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Research article

Extracellular matrix composition determines astrocyte responses to mechanical and inflammatory stimuli

Kasey M. Johnson[a], Richard Milner[b], Stephen J. Crocker[a,*]

[a] Department of Neuroscience,University of Connecticut School of Medicine, Farmington, CT, USA
[b] Department of Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA

HIGHLIGHTS

- Astrocyte wound recovery to mechanical injury varied on different ECM substrates.
- Astrocyte wound responses were differentially affected by IL-1β.
- β1 integrin mediated ECM dependent astrocyte wound responses.
- Rapamycin blocked wound responses of astrocytes grown on TnC.
- ECM changes in disease may impact astrocytic function.

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ABSTRACT

Astrocytes perform critical homeostatic physiological functions in the central nervous system (CNS) and are robustly responsive to injury, inflammation, or infection. We hypothesized that the components of the extracellular matrix (ECM), which are known to vary during development and in response to disease, determine astrocytic responses to injury and inflammation. We examined the response of primary astrocyte cultures grown on different ECM proteins to a mechanical wound (i.e., scratch). ECM substrates selected were laminin (Ln), vitronectin (Vn), fibronectin (Fn) or Tenascin C (TnC). We found that regrowth of the scratch wound was ECM dependent: recovery was arrested on fibronectin (Fn), almost complete on either Vn, Ln, or TnC. To determine whether ECM responses were also influenced by inflammation, we treated ECM plated cultures with interleukin-1β (IL-1β). We found that IL-1β arrested astrocyte growth on Ln, accelerated astrocyte growth on Fn and had no significant effect on astrocyte growth on TnC or Vn. We also determined that blocking β1integrins, the major class of receptors for all ECM proteins tested, prevented the robust response of astrocytes exposed to TnC, Ln and Vn, and also inhibited the robust effect of IL-1β to stimulate astrocyte growth on Fn. In addition, we evaluated downstream targets of integrin signaling, specifically the mammalian target of rapamycin (mTOR), and determined that activation of this pathway contributed to the response of astrocytes grown on TnC, but not on Ln, Vn or Fn. These findings provide new insights into the role of ECM as a source of heterogeneity of glial responses that may have important implications for neuropathological sequelae.

1. Introduction

The extracellular microenvironment provides fundamental cues that guide cellular responses. The protein composition of the extracellular matrix (ECM) exhibits regional variation and dynamic changes related to age, injury or disease [1]. These changes in the ECM play an important role in molding central nervous system (CNS) development and influence cellular responses within the adult CNS [2]. For instance, tenascin C (TnC) is a secreted ECM glycoprotein that is expressed prominently during development and down-regulated in the mature CNS. However, TnC levels are up-regulated during neuroinflammation, specifically in response to pro-inflammatory cytokines [3]. Laminins (Ln) and fibronectins (Fn) are highly expressed during regeneration and have been shown to promote regeneration [4]. Specific changes in ECM protein expression, such as the accumulation of Fn or TnC in demyelinated plaques in multiple sclerosis, have also been shown to impede

Abbreviations: ECM, extracellular matrix; Ln, laminin; Vn, vitronectin; Fn, fibronectin; TnC, tenascin C; IL-1β, interleukin-1β; mTOR, mamalliam target of rapamycin.

* Corresponding author.
E-mail address: crocker@uchc.edu (S.J. Crocker).

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remyelination in this disease [5,6]. Similarly, up-regulation of TnC in plaques of Alzheimer's mouse brain has lead to a suggested role of TnC as an integral part of the inflammatory pathology in this disease [7]. Studies on how ECM influences the function of cells within the CNS have revealed a prominent role for ECM regulation of astrocytic functions [8,9].

Astrocytes are the most abundant cell type in the CNS and provide homeostatic and sentinel functions when they become activated in response to CNS injury, inflammation or disease [9]. Astrocytes have a spectrum of resting and activation states that are influenced by the extracellular environment [9]. This extracellular influence on the activation of astrocytes likely underlies whether astrocytes contribute to CNS pathology or promote CNS recovery [10]. ECM molecules activate intracellular signaling cascades through interactions with transmembrane protein receptors called integrins. Integrins are members of a family of glycoproteins that regulate many cellular behaviors, including proliferation and differentiation [11]. Integrin α and β subunits form heterodimers that endows a range receptor diversity and specificity for ECM proteins. Study on how different ECM molecules influence the behavior of astrocytes is expected to enable a better understanding on the role(s) of astrocytes in pathological settings.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase and mTOR signaling has been implicated in regulation of cell growth, proliferation, motility, survival, as well as promoting protein synthesis, and transcriptional activation [12–14]. mTOR has two distinct complexes, mTORC1 and mTORC2, that regulate distinct signal transduction pathways. In the CNS, mTOR has been shown to enhance protein synthesis and promote neural cell differentiation, therefore, promoting an importance of mTOR signaling in the health CNS. Rapamycin is an inhibitor of mTOR activation, specifically mTORC1, and has been shown to attenuate glial cell activation [15]. Integrin signaling has been reported to engage a variety of downstream targets in the mTOR pathway. Given that astrocytes can exhibit disparate reactions to specific ECM proteins [16], we reasoned that activation of mTOR signaling may be one mechanism underlying these differences.

