objective distance-light collection function and the intensity ratio between the adsorbed protein layer and a protein solution film of known thickness and concentration. All images were corrected for dark counts and uneven specimen illumination. Image Pro v4.1 software (MediaCybernetics, Silver Spring, MD) was used to process and quantify images. Average protein layer density was calculated from 8 to 10 locations on each of two to three samples produced for each type of pattern.

The various protein patterns and homogeneously adsorbed protein layers were produced with non-labeled species then incubated in rat anti-mouse laminin IgG antibody solution (AL-4, 1:200 v/v, Chemicon, Temecula, CA) for 30 minutes at room temperature. The samples were

rinsed 3x in PBS then incubated in AF488 labeled goat anti-rat IgG (H&L) (1:500 v/v, Invitrogen) for 30 minutes at room temperature. Because this assay was performed to interrogate possible masking of LN epitopes by AG or HSA, no blocking agent was used out of concern that introduction of additional proteins could cause artifacts due to adsorption from the blocking solution. By applying this immunostaining scheme to patterns with segregated protein fields, it was found that negligible adsorption of the primary (AL-4) or secondary (IgG) antibodies occurred on AG and HSA layers in the absence of blocking agents. Final epitope presentation, as measured by secondary antibody fluorescence intensity, was normalized by LN density as quantified in AF488-LN patterned layers. Image Pro v4.1 software was used to process and quantify images. Intensities were averaged from two to three samples of each pattern or homogeneously adsorbed protein layer measured at 8 to 10 locations for each sample.

Stamped AG, HSA, and LN and adsorbed LN layers were probed using various scanning force microscopy (SFM) techniques including constant force and lateral force modes using a Topometrix Explorer instrument (Veeco, Woodbury, NY) and Digital Pulsed Force Mode (DPFM) using a Nanoscope III instrument (Veeco) equipped with a WITec (Savoy, IL) driving and analysis system. Ultrasharp tip triangular cantilevers (MikroMasch, Wilsonville, OR) used to probe the various protein layers were chemically modified with hydrophobic organosilane chains in a previously described procedure [20] prior to taking measurements.

2.5. CS digestion

Chondroitinase ABC (ChABC, Sigma) was tested for its activity against AG using a protocol previously described [21]. Briefly, 5 mUnits of ChABC dissolved in 50 mM Tris buffer, with 60 mM sodium acetate as an activator, were mixed with 50 μ g of AG in PBS and the change in absorbance at 232 nm due to cleaved chondroitin sulfate residues was monitored using a UV/Vis spectrophotometer. Patterned AG films stamped on adsorbed LN were then incubated in 0.2 Units ChABC/sample or in PBS as a control for 90 minutes at 37°C.

Digested and untreated control AG patterned films were blocked in 4% goat serum for 30 minutes then incubated in antibodies against CS chains (CS-56 IgM, 1:100 v/v, Sigma) and against completely digested CS chain protein core stubs (Anti-Chondroitin-4-Sulfate IgG1, 1:200 v/v, Chemicon) for 90 minutes. Samples were rinsed 3x in PBS and once with blocking solution then incubated for 30 minutes in secondary antibody solutions either against IgMs (AF488-Goat anti-Mouse IgM, 1:500 v/v, Invitrogen) or against IgG1s (AF488-Goat anti-Mouse IgG1, 1:500 v/v, Invitrogen). Two ChABC treated and two control samples for each antibody treatment were imaged using a Leitz 100x, 1.25 N.A. objective to verify complete CS chain removal.

2.6. DRG isolation and culture

Postnatal day 1–4 Fisher rat dorsal root ganglion (DRG) neurons were explanted under aseptic conditions then cleaned and purified for substratum seeding. Briefly, ganglia were treated with collagenase, trypsin, and DNase to remove debris and to dissociate cells, and then were triturated through a fire polished Pasteur pipette in DNase and soybean trypsin inhibitor. Contaminating Schwann cells were selectively killed by the addition of O4 antibodies and rabbit complement. The final cell supernatant was resuspended in serum free SATO⁺ medium [22] containing 10 ng/ml nerve growth factor. DRGs were counted and seeded at a density 1.5 k cells/sample. All surfaces were treated with 500 μ g/ml gentamicin in sterile PBS for 15 to 30 minutes then rinsed 3x before cell seeding. After incubation at 37°C, allowing sufficient time for cells-substrate adhesion and neurite outgrowth, DRGs were fixed in 2% paraformaldehyde then stored refrigerated in PBS containing 0.1% NaN₃.

2.7. Stripe assays and cell adhesion

PDMS devices were fabricated to produce 50 μm wide stamped AG (or HSA) lanes separated by 40 μm wide LN lanes. DRGs were seeded on samples containing stamped AG or HSA lanes colocalized with a homogeneously adsorbed LN layer; stamped AG or HSA lanes segregated from μFN deposited 40 μm wide LN lanes; or on homogeneous single protein field controls of stamped AG or HSA or adsorbed LN on glass. DRGs were also seeded on AG and LN segregated striped layers patterned on tissue culture polystyrene (PS). DRGs were incubated and allowed to extend processes for 20 hours before being fixed in paraformaldehyde. Randomly selected fixed samples were incubated in 0.3% Triton containing PBS for 10 minutes, rinsed 3x in PBS then labeled with AF488-phalloidin (1:200 v/v in PBS, 30 minute incubation, Invitrogen) to identify neurite structures.

