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Astrocytes Specifically Remove Surface-Adsorbed Fibrinogen and Locally Express Chondroitin Sulfate Proteoglycans

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Abstract

Surface-adsorbed fibrinogen (FBG) was recognized by adhering astrocytes and removed from the substrates in vitro by a two-phase removal process. The cells removed adsorbed FBG from binary proteins surface patterns (FBG + laminin, or FBG + albumin) while leaving the other protein behind. Astrocytes preferentially expressed chondroitin sulfate proteoglycan (CSPG) at the loci of fibrinogen stimuli; however no differences in overall CSPG production as a function of FBG surface coverage were identified. Removal of FBG by astrocytes was also found to be independent of transforming growth factor type β (TGF- β) receptor based signaling as cells maintained CSPG production in the presence of TGF- β receptor kinase inhibitor, SB 431542. The inhibitor decreased CSPG expression, but did not abolish it entirely. Because blood contact and subsequent FBG adsorption are unavoidable in neural implantations, the results indicate that implant-adsorbed FBG may contribute to reactive astrogliosis around the implant as astrocytes specifically recognize adsorbed FBG.

Keywords

astrocytes; fibrinogen; chondroitin sulfate proteoglycan; TGF- β ; TGF- β inhibitor SB 431542

1. Introduction

Neural implants elicit the foreign body response of the central nervous system (CNS) leading to a loss of local neurons [1,2]. The CNS response to implants is characterized by acute activation of microglia and astrocytes within one day of injury. Edema and debris are lessened within one week, however, chronic inflammation persists around these devices, and they become encapsulated by macrophage-like cells after four weeks [3]. This persistent, stable sheath of tissue, termed the glial scar, is composed of astrocytes and microglia. In the case of recording or stimulating electrodes that are implanted in the brain, degradation of electric signal quality has been attributed to this foreign body response and associated neuronal loss [1].

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Initial CNS response to the neural implant can provide insight into the mechanism of glial scar formation. A universal feature of neural implants is that the initial surgical intervention during implantation causes hemorrhage and/or leakage of the blood-brain barrier (BBB). This effect may be compounded if the BBB remains compromised allowing continuous contact of the device with blood. Like all materials exposed to blood, surfaces of such implants quickly adsorb plasma proteins at the blood-implant interface. Among the dozen plasma proteins with concentrations greater than 1 mg/mL, fibrinogen (FBG, 2–3 mg/mL in blood plasma) plays a key role in hemostasis after injury and is known to be a surface active protein that readily adsorbs to surfaces [4]. In circulation FBG adsorbs to the surface of implants and often undergoes conformational changes that lead to adhesion and activation of platelets [5]. Fibrinogen adsorbed onto the surface of neural implants is ultimately exposed to the CNS tissue and not to blood. The question is whether CNS astrocytes recognize adsorbed FBG and react by mounting CNS inflammatory response. Such recognition could be one of the initial steps in the formation of a glial scar that ultimately results in an inhibition of neuronal activity around implants.

Astrocytes *in vivo* have minimal glial fibrillary acidic protein (GFAP) expression in their quiescent state [3,6]. In reactive astrogliosis, however, GFAP expression is increased and astrocytes display hypertrophied morphology. Interestingly, astrocytes cultured in 3D collagen gels mimic more closely their *in vivo* state, while in 2D cultures *in vitro* they appear as being reactive [7]. In addition to their morphological changes, reactive astrocytes proliferate and secrete various factors and macromolecules. Of these secreted species, chondroitin sulfate proteoglycan (CSPG) is of particular interest as it has been implicated as a potential inhibitor of neuronal regeneration [8,9]. The modes of astrocyte expression of CSPGs can be varied. For example, brevican CSPGs are attached to the astrocyte membrane [9,10] while other CSPGs, like neurocan, are shed into cell media and were also found bound to underlying substrates [11].

Soluble chemical signals are also potent contributors to astrogliosis [12,13]. For example, transforming growth factor type β (TGF- β) is known to trigger astrocyte activation. In this role TGF- β has been utilized to enhance *in vitro* models of the glial scar for neuronal outgrowth studies [14]. It has recently been shown that soluble blood FBG is a source of latent TGF- β ; the BBB leakage of soluble FBG into the CNS causes an increased CSPG expression in astrocytes both *in vivo* and *in vitro* by the TGF- β -mediated *smad* signaling pathway [15]. While it has been demonstrated that astrocytes can become reactive without BBB leakage by addition of a neurotoxicant [16], the FBG/TGF- β /*smad* activation pathway seems also likely when neural implants covered with adsorbed blood proteins, including FBG, interface with CNS tissue.