In this study, we hypothesized that the ECM substrate would impact the response of astrocytes to a mechanical injury and an inflammatory stimulus. For these experiments we used the canonical pro-inflammatory cytokine, interleukin-1β (IL-1β) because it is known to activate astrocytes and is prominently produced in a variety of neural injury settings [17]. We report that astrocytic behavior was dramatically influenced by the ECM protein substrate and that this determined the activation and contribution of mTOR signaling to these astrocytic responses. Together, these finding provide new information on the regulation of astrocytes that may serve to define the extracellular cues that contribute to astrocytic heterogeneity in responses to CNS injury and inflammation.

2. Material and methods

2.1. Primary glial cultures

Cultures were generated from cerebral cortices of neonatal C57BL/6 mouse pups (P0-P3), using a neural tissue dissociation kit (Miltenyi Biotec) [18]. Cells were plated into T175 flasks for up to 2 weeks before detachment using trypsin (Sigma) and then replated onto coverglass coated with Ln (10 μg/μL; Sigma–Aldrich), TnC, (100 μg/μL; EMD Millipore), Fn (10 μg/μL; Sigma–Aldrich), or Vn (25 μg/μL; Sigma–Aldrich) in 24-well plates. The purity of each culture system was consistent with previous reports of 90–97% GFAP+cells [19], as verified by immunocytochemistry (ICC) for GFAP for astrocytes (1:1000, Chemicon), and, Iba-1 for microglia (1:1000, WAKO).

2.2. Scratch Injury model

Confluent astrocyte monolayers were scratched once across the diameter of the coverglass using a sterile P200 pipette tip to produce an injury ~600 μm in diameter [20]. Cells were fixed at different timepoints and ICC performed. IL-1β (10 ng/ml; Peprotech) was added to the cultures as a prototypic inflammatory stimulus, as previously described [21]. The role of β1integrin was tested using a function blocking antisera (Ha2/5; 5 μg/mL) and compared with control, isotype-matched, IgG (both from BD Pharmigen). Effects of mTOR were assessed using rapamycin (5 μg; Santa Cruz Biotech) dissolved in DMSO (10 nM, Sigma). Scratch injuries were measured perpendicular to the longitudinal axis of the scratch at a minimum of three points spanning from the edges of the scratch using image analysis software (Northern Eclipse software; Empix Imaging). Western blotting was performed on cultures grown in 6 well plates coated with Ln, TnC, Fn, or Vn, and scratched 4 times per well using a sterile P200 pipette tip as described above.

2.3. Immunocytochemistry (ICC)

ICC was performed as previously described [19]. Cultures were fixed in 4% paraformaldehyde, washed and then incubated with primary fluorescent conjugated antisera for Glial Fibrillary Acidic Protein (GFAP-Cy3; 1:1000, Sigma). 4′,6-diamidino-2-phenylindole (DAPI) was added after incubation to identify nuclei. Immunoreactivity was visualized using a fluorescent microscope (Olympus, IX71) and image analysis software (Empix Imaging).

2.4. Western blot analysis

Lysates from primary cultures grown on Ln, TnC, Fn, or Vn were prepared as previously described [19]. Cells were scraped into PBS, after 0, 4, or 24 h after treatment, centrifuged (6000 × *g*), the pellet was resuspended in RIPA buffer [Triton X-100, NaCl, Tris–HCl base, Deoxycholic Acid, SDS, H_2O] and mechanically dissociated using a motorized pellet pestle (Fisher Scientific). Each sample was prepared with 2× loading buffer [Tris–HCl, Glycerol, SDS, H_2O, BPB, DTT] and 15 μg of total protein was loaded into a graduated 5–15% acrylamide gel (Biorad). After PAGE, protein was transferred onto PVDF membrane (Thermo Scientific). Blots were blocked and then incubated overnight in primary antisera. Primary antisera included pAktS473 (1:1000, Cell Signaling), panAkt (1:1000, Cell Signaling) S6KpThr389 (1:1000, Cell Signaling), p70S6Kinase (1:1000, Cell Signaling), and β-actin (1:2000, Sigma). Secondary antisera were HRP-conjugated anti-mouse (1:10,000, Vector Labs), or HRP-conjugated anti-rabbit (1:10,000, Vector Labs) for detection using ECL reagent (GE Biosciences). Exposed films were developed and quantified relative to β-actin loading for each sample [19].

2.5. Statistics

Experiments were performed in quadruplicate replicates and repeated in triplicate. Comparisons between treatments on the same substrate were made using *t*-tests, or one-way ANOVA to evaluate changes over time. Inter-group comparisons for differences on each ECM over time, or treatment were made two-way ANOVA. Data are presented as mean ± SEM. The null hypothesis for all experiments was $P < 0.05$.

3. Results

3.1. Astrocyte response to scratch is ECM dependent

We hypothesized that the composition of the ECM may influence the behavior of astrocytes and their ability to recover from