Phase contrast images of cells on the various patterns were recorded using a Nikon Diaphot 200 microscope equipped with a Nikon 10x Plan Phase 1 objective, a Princeton Instruments CCD camera, and an automated microscope stage controlled by OpenLab v3.1.5 software (Improvision Inc., Lexington, MA). In all samples, single dissociated viable DRGs, for which neurite outgrowth exceeded at least one cell diameter and did not cross or fasciculate with neurites of adjacent DRGs, were chosen as candidates for the following three measurements: (1) number of cell bodies adhered to LN, AG, or HSA in each pattern type, (2) trace length of the longest neurite of each DRG, and (3) number of AG or HSA lanes crossed by the longest neurite. Neurite lengths were measured using Image Pro v4.1. Duplicates of each pattern from three separate experiments and one sample of each control from each of three experiments were imaged and measured for DRG adhesion and outgrowth parameters. Four patterned polystyrene substrates were produced in one experiment and imaged and measured for the three listed DRG parameters.

DRGs seeded on ChABC-treated and control patterned surfaces were incubated for 18 hours before cell fixation. DRGs were measured following the same criteria as described above for cell body adhesion on AG or LN and for the number of AG lanes crossed. Three ChABC digested samples and three control samples in one experiment were imaged and measured to quantify the effects of CS removal on DRG outgrowth.

2.8. Statistical analysis

All statistical analyses were performed with SPSS v12.0 software (SPSS Inc., Chicago, IL). T-test p values were two-tailed and found assuming unequal variance. One-way Anova analyses were conducted and are reported where applicable. Correlations were performed using the Spearman's Rho one-tailed test.

3. Results

3.1. Protein layer densities

We deposited three proteins, laminin, aggrecan, and albumin, onto glass by various techniques (Fig. 1 and 2) to investigate the effect of protein presentation on DRG cell body substrate attachment, neurite outgrowth, and growth cone decisions at choice boundaries. To understand the magnitude of each protein signal encountered by DRGs, we used fluorescence microscopy to quantify the surface density of each protein in each pattern type (Fig. 3). Aggrecan was stamped onto adsorbed LN layers by μCP at a density of 285 ng/cm^2 , but deposited at a significantly higher density of 342 ng/cm^2 on glass (Fig. 3A) ($p < 0.001$, t-test). Albumin transferred at a significantly lower density onto glass at 70.8 ng/cm^2 than onto adsorbed LN layers where the average albumin density was 110 ng/cm^2 (Fig. 3A) ($p < 0.001$, t-test).

Laminin deposition density was adversely affected by the surface to which it was adsorbed and by the method of deposition. Laminin was deposited onto glass by μ CP at a density of 811 ng/cm², which was significantly greater than by all other laminin deposition methods (Fig. 3G) ($p < 0.001$, t-test, pair-wise comparisons). LN adsorbed at a significantly greater density to glass in microfluidic channels than from a thin liquid film ($p < 0.02$, t-test pair-wise comparisons), and microchannels coated with AG yielded significantly greater densities of LN on glass than channels coated with either LN or HSA ($p < 0.001$, t-test, pair-wise comparisons) suggesting that protein coated channels affect LN μ FN patterning on glass.

To better understand how possible protein-protein and protein-substrate interactions may have affected LN and AG co-adsorption onto glass substrata, samples were created in which LN was either co-adsorbed with AG onto glass or adsorbed onto an AG layer on glass. Laminin adsorption to glass was significantly affected by either the presence of soluble or bound AG ($p < 0.001$, t-test, both cases). Glass surfaces already coated with an AG layer adsorbed 72% less LN than clean glass surfaces, while LN adsorption on glass in the presence of soluble AG was reduced by 34% at the concentrations tested. These results indicate that LN and AG compete for adsorption sites on glass and that LN adsorbs less well to an AG layer than to acid cleaned glass.

3.2. Laminin epitope presentation

It has been demonstrated that the method of LN deposition and subsequent surface presentation affects LN activity in cell attachment assays, and in addition, AG deposition appears to interfere with LN activity in an adsorptive scheme [23]. We found that stamped AG on a uniform LN field does indeed mask approximately 48% of the available LN epitopes to AL-4 antibody binding, while surprisingly, stamped HSA on a LN field stabilizes approximately 54% more epitopes than when LN is presented alone (Fig. 3B). Investigation of the underlying LN field density during antibody incubation ruled out the possibility of differential LN desorption from non-stamped areas of the pattern. Additionally, it was found that, in the segregated protein patterns, neither AG nor HSA stamped layers nonspecifically interacted with or bound the AL-4 antibody or its secondary antibody.

Laminin epitope presentation was greatly affected by the method of LN deposition (Fig. 3H), where stamping LN and adsorbing LN from AG coated microchannels had the most adverse effect on epitope presentation. Stamping LN reduced available epitope density by 70-fold over the adsorbed control while LN adsorbed from AG coated microchannels increased available epitope density 5-fold over the adsorbed control. Interestingly, LN adsorption from HSA coated microchannels had no noticeable effect on LN epitope presentation density while adsorption from LN coated microchannels reduced epitope presentation density two-fold.

Laminin epitope presentation was also greatly affected by adsorbing laminin in the presence of soluble AG or onto a pre-adsorbed AG layer on glass. Surprisingly, but in line with the microfluidic data, adsorbing LN in the presence of AG resulted in the stabilization of LN epitope presentation compared to LN adsorbed alone to glass. Co-adsorbing LN with AG increased AL-4 targeted epitope presentation two-fold while adsorbing LN onto a preadsorbed AG layer increased LN epitope presentation by more than three-fold.