The objective of the present study was to find how astrocytes respond to adsorbed FBG and determine if this causes astrocytes to become more reactive and change their CSPG production. To investigate this particular scenario, we cultured astrocytes on surface-adsorbed FBG layers at different protein surface coverage. We found that the cells, after some initial incubation period, start to actively remove adsorbed FBG and locally produce CSPG. To test the role of latent TGF- β on adsorbed FBG recognition, we blocked the TGF- β receptors by an inhibitor of TGF- β receptor kinase. This blocking did not alter FBG removal by astrocytes nor completely abolish expression of CSPG *in vitro* thereby indicating that signaling pathways other than TGF- β /*smad* may be involved.

2. Materials and Methods

2.1 Cell Culture

Primary cortical astrocytes were harvested from P1 Sprague-Dawley rats according to the protocol approved by University of Utah Institutional Animal Care and Use Committee [17]. Briefly, cortical tissue with removed meninges was broken up and digested in collagenase (1.33%, Worthington) for 30 minutes. Cortices were then treated with trypsin (0.25%, Worthington) for 30 minutes, triturated, suspended, and plated in 75 cm² tissue culture flasks. To purify astrocyte cultures, flasks were shaken overnight at 175 rpm after one week of culture post-dissection. After shaking, astrocytes were dissociated with 0.25% trypsin-EDTA (Gibco) and either frozen in liquid N₂ or prepared for use. Cultures were verified for astrocyte populations (Fig S1) with anti-GFAP immunostaining (1:1000, Chemicon). Cells were maintained in DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma). Astrocytes had media exchanged once every two days and were cultured for one week prior to use in experiments.

Cells were seeded onto prepared coverslips in a 12-well tissue culture plate (CellTreat) at a density of approximately 10,000 cells/cm². This density was used to maintain sparse culture and minimize intercellular interactions. Astrocytes were seeded and cultured in DMEM/F12 with three types of conditions: a) 10% FBS, b) 10% FBS with 1% DMSO (Sigma), and c) 10% FBS with 10 mM TGF- β receptor kinase inhibitor (SB 431542, Sigma) in 1% DMSO to block TGF- β signaling. Cells were cultured on experimental substrates for 48 hours and then fixed with a 4% paraformaldehyde (PFA, Sigma) solution for 15 minutes.

2.2 Substrate Preparation

Human plasma fibrinogen (plasminogen depleted, Calbiochem) was fluorescently labeled with Alexa Fluor 594 (A-20004, Invitrogen) by reacting at room temperature for 2 hours in 0.1 M sodium bicarbonate solution. The solution was eluted in phosphate buffered saline (PBS) through a PD-10 Sephadex column (GE Healthcare) to separate labeled protein from free dye. Collected fibrinogen solution (2 mg/mL) was then filtered through a 0.2 μ m syringe filter (Sarstedt), divided into 100 μ L aliquots, and stored at -20°C until use. Laminin (L2020, Sigma-Aldrich) solution was prepared in a 100 μ g/mL concentration in PBS. Laminin was fluorescently labeled using Alexa Fluor 488 (A-20000, Invitrogen). For albumin studies, bovine serum albumin (68700, Proliant) was dissolved in PBS at 4.5 mg/mL and was also labeled with Alexa Fluor 488.

Glass coverslips (Fisherbrand, 18 mm #2, Fisher) were rinsed in acetone, ethanol, and DDI water before being sonicated in DDI water for 15 minutes. Coverslips were then dried in N₂ and autoclaved. For samples with single adsorbed protein (fibrinogen or laminin), protein solution was applied over the surface and allowed to incubate at room temperature for one hour. Autoclaved coverslips without adsorbed proteins were used as controls. For samples with added soluble TGF- β , human TGF- β 1 (R&D Systems) was added to culture media at 20 ng/mL at time of seeding.

