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Transient acidification and subsequent proinflammatory cytokine stimulation of astrocytes induce distinct activation phenotypes

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Abstract

The foot processes of astrocytes cover over 60% of the surface of brain microvascular endothelial cells, regulating tight junction integrity. Retraction of astrocyte foot processes has been postulated to be a key mechanism in pathology. Therefore, movement of an astrocyte in response to a proinflammatory cytokine or even limited retraction of processes would result in leaky junctions between endothelial cells. Astrocytes lie at the gateway to the CNS and are instrumental in controlling leukocyte entry. Cultured astrocytes typically have a polygonal morphology until stimulated. We hypothesized that cultured astrocytes which were induced to stellate would have an activated phenotype compared with polygonal cells. We investigated the activation of astrocytes derived from adult macaques to the cytokine TNF- α under resting and stellated conditions by four parameters: morphology, intermediate filament expression, adhesion, and cytokine secretion. Astrocytes were stellated following transient acidification; resulting in increased expression of GFAP and vimentin. Stellation was accompanied by decreased adhesion that could be recovered with proinflammatory cytokine treatment. Surprisingly, there was decreased secretion of proinflammatory cytokines by stellated astrocytes compared with polygonal cells. These results suggest that astrocytes are capable of multiple phenotypes depending on the stimulus and the order stimuli are applied.

Keywords

Morphology; Neuroscience; inflammation

INTRODUCTION

Astrocytes form an essential signaling link between the blood-brain barrier (BBB) and neurons (Gourine et al., 2010). They are commonly thought of as playing a supportive role to neurons, primarily by maintaining physiological conditions in the brain through a variety of functions: secretion of trophic factors, reuptake of neurotransmitters released at neuronal

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synapses, maintaining appropriate ionic levels for neuronal function, removing waste products from the extracellular space and interacting with endothelial cells to maintain the BBB. Astrocyte end-feet processes not only sense what is happening in the BBB, but also transmit signals from the parenchyma to the BBB.

Astrocytes can be activated to serve a pivotal role in neuroinflammation. Gliosis-related changes in astrocyte physiology occur in a context-specific manner (Sofroniew and Vinters, 2010) through loss or gain of function. These changes, in addition to detrimental effects, can also have beneficial impacts on neuronal (Ridet et al., 1997) and non-neuronal cells (Al-Ahmad et al., 2011), including astrocytes (Lau et al., 2012; Leung et al., 2010). Reactive astrocytes can protect CNS tissues through a variety of mechanisms including: glutamate uptake, facilitating BBB repair, reducing vasogenic edema, limiting the spread of inflammatory cells or infectious agents (Fitch and Silver, 2008; Sofroniew and Vinters, 2010). It is, therefore, important to distinguish beneficial from detrimental functions.

In vivo, primate astrocytes are known for their stellate morphology, with high expression of glial fibrillary acidic protein (GFAP) (Oberheim et al., 2009). In contrast, *in vitro* cultures of astrocytes typically exist as polygonal cells and express only low levels of GFAP (Sasaki and Endo, 2000). Astrocyte stellation has previously been defined as “cells in which there was clear evidence of full retraction of the cell body together with emission of processes, independent of length of the process” (Gottfried et al., 2003). Transient acidification of the culture media induces astrocytes to alter their phenotype, taking on a stellate morphology with robust GFAP expression (Cechin et al., 2002; Gottfried et al., 2003). Stellation of astrocytes can be induced with extracellular matrix proteins (Al-Ahmad et al., 2011) or blocking actin signaling intermediaries (Lau et al., 2012) which modulate astrocyte physiology, including many changes in the actin cytoskeleton (Lau et al., 2012), and extension of processes (Olk et al., 2010). How astrocytes react to sequential proinflammatory stimuli in these disparate phenotypes has yet to be examined in detail.

Disruption of intermediate filaments, including GFAP, alters vesicle motility in astrocytes (Potokar et al., 2007). Therefore intermediate filament changes could logically be linked with altered cytokine secretion in addition to morphological changes. This hypothesis is supported by several lines of evidence: astrocytes show altered morphology concomitant with inflammation (Xing et al., 2008), associated with altered expression of intermediate filaments, including nestin (Pekny et al., 1998; Strong et al., 2004) and vimentin (Al-Ahmad et al., 2011). Astrogliosis has been associated with bacterial (Krum et al., 2008; Ramesh et al., 2003; Samartino et al., 2010) and viral infection of brain, including HIV (Bethel-Brown et al., 2011; Cook-Easterwood et al., 2007), potentially in response to proinflammatory cytokines including TNF- α (Orandle et al., 2002).

Astrocytes express altered levels of integrins on activation *in vivo* (Li et al., 2010; Rubio et al., 2010) and *in vitro* (MacLean et al., 2004a; MacLean et al., 2004b). It is, therefore, important to monitor changes in astrocyte adhesion when astrocytes switch from low GFAP expression to more robust GFAP expression. Measuring cytokines, adhesion, shape change and cytoskeletal protein content are all relevant for modeling astrogliosis *in vitro*.

We found that transient acidification induced irreversible changes in the morphology of astrocytes with increased GFAP expression. Adhesion was reduced as measured by electrical impedance and decreased expression of integrins. Cytokine secretion was also lowered in stellated astrocytes. On subsequent activation with TNF- α , there was an increase in the astrocyte arbor, an identical increase in adhesion with a very similar increase in cytokine secretion. This stepwise activation models neuroinflammation; for example in stroke, where a transient acidosis precedes an increase in inflammatory cytokines.

MATERIALS & METHODS

Cell culture

All monkeys were housed at the Tulane National Primate Research Center in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Research Council, National Academies Press, Washington, DC. The Tulane Institutional Animal Care and Use Committee approved all studies. Brains were obtained from normal rhesus macaques (*Macaca mulatta*) at necropsy and astrocytes cultured by standard techniques (Guillemin et al., 1997; MacLean et al., 2002; MacLean et al., 2004a; MacLean et al., 2004b). Animals used included both sexes, with an age range from 1–5 years of age.