3.3. Protein film morphology

We employed scanning force microscopy (SFM) techniques to image adsorbed LN layers and stamped AG and HSA patterns on glass to discover protein morphology at a nanoscale resolution. As expected, due to the large dimensions of the aggrecan molecule, AG stamped lanes had a significantly larger height profile of 7.2 nm compared to HSA lanes, which had an average height of 3.8 nm ($p < 0.001$, t-test) (Fig. 4). It was surprising however to find that,

although adsorbed and stamped LN and stamped HSA produced smooth films of seemingly uniform coverage, stamping AG produced rough films of uneven coverage, which were composed of an apparent web structure of AG molecules. Using Digital Pulsed Force Mode (DPFM) SFM, we were able to resolve the structure of stamped AG layers in both topography and adhesion maps revealing an intertwined mesh of individual AG molecules separated by relatively large layer voids (Fig. 4G and 4H). The web structure persisted in the presence of water and could also be detected by fluorescence microscopy imaging of the stamped AG layers (Fig. 4F).

3.4. DRG adhesion and stripe assays

DRG cell body adhesion to the various patterns and homogeneous single protein controls exhibited that clear differences exist between adhesion to AG, HSA, and LN and that substratum material further affects adhesion to LN. In the control samples, far fewer cells adhered to AG and HSA layers than to the LN controls (Hodgkinson and Hlady, unpublished observations). More interesting, however, is how DRG adhesion proceeded at the various choice points in the patterned surfaces. As expected, in the LN and AG patterns a majority of DRG cell bodies preferentially adhered to LN lanes, with a maximum of 98% of DRGs adhering to LN lanes patterned by μ FN adsorption on polystyrene (Fig. 5B). Unexpectedly, however, a majority of DRG cell bodies preferentially adhered to HSA lanes in both pattern types where HSA was either colocalized or spatially segregated from LN, with 79% of DRGs preferentially adhering to stamped pure HSA lanes over pure LN lanes.

DRG behavior on the various patterns suggests that neurite outgrowth and pathfinding function by a different set of mechanisms than cell body attachment, although substratum material again affected neurite outgrowth behavior. DRG neurites crossed AG and HSA lanes in all patterned surfaces although neurite outgrowth was largely restricted to LN lanes on segregated protein patterns and when AG lanes were colocalized on an adsorbed LN layer (Fig. 5A). Neurite crossing over AG lanes was significantly reduced when AG fields were completely segregated from LN on glass ($p < 0.001$, t-test) and was significantly reduced further when the substratum material was polystyrene ($p \leq 0.001$, t-test). Unlike in the case of cell body adhesion, pure HSA lanes were nearly as effective at reducing neurite crossing events as AG lanes. However, when HSA was stamped on LN, it presented no barrier to neurite outgrowth, and the resulting neuron morphologies on these patterns were indistinguishable from DRGs grown on uniform LN control layers (Fig. 6).

Although it has been shown that DRG neurite outgrowth lengths are largely insensitive to LN concentration, it has been observed that LN activity can affect neurite outgrowth [24]. We thus quantified neurite outgrowth length on the various patterns and searched for a correlation with AL-4 targeted epitope presentation density. It was found in cases where LN was adsorbed or patterned by μ FN that increasing neurite outgrowth distances significantly correlated with diminishing AL-4 targeted epitope presentation density (-0.872 correlation, $p < 0.03$, Spearman's Rho Test). Average neurite outgrowth lengths on all μ FN patterned surfaces were significantly less than on the adsorbed LN control (Fig. 5C) ($p \leq 0.005$, t-test, pair-wise comparisons) while outgrowth distances were significantly reduced in either stamped AG ($p < 0.001$, t-test) (Fig. 6A) or stamped HSA ($p \leq 0.004$, t-test) (Fig. 6D) patterns on glass when LN was deposited by μ FN patterning. We additionally discovered in cases where LN was stamped onto glass, which resulted in an almost completely abolished presentation of AL-4 targeted epitopes, that neurites completely avoided stamped LN lanes as if they had been rendered inhibitory by the micropatterning process (Fig. 6G).

It has previously been shown that AG inhibition on neurite outgrowth is a composite effect of both the GAG chains and the protein core [25]. A ChABC treatment of AG stamped on LN was thus used to investigate whether stamping AG adversely affects the activity of either the

CS chains or the core protein on neurite outgrowth inhibition. Antibody staining was used to verify the complete digestion of CS chains by ChABC treatment (See Materials and Methods). It was found that ChABC digestion significantly increased the average number of neurite crossing events over stamped AG lanes, demonstrating that CS chain activity was retained after stamping (Fig. 7A) ($p < 0.001$, t-test). We also found that the core protein activity (and possibly keratan sulfate chain activity) was retained as evidenced by fewer average AG crossing events for the longest neurite in each cell (1.76 ± 0.2 lanes/cell) compared to neurite crossing over the inert protein HSA stamped on LN (2.74 ± 0.3 lanes/cell), and by visual evidence that many neurites continued to turn at AG boundaries and were generally directed by the AG lanes (Fig. 7C) ($\pm 95\%$ conf levels, t-test). Additionally, DRG cell body attachment to AG lanes did not significantly increase on samples treated for CS chain removal, with $3.7 \pm 2.1\%$ DRGs adhering to AG lanes on CS containing controls and $10.7 \pm 8.3\%$ on ChABC treated AG lanes ($\pm 95\%$ conf levels, t-test) suggesting that the mechanism of cell adhesion inhibition by AG may not depend on CS chains.