Microcontact printing (μ CP) was used to create surfaces with different FBG coverage [18]. Patterns of randomly distributed μ m-sized islands with coverage of 30% and 50% were made using soft lithography [19]. The 30% and 50% random distributions of pixels were first created in Mathematica (Wolfram). These pixels were then converted to a template for a photolithography mask where each pixel equated to a $\sim$ 1 μ m² feature. The mask served as a mold for polydimethylsiloxane (PDMS) casting. PDMS (Sylgard 184, Dow Corning) was poured over the mask and allowed to cure at 100°C. Cast PDMS was peeled from the mask and soaked in hexane, acetone, and ethanol to remove unreacted siloxane molecules, dried in an oven and cut into stamps containing the desired coverage pattern. The stamps were then

sonicated in detergent solution (Alconox), rinsed with DDI water, and stored in DDI water prior to use. For use in μ CP, the stamp was incubated in FBG solution (2 mg/mL) at room temperature for 20–30 minutes, then briefly rinsed with DDI water and dried with an N_2 stream. Stamps were brought into conformal contact with sterilized coverslips for approximately 1 minute for protein transfer. Upon stamp removal, the patterned coverslips were backfilled with laminin solution. The created FBG patterns are shown in Figure 1. The coverage of proteins was 100% FBG (FBG100/LN0), 50% FBG with LN backfill (FBG50/LN50), 30% FBG with LN backfill (FBG30/LN70), or 100% LN (FBG0/LN100). This same protocol was followed to create binary FBG and albumin surfaces except that the labeled albumin solution was used for backfill after stamping. All substrates were then immediately used for cell culture.

2.3 Time-Lapse Microscopy

Astrocytes were seeded onto sterile glass-bottomed culture dishes (Fluorodish, WPI Inc.) that had been covered with micro-contact printed and/or adsorbed proteins as described above. Cells were allowed to attach for 4 hours and then imaged with an Olympus IX81 microscope on a temperature-controlled stage. Metamorph software (Molecular Devices) was used to take images of multiple stage positions once every 6 minutes for 20 hours. Live cell images were taken with a 20x DIC objective and overlaid with corresponding fluorescence images.

Time-lapse fluorescence images were brought in register manually to compensate for sample drift and were used in calculating fibrinogen removal kinetics. Areas of 5 x 5 pixels from five different cell regions of a given cell were tracked through time for analysis and used in calculation of the FBG removal rate according to the equation:

$$\theta(t) = \theta_0 + A e^{\frac{t_0 - t}{\tau}} \quad (1)$$

where $\theta(t)$ is the surface coverage of FBG (assumed to be proportional to the fluorescence intensity) measured as a function of time, t ; θ_0 is an initial FBG coverage at the time t_0 , A is a removal coefficient, and τ is a characteristic time for this removal process (τ = the inverse of the FBG removal rate constant). The initial time, t_0 , is defined as the time when the cell began to *rapidly* remove FBG and was determined as the point where there was a rapid fluorescence intensity change in the $\theta(t)$ curves. Coefficients for Equation 1 were found by fitting the $\theta(t)$ curves using Igor Pro (WaveMetrics).

2.4 Immunocytochemistry

Astrocytes were fixed with 4% PFA for 15 minutes and rinsed in PBS with 0.1% sodium azide prior to immunocytochemical staining. All procedures were carried out at room temperature. Samples were first blocked with 4% goat serum in PBS for 1 hour then rinsed thrice in PBS with azide. Primary anti-chondroitin sulfate (CS-56) antibody (C8035, Sigma) was applied to the fixed astrocytes for one hour at a 1:500 dilution in block solution. Samples were again rinsed 3 times in PBS/azide and the secondary goat anti-mouse IgM antibody labeled with Alexa Fluor 488 (A21042, Molecular Probes) was subsequently applied to the samples for 1 hour. Following three more PBS/azide rinses, DAPI (Invitrogen) at a 1:100 dilution was added for 15 minutes to stain for nuclei. Samples were then rinsed in DDI water, allowed to dry, and mounted onto 3" glass microscope slides (VWR) with Fluoromount-G (Southern Biotech) for fluorescence imaging.