In brief, meninges were removed and frontal cortices were diced using a pair of scalpels. The tissue was digested using 0.25% trypsin (Invitrogen, Carlsbad, CA) and DNAase (4 U/ml, Worthington, Lakewood, NJ) at 37°C for 60 minutes before trituration and filtration through 110 µm pore filters (Sigma). The cell rich slurry was centrifuged three times at 1,000rpm, washed and plated in M199 supplemented with 0.7mM sodium bicarbonate and 5% fetal calf serum.

When cells reached 80% confluence, contaminating microglia were removed by vigorous shaking for 10 minutes at room temperature (Renner et al., 2011a). Purified astrocyte cultures were allowed to recover for 48 hours before use. These cultures were uniformly free from neurons as determined by MAP-2 staining (Supplemental Figure 1).

Experimental design

Purified astrocyte cultures were incubated with either control media or media where the supplemental sodium bicarbonate was replaced with 8 mM HEPES (final concentration) as described in Figure 1. On incubation in 5% CO₂, this lowered the pH of the media from 7.6 to 7.1 within an hour. There was no such decrease with bicarbonate-buffered media (not shown). Astrocytes were monitored for the next 48 hours before addition of bicarbonate-buffered media containing 100 U/ml TNF-α (R&D Systems, Minneapolis, MN). Cells were fixed or proteins and mRNA extracted 48 hours following TNF-α stimulation (Figure 1).

Imaging of astrocytes

Astrocytes were fixed and imaged using either phase contrast objectives or fluorescence microscopy. For fluorescence imaging, cells were permeabilized using 0.1% Triton X-100 in PBS and stained using antibodies at the concentrations specified in Table 1.

Statistical analyses were performed to determine the proportion of GFAP expressing cells in each treatment. For these studies, low power images (10X objective) were collected from five non-overlapping fields per treatment, all cells in each field were counted, and determined to be expressing, or not expressing, GFAP. Data were analyzed by Mann-Whitney non-parametric test using InStat version 3.0a for Macintosh (GraphPad Software, San Diego California USA). Reported results are typical from five independent astrocyte cultures.

Quantitative analyses of astrocyte morphology

Astrocytes were identified using light microscopy at 20–40x magnification for process morphology and analyzed using Neurolucida (MBF Biosciences, Williston, VT), using similar methods to those described previously (Prithviraj et al., 2008). Among the analyses, we measured size of cell body, total arbor length and field size.

Fluorescently stained and phase contrast images of astrocytes were captured following fixation using 20–40x objective lenses. The following parameters for each astrocyte were analyzed: soma size, modified Sholl analysis and total arbor length. Statistical variance among treatment groups was analyzed using Kruskal-Wallis test, with pairwise comparisons between treatment groups performed using Dunn's post test. Forty cells were counted for each condition, from 3 independent experiments.

Automated measure of cell adhesion

Purified astrocytes were plated on xCELLigence E-plates at 20,000 cells per well. 24 hours after plating, wells were treated with either control media or media where HEPES replaced the sodium bicarbonate for 48 hours (as described in Figure 1). At this time the cells were treated with either control media or media containing TNF- α . Traces were plotted automatically using the installed software. To determine the degree of change in impedance compared with control media, the traces are normalized such that the negative control has a value of zero, again using the installed software. Data were analyzed by Friedman Test (non-parametric repeated measures ANOVA) with Dunn's post test using InStat version 3.0a for Macintosh (GraphPad Software, San Diego California USA) as we have recently described (Sansing et al., 2012).

This system is unique in that the wells are in a standard 96 well format and a high percentage of the surface is electrode (80%). Therefore, the system is capable of unbiased, automated measurement of essentially all cells that come into contact with the base of the well. As these studies are performed in real time, each sample has its own internal control (measured at time zero).

Secretion of proinflammatory cytokines

To determine the effect of HEPES pretreatment on subsequent stimulation with TNF- α , supernatants were harvested at intervals over 48 hours. Aliquots were frozen at -80°C until use. Cytokines were measured using a 15-plex non-human primate-specific cytokine bead array kit from Millipore (Billerica, Massachusetts), per manufacturer's instructions. Results from two independent runs were used.

RNA extraction and DNA microarrays

Purified astrocytes were grown in T75 flasks (Corning, Lowell, MA) and treated for 48 hours with either control media or media where the bicarbonate was replaced with HEPES. Following this pretreatment, media was replaced with bicarbonate-buffered media containing TNF- α (100U/ml) for 48 hours or vehicle. Media contained 5% FBS throughout the course of the study.

Total RNA was extracted from astrocytes using the RNeasy lipid tissue mini kit, following the manufacturer's instructions (Qiagen Inc., Valencia, CA). Possible DNA contamination was removed by subjecting the RNA to DNase treatment (DNA-free kit; Ambion, Austin, TX). Approximately 1 to 2 μg of each RNA sample was electrophoresed through a 1% agarose-0.1% sarkosyl gel to verify the quality of the preparations.

Microarray analysis was performed as previously published (Dutta et al., 2012; Mehra et al., 2010). Briefly, cDNA derived from total RNA was labeled with Cy3 (control) and Cy5 (experimental) fluorescent dyes using the Agilent Low RNA Input Linear Amplification Kit (LRILAK) (Agilent Technologies). Equimolar quantities of Cy-labeled cDNA samples were mixed together and hybridized to rhesus macaque microarrays in a 4x44k format (Agilent Technologies). Arrays were hybridized overnight in a SciGene rotating oven at 65°C , washed according to manufacturer's protocols and scanned using GenePix Pro. Raw data

was subjected to Locally Weighted Scatterplot Smoothing (LOWESS) normalization using Spotfire DecisionSite and fold changes calculated as described (Dutta et al., 2012; Mehra et al., 2010). In order to identify gene-ontologies significantly overrepresented in our data, we employed the functional analysis tool of DAVID (Database for Annotation, Visualization and Integrated Discovery v6.7 (<http://david.abcc.ncifcrf.gov/>)).