4. Discussion

We have used several protein-surface pattern methods to improve our understanding of how protein-protein and protein-substratum interactions affect cell behavior in choice assays. In studying DRG behavior in these patterns, we have found a number of results that help explain in vitro DRG behavior reported in the literature, laminin activity sensitivity to deposition, and mechanisms underlying aggrecan inhibitory properties. Table 1 lists the major findings deduced from protein patterning, protein layer characterization, and DRG cell choice assays. We observed that non-specific adhesion affects DRG cell body substratum attachment to a greater extent than specific interactions with laminin, and that aggrecan with or without intact CS chains is largely resistant to DRG cell body nonspecific attachment even in the presence of laminin. Surprisingly, in an adsorptive scheme, aggrecan interacts with laminin to stabilize the presentation of an epitope located within known neurite outgrowth promoting domains, while stamping aggrecan onto an adsorbed laminin layer decreases presentation of the same epitope. Laminin neurite outgrowth promoting potency seemingly decreases with increasing epitope density over the range tested in this study, and abolishing presentation of the epitope through μ CP results in a nonpermissive laminin layer. Additionally, aggrecan can be patterned by μ CP without adversely affecting its neurite inhibiting properties while laminin epitope presentation is highly sensitive to its method of deposition.

4.1. Laminin-aggrecan interactions at interfaces

Condic et al. [23] found that the order of aggrecan and laminin deposition affected the ability of laminin to support DRG cell body attachment. It was demonstrated that the co-adsorption of laminin and aggrecan produced the most pronounced inhibitory effect on DRG cell body attachment and that aggrecan adsorbed onto a laminin layer had the least inhibitory effect. These results suggest that aggrecan interacts with laminin to decrease its activity to cell adhesion but through a process other than masking laminin cell binding sites. However, Snow and Letourneau [5] showed in a step gradient assay that CSPGs were equally inhibitory to neurite outgrowth whether adsorbed to the substrate before or after laminin deposition.

We propose, based on results presented in this study, that DRG cell attachment on laminin is easily overridden by nonspecific events, as evidence by preferential DRG attachment to pure albumin fields in laminin-albumin stripe choice assays. Although aggrecan apparently exerts an inhibitory effect on DRG attachment to laminin, we believe that the robustness of this effect remains to be proven in light of evidence from DRG adhesion to control samples in this study in which cell bodies attached to homogeneous albumin layers and in some cases to stamped aggrecan layers containing no laminin.

We found that aggrecan interacts with laminin at the glass-aqueous interface but that aggrecan stabilizes a laminin epitope seemingly important to neurite outgrowth. This result suggests that the presence of aggrecan improves native laminin structure retention during adsorption to glass, and we found that this effect was most pronounced when laminin was adsorbed to a pre-adsorbed aggrecan layer. These findings suggest that the inhibitory effect produced by aggrecan on neurite outgrowth may be purely intrinsic and not the result of disruption or blocking of important laminin neurite growth promoting epitopes.

Importantly, we found that stamped aggrecan layers, although porous in structure, mask AL-4 recognized laminin epitopes suggesting that the micropatterning techniques employed in this study alter resulting laminin presentation differentially from the typical deposition scheme in which aggrecan and laminin are allowed to interact during adsorption. Although stamping apparently limited typical aggrecan-laminin interactions, aggrecan remained inhibitory to DRG cell adhesion and neurite outgrowth further suggesting that aggrecan is intrinsically inhibitory. Indeed, neurite crossing over pure aggrecan lanes was significantly less than crossing over pure albumin lanes in segregated stripe patterns suggesting that aggrecan is not simply non-permissive to neurite outgrowth but continues to exert an inhibitory effect on outgrowth even in the absence of interactions with laminin.

4.2. Microcontact printing and retention of laminin and aggrecan protein structure and activity

We observed that laminin density and epitope presentation were sensitive to the method of substratum deposition. In particular, laminin solution flowed through aggrecan coated microchannels yielded laminin layers on glass 59% more dense than layers produced from laminin coated channels. More intriguing was the finding that these patterned laminin layers contained a more than 9-fold greater density of AL-4 recognizable epitopes than layers produced from laminin coated channels. We propose that the observed differences between μ FN patterned laminin layers can be explained by differential adsorption rates [26–29], in which laminin interactions with microchannel adsorbed aggrecan molecules resulted in slower adsorption rates to glass and more favorable laminin packing in the adsorbate layer. Laminin epitope presentation was also affected by μ CP, which resulted in the disruption of nearly all native AL-4 targeted epitopes. The laminin denaturation observed in stamped layers was most likely caused by strong non-specific adsorption to the hydrophobic PDMS stamps [28] and by the physical nature of the stamping process.

Although laminin epitope presentation is sensitive to μ CP, our results show that aggrecan inhibitory properties were preserved during microprinting. This finding is evidenced not only by the ChABC assay in this study but also by comparisons with results in the literature. Snow et al., [5,6] found that neurites tend to cross CSPG fields containing a nearly 1:1 CSPG to laminin mass ratio. In one study, it was shown that DRG neurons grew into adsorbed CSPG fields 22% of the time when the fields contained a 1:1 CSPG to laminin mass mixture [5]. We found a similar crossing event probability of 55% in our patterns with a 1.2:1 laminin to aggrecan mass ratio. The higher crossing probability that we observed may be due in part to the limited width of the aggrecan lanes used in this study compared to the relatively wide CSPG fields produced in other comparative studies [5,6].

