Published in final edited form as:

Neuroscience. 2009 January 23; 158(2): 885–895. doi:10.1016/j.neuroscience.2008.10.047.

Monocyte Chemoattractant Protein-1 in the choroid plexus: a potential link between vascular pro-inflammatory mediators and the CNS during peripheral tissue inflammation

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Abstract

During peripheral tissue inflammation, inflammatory processes in the CNS can be initiated by blood-borne pro-inflammatory mediators. The choroid plexus, the site of CSF production, is a highly specialized interface between the vascular system and CNS, and thus, this structure may be an important element in communication between the vascular compartment and the CNS during peripheral tissue inflammation. We investigated the potential participation of the choroid plexus in this process during peripheral tissue inflammation by examining expression of the SCYA2 gene which codes for monocyte chemoattractant protein-1 (MCP-1). MCP-1 protein was previously reported to be induced in a variety of cells during peripheral tissue inflammation. In the basal state, SCYA2 is highly expressed in the choroid plexus as compared to other CNS tissues. During hind paw inflammation, SCYA2 expression was significantly elevated in choroid plexus, whereas it remained unchanged in a variety of brain regions. The SCYA2-expressing cells were strongly associated with the choroid plexus as vascular depletion of blood cells by whole-body saline flush did not significantly alter SCYA2 expression in the choroid plexus. In situ hybridization suggested that the SCYA2-expressing cells were localized to the choroid plexus stroma. To elucidate potential molecular mechanisms of SCYA2 increase, we examined genes in the NF- κ B signaling cascade including TNF- α , IL-1 β and I κ B α in choroid tissue. Given that we also detected increased levels of MCP-1 protein by ELISA, we sought to identify potential downstream targets of MCP-1 and observed altered expression levels of mRNAs encoding tight junction proteins TJP2 and claudin 5. Finally, we detected a substantial up-regulation of the transcript encoding E-selectin, a molecule which could participate in leukocyte recruitment to the choroid plexus along with MCP-1. Together, these results suggest that profound changes occur in the choroid plexus during peripheral tissue inflammation, likely initiated by blood-borne inflammatory mediators, which may modify events in CNS.

Keywords

BBB; BCSFB; cox-2; cytokines; glial cells; MCP-1

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Section Editor: Dr. Linda S. Sorkin (Pain Mechanisms)

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Excitation of nociceptive primary afferent endings in the periphery results in action potentials along primary afferent fibers of dorsal root ganglia neurons. The nociceptive signals are relayed to and processed by neurons of the spinal cord and then transmitted to the brain. This neural circuit, which is essential for normal withdrawal responses, protection from injury and the sensation of pain, is hyperexcitable in patients suffering from chronic pain. Therefore considerable attention has been spent on understanding the molecular and cellular events associated with pain hypersensitivity (Woolf and Costigan, 1999, Lacroix-Fralish et al., 2007).

During peripheral tissue inflammation, infiltrating leukocytes contribute to pain hypersensitivity by releasing inflammatory molecules at the inflamed site which sensitize peripheral nociceptive primary afferent terminals. Recent research has also suggested that inflammatory mediators can become blood-borne during peripheral tissue inflammation where they can influence events in the CNS (Samad et al., 2001, Ibuki et al., 2003, Oka et al., 2007). Cox-2, which converts arachidonic acid into prostaglandins and contributes to nociceptive sensitization, has been reported to be induced along the entire longitudinal axis of the spinal cord and in several brain regions during inflammation of the hind paw (Laflamme et al., 1999, Samad et al., 2001, Ibuki et al., 2003). Prolonged blockade of the nociceptive neural signals during peripheral tissue inflammation or surgical incisions does not fully block cox-2 induction, which is consistent with the idea that cox-2 upregulation is partially mediated by non-neuronal factors (Samad et al., 2001, Kroin et al., 2004). In an independent report, it was suggested that circulating IL-6, which is significantly elevated in plasma during peripheral tissue inflammation, mediates the widespread induction of cox-2 (Oka et al., 2007). Together, these studies suggest that activated immune cells located at the inflamed site or in the general or CNS circulation (Mitchell et al., 2008) release inflammatory mediators into the bloodstream which may directly influence events in the CNS during peripheral tissue inflammation.