RESULTS

HEPES INDUCED STELLATION

For clarity, the results are divided into 2 sections. First, we will describe changes induced through transient acidification. This will be followed by further changes induced in stellated astrocytes by the proinflammatory cytokine TNF- α .

Induction of astrocyte stellation by transient acidification

When examined under phase contrast objectives, control astrocytes have a polygonal morphology (Figure 2A). Transient acidification induced irreversible stellation (Figure 2B). Images were imported into Neurolucida (MBF Biosciences, Williston, VT), and traced. Measurements of cell field size, total arbor length, and cell body size were collated, analyzed and plotted (Figure 2E). Collectively, these measurements tell how cell shape change is occurring. Stellation of astrocytes induced a decrease in soma size. The mean soma size dropped from 275.22 μm ($\pm 127 \mu\text{m}$) for control astrocytes to 154.41 μm ($\pm 77 \mu\text{m}$) for HEPES treated ($p < 0.001$ compared with control cells).

The total length of the arbor was not significantly increased above control values in the transiently acidified group (199.9 μm $\pm 175 \mu\text{m}$ to 246.9 μm $\pm 112 \mu\text{m}$, $p > 0.05$). As measured by modified Sholl analysis, there was essentially no change to the field size of the cells (109 μm to 111 μm) after transient acidification. This suggests that there is retraction of cytoskeleton and cytoplasm rather than extension of processes in response to transient acidification.

Altered cytoskeletal filament expression is coincident with stellation

To determine if stellation was associated with changes in cytoskeletal proteins, we stained astrocytes with phalloidin and antibodies to GFAP and other intermediate filament proteins (see Table 1 for antibody concentrations). Polygonal astrocytes have low expression of GFAP (Figure 2F, red). Following transient acidification (H), there was a significant increase in the percentage of GFAP expressing cells from 20.72% ($\pm 5\%$) GFAP $^{+}$ cells in control cultures, to 30.22% ($\pm 10\%$) GFAP $^{+}$ in acidified cultures (Mann-Whitney test, $p = 0.045$). For each treatment, $n > 110$ cells.

In control astrocytes, there was low, predominately perinuclear expression of vimentin (G). Vimentin expression was increased on stellation (I), mainly in processes. F-actin was disseminated across polygonal astrocytes (F,G). Following transient acidification, the F-actin was slightly condensed with F-actin predominately in the cell body, with occasional filaments extending into processes (H, I).

Transient acidification-induced stellation is concomitant with changes in cytoskeletal genes

To examine which other cytoskeletal-associated genes were differentially regulated following transient acidification, analyses of gene array data were performed as described in Materials and Methods. Several actin and actin binding genes were down regulated (Table 2). This was coupled with increased expression of tubulins (gamma (+2.11 fold), alpha 2 (+3.11 fold) and beta 4 (+2.66)), and decreased dynein (−8.57 fold) expression.

Real time analyses of astrocyte adhesion

To examine how changes in morphology were translated into altered adhesion by astrocytes, we used the xCELLigence system (Roche) to monitor alterations in adhesion following transient acidification in real time (Figure 3A). Similar methods have been used to measure altered morphology and adhesion in non-monolayer forming cells (Ramachandran et al., 2011), including astrocytes (Moodley et al., 2011) and astrocyte cell lines (Meshki et al., 2011). Stellated astrocytes (blue and purple traces in Figure 3A) have dramatically reduced adhesion compared with controls (red and green traces in Figure 3A, and Figure 3B). The decreased adhesion was apparent within one hour, peaked five hours after transient acidification and remained lower than control cells for at least 20 hours. The rate of decrease over the first 2 hours was measured using the installed software (B), indicating a decrease cell index of 0.8hr^{-1} relative to the untreated controls. The traces are representative of three repeat experiments.

Transient acidification-induced stellation is concomitant with changes in adhesion-related genes

Highlights for changes in adhesion-related genes following stellation induced by transient acidification are presented in Table 3. We noted down regulation of five integrins including: alpha 11 (−2.31-fold), 8 (−3.58-fold) & 10 (−2.49-fold) and beta 1 (−5.05-fold)). Extracellular matrix proteins were also downregulated including: fibronectin (−2.56-fold), Versican (−6.8-fold), aggrecan (−7.29-fold), 17 collagen and collagen-related genes, including the basement membrane-specific Type IV collagen (−2.33 fold), and ADAMTS1 (2.1-fold). This was concomitant with up regulation of MMP3 (stromelysin, +23.86-fold). Combined, these would lead to decreased adhesion of astrocytes to the basement membrane, consistent with real time adhesion data (Figure 3).

In addition to alterations in adhesion between astrocytes and the extracellular matrix, Connexin 43 was downregulated 2.74-fold. Connexin 43 has been shown to be vital for formation and function of gap junctions between astrocytes. This allows for passage of calcium signals through the “astrocyte syncytium” (Sun et al., 2012). Combined with decreased expression of glutamate receptor 8 (−2.07 fold) and aquaporin 1 (−2.69 fold) this would also impact water balance, an essential function of astrocytes in normal neural function.

Secretion of cytokines and chemokines

In order to measure cytokine secretion from the various treatments, media samples were analyzed by multiplex bead assay. 48 hours after stellation, samples were taken immediately after washing (time zero) and after 20, 60, 120, and 240 minutes. Longer time point samples were taken at 24 and 48 hours. Of the 15 cytokines analyzed, 6 showed differential expression between treatments in the measureable range of each analyte.