We postulate that aggrecan activity is unaffected by μ CP because of the presence of CS and keratin sulfate GAG chains, which comprise ~90% of aggrecan's mass [30–32]. Based on our observations, we further postulate that a majority of aggrecan's inhibitory properties are derived from GAG chains and that the stamping process does not disrupt CS chain structure as evidenced by strong staining of stamped aggrecan films using a CS specific antibody and full removal of CS sugars by ChABC digestion. These latter results suggest that aggrecan

substratum adhesion after stamping is mediated by the proteoglycan core and that CS chains remain accessible in solution to enzymatic cleavage.

4.3. Laminin activity dependence on epitope presentation

We have shown in this study that aggrecan acts to stabilize a laminin epitope that can be blocked against $\alpha_6\beta_1$ integrin receptor attachment by the antibody AL-4 [33]. We found that the magnitude of laminin epitope presentation recognized by AL-4 was a strong predictor of neurite outgrowth length on laminin. Although unexpected, we believe that there is a valid explanation for the significant negative correlation between laminin epitope density and neurite outgrowth length. It has been shown that neonatal rat superior cervical ganglia neurons exhibit a bimodal outgrowth length behavior when cultured on increasing densities of laminin and that the inflection point for maximum length is in the range of laminin densities generated in this study [34]. Additionally, it has been shown that migratory cells tend to slow in speed in vitro with increasing substratum bound adhesion protein density [35–37]. Thus a correlation between outgrowth length and epitope presentation suggests that neurite attachment and outgrowth length may more accurately be predicted by intact integrin density as identified by AL-4 binding than by laminin density alone.

Bolstering the importance of the AL-4 targeted epitope for neurite outgrowth, we unsuccessfully attempted on several occasions to pattern DRG outgrowth on stamped laminin lanes on glass and subsequently discovered that the stamping process had almost completely abolished the AL-4 targeted laminin epitope. Significantly, it has been shown elsewhere that denaturation of the $\alpha_6\beta_1$ laminin binding domain, which is recognized by the AL-4 antibody, is sufficient to arrest neuronal cell attachment and neurite extension in the absence of other laminin sub-domains [18]. Curiously, others have demonstrated the successful outgrowth of neurons on μ CP laminins, but only in cases where polar groups were introduced into the stamp material, onto the pattern substratum, or directly into the laminin molecule [38,39], indicating the importance of electrostatic interfacial interactions on laminin structure and resulting activity.

5. Conclusion

Evidence is presented in this study that μ CP and μ FN patterning can be used to segregate or combine dual protein signals while limiting protein-protein interactions. Based on our observations, we conclude that aggrecan possesses intrinsic inhibitory properties that cannot be explained alone by interactions with laminin and that DRG cell body attachment is dictated largely by non-specific interactions. We additionally conclude that laminin AL-4 targeted epitope presentation is highly sensitive to the method of deposition and is a strong predictor of neurite outgrowth behavior. We propose that the assays presented in this study can be generated using other dual protein signals and applied to cell types that demonstrate behavior seemingly influenced by protein-protein interactions. Such assays could aid in unraveling the nature of dual protein interactions at interfaces and improve our ability to interpret cell behavior influenced by multiple substratum bound factors.

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References

1. Challacombe JF, Snow DM, Letourneau PC. Actin filament bundles are required for microtubule reorientation during growth cone turning to avoid an inhibitory guidance cue. *J Cell Sci* 1996;109:2031–2040. [PubMed: 8856499]
2. Hynds DL, Snow DM. Neurite outgrowth inhibition by chondroitin sulfate proteoglycan: stalling/ stopping exceeds turning in human neuroblastoma growth cones. *Exp Neurol* 1999;160:244–255. [PubMed: 10630209]
3. Hynds DL, Snow DM. Fibronectin and laminin elicit differential behaviors from SH-SY5Y growth cones contacting inhibitory chondroitin sulfate proteoglycans. *J Neurosci Res* 2001;66:630–642. [PubMed: 11746383]
4. Yu X, Bellamkonda RV. Dorsal root ganglia neurite extension is inhibited by mechanical and chondroitin sulfate-rich interfaces. *J Neurosci Res* 2001;66:303–10. [PubMed: 11592128]
5. Snow DM, Letourneau PC. Neurite outgrowth on a step gradient of chondroitin sulfate proteoglycan (CS-PG). *J Neurobiol* 1992;23:322–36. [PubMed: 1624935]
6. Snow DM, Smith JD, Gurwell JA. Binding characteristics of chondroitin sulfate proteoglycans and laminin-1, and correlative neurite outgrowth behaviors in a standard tissue culture choice assay. *J Neurobiol* 2002;51:285–301. [PubMed: 12150504]
7. Jones LL, Sajed D, Tuszynski MH. Axonal regeneration through regions of chondroitin sulfate proteoglycan deposition after spinal cord injury: a balance of permissiveness and inhibition. *J Neurosci* 2003;23:9276–9288. [PubMed: 14561854]
8. Cummings BJ, Su JH, Geddes JW, Van Nostrand WE, Wagner SL, Cunningham DD, Cotman CW. Aggregation of the amyloid precursor protein within degenerating neurons and dystrophic neurites in Alzheimer's disease. *Neuroscience* 1992;48:763–777. [PubMed: 1378573]
9. Kuhn TB, Schmidt MF, Kater SB. Laminin and fibronectin guideposts signal sustained but opposite effects to passing growth cones. *Neuron* 1995;14:275–285. [PubMed: 7531986]
10. Tanaka E, Sabry J. Making the connection: cytoskeletal rearrangements during growth cone guidance. *Cell* 1995;83:171–6. [PubMed: 7585934]
11. Dickson BJ. Molecular mechanisms of axon guidance. *Science* 2002;298:1959–1964. [PubMed: 12471249]
12. Skubitz AP, McCarthy JB, Charonis AS, Furcht LT. Localization of three distinct heparin-binding domains of laminin by monoclonal antibodies. *J Biol Chem* 1988;263:4861–8. [PubMed: 3350814]
13. Friedlander DR, Milev P, Karthikeyan L, Margolis RK, Margolis RU, Grumet M. The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/ NILE and N-CAM, and inhibits neuronal adhesion and neurite outgrowth. *J Cell Biol* 1994;125:669–680. [PubMed: 7513709]
14. Milev P, Friedlander DR, Sakurai T, Karthikeyan L, Flad M, Margolis RK, Grumet M, Margolis RU. Interactions of the chondroitin sulfate proteoglycan phosphacan, the extracellular domain of a receptor-type protein tyrosine phosphatase, with neurons, glia, and neural cell adhesion molecules. *J Cell Biol* 1994;127:1703–15. [PubMed: 7528221]
15. Braunewell KH, Martini R, LeBaron R, Kresse H, Faissner A, Schmitz B, Schachner M. Up-regulation of a chondroitin sulphate epitope during regeneration of mouse sciatic nerve: evidence that the immunoreactive molecules are related to the chondroitin sulphate proteoglycans decorin and versican. *Eur J Neurosci* 1995;7:792–804. [PubMed: 7542529]
16. Dou CL, Levine JM. Inhibition of neurite growth by the NG2 chondroitin sulfate proteoglycan. *J Neurosci* 1994;14:7616–7628. [PubMed: 7996200]
17. Dou CL, Levine JM. Differential effects of glycosaminoglycans on neurite growth on laminin and L1 substrates. *J Neurosci* 1995;15:8053–8066. [PubMed: 8613742]
18. Deutzmann R, Aumailley M, Wiedemann H, Pysny W, Timpl R, Edgar D. Cell adhesion, spreading and neurite stimulation by laminin fragment E8 depends on maintenance of secondary and tertiary structure in its rod and globular domain. *Eur J Biochem* 1990;191:513–522. [PubMed: 2200677]
19. Hall DE, Reichardt LF, Crowley E, Holley B, Moezzi H, Sonnenberg A, Damsky CH. The alpha 1/ beta 1 and alpha 6/ beta 1 integrin heterodimers mediate cell attachment to distinct sites on laminin. *J Cell Biol* 1990;110:2175–2184. [PubMed: 2351695]