2.5 Astrocyte Surface CSPG Expression

Stained CSPG samples were imaged using a Nikon Eclipse E600 epifluorescence microscope with a 20x PlanApo objective and CCD camera (CoolSNAP, Photometrics)

using identical exposure times with blank images subtracted. Ten sample images were taken per condition. Each image was divided into 6 equal regions for quantification. The fluorescence intensity in each region was quantified using ImageJ (NIH). The integrated CSPG fluorescence intensity was normalized by dividing it by the number of cells per region as identified by DAPI nuclear stain. To correct for regions where no cells were present and also regions with high densities of cells, 10 regions with the highest and 5 regions with the lowest CSPG per cell values were removed from the analysis. An ANOVA with a Tukey post hoc test ($\alpha = 0.05$) was used to determine the significance of data differences. To assess the 3D distribution of CSPG fluorescence, a confocal microscope (Olympus BX61WI, 40x PlanFLN, NA 1.30) was used to image samples using multiple slices in the vertical, z-direction. Image stacks were then compiled using Olympus Fluoview software. Vertical distribution of FGB and CSPG was measured at nuclear (as confirmed by DAPI), cell periphery and regions where cells deposited CSPG and then migrated away. For each region the fluorescence intensity was averaged over 7 areas of 25 by 25 pixels in each z-slice.

2.6 Expression of Shed CSPG

Radioactive sulfur assay was used to determine the amount of glycosaminoglycans (GAGs) shed by astrocytes into media vs. the GAG amount present on the cell membrane [20]. Astrocytes were cultured for 48 hours in F-12 nutrient mixture (Gibco) with 10% dialyzed FBS supplemented with 700 μCi of [^{35}S] Na_2SO_4 (Perkin Elmer Life Sciences). Cells were cultured in T75 flasks on tissue-culture polystyrene (TCPS) with or without pre-adsorbed FBG layer. After the culture period, the conditioned medium was removed for GAGs analysis and the remaining cells were treated with 1 mg/mL pronase solution (Pronase *Streptomyces griseus* was, Sigma-Aldrich) for endogenous GAG quantification. Each sample was purified for GAG chains using a DEAE-sepharose (Amersham Biosciences) column, and ^{35}S radioactivity was measured using a scintillation counter (Beckman).

3. Results

3.1 Astrocyte Removal of Adsorbed Fibrinogen

Astrocytes adhered to fibrinogen-covered surfaces and subsequently removed the fibrinogen, as evidenced by progressive loss of fluorescence over time. This behavior was observed in real time via time-lapse microscopy and was also evident in fixed samples (Fig. 2). Figure 2A–C (DIC + FBG fluorescence) shows the progress of fibrinogen removal from 4 to 23 hours post seeding. Astrocyte adhesion to FBG-coated surfaces was a dynamic process with cells attaching to, detaching from, and migrating across the surface throughout 24 hours of culture. The initial 4 hours of culture were used to allow for cells to attach to the substrates prior to imaging. At the 4-hours time point, however, some regions depleted of FBG were already present on the substrates. Some of these sites had astrocytes attached, while others did not have any cells remaining on the FBG depleted sites.

Adsorbed FBG removal by astrocytes was found to be a two-step process characterized by an initial gradual decrease of FBG fluorescence followed by a more rapid decay of fluorescence intensity (Fig. 3). The rapid FBG removal phase, when modeled as exponential intensity decrease (Eq. 1), had a characteristic time, $\tau = 1.69 \pm 0.46$ hr (mean $\pm$ st. deviation) over the five sub-areas across the cell (Fig. 3B,E). Similar FBG removal phases were found on patterned surfaces as well (Fig 3C,D). The rapid removal only occurred after an incubation period that varied as the cell migrated along the substrate (which contributed to dispersion in τ values). The initial slow FBG removal had an average $d\theta/dt$ slope of -0.0064 ± 0.0033 hr^{-1} (mean $\pm$ st. deviation) and the duration of the slow FBG removal phase varied from cell to cell.

In fixed and stained samples, FBG was seen as being selectively removed in the areas that were frequently co-localized with adhered cells. Such astrocyte behavior was independent of blocking TGF- β receptor type I, as astrocytes cultures treated with SB 431542 showed similar substrate modification. There was also sporadic evidence of cellular uptake of FBG in fixed samples as shown by areas of increased fluorescence associated with astrocytes (Fig. 4A,B).

3.2 FBG Removal is a Protein Specific Astrocyte Response

The specificity of astrocyte recognition and removal of adsorbed FBG was tested by comparing the effect of astrocytes on laminin (LN) and albumin (ALB) coated surfaces. These two proteins also provided a test for proteins larger and smaller than FBG. Adsorbed laminin is known to present the sites for astrocytes adhesion [21], and the cells spread more readily on laminin surfaces when compared to surfaces covered with FBG or serum proteins alone (Fig S2, Supplementary Data). Unlike FBG, surface adsorbed laminin remained minimally modified by astrocytes over the culture period (Fig. 2D). The modification of surface fibrinogen by astrocytes was therefore not merely a result of cellular adhesion.