CCL2 (Figure 4A) was by far the cytokine with the highest level of secretion, secreting nanogram quantities by 48 hours. Control cultures secreted almost 95 ng/ml by 48 hours in culture (closed circle). Interestingly, transiently acidified astrocytes produced substantially less CCL2 peaking at 61 ng/ml by 48 hours (closed triangle).

GM-CSF is an important cytokine in glial signaling (Guillemin et al., 1997; Renner et al., 2012). Both control and transient acidification treatments remained essentially below the limit of detection at all time points measured (Figure 4B).

VEGF secretion was essentially linear, with a slow, but continuous secretion (Figure 4C). For the first 4 hours, the secretion was identical between treatments. By 24 hours, the

transiently acidified astrocytes secreted less VEGF (1205 pg/ml) than untreated astrocytes (1661 pg/ml). The same pattern was observed at 48 hours, with transient acidification inhibiting the level of secretion by approximately 40% (2481 pg/ml vs 1795 pg/ml, respectively).

IL-6 levels in control (Figure 4E), and transiently acidified astrocytes reached 1468, and 942 pg/ml respectively. Control astrocytes constitutively secreted IL-8, reaching 8420 pg/ml by 48 hours (Figure 4F). Transiently acidified astrocytes secreted lower levels, 7103 pg/ml at the same time point.

Of the analytes tested that had differential expression, IL-10 is the only anti-inflammatory cytokine. Although levels were low throughout the course of the experiment, control cells secreted detectable quantities at 48 hours, while transiently acidified astrocytes secreted barely detectable levels throughout the 48 hours examined. This is intriguing to note because in the context of the six analytes measureable, the HEPES treatment was associated with lower levels of secretion, suggesting that this treatment results in an altered cytokine phenotype, and that antiinflammatory mechanisms are probably not involved. The decreased levels of cytokine secreted could be linked to the altered cytoskeletal structure of the cells, as has been noted by others (Potokar et al., 2010).

Transient acidification-induced stellation is concomitant with changes in cytokine genes

To determine the effects of stellation on cytokine expression, gene array analyses were performed for cytokine pathways. We were intrigued that only eight cytokine genes were differentially regulated in transiently acidified astrocytes (Table 4). Of those genes differentially regulated, three were up regulated: including CCL24 (+3.64 fold) and MCP-3/CCL7 (+2.37 fold). In contrast, interleukin 6 (IL-6) was down regulated 5.86 fold in transiently acidified astrocytes compared with control cells, in agreement with the multiplex data.

EFFECTS OF STELLATION ON DOWNSTREAM ACTIVATION

To study the effects of downstream activation on astrocytes subsequent to stellation, we stimulated stellated astrocytes with TNF- α as described in Figure 1. This proinflammatory cytokine has been observed in viral encephalitides (Orandle et al., 2002), bacterial infection (Phulwani et al., 2008), and stroke (Tuttolomondo et al., 2012). By treating with TNF- α with and without transient acidification we are able to model a broad-spectrum of disease states.

Analyses of astrocyte morphology

Incubation with TNF- α resulted in appreciably altered morphology (Figure 2C), which was even more pronounced in astrocytes that had been stellated previously by transient acidification (Figure 2D). All images were captured 48 hours following TNF- α treatment. The soma size of polygonal astrocytes treated with TNF- α was reduced from 275 μm $\pm$ 127 μm to 211 μm $\pm$ 75 μm (Figure 2E, not significant) and to 162.65 μm $\pm$ 65 μm for transient acidification followed by TNF- α treatment ($p < 0.01$ compared with control cells). As stellation induced by transient acidification induced a significant decrease in soma size, the further effect of TNF- α on the soma of stellated cells was not significant. Likewise, there was essentially no change to the field size of the cells for any of the treatments, with means ranging from 109 μm to 116 μm . However, the total length of the arbor was significantly increased in transiently acidified astrocytes subsequently treated with TNF- α compared to transiently acidified astrocytes ($p < 0.01$), with the length of the arbor increasing from 199.87 μm $\pm$ 175 μm to 290.06 μm $\pm$ 136 μm . Therefore, only cells which had

been transiently acidified and subsequently treated with proinflammatory cytokine had a significantly increased cell arbor.

Cytoskeletal filament expression following TNF- α treatment

TNF- α treatment of non-stellated astrocytes (Figure 2J) did not significantly alter the percentage of cells expressing GFAP, (20.72% (+/-5%) to 22.83% (+/-14%) $p=0.3477$). However, F-actin filaments labeled with phalloidin (green), did appear to coalesce somewhat (Figure 2J-L). Treatment with TNF- α in astrocytes previously stellated by transient acidification (Figure 2L) resulted in a no significant increase in the percentage of cells expressing GFAP, (30.22% (+/-14%) to 31.09% +/- 7% $p=0.4329$). However, it should be noted that the percentage of GFAP expressing cells had already been significantly increased during the stellation induced by transient acidification (Figure 2H). For each treatment, $n>110$ cells.

In astrocytes previously stellated by transient acidification, TNF- α treatment reorganized vimentin from whorls around the periphery of the cells into a pattern more similar to spokes on a wheel (Figure 2K).

It was noted in both TNF- α treatments (Figure 2 J&K), F-actin-labeled filaments, as labeled by phalloidin appeared to be more organized, forming thicker filaments when compared with either the acidified or control cells (Figure 2 F&G).

Molecular analyses of cytoskeletal components on TNF- α stimulation

TNF- α altered fewer cytoskeletal genes than did transient acidification (6 vs 18 in Table 2). This suggests that transient acidification has a stronger effect on structural (cytoskeletal) genes than TNF- α . As with the astrocytes induced to stellate by transient acidification, TNF- α stimulation induced changes in a number of actin genes (Table 2). Curiously, the two Rho gap genes altered by transient acidification were not altered with any of the other combinations of treatments used.