It is well documented that systemic injections of inflammatory agents leads to behavioral changes including fatigue, lethargy and malaise (Dantzer et al., 1998), some of which are observed during chronic pain. Examination of molecular alterations in brain has led investigators to consider the role of the choroid plexus during systemic inflammation. Indeed, the choroid plexus, which produces cerebrospinal fluid (CSF) and forms the blood-CSF barrier (BCSFB) in the lateral and fourth ventricles, possesses numerous receptors relating to inflammation including TLR4 and CD14, which are activated by LPS, and for various cytokines (Ericsson et al., 1995, Lacroix et al., 1998, Nadeau and Rivest, 1999, Laflamme and Rivest, 2001). Accordingly, systemic injection of inflammatory agents such as LPS, TNF- α and IL-1 β can significantly elevate the expression of many inflammation-related genes in the choroid plexus including TNF- α , IL-1 β , cox-2, I κ B α and SCYA2 (Quan et al., 1997, Lacroix and Rivest, 1998, Thibeault et al., 2001, Proescholdt et al., 2002) and LPS administration can significantly elevate levels of cytokine proteins in the CSF (Sanna et al., 1995, Marques et al., 2007).

In the present study, we examined whether inflammatory mediators and associated signaling pathways were upregulated in the choroid plexus during peripheral tissue inflammation. We chose to examine the expression of SCYA2 and its protein product, MCP-1, based on the regional expression pattern we observed in the brain and its expression in multiple cell types during peripheral tissue inflammation including endothelial cells, activated immune cells, and neurons of the dorsal root ganglia (Yang et al., 2007). A detailed analysis of SCYA2/MCP-1 expression and regulation (as well as the regulation of potential upstream and downstream targets of SCYA2/MCP-1), suggests that the choroid plexus may be critical in relaying inflammatory information from the blood to CNS during peripheral tissue inflammation.

Experimental Procedures

Animal used, carrageenan treatment, animal perfusion and tissue dissection

Male Sprague Dawley rats weighing 250-350 g were housed with a 12-h light/dark cycle and given water and food *ad libitum*. Procedures for all animals used were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the National Institute of Dental and Craniofacial Research.

Inflammation was induced by injection of carrageenan Lambda type IV (Sigma, St Louis, MO, USA), 8 mg in 200 μ l of phosphate buffered saline per paw, into the plantar surface of the rat hind paw bilaterally. This concentration was used to ensure uniform activation of inflammation at early time periods (e.g. 2h). Untreated rats were used as controls. In some experiments, rats were anesthetized with ketamine and xylazine and perfused transcardially with cold phosphate buffered saline to flush the blood leukocytes out of the vascular tree. To determine the regional distribution of SCYA2 mRNA, rats were sacrificed by exposure to CO₂ and the brain and choroid plexus rapidly removed. Brains were dissected into striatum, frontal cortex, nucleus accumbens, periaqueductal gray and hypothalamus as described (Naranjo et al., 1986). All of the tissues were frozen in dry ice and stored at -80 °C until processed for RNA extraction. For the choroid plexus studies, control or carrageenan injected rats were sacrificed by CO₂ exposure followed with decapitation at 2, 4, 6, 8 or 24 h and the choroid plexus was rapidly removed bilaterally as follows. The lateral ventricle was exposed on one side and the choroid plexus could be seen lying along the curvature of the striatum. It was grasped at its posterior end near the ventral hippocampus with a fine forceps and gently lifted away from the tissue. The rostral end where it emerges from near the septum was then grasped with a second fine forceps and the tissue was removed from the brain, frozen in dry ice and stored at -80 °C until processed for RNA or MCP-1 protein extraction. We generally used a dissecting microscope and the dissection was relatively easy when the choroid vasculature contained blood. After the blood was flushed out with saline, the choroid plexus was difficult to see and the dissecting microscope was a good aid. In all cases, care was taken to remove only the choroid plexus and not any brain tissue.