Similarly, astrocyte culture on a mixed albumin-fibrinogen substrate with 50% ALB and 50% FBG coverage left albumin largely intact while still removing FBG (Fig 4C,D). Thus astrocytes specifically removed the FBG from amongst the albumin, and left albumin in its original surface patterns. Adsorbed albumin and laminin results indicated that astrocyte did not use any nonspecific, broad-target proteolysis to remove FBG. Instead, the removal of FBG by astrocytes appeared to be a selective process that was unaffected by presence of other proteins.

3.3 CSPG Expression in Response to Surface Coverage of Fibrinogen

The cell-associated CSPG production was not significantly dependent on the amount of adsorbed fibrinogen presented to the cells (Fig. 5). Astrocytes expressed CSPG on all four substrates used for cell culture: full-coverage fibrinogen (FBG100/LN0), 50% coverage FBG backfilled with LN (FBG50/LN50), 30% coverage FBG backfilled with LN (FBG30/LN70), and full-coverage laminin (FBG0/LN100). The levels of CSPG production were comparable to those found on astrocytes cultured in culture media on glass coverslips alone (data not shown). However, astrocytes seemed to produce CSPG in a local response to adsorbed fibrinogen patterns. For example, on FBG50/LN50 and also on FBG30/LN70 substrates, astrocytes were often found to produce the higher levels of CSPG by depositing it in between the random FBG patches (Fig. 6A, arrows). Astrocyte production of cell-associated CSPG was largest in the areas where cell membrane made contact with FBG. This behavior was consistent across both nuclear and peripheral regions of the cell. Confocal 3D imaging showed that the vertical z-position of expressed CSPG coincided with the z-position of fibrinogen (Fig. 6B–D)). Cells also produced CSPG throughout their intracellular space in addition to membranes. CSPG was also found deposited on surfaces where the cells had previously been but were no longer present, as evidenced by a footprint of removed FBG but the lack of positive DAPI staining (Fig 6D). Once FBG was removed from a region, CSPG expression by the adherent cell became more uniform and was similar to CSPG expression by cells on homogenous substrates. Importantly, CSPG was not found on areas without either a footprint or a nucleus of cells, indicating that CSPG was not merely shed into the solution and then re-adsorbed to the surface from the medium.

3.4 TGF- β Contribution to CSPG Production *in vitro*

To clarify the role of adsorbed FBG in TGF- β signaling of CSPG production by adherent astrocytes, a small molecule inhibitor, SB 431542, was used to inhibit TGF- β receptors with superfamily type I activin receptor-like kinase [22]. These receptors have been shown to

mediate CSPG expression in astrocytes when latent TGF- β was provided by soluble fibrinogen [15]. Figure 7 shows that CSPG production was somewhat attenuated in cultures with added inhibitor but not eliminated. The only significant decrease in CSPG production per cell with inhibitor treatment was found on cells grown on substrates with low or no fibrinogen (i.e. on FBG30/LN70 and FBG0/LN100 substrates). In general, the cells also maintained their ability to remove FBG from surfaces even in the presence of the inhibitor.

Adding extraneous, soluble TGF- β to the sparse astrocyte cultures in 10% serum showed no significant differences in the overall CSPG produced per cell on fibrinogen (FBG100/LN0) and laminin (FBG0/LN100) covered substrates (Fig. 8). For clearer understanding as how the produced CSPGs were distributed, scintillation counts of [35 S] from astrocytes cultured for 48 hours on TCPS coated with FBG were taken. The radioactivity counts showed that around 4 times as many GAGs were secreted into the culture media (206556 counts per minute, cpm) as compared to remaining CSPGs associated with the cell (49755 cpm on FBG-coated TCPS). Similar [35 S] counts were also found for TCPS control surfaces coated with serum proteins from 10% FBS (211977 cpm for secreted vs. 58950 cpm for cell-bound GAGs).