Real time analyses of astrocyte adhesion

Addition of TNF- α (48 hours after transient acidification, Figure 1) induced an increase in adhesion (Figure 3C) consistent with cellular activation and increased cell adhesion protein expression (Li et al., 2010). Transient acidification did not alter the adhesion response to TNF- α (Figure 3D, measured using integrated software, $p<0.01$). The response to TNF- α was of the same magnitude regardless of pretreatment. The rate of increase over the first 2 hours was measured using the installed software (D), indicating an increased cell index of 0.04hr^{-1} relative to the untreated controls.

Molecular analyses of adhesion-related genes altered by TNF- α treatment

As with the cytoskeletal changes noted above (Table 2), transient acidification altered the expression of three times as many adhesion-related genes as TNF- α treatment (Table 3). The major changes noted with TNF- α treatment were: VCAM-1 increased (+18.3 fold in TNF- α and +12.56 fold in transiently acidified astrocytes with subsequent TNF- α treatment). Integrin $\alpha 6$ was decreased 3.4 fold in both treatments, as were fibronectin III (-2.75 fold and -4.97 fold). PTK2 was downregulated 2.76 fold in TNF- α treated astrocytes compared with controls and 2.97 in transient acidification with subsequent TNF- α treatment versus transient acidification treatment. The similar increase in VCAM-1 expression in both TNF- α -stimulated samples agrees with the increased adhesion by xCELLigence (Figure 3C). There was also a similar increase in PKCO (2.65-fold).

TNF- α -induced Secretion of cytokines and chemokines

As with measurement of cytokines secreted by transiently acidified astrocytes, samples were taken immediately after the addition of TNF- α (time zero), and after 20, 60, 120, and 240 minutes. Longer time point samples were taken at 24 and 48 hours post TNF- α exposure. For easy comparison with stellated astrocytes receiving no TNF- α treatment, these traces are superimposed.

CCL2 (Figure 4A) was rapidly upregulated in TNF- α treated samples (open circle). Both TNF- α treatments induced CCL2 secretion exceeding the theoretical limit of detection in our system of 115 ng/ml by 24 hours. Curiously, secretion of CCL2 by astrocytes in both of the transiently acidified samples started off slower than the control or TNF- α samples, taking over 4 hours to “catch up”. As noted earlier, this could be because of the link between intermediate filament rearrangement and vesicle motility (Potokar et al., 2010). Of the analytes examined CCL2 had by far the highest levels of basal secretion, suggesting that CCL2 is a major component of the astrocyte secretome, and that it can be rapidly upregulated following an inflammatory stimulus (McKimmie and Graham, 2010; Muratori et al., 2010).

GM-CSF (B) was upregulated in both TNF- α treatments, by the same magnitude. By 48 hours, both TNF- α treatments induced approximately 10 pg/ml.

By 24 hours, the TNF- α -treated samples were secreting approximately 50% more VEGF than the controls, either with transient acidification (1978 pg/ml vs 1205 pg/ml) or without (2337 pg/ml vs 1661 pg/ml). The same pattern was observed at 48 hours, with transiently acidified astrocytes secreting approximately 40% lower levels of VEGF (2481 pg/ml or 3798 pg/ml vs 1795 pg/ml or 2387 pg/ml, for control or TNF- α incubations, respectively). It is curious that this change in secretion of VEGF occurred at the same time as the TNF- α was either degraded, or absorbed by the astrocyte cultures (D), implying a downstream signaling mechanism (Chiarini et al., 2010).

IL-6 levels (Figure 4E), was secreted in high quantities by both TNF- α treated samples. Transiently acidified astrocytes with subsequent TNF- α treatment showed a more rapid upregulation, with 6909 pg/ml secreted by 24 hours compared to 4145 pg/ml in nonstellated cells, but this was mitigated by 48 hours, when the TNF- α treated astrocytes had essentially caught up (8603 pg/ml vs 8094 pg/ml, respectively).

Both TNF- α treatments induced approximately 670 pg/ml IL-8 at 60 minutes (F) with substantial secretion occurring beyond that, reaching 180,000 pg/ml at 48 hours for the TNF- α and 155,530 pg/ml for the transiently acidified astrocytes with subsequent TNF- α treatment.

TNF- α treated astrocytes showed a small (but significant) increase in IL-10 secretion (Figure 4G).

TNF- α -induced changes in cytokine genes

Stimulation with TNF- α induced increased expression of cytokines in both transiently acidified and conventional cultures (Table 4). Stellated astrocytes further stimulated with TNF- α altered the expression of mRNA for IL-6 (+24.24 fold v +8.55 fold, respectively), CXCL6 (+9.65 fold v +23.17 fold), CXCL12 (−4.61 fold, only in transiently acidified astrocytes with subsequent TNF- α treatment). Therefore, expression of mRNA for select cytokines appears to be differentially regulated in astrocytes following stellation. Other cytokine genes were upregulated to a similar magnitude irregardless of transient acidification pretreatment. Of note, CCL2/MCP-1 (+4.14 fold v 6.43 fold) and IL-8 (3.99

fold v 9.73 fold) were both upregulated. It was also noted that CCL5 was upregulated by over twenty-five fold. It will be of interest to determine if differential regulation of cytokine production and adhesion are intrinsic to priming of astrocytes.

In summary, these experiments add further evidence of a multistep activation cascade for astrocytes in neuroinflammation. Transient acidification and stimulation with cytokines represents two distinct activation pathways. Indeed these two mechanisms may be additive: initial activation through transient acidification would model ischemic conditions. Subsequent activation through cytokines would be more similar to post ischemic inflammation. Acidification induced a stellate morphology with altered cytoskeletal proteins, and changes in extracellular matrix related genes. TNF- α , in contrast, had little affect on morphology and the cytoskeleton; instead upregulating cytokine expression, and increasing adhesion to the culture substrate.