RNA extraction and RT-PCR

Total RNA was isolated from brain regions or choroid plexus tissue using RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA) with an additional step of DNase treatment. RNA was quantified by the RiboGreen reagent (Invitrogen, Carlsbad, California, USA) and then used for RT-PCR analysis.

Gene expression analysis was performed with RT-PCR using the access RT-PCR system (Promega, Madison, WI, USA). The PCR primers and product sizes are listed in Table 1. RT-PCR analysis was carried out according to the manufacturer's instructions in 25 μ l reaction mixture containing 2-8 ng of RNA. Briefly, RT-PCR steps were: 45 min at 45°C for reverse transcription, 2 min at 94°C for inactivation of transcriptase, 28-36 cycles of 30 s at 94°C for denaturation, 1 min at 55°C for annealing, and 2 min at 68°C for extension. After the final cycle, an additional extension was done for 7 min at 68°C.

The RT-PCR products were separated by 2% agarose/ethidium bromide gel electrophoresis and fluorescent DNA bands were acquired using a CCD camera (Alpha Innotech, San Leandro, CA, USA). The intensities of the specific DNA bands were analyzed quantitatively using ImageQuant 5 software (Molecular Dynamics, Piscataway, NJ, USA). The results were normalized to glyceraldehyde-3-phosphate dehydrogenase or β -actin, housekeeping genes, neither of which were altered by hind paw inflammation.

MCP-1 determination

Rats were perfused transcardially with phosphate buffered saline to flush out the blood from the choroid plexus. The choroid plexus was removed from the brain and stored at -80°C as described above. Frozen samples from controls and 24 h carrageenan-treated animals were homogenized in phosphate buffered saline (30 µl/pair of choroid plexii) containing protease inhibitors (Halt Protease Inhibitor Cocktail Kit, Pierce, Rockford, IL., USA). The homogenates were centrifuged at 15,000 g for 20 min at 4°C and the supernatants were used for assay of MCP-1 and protein content. MCP-1 was analyzed using a MCP-1 immunoassay kit (R & D system, Minneapolis, MN, USA) and protein determination was carried out with the Bradford Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

In situ hybridization

Three control and three hind paw inflamed (6 h) animals were sacrificed by carbon dioxide exposure. The brain was removed from each animal and immediately frozen on dry ice and then stored at -80 °C until cryostat sectioning. 12 µm sections were cut and placed on Colorfrost®/plus slides (Fisher Scientific, Pittsburg, PA) and stored at -80 °C. To prepare sections for in situ hybridization, slides were warmed at room temperature (10 min), fixed in 4% buffered formaldehyde (10 min), rinsed twice in PBS (5 min) and treated with acetic anhydride in TEA buffer, pH 8.0 (10 min). The slides were then dehydrated for 1 min each in increasing concentrations of ethanol: 70%, 80%, 95%, 100% and 95%. SCYA2 labeled-riboprobes were added to dried sections.

The generation and detection of the SCYA2 digoxigenin-labeled antisense riboprobe was performed as previously described (Yang et al., 2007). As an additional control for non-specific reactions from the secondary reagents, the riboprobe was omitted; no signal was observed upon omission of the digoxigenin-labeled antisense riboprobe.

Statistical Analyses

Differences in transcript densitometric values and levels of MCP-1 in samples from control versus hind paw inflamed animals were tested for statistical significance using Student's t-test when comparing a single time point versus control. When more than one inflammation time point was examined, a one-way ANOVA followed by a Bonferroni correction was used.

Results

Distribution of SCYA2 mRNA

The regional distribution of SCYA2 gene expression in the rat CNS was examined by RT-PCR and results are shown in Fig 1. The highest level of SCYA2 transcript is in the choroid plexus. In order to determine whether the source of SCYA2 mRNA in the choroid plexus and brain is from blood cells within the lumen of blood vessels, rats were perfused with PBS before harvesting of the tissues. Perfusion did not significantly change the expression profile; similar to the non-perfused rats (Fig 1b), low levels of expression were found in the brain regions and the highest levels of SCYA2 mRNA were found in choroid plexus (Fig 1a).