4. Discussion

It has been shown that astrocytes respond to soluble fibrinogen leaked into the CNS by cleaving latent TGF- β that subsequently activates the *smad* signaling pathway leading to increased CSPG expression [15]. The present study showed that surface-adsorbed FBG is also recognized by astrocytes and removed away from surfaces as a part of the CNS inflammatory response. Furthermore, astrocytes removed adsorbed FBG both with and without TGF- β signaling inhibitor, SB 431542. Therefore, when present on the surfaces of neural implants and CNS biomaterials, adsorbed FBG is sufficient to incite astrocytes to respond by FBG recognition and subsequent removal. The FBG removal was found to be a two-step process: an initial gradual decline followed by a more rapid phase. The rapid removal phase had similar characteristic time constant, $\tau \sim 1 - 2$ hours, when measured at different locations. The rapid removal only occurred after an initial period that varied as the cells migrated along the substrate¹. No consistent evidence for cellular uptake of FBG was found in time-lapse experiments. Occasionally increased intracellular FBG fluorescence signals were seen due to cells that detached from the surface and passed through the field of view while still being suspended in medium. In studies where the cells were fixed, there were also few examples of FBG cellular uptake (Fig 4A,B). At this stage, there is no information about the mechanism by which astrocytes removed and potentially digested adsorbed FBG, however it is clear that the removal process was specific to FBG. The proteases present in the serum-containing media did not appear to influence the specific FBG removal. FBG used was plasminogen depleted to minimize the addition of excess plasmin. The characteristic patterns of FBG removal from the substrates were associated with astrocytes. Even if there was any removal of surface protein mediated by serum proteases, it was much less than what occurred with the cellular removal of FBG. Furthermore, the FBG removal was independent of presence of TGF- β receptor inhibitor. The phases of FBG removal indicated that the cells need some initial period for FBG recognition and mounting of the digestion apparatus for rapid FBG removal.

Serum albumin uptake by astrocytes after BBB disruption has been implicated in altering astrocyte behavior and causing pathological neuronal outcomes[23]. Astrocytes have been shown to uptake soluble albumin both *in vivo* and *in vitro* by Ivens et al [24]. Such albumin

¹To reliably observe cells behavior, initial 4 hours of culture was used to allow for cells to attach to the substrates. In that initial time-period, however, some regions of depleted fibrinogen but lacking cells were already present on the surface.

uptake led to epileptiform activity in brain tissues. The same authors also showed that both type I and type II TGF- β receptors were involved in albumin uptake into the brain. There may be a role for FBG in epileptogenesis as soluble FBG also triggers the same *smad* pathways [15]. In the current study, some evidence of astrocytes uptake of labeled albumin was seen when the cells were visualized by albumin fluorescence (Fig. 4C–D), but any uptake of adsorbed albumin from substrates was much smaller than the extent of FBG removal. This may potentially be due to cells being cultured in the presence of 10% serum and thus having an abundance of soluble albumin to uptake. Similarly, the differences in albumin conformation when adsorbed to the surface may have changed its bioavailability. However, in all samples astrocytes maintained FBG recognition and removal in the presence of serum.

The experiments showed that the responses of astrocytes to the FBG patterns were local: the cells that recognized and removed adsorbed FBG preferentially expressed CSPG on the locations of the FBG stimuli (Figs. 5,6). However, there was no apparent dose-dependence of CSPG expression on adsorbed FBG coverage (Fig. 7). On these samples, laminin substrates had similar levels of CSPG production to the substrates with adsorbed FBG even though there was no evidence of laminin uptake by astrocytes. This was possibly due to CSPG binding to surface laminin which is known to occur and has been well characterized *in vitro* [25,26]. The effect of such binding is likely minor in the present studies as the CSPG expression on LN substrates were not significantly greater than expression on FBS-coated glass substrates (data not shown). The binding of CSPG to LN could explain the presence of CSPG deposited in between the FBG patterns, but one would then expect that the bound CSPG would remain in such patterns even after FBG removal as the LN remains unchanged. On the contrary, CSPG expression by cells becomes more uniform after the underlying patterns are removed (Fig. 6). This suggests a specific deposition of CSPG in response to FBG that diminishes as the stimulus wanes. Another potential contributor to the similar levels of CSPG expression across surface types could be due to the presence of 10% serum in cell culture medium. The effects of growth factors, albumin, and other blood proteins may have affected astrocyte phenotypes to the point where the influence of substrate proteins was less effective. In the CNS wound environment, however, the same serum proteins would also be present. Additionally, the effect of using SB 431542 to inhibit TGF- β could have been diminished if those same TGF- β receptors were inundated with albumin. This inhibitor treatment, however, did yield significant differences in CSPG expression for some samples (Fig. 7).