DISCUSSION

Our hypothesis was that stellated astrocytes would have an activated phenotype compared with polygonal astrocytes. We assessed morphology, adhesion and cytokine secretion through function, protein, and gene expression as a way to evaluate whether astrocytes respond to transient acidification by activating an inflammatory profile. Transient acidification of the culture media resulted in stellation of cultured astrocytes. This coincided with increased GFAP and vimentin expression and decreased adhesion combined with decreased integrin and FAK expression. Stimulation with TNF- α following this transient acidification resulted in significantly increased adhesion with increased cytokine expression/secretion. Thus, generally, transient acidification and TNF- α stimulation represent two distinct activation phenotypes: structural changes dominated the transient acidification response; whereas cytokine secretion was the principal response to TNF- α .

While other studies have examined stellation of rodent astrocytes in culture, it was important to determine the effects of stellation in primate astrocytes (Oberheim et al., 2009). In our cells, altered morphology was apparent following transient acidification, with approximately 50% increase in the number of GFAP-positive astrocytes. It should be noted that treatment with TNF- α alone gave a subtle shift in the expression of GFAP expression of approximately 10%. This was enough to make the difference between TNF- α alone and transient acidification with subsequent TNF- α treatment not significant (22.83% to 31.09%, $p=0.539$).

Combining the data in the morphometric studies and gene array analyses, it is apparent that transient acidification of astrocytes induced a combination of both decreased cell body size (Figure 2) and integrin expression (Table 3), resulting in stellated cells (Edwards et al., 1993) and decreased electrical resistance (Figure 3). Subsequent TNF- α treatment recovered some of this electrical resistance, in the absence of increased cell body size (Figure 2), but with increased VCAM-1 expression (Table 3). Increased VCAM-1 expression has been observed on astrocytes in stroke models (Yamagata, 2012), and several proinflammatory conditions including viral encephalitides (MacLean et al., 2004a; MacLean et al., 2004b; Rubio et al., 2010; Song et al., 2011).

Stimulation of astrocytes with TNF- α induced an increase in secretion of numerous cytokines. By comparing differential gene array analyses of astrocytes treated with TNF- α with prestellated astrocytes treated with TNF- α , it was apparent that the only cytokine upregulated was CCL7 which we have previously shown to be important in HIV neuropathogenesis (Renner et al., 2011b). Potentially, astrocytes may provide a role for the resolution of inflammation by reducing the secretion of pro-inflammatory cytokines and

increasing anti-inflammatory processes (Hauwel et al., 2005; Kielian, 2004; Park et al., 2003). In our studies, polygonal astrocytes stimulated with TNF- α expressed higher levels of cytokines, including VEGF (Figure 4) than TNF- α stimulated stellated astrocytes at the time points examined. Thus, the initial activation of astrocytes through transient acidification may “prime” them for a diminished cytokine response rather than an increased one.

Collagen deposition is a hallmark of scar formation *in vivo* (Sofroniew and Vinters, 2010). However, on stellation with HEPES, expression of several collagen and other ECM components was decreased, indicating a potential “good” reactive gliosis that would be permissive to neurite outgrowth (Leung et al., 2010). Recent studies have shown that facilitating a limited number of macrophages into neural tissues is important for repair following injury (London et al., 2011). Combined with increased secretion of MMPs, which would degrade the ECM further facilitating “atypical” gliosis (Al-Ahmad et al., 2011).

However, we also observed a number of genes expressed during these studies that would be thought of as being “disease-associated”. For example, decreased connexin expression (3-fold following transient acidification), combined with decreased aquaporin expression (decreased 2-fold in HEPES-treated versus control-Table 2), could reduce potassium ion syphoning and increased water levels in brain (Lichter-Konecki et al., 2008). By removing select astrocytes from the connexin-linked syncytia, this could facilitate the walling-off of lesion areas observed in bacterial infection of brain (Kielian, 2004; Sofroniew and Vinters, 2010). Previously, the brain-saving attributes of gliosis have been overlooked. In this case, transient acidification could also be analogous to acidosis in stroke.

Combined these studies add to the increasing body of literature demonstrating that astrocytes are not just ‘on’ or ‘off’. They display a range of activation states, and that the previous activation state can affect how the cells respond to further inflammatory insults.

It is becoming increasingly apparent that “reactive” astrocytes covers a spectrum of activation phenotypes, and the term “gliosis” may, perhaps, be inadequate to describe astrocytes in neuroinflammatory lesions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Timeline describing transient acidification and subsequent stimulation

48 hours after plating, astrocytes were transiently acidified with media where the bicarbonate buffer was replaced with 8mM HEPES. 48 hours after pretreatment, astrocytes were returned to sodium bicarbonate buffered media containing TNF- α or vehicle. The experiment then continued for 48 hours.

Figure 2. Morphometric and structural changes induced by transient acidification

48 hours after addition of TNF- α or vehicle, astrocytes cultures were fixed. Phase contrast imaging of astrocytes demonstrates the characteristic change in morphology of astrocytes on activation from polygonal (A) to stellate upon transient acidification (B). The scale bar represents 20 μ m. The cell body size was significantly decreased with transient acidification (E). There was no apparent change in total cell field size, and while the arbor was increased, this was not significant. Control cultures of astrocytes have low expression of GFAP (F, red), with filamentous actin throughout (green). On stellation (H), GFAP is expressed in high quantities by some, but not all cells. Coincident with these changes is a tendency towards more diffuse F-actin staining in stellate/GFAP+ cells. Control astrocytes had low levels of vimentin, expressed predominately perinuclearly (G). Transient acidification induced rearrangement of vimentin to a more peripheral expression pattern, often noted in processes extending from the soma (I). Incubation with TNF- α (100U/ml for 2 days) did not induce stellation in control (C) or transiently acidified cultures (D). While there was partial retraction of the cell body, it does not meet the definition of the Rodnight group (Gottfried et al., 2003). TNF- α treatment of previously stellated astrocytes had no effect on soma size (E). Nor was there a change in total cell field size. Although the total arbor was significantly increased in stellated astrocytes subsequently treated with TNF- α compared to controls, the difference between TNF- α treated cells and stellated cells treated with TNF- α . Treating polygonal cells with TNF- α (J) increased GFAP expression to 23% (not significant). The F-actin staining was noted to be more robust in stellated cells following TNF- α treatment (L). TNF- α treatment induced rearrangement of vimentin to a more peripheral expression pattern

(K), and increased GFAP expression to 31. Vimentin expression often followed processes radially from the soma (M).