Effects of peripheral tissue inflammation on SCYA2 system in choroid plexus

To explore the role of choroid plexus SCYA2 during peripheral inflammation, rats were injected with carrageenan into the hind paws bilaterally and a regional analysis was performed for SCYA2 gene expression at the 24 h time-point. Two groups of rats were used, the first group was not perfused and the second group was perfused to flush out intra-luminal blood cells. Upregulation of SCYA2 gene expression in choroid plexus was observed in both groups of rats while other brain regions were not affected (Fig 2a and 2b). As shown in Fig 3a, a small

but significant upregulation of choroid plexus SCYA2 expression was observed 2 hours after carrageenan treatment. By 6 h post-carrageenan, a marked upregulation had occurred and the elevation lasted at least 24 hours (Fig 3b) (the peripheral inflammation is sustained throughout this period (Iadarola et al., 1988)). The SCYA2 gene product MCP-1 was analyzed in the choroid plexus at 24 h after hind paw carrageenan inflammation and found to be significantly increased (Fig 3c). The known receptors for MCP-1, CCR2, CCR7 and CCR4, were analyzed for their gene expression by RT-PCR (data not shown). CCR2 was below the level of detection under the conditions used (8 ng total RNA and up to 37 PCR cycles). CCR7 was found to have a very low level of expression, while a comparatively high level of CCR4 mRNA was detected. The expression level of both detected genes was not affected by hind paw carrageenan inflammation.

Localization of SCYA2-expressing cells in choroid plexus during peripheral tissue inflammation

To determine which cell(s) express SCYA2, we performed in situ hybridization. In control animals (Fig 4a), we rarely detected labeling using in situ hybridization for SCYA2 in the choroid plexus. This is consistent with previous studies that failed to detect this transcript in choroid plexus of unmanipulated animals (Thibeault et al., 2001, Proescholdt et al., 2002). In contrast to control animals, hind paw inflamed animals (6 h) consistently yielded cellular labeling in the choroid plexus (Fig 4b). The SCYA2-positive cells appeared randomly scattered throughout the choroid plexus and higher magnifications of the images (Fig 4c) suggest that the labeling is located in cells in the choroid plexus stroma and not in epithelial cells or ependymal cells. The SCYA2-expressing cells were still detected in the same stromal location, even when the animals were perfused with saline (data not shown), consistent with our RT-PCR and perfusion data (Fig 1).

Genes involved in NF- κ B signaling in the choroid plexus of rats with or without peripheral tissue inflammation

To explore the mechanisms underlying the upregulation of MCP-1 in the choroid plexus, genes in the signaling pathway for nuclear factor kappa B (NF- κ B) were analyzed and the results are shown in Fig 5. TNF- α , caspase 1 (IL-1 β -converting enzyme) and IL-1R2 were upregulated with the peak elevation occurring at 6 h after hind paw carrageenan injection. Upregulation of IL-1 β was also observed 6 h after carrageenan treatment but its expression was further elevated by 8 h. The expression of the cytokine-responsive immediate early gene I kappa B alpha (I κ B α) mRNA was upregulated 4 h after the hind paw carrageenan injection and this elevation quickly declined to the basal level by 8 hours. The expression of Adam 17, the TNF- α convertase, was not affected by the peripheral inflammation for up to 8 hours. Cytokine genes which are (a) constitutively expressed in the choroid plexus, (b) not removed by the saline flush and (c) not affected by peripheral inflammation include TNF-R1, TNF-R2 and IL-1R1 (data not shown).