CSPG expression is expected to be dynamic process in the sense that the acute CSPG response to local FBG might have been attenuated over time. Note that in the present study CSPG production was not observed in real time, but only after 48 hours of culture and subsequent cell fixing and staining. In an *in vivo* study of CSPG production in a stab-wound induced glial scar in the spinal cord, it was shown that 130 kD neurocan production actually decreased in the first 48 hours post injury in the spinal cord, although this behavior was different for different types of CSPG macromolecules [27]. The peak level of RNA expression for neurocan in astrocytes *in vitro* occurred at 12 hours after soluble fibrinogen treatment and this expression then diminished to baseline levels by 24 hours though neurocan secretion into media persisted up to 7 days [15].

Astrocytes migrated while removing FBG and were even seen detaching after modifying the surface. Because of cell detachment, there likely was even higher CSPG production than what was captured after sample fixation and antibody staining. The radioactive sulfur measurements indicated that the ~4 times as much GAGs are produced by astrocytes and released in the medium, though no differences in secreted and membrane bound CSPG were found for astrocytes exposed to TCPS-adsorbed FBG vs. FBS-only proteins controls. It is

apparent, however, that there was local, membrane bound CSPG expression in the presence of FBG. Confocal imaging indicated that CSPG vertical position was coincident with the position of surface FBG (Fig. 6). This local expression on the μm -scale suggests that the astrocytic response to FBG was not a sustained, global upregulation of CSPG but rather a defined, controlled increase on membrane regions exposed to adsorbed FBG

Significant decrease in CSPG production by blocking TGF- β receptors with SB 431542 in astrocyte cultures was only found for the substrates with predominant laminin coverage (FBG0/LN100 and FBG30/LN70). Decreases in CSPG production were found on the other substrate types but were not statistically significant (Fig. 7). It has been shown that astrocytes alone produce TGF- β [28] available for intercellular signaling which would be affected by adding of the inhibitor. This autocrine TGF- β functionality of astrocytes may potentially be more causal in determining overall CSPG production than the surface adsorbed FBG or externally added TGF- β , as these particular experiments yielded no significant changes in CSPG production. Although TGF- β contributed to CSPG production, blocking of the receptor did not abolish *all* CSPG production. Reducing the amount of expressed CSPG is an active area of research as potential CNS injury treatment. One approach is the digestion of CSPGs by chondroitinase, which leads to improved neuronal regeneration and function [29,30]. Similarly, SB 431542 might be considered for local treatment of CNS injuries and implants to reduce CSPG expression and astrogliosis, but the myriad roles of TGF- β signaling in the CNS wound environment must first be further understood [31]. The results presented here indicate that TGF- β signaling pathway was not entirely responsible for astrocyte activation and CSPG expression *in vitro* on substrates with predominant fibrinogen coverage as the cells continued to produce CSPG even with SB 431542 treatment. In fact, a large fraction ($> 75\%$) of the astrocytic CSPG expression remained after inhibitor treatment on all types of substrates. In the case of neural implants, one can infer that astrocytes will respond to the foreign stimulus and continue to present inhibitory CSPGs even without TGF- β signaling. The impact that fibrinogen has on long term CNS implants has yet to be determined. It has been shown that BBB leakage continues 12 weeks after implantation as demonstrated by continued presence of IgG in brain tissue [1]. While FBG is approximately twice as large as IgG, it is likely that, if the BBB is compromised, various proteins could enter and potentially adsorb to the implant surface. It is important to note, however, that after long-term implantation, astrocytes are found at a distance of approx. 50 μm from the implant/tissue interface that is typically populated with microglia and macrophages [2]. Thus it is unclear whether astrocytes would be able to maintain long-term contact with implant surfaces. Regardless, based on the evidence presented here, astrocytes mount an initial removal of FBG from and deposition of CSPG on implant surfaces; the events that may trigger the neuronal inhibition caused by CSPG.