Figure 3. Real time analyses of astrocyte adhesion

Astrocytes were cultured for 36 hours before transient acidification (A, blue & purple traces). Electrical impedance was noted to drop quickly after transient acidification (arrowhead at top of graph at 37 hours post plating). Decreased adhesion was evident within 1 hour. Adhesion was decreased maximally within 6 hours and remained lower for over 20 hours. Inbuilt software (Roche, Indianapolis, IN) was used to normalize the traces to the internal controls (red and green traces) and determined the rate of change in adhesion (B). Thirty-six hours after stellation was induced through transient acidification, the addition of TNF- α (100U/ml) induced an increase in electrical impedance (C). Transient acidification did not alter the magnitude nor duration of adhesion in response to subsequent TNF- α treatment (D). Each trace represents 4 identically-treated wells. The results are representative of three repeat experiments.

Figure 4. Cytokine secretion induced in astrocytes stellated by transient acidification

Forty-eight hours after transient acidification, there were reduced levels of secretion of all six cytokines with measurable secretion. Most cytokines had increased secretion four hours after addition of fresh media. GM-CSF (B) and IL-10 (G) were at or below the limit of detection for either control or HEPES treatment. CCL2, GM-CSF, VEGF, IL-6, IL8 and IL-10 were all upregulated in astrocytes following treatment with TNF- α . In general, there was less cytokine secreted in HEPES-treated compared with control astrocytes. The level of TNF- α in the cultures decreased at a steady rate over time.

Table 1

Antibodies used and concentrations.

Antibody	Source	Catalog #	Clone	Dilution
GFAP	Thermo	MS-280-P	GA5	1:500
Vimentin	Santa Cruz	SC-6260	V9	1:250
MAP2	Sigma	M4403	HM2	1:50
Phalloidin	Invitrogen	O7466	NA	1:500

Table 2

Genes associated with the cytoskeleton with expression changes of at least two fold in at least one treatment group.

	Astrocytes v HEPES astrocytes		TNF v HEPES TNF		Astrocytes v TNF		HEPES v HEPES TNF	
	fold change	Std Dev	fold change	Std Dev	fold change	Std Dev	fold change	Std Dev
Actin α2	-4.19	1.82	-6.67	0.21	-2.48	0.09	-4.13	0.35
Actin γ2	-3.24	0.68	-6.8	0.7	-3.3	0.15	-7.58	0.18
Actin cytoplasmic 2	--	--	-2.46	0.48	--	--	--	--
Tubulin α	--	--	--	--	--	--	-2.9	0.03
Tubulin γ	2.11	0.12	--	--	--	--	--	--
Tubulin α1	3.15	0.49	--	--	--	--	--	--
Tubulin α2	3.11	0.19	--	--	--	--	--	--
Tubulin β	--	--	--	--	--	--	-2.4	0.56
Tubulin β4	2.66	0.04	--	--	--	--	--	--
Tubulin α6	--	--	--	--	--	--	--	--
Tubulin α	--	--	-2.47	0.04	--	--	-8.44	0.44
Tubulin δ4	--	--	--	--	2.94	0.94	--	--
Dynein	-8.57	1.13	--	--	-4.06	0.39	--	--
Plectin	-3.02	0.4	--	--	--	--	--	--
Tropomyosin 2	-2.41	0.12	-2.94	0.21	--	--	--	--
Tropomyosin 1	-2.12	0.06	--	--	-2.77	0.01	--	--
RHO Gap	-2.38	0.47	--	--	--	--	--	--
RHO Gap22	3.31	0.37	--	--	--	--	--	--
PTK2	--	--	--	--	-2.76	0.18	-2.97	0.04
MLCK	--	--	--	--	--	--	--	--
PKC μ	--	--	--	--	--	--	--	--
PKC ζ	2.65	0.12	--	--	--	--	--	--
PI4,5 Ki-se	-2.71	0.57	--	--	--	--	--	--
PKC B	--	--	-4.77	0.21	--	--	--	--
Sorting nexin 7	-2.51	0.16	--	--	--	--	--	--
Palladin	-4.43	1.06	--	--	--	--	--	--
Filamin A	-1.55	1.26	--	--	--	--	--	--

	Astrocytes v HEPES astrocytes		TNF v HEPES TNF		Astrocytes v TNF		HEPES v HEPES TNF	
	fold change	Std Dev	fold change	Std Dev	fold change	Std Dev	fold change	Std Dev
Vinculin	-2.82	0.66	--	--	--	--	--	--
Vilin	--	--	--	--	--	--	--	--

Relative fold change for mRNA extracted from astrocytes.

Table 3

Genes associated with adhesion and the extracellular matrix with expression changes of at least two fold in at least one treatment group.