20. Hodgkinson G, Hlady V. Relating material surface heterogeneity to protein adsorption. I. The effect of annealing of micro-contact-printed OTS patterns. *J Adh Sci Technol* 2005;19:235–255.
21. Yamagata T, Saito H, Habuchi O, Suzuki S. Purification and properties of bacterial chondroitinases and chondrosulfatases. *J Biol Chem* 1968;243:1523–35. [PubMed: 5647268]
22. Hayashi I, Sato GH. Replacement of serum by hormones permits growth of cells in a defined medium. *Nature* 1976;259:132–134. [PubMed: 813153]
23. Condic ML, Snow DM, Letourneau PC. Embryonic neurons adapt to the inhibitory proteoglycan aggrecan by increasing integrin expression. *J Neurosci* 1999;19:10036–10043. [PubMed: 10559411]
24. Hammarback JA, Palm SL, Furcht LT, Letourneau PC. Guidance of neurite outgrowth by pathways of substratum-adsorbed laminin. *J Neurosci Res* 1985;13:213–220. [PubMed: 3973933]
25. Lemons ML, Sandy JD, Anderson DK, Howland DR. Intact aggrecan and chondroitin sulfate-depleted aggrecan core glycoprotein inhibit axon growth in the adult rat spinal cord. *Exp Neurol* 2003;184:981–90. [PubMed: 14769391]
26. Pitt WG, Grasel TG, Cooper SL. Albumin adsorption on alkyl chain derivatized polyurethanes II The effect of alkyl chain length. *Biomaterials* 1988;9:36–46. [PubMed: 3349120]
27. Ge S, Kojio K, Takahara A, Kajiyama T. Bovine serum albumin adsorption onto immobilized organotrichlorosilane surface: Influence of the phase separation on protein adsorption patterns. *J Biomater Sci Polym Ed* 1998;9:131–150. [PubMed: 9493841]
28. Noinville S, Revault M, Baron MH, Tiss A, Yapoudjian S, Ivanova M, Verger R. Conformational changes and orientation of Humicola lanuginosa lipase on a solid hydrophobic surface: An in situ interface Fourier transform infrared-attenuated total reflection study. *Biophys J* 2002;82:2709–19. [PubMed: 11964257]
29. Choi EJ, Foster MD, Daly S, Tilton R, Przybycien T, Marjkrzak CF, Witte P, Menzel H. Effect of flow on human serum albumin adsorption to self-assembled monolayers of varying packing density. *Langmuir* 2003;19:5464–5474.
30. Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. *FASEB J* 1992;6:861–870. [PubMed: 1740236]
31. Ernst S, Langer R, Cooney CL, Sasisekharan R. Enzymatic degradation of glycosaminoglycans. *Crit Rev Biochem Mol Biol* 1995;30:387–444. [PubMed: 8575190]
32. Kiani C, Chen L, Wu YJ, Yee AJ, Yang BB. Structure and function of aggrecan. *Cell Res* 2002;12:19–32. [PubMed: 11942407]
33. Furtado GC, Cao Y, Joiner KA. Laminin on Toxoplasma gondii mediates parasite binding to the beta 1 integrin receptor alpha 6 beta 1 on human foreskin fibroblasts and Chinese hamster ovary cells. *Infect Immun* 1992;60:4925–4931. [PubMed: 1399003]
34. David S, Braun PE, Jackson DL, Kottis V, McKerracher L. Laminin overrides the inhibitory effects of peripheral nervous system and central nervous system myelin-derived inhibitors of neurite growth. *J Neurosci Res* 1995;42:594–602. [PubMed: 8568945]
35. DiMilla PA, Stone JA, Quinn JA, Albelda SM, Lauffenburger DA. Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. *J Cell Biol* 1993;122:729–737. [PubMed: 8335696]
36. Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, Horwitz AF. Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature* 1997;385:537–40. [PubMed: 9020360]
37. Webb K, Hlady V, Tresco PA. Relationships among cell attachment, spreading, cytoskeletal organization, and migration rate for anchorage-dependent cells on model surfaces. *J Biomed Mater Res* 2000;49:362–8. [PubMed: 10602069]
38. Kam L, Shain W, Turner JN, Bizios R. Axonal outgrowth of hippocampal neurons on micro-scale networks of polylysine-conjugated laminin. *Biomaterials* 2001;22:1049–1054. [PubMed: 11352086]
39. Schmalenberg KE, Buettner HM, Urich KE. Microcontact printing of proteins on oxygen plasma-activated poly(methyl methacrylate). *Biomaterials* 2004;25:1851–7. [PubMed: 14738849]