Effects of peripheral tissue inflammation on genes encoding cell adhesion molecules and junction proteins in choroid plexus

To examine potential alterations in choroid plexus function during peripheral tissue inflammation, such as an alteration of the blood-cerebrospinal fluid barrier, we measured the transcript levels of various adhesion molecules and junctional proteins in the choroid plexus under normal conditions and during inflammation induced by hind paw carrageenan injection. As shown in Fig 6, ICAM-1, VCAM-1, PECAM-1 and E-selectin but not MADCAM-1 (data not shown) are constitutively expressed in choroid plexus. Among these adhesion molecules, only E-selectin transcripts were found to be upregulated. The elevation was observed at 6 h after carrageenan treatment and the effect lasted for at least 24 hours.

Tight junction proteins (TJP1 and TJP2), claudin 5 and occludin transcripts were analyzed by RT-PCR in the choroid plexus from control rats and rats with hind paw inflammation induced by carrageenan (Fig 7). The expression of TJ2 was upregulated while that of claudin 5 was down-regulated by peripheral inflammation. In contrast TJP1 and occludin transcripts were not affected for at least 8 hours after carrageenan injection of hind paws. These data demonstrate multiple, distinct patterns of gene induction that are rapidly engaged to modify choroid plexus function.

Discussion

The present study provides a temporal and spatial analysis of SCYA2/MCP-1 expression in the choroid plexus during peripheral tissue inflammation induced by carrageenan injection of rat hind paws. We show that SCYA2/MCP-1, which in basal state is expressed at high levels in choroid plexus as compared to other CNS regions, is significantly and substantially increased during peripheral tissue inflammation. The greatest increase in SCYA2/MCP-1 occurs in cells that appear to be located in the choroid plexus stroma. We also show that genes in the NF- κ B cascade, which are potential upstream factors mediating increased SCYA2 expression, and genes encoding tight junction proteins, which are potential downstream candidates of MCP-1 activity, are altered in choroid plexus during peripheral tissue inflammation. These data are consistent with the idea that focal inflammation in peripheral tissues is sufficient to elicit an upregulation of cytokines, adhesion factors and signaling pathway genes in the choroid plexus and suggests that the SCYA2/MCP-1-expressing cells may function as effector cells in this response.

In the normal state, SCYA2 mRNA expression is high in choroid plexus and comparatively much lower in other CNS regions as determined by RT-PCR (Fig. 1). According to previous studies that primarily used in situ hybridization to detect SCYA2, the gene was reported to be below the level of detection in choroid plexus as well as in other CNS regions (Thibeault et al., 2001, Proescholdt et al., 2002). In the present study, we also failed to detect basal expression of SCYA2 in the choroid plexus using in situ hybridization even though we clearly detected high transcript levels via RT-PCR with multiple different primer pairs. It can be reasoned then, that RT-PCR analysis is more sensitive than in situ hybridization, and that in situ hybridization analysis, under conditions used here and in prior studies, only detected newly induced SCYA2 transcripts. Our present RT-PCR analyses support the idea that SCYA2 transcripts are endogenously expressed at a low basal level in discrete regions throughout the CNS and corroborates a recent report showing basal expression of SCYA2/MCP-1 in the brain as determined by RT-PCR, immunocytochemistry and ELISA (Banisadr et al., 2005). In contrast, the high endogenous level of SCYA2 expression in choroid plexus, which to our knowledge has not been previously reported, suggests that the SCYA2 gene product, MCP-1, may have a significant role in normal choroid plexus function.

Under conditions of peripheral tissue inflammation we observed a dramatic increase in the levels of SCYA2 in the choroid plexus. The upregulation was detected as early as 2 h, peaked by approximately 6 h and remained elevated for at least 24 h. The temporal pattern of SCYA2 induction during peripheral tissue inflammation differs from that seen with other models reported to provoke an elevation of SCYA2 expression in choroid plexus. For example, following systemic injection of LPS, TNF- α , or IL-1 β , a rapid upregulation of SCYA2 was reported (between 1.5 and 3 h), followed by an immediate return to baseline (Thibeault et al., 2001). Also, after intracerebroventricular injection of IL-1 β , a rapid onset of SCYA2 induction was also detected, which returned to baseline by 8 h (Proescholdt et al., 2002). An important observation from these studies is that the kinetics of SCYA2 gene expression appear to be rapid in terms of both onset and return to baseline levels once the stimulus is cleared from the body, as the cited studies only gave a single bolus of the stimulant. In the present study, the paws are

inflamed throughout the study, and thus, the SCYA2-inducing factor(s) may be present for longer periods of time.