	Astrocytes vHEPES astrocytes			TNF v HEPES TNF			Astrocytes v TNF			HEPES v HEPES TNF		
	fold change	Std Dev		fold change	Std Dev		fold change	Std Dev		fold change	Std Dev	
Integrin α6	--	--	--	--	--	--	-3.37	0.91	--	-3.41	0.9	--
Integrin α8	-3.58	0.66	--	-3.65	0.54	--	--	--	--	--	--	--
Integrin α10	-2.49	0.19	--	--	--	--	--	--	--	--	--	--
Integrin α11	-2.31	0.18	--	-2.71	0.25	--	--	--	--	--	--	--
Integrin β1	-2.44	0.01	--	--	--	--	--	--	--	--	--	--
Integrin β1	-6.54	2	--	--	--	--	--	--	--	--	--	--
Integrin β1	-6.28	1.44	--	--	--	--	--	--	--	--	--	--
Integrin β2	--	--	--	-2.1	0.04	--	--	--	--	--	--	--
Integrin β5	-0.75	0.21	--	--	--	--	--	--	--	--	--	--
Cadherin 2	--	--	--	--	--	--	--	--	--	-2.6	0.25	--
Cadherin 13	-3.21	0.61	--	--	--	--	--	--	--	--	--	--
VCAM-1	--	--	--	--	--	--	18.3	3.94	--	12.56	5.41	--
Fibronectin	-2.56	0.35	--	--	--	--	--	--	--	--	--	--
Fibronectin III	--	--	--	--	--	--	-2.75	0.08	--	-4.97	1.04	--
Collagen I α2	-3.61	0.58	--	-2.37	0.04	--	--	--	--	-2.28	0.26	--
Collagen I α1	-2.45	0.26	--	-2.13	0.13	--	-3.35	0.25	--	-2.6	0.21	--
Collagen III	-3.42	0.38	--	--	--	--	--	--	--	--	--	--
Collagen IV	-2.33	0.46	--	--	--	--	--	--	--	--	--	--
Collagen 5 α1	--	--	--	--	--	--	--	--	--	--	--	--
Collagen V	--	--	--	--	--	--	--	--	--	--	--	--
Collagen VI	-2.42	0.07	--	--	--	--	--	--	--	--	--	--
Collagen XI	-3.12	0.41	--	--	--	--	--	--	--	--	--	--
Collagen XIV	-3.56	0.72	--	--	--	--	--	--	--	--	--	--
Collagen XV α1	--	--	--	--	--	--	--	--	--	--	--	--
Collagen XVI	-3.86	0.76	--	--	--	--	--	--	--	--	--	--
Collagen XVIII	--	--	--	--	--	--	--	--	--	--	--	--
Aggrecan	-7.29	0.43	--	-3.63	0.21	--	-2.86	0.33	--	--	--	--

	Astrocytes vHEPES astrocytes		TNF v HEPES TNF		Astrocytes v TNF		HEPES v HEPES TNF	
	fold change	Std Dev	fold change	Std Dev	fold change	Std Dev	fold change	Std Dev
Versican	-6.8	0.2	--	--	3.72	0.85	--	--
ADAM TS1	-2.1	0.05	-3.03	0.47	--	--	--	--
ADAM TS2	-2.37	0.32	--	--	--	--	--	--
ADAM 19	-2.56	0.09	-3.05	0.28	-2.57	0.07	--	--
MMP3	23.86	11	2.72	0.43	7.77	0.7	--	--
Aquaporin 1	-2.69	0.54	--	--	--	--	--	--
Connexin 40	--	--	-3.24	1.24	--	--	--	--
Connexin 32	2.42	0.29	--	--	--	--	--	--
Connexin 43	-2.74	0.08	--	--	--	--	--	--

Relative fold change for mRNA extracted from astrocytes.

Table 4
Genes associated with cytokines with expression changes of at least two fold in at least one treatment group.

	Astrocytes v HEPES astrocytes			TNF v HEPES TNF			Astrocytes v TNF			HEPES v HEPES TNF		
	fold change	Std Dev		fold change	Std Dev		fold change	Std Dev		fold change	Std Dev	
CCL2	--	--	--	--	--	--	6.43	0.41	--	4.14	0.94	--
CCL5	--	--	--	--	--	--	30.93	6.81	--	27.23	3.38	--
CCL7	2.37	0.03	--	2.07	0.89	--	--	--	--	--	--	--
CCL13	--	--	--	--	--	--	--	--	--	--	--	--
CCL24	3.64	1.77	--	--	--	--	--	--	--	--	--	--
CXCL1	--	--	--	--	--	--	--	--	--	7.82	1.84	--
CXCL2	--	--	--	--	--	--	--	--	--	4.3	1.5	--
CXCL3	--	--	--	--	--	--	--	--	--	13.67	6.57	--
CXCR4	--	--	--	--	--	--	5.31	2.14	--	5.49	0.12	--
CXCL6	--	--	--	--	--	--	23.17	3.3	--	9.65	1.88	--
CXCL12	--	--	--	--	--	--	--	--	--	-4.61	1.18	--
CXCL14	--	--	--	-4.02	0.41	--	2.87	0.13	--	-3.87	0.36	--
Interleukin 6	-5.86	1.08	--	--	--	--	8.55	0.94	--	24.24	9.57	--
Interleukin 8	--	--	--	--	--	--	9.73	0.12	--	3.99	0.71	--
Interleukin 11	--	--	--	--	--	--	11.34	0.38	--	6.6	1.59	--
TNFRSF11b	-3.14	0.13	--	--	--	--	3.16	0.16	--	3.28	0.03	--
TNFRSF10c	--	--	--	--	--	--	4	1.82	--	--	--	--
TNFRSF18	--	--	--	--	--	--	2.19	0.02	--	--	--	--
TNFSF18	--	--	--	-2.63	0.59	--	2.83	0.22	--	--	--	--
TNFSF 6 (FAS)	--	--	--	--	--	--	2.69	0.57	--	--	--	--
TNFSF4	-4.03	2.37	--	--	--	--	--	--	--	--	--	--
CKLSF4	--	--	--	--	--	--	--	--	--	--	--	--
CCRL1	-2.4	0.2	--	-2.77	0.16	--	--	--	--	--	--	--
IL17RC	3.45	1.85	--	--	--	--	--	--	--	--	--	--
VEGF C	--	--	--	--	--	--	--	--	--	3.11	0.22	--

Relative fold change for mRNA extracted from astrocytes.