Figure 1.

Micropatterning methods used to create colocalized and segregated dual LN + AG or LN + HSA patterns. A – Segregated layers produced by combination μ FN/ μ CP patterning. Each protein species was deposited in segregated, adjacent locations. B – Colocalized layers produced by combined adsorption and μ CP patterning. A heterogeneous stamped protein layer was colocalized onto a second adsorbed homogeneous protein layer.

Figure 2.

Representative fluorescence images of dual AF488-laminin (green) and AF594-aggrecan (red) layers created by combination μ FN/ μ CP to generate segregated dual protein patterns or by combined adsorption and μ CP (Ads/ μ CP) to generate colocalized dual protein patterns. Protein layers remained completely segregated in the combination μ FN/ μ CP patterns even after rinsing. The laminin film in the Ads/ μ CP case appears patterned due to fluorescence resonance energy transfer from AF488 to AF594 fluorophores in the colocalized layers (dim green areas). Arrows in the Ads/ μ CP images indicate areas of low AG density where less energy transfer resulted from the AF488-LN layer. Scale bars – 100 μ m

Figure 3.

Protein densities and laminin epitope availability were affected by the substratum layer and deposition method. A – Stamped AG and HSA layer densities on glass or stamped on an adsorbed LN layer; Both AG and HSA transferred onto LN layers at densities significantly different from those produced by glass substratum deposition * ($p < 0.001$, t-test). B – LN epitope availability for adsorbed LN layers and LN molecules exposed through HSA or AG stamped layers; Stamped AG layers masked LN epitopes while stamped HSA layers enhanced LN epitope presentation over adsorbed LN alone. C – Fluorescence image of LN epitope availability (AL-4 antibody + AF488 IgG staining) in a colocalized adsorbed LN and stamped AG pattern. D – Fluorescence image of LN epitope availability in a colocalized adsorbed LN and stamped HSA pattern. E – Fluorescence image of alternating stamped and μFN deposited AF488-LN lanes; LN density was greater in stamped lanes than in μFN deposited lanes. F – Fluorescence image of immunostained LN epitope presentation in alternating stamped and μFN deposited LN lanes; LN epitope presentation was more pronounced in μFN deposited lanes despite LN being deposited at a lower density than in stamped LN lanes. G – LN densities produced by various modes of deposition: Stamped LN (Stamped); μFN deposited LN with stamped AG (St AG), stamped HSA (St HSA), or stamped LN (St LN); adsorbed LN (Adsorbed); LN co-adsorbed with AG (Co-Ads w/ AG); and LN sequentially adsorbed onto a pre-adsorbed AG layer (Seq Ads on AG). Anova analysis of all LN densities returned a p value < 0.001 . * Significantly different from all others ($p < 0.001$, t-test pair-wise comparisons), X

significantly different ($p < 0.04$, t-test), + significantly different ($p < 0.02$, t-test). H – Normalized LN epitope availability for the various modes of LN deposition; μ FN LN deposition from AG coated channels produced layers with the greatest epitope density while stamping LN nearly abolished all epitope presentation. Adsorbing LN onto an AG layer or co-adsorbing LN with AG resulted in greater LN epitope density than adsorbing LN alone to glass. Scale bars: C&D – 50 μ m (same scale), E&F – 40 μ m (same scale). Error bars $\pm 95\%$ confidence intervals. n = total number of imaged areas.

Figure 4.

Stamping produced widely different protein layer morphologies for AG and HSA. A – SFM topography of stamped AG lanes on glass measured in air. B – SFM topography of stamped HSA lanes on glass measured in air. C – Friction map of stamped AG on glass measured in water. D – Friction map of stamped HSA on glass measured in water. E – Fluorescence image of adsorbed AF488-AG on glass. F – Fluorescence image of stamped AG on glass immunostained with CS-56 antibody + AF488 IgG. G – DPFM adhesion map of stamped AG layer on glass. Lighter areas correspond to higher adhesion of glass. One AG molecule is outlined in white in the image. H – Corresponding DPFM topography map of stamped AG film. Both stamped and adsorbed AG layers took on a web structure while stamped HSA layers appeared more continuous and smooth. Scale bars A, B, E,&F – 5 μm , C&D – 1 μm , G&H – 400 nm (Same scale).