The present study also shows that peripheral tissue inflammation modulates molecular targets in the signaling pathway upstream of SCYA2. During peripheral tissue inflammation, we detected elevated transcript levels in choroid plexus of TNF- α and IL-1 β transcripts as early as 6 h as well as caspase 1 transcripts. The induction of the latter gene is significant because it suggests that caspase 1 *protein* is needed to proteolytically cleave the precursor form of IL-1 β into its mature, active form, which could then act locally on choroid plexus cells or could act more globally (Samad et al., 2001) if released into the CSF. The mRNA for IL-1R2, which encodes a soluble decoy receptor that functions to buffer the actions of IL-1 β , is also upregulated, again consistent with active IL-1 β being present in the choroid plexus during peripheral tissue inflammation. We also found an increase in the expression of I κ B α mRNA in choroid plexus during peripheral tissue inflammation. This gene, whose protein product forms a complex with NF- κ B (thereby inhibiting NF- κ B in unstimulated states), is itself a target of activated NF- κ B and its induction has been reported to tightly mirror the activation state of NF- κ B (Quan et al., 1997). Inflammatory mediators(s), which increase NF- κ B, may then be responsible for the augmented expression of choroid plexus SCYA2 during peripheral tissue inflammation. In terms of coordinate regulation, TNF- α , IL-1 β and SCYA2 all have NF- κ B sites in the promoter region of their corresponding DNA sequences, suggesting that NF- κ B mediated processes may participate in the induction of all three genes in choroid plexus during peripheral tissue inflammation.

In situ hybridization revealed that SCYA2 is increased in a subpopulation of cells located on the basolateral side of the choroid plexus epithelial cells. The present technique could not discriminate, however, whether the SCYA2-expressing cells are leukocytes located in the vascular lumen or if they are stromal cells (e.g., macrophages). To further elucidate the nature of the SCYA2-expressing cells, we examined whether they could be flushed out of the choroid plexus by perfusing the animals with saline. We observed that most (if not all) of the SCYA2-expressing cells remained after perfusion, indicating that they are not lightly attached to the capillary wall. Thus the data suggest that the SCYA2-expressing cells are leukocytes that have become firmly attached to (or are in the process of crossing) the capillary wall *or* they are cells which were present in the stroma before peripheral tissue inflammation and afterwards became strongly SCYA2 positive. One group of cells which are thought to become activated in the presence of inflammatory mediators is the stromal macrophages (Matyszak et al., 1992). These cells have been reported to lie at the basal surface of the epithelium (Matyszak et al., 1992, Nataf et al., 2006), consistent with our present observation of SCYA2-expressing cells lying adjacent to the epithelium. Finally, although the location and morphology of the SCYA2-expressing cells indicate that they are not epithelial cells, it cannot be ruled out that a smaller induction of SCYA2 also occurs in epithelial cells during peripheral tissue inflammation, which may not have been detected by our present in situ hybridization procedures.

Using ELISA assays, we observed that MCP-1 protein levels are dramatically increased in choroid plexus during peripheral tissue inflammation. MCP-1, like other chemokines, can be released into the extracellular milieu, and its interaction with other cells can lead to molecular and physiological changes. Due to the fenestrated properties of choroid endothelial cells, molecules released even intraluminally can interact with a variety of cells including the epithelial cells that face the ventricular space and manufacture the CSF. These facts led us to investigate whether choroid epithelial cells also undergo changes in gene expression during peripheral tissue inflammation. The present observation that mRNA encoding tight junction proteins, TJP2 and claudin 5, are altered during peripheral tissue inflammation, not only indicates that signaling molecules bind to and influence the expression of genes in epithelial cells, but suggests that the BCSFB may be changed during peripheral tissue inflammation.

Indeed, the blood-brain barrier (BBB) has been reported to be altered following hind paw inflammation (Rabchevsky et al., 1999, Huber et al., 2001, Huber et al., 2002, Huber et al., 2006), but it is currently unknown whether the BCSFB is opened (or tightened) during carrageenan-induced hind paw inflammation. Notwithstanding, it is conceivable that MCP-1 may have been upstream of the changes in mRNA expression for TJP2 and claudin 5, given that that intracerebral and intracerebroventricular administrations of MCP-1 have been shown to open the BBB by altering expression of endothelial tight junction proteins (Stamatovic et al., 2005). Cytokines such as TNF- α or IL-1 β have also been reported to be upstream of the tight junction transcript changes (Yang et al., 1999, Blamire et al., 2000). The cytokines can also be transported across the BBB through saturable carrier systems (Banks et al., 1995). This may have significance to peripheral tissue inflammation since it has been reported that IL-1 β and TNF- α are increased in CSF after injection of rat hind paws with complete Freund's adjuvant (Samad et al., 2001, Bianchi et al., 2007) and after hip replacement surgery in humans (Buvanendran et al., 2006). An implication from our present demonstration of upregulated cytokine/chemokines is that the choroid plexus may be one source of cytokines in the CSF during peripheral tissue inflammation. Cytokines may then act on brain targets which have already been sensitized by persistent activation of nociceptive neural pathways (Dantzer et al., 2008).

There are other potential routes which may lead to increased levels of cytokines/chemokines in the CNS during peripheral tissue inflammation with most focusing on glial cells, which are reported to be globally activated in brain and spinal cord during peripheral tissue inflammation (Fu et al., 1999, Raghavendra et al., 2004, Huber et al., 2006, Clark et al., 2007). First, it is possible that molecules released at nociceptive circuits in spinal cord and brain during inflammatory pain can stimulate cells in the vicinity, including glial cells, which through the release of cytokines and other factors, could initiate a progressive effect on the remainder of the CNS. A second alternative is that the neurally-released mediators enter the CSF during inflammatory pain which could then globally activate glial cells. It has also been speculated that, for nerve injury models, neuronal factors can alter the BBB, thereby allowing inflammatory mediators in the blood access to the CNS where they could trigger glial cell activation (Banks and Watkins, 2006, Gordh et al., 2006). A prior study which demonstrated that glial cell activity was more closely associated with tissue damage rather than afferent nerve input during hind paw inflammation (Fu et al., 2000) indicates, however, that mechanisms involving neuronal factors may not be so critical in initiating global glial cell activation during peripheral tissue inflammation. Another potential route for widespread glial activation is that inflammatory mediators may be released into the bloodstream during peripheral tissue inflammation which could be transported across the BBB or globally stimulate BBB endothelial cells to produce molecules that cross (or alter the BBB) or that are secreted into the CSF where they can have widespread effects on the CNS (Clark et al., 2007, Oka et al., 2007). Also the possibility cannot be excluded that the global CNS changes are mediated in part by hormones released from one or more members of the hypothalamo-pituitary-adrenal axis, whose activity has been reported to positively correlate with hind paw inflammation (Fecho et al., 2007). Given that it has been proposed that cytokines released from activated glial cells are required for sickness response (Watkins and Maier, 2005) as well as alterations in BBB during peripheral tissue inflammation (Willis and Davis, 2008), elucidating the mechanism(s) leading to cytokine increases may lead to a better understanding of the connection between pain physiology and broader actions on the CNS.

Our present observation of increased levels of E-selectin expression indicates that choroid plexus endothelial cells are activated during peripheral tissue inflammation. We speculate that the endothelial cell activation is initiated by inflammatory mediators that have been released into the bloodstream as immune cells respond to inflammation and possibly to stress, which can also activate the immune system (LeMay et al., 1990). In contrast, the molecular changes

that occur in BBB endothelial cells during hind