5. Conclusions

It was found that surface-adsorbed FBG is specifically recognized and removed by astrocytes. Thus, in addition to its roles in coagulation and carrying latent TGF- β in circulation, adsorbed fibrinogen also initiates cellular responses by CNS astrocytes. Astrocytes removed adsorbed FBG over the course of several hours, and such behavior was maintained regardless of the presence of a TGF- β signaling pathway inhibitor. This indicated that astrocytes maintain sensitivity to adsorbed FBG by some, yet unknown, TGF- β receptor-independent mechanism. While the CSPG expression was not dependent on the amount of FBG presented on the substrates, there was evidence that astrocytes preferentially produce CSPG at FBG contacting parts of their cell membranes. Additionally, CSPG production was also maintained in the presence of the inhibitor. Based on the fact that blood contact and subsequent FBG adsorption is unavoidable in neural implantations, the present results indicate that implant-adsorbed FBG will trigger astrocyte removal of FBG. This

alone may lead to local CSPG expression at implant-CNS interface and thus contribute to subsequent inhibition of neuronal activity around the implant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Three substrates created by microcontact printing or adsorption of FBG. (A) 30% fibrinogen coverage with laminin (unlabeled) backfill (FBG30/LN70) and (B) 50% fibrinogen coverage with laminin backfill (FBG50/LN50) surfaces. (C) 100% coverage created by fibrinogen adsorption (FBG100/LN0). Scale bar indicates 50 μm for A–C. (D) Histograms of the fluorescence intensity from fluorescently labeled FBG images showing differences in FBG deposition on different substrates.

Figure 2.

Astrocyte modification of FBG and LN coated substrates. (A–C) FBG removal (DIC + fluorescence) by astrocytes at 4 hr (A), 16 hr (B), and 23 hr (C) post-seeding. (D) Laminin 100% substrate (FBG0/LN100) remains largely unchanged after 48 hours of astrocyte culture. (E,F) FBG layers modified by astrocytes after 48 hours of culture on FBG100/LN0 (E) and FBG50/LN50 (F) substrates. Scale bars (in C for A–C and in F for D–F): 50 μm

Figure 3.

Two phase FBG removal process. (A) Initial (4 hr post seeding) FBG100/LN0 substrate with cell outline. (B) Same region of FBG100/LN0 surface at 24 hours post seeding with updated cell outline. Five regions (5 by 5 pixels) used in analysis of removal kinetics are indicated. (C) Initial FBG50/LN50 substrate (LN unlabeled) with outlined cell in early phases of spreading. (D) Same region FBG50/LN50 surface after 14.5 hr of culture showing cell outline and removal of underlying FBG layer. Scale bar: 25 μm . (E) Plot of normalized fluorescence intensity as a function of time from five indicated cell locations in (B).

Figure 4. Confocal images of fixed and stained samples showing astrocyte removal of FBG taken 48 hours after seeding. (A) Majority of cells removed FBG (red) without any visible intracellular uptake. (B) Smaller fraction of cells showed increased FBG (red) fluorescence in otherwise dark cell footprint regions. (C–D) On a binary protein surface pattern (FBG + albumin) astrocytes did not alter albumin (green) to the degree of removing FBG (red) over 48 hours. Cell nuclei were DAPI stained (blue). Yellow square in D indicates location of C. Scale bars: 25 μm for A–C, 50 μm for D.

Figure 5. Fluorescence images of fixed and stained samples showing the removal of FBG (red) and expression of CSPG (green) on FBG100/LN0 (A), FBG50/LN50 (B), FBG30/LN70 (C) and on FBG0/LN100 (D) substrates. Cell nuclei were DAPI stained (blue) and laminin was unstained. Scale bar: 50 μ m.

Figure 6.

(A) Confocal images stack of a representative fixed and stained sample showing CSPG (green) production on the FBG50/LN50 substrate (FBG in red, nuclei in blue). Yellow lines indicate the planes for z-projections shown beside and below in A. CSPG deposition was high in between the FBG patches prior to FBG removal (arrows). Scale bar: 25 μm . (B–C) Normalized vertical (z) intensity profiles show the peak CSPG intensity coinciding with peak FBG intensity at both the nucleus position (marked *1 in A, plot B), the periphery of cell (marked *2 in A, plot C), and in the region where the cell was no longer present (marked *3 in A, plot D).

Figure 7.

The effect of TGF- β receptor kinase inhibitor SB 431542 on CSPG expression per cell on substrates with different FBG/LN coverage. Solid horizontal lines indicate the mean, and filled or empty points denote absence or presence of inhibitor, respectively. Asterisks denote $p < 0.005$, $n = 90$.

Figure 8.

The effect of solution added TGF- β in DMEM/F12/10% FBS media + DMSO on CSPG production. CSPG staining per cell data were normalized to FBG0/LN100 sample. Error bars indicate $\pm$ SEM. Asterisk denotes $p < 0.05$, $n = 10$.