Figure 5.

Neurite outgrowth and cell body attachment varied according to pattern type and pattern substratum: Homogeneous stamped AG layer (St AG Control) or HSA layer (St HSA Control); homogeneous adsorbed LN field (Ads LN Control); colocalized stamped AG (St AG Ads LN) or HSA (St HSA Ads LN) stripes on homogeneous adsorbed LN field; segregated LN and AG (St AG μ FN LN), LN and HSA (St HSA μ FN LN) stripes, segregated LN and AG patterned on polystyrene (St AG μ FN LN on PS). A – Neurite crossing over AG and HSA lanes on both μ FN/ μ CP and Ads/ μ CP cases resulted in reduced crossing when AG or HSA stripes were segregated from LN. Crossing was further diminished when segregated LN and AG patterns were deposited on polystyrene. All populations were significantly different from all others ($p < 0.025$, t-test pair-wise comparisons). Anova analysis of all neurite crossing events returned a p value < 0.001 . B – Cell body attachment to pure LN, AG, or HSA lanes or to LN+AG or LN+HSA lanes in the various patterns resulted in preferential adhesion to LN versus AG and to HSA versus LN. C – Neurite outgrowth lengths for the various patterns and controls. Neurite lengths were greatest for LN deposited by adsorption. * Significantly less than the adsorbed LN control ($p \leq 0.005$, t-test), + significantly less than the corresponding Ads/ μ CP case ($p < 0.005$, t-test). Anova analysis of all neurite outgrowth lengths returned a p value < 0.001 . Error bars $\pm 95\%$ confidence intervals. n = number of measured DRGs.

Figure 6.

AG and HSA lanes segregated from LN lanes reduced neurite crossing and stamped LN lanes were non-permissive to neurite growth. (A-C) Fluorescence images of DRGs stained for actin (AF488-phalloidin, green) on stamped AF594-AG (red) and non-labeled LN (black) lanes. (A) Segregated stamped AG and μ FN-deposited LN on glass; cell body adhesion and neurite outgrowth were largely restricted to LN lanes. (B) Colocalized stamped AG and adsorbed LN on glass; neurite crossing over AG lanes was observed (arrows). (C) Segregated stamped AG and μ FN-deposited LN on polystyrene; cell body adhesion and neurite outgrowth were almost exclusively restrained to LN lanes on polystyrene. (D and E) Combined fluorescence and phase images of DRG outgrowth on stamped FITC-HSA (bright stripes) and LN patterns. (D) Segregated stamped HSA and μ FN deposited LN on glass; neurite outgrowth was largely restrained to LN lanes, however, a majority of cell bodies adhered to HSA lanes. (E) Colocalized stamped HSA and adsorbed LN; neurite outgrowth proceeded unrestrained over both LN and HSA lanes. (F) Phase image of DRGs on an adsorbed LN control; Robust cell attachment and neurite outgrowth were observed for the LN control. (G) Combined fluorescence and phase image of DRG outgrowth on stamped AF488-LN lanes (bright stripes) on glass; stamped LN was non-permissive to DRG cell body attachment and neurite outgrowth. Glass lanes presumably contained a low-density coverage of laminin carried over from stamped lanes during a rinsing step before cell seeding. Scale bars A-E, G=100 μ m and F=200 μ m.

Figure 7.

DRG outgrowth proceeded differentially on ChABC treated and control colocalized stamped AG patterns on adsorbed LN fields. A – Neurite crossing over AG lanes was more pronounced after ChABC treatment, with a majority of cells exhibiting neurites that crossed multiple AG lanes. Error bars $\pm 95\%$ confidence intervals. n = number of measured DRGs. B – Fluorescence image of actin stained DRGs on control stamped AG pattern. DRG cell body attachment and neurite outgrowth were mostly constrained to pure LN lanes. C – Fluorescence image of actin stained DRGs on ChABC treated AG pattern. Although neurites more freely crossed AG core proteins after CS chain removal, AG + LN lanes continued to exert an inhibitory effect on

outgrowth and to constrain a majority of growth cone contacts to pure LN lanes. Scale bar – 100 μm (Same scale for both images).

Table 1

Summary of major findings derived from protein patterning, protein layer characterization, and DRG cell choice assays.

Major Findings	LN AL-4 targeted epitope presentation is predictor of neurite outgrowth promoting activity	Proof that AG inhibition is independent of interactions with LN	Non-specific interactions override DRG cell body attachment to LN	Substrate material-protein interactions affect resulting DRG behavior
Key Observations	a. LN density and epitope presentation affected by deposition method (Fig. 3) b. Inverse correlation between neurite length and epitope density (Figs. 5C & 3H) c. Epitope presentation removal through stamping abolished LN neurite promoting activity (Fig. 6G)	d. Pure AG lanes significantly reduced neurite crossing events vs. pure HSA lanes (Fig. 5A) e. AG – LN interactions stabilized LN neurite promoting epitope (Fig. 3H) f. Stamped AG retained core protein and CS chain inhibitory properties (Fig. 7)	g. DRGs preferentially adhered to pure HSA lanes vs. pure LN lanes (Figs. 5B & 6D) h. DRGs adhered to homogeneous AG and HSA fields	i. Neurite crossing events over pure AG lanes were significantly reduced on tissue culture polystyrene vs. glass substrata (Figs. 5A & 6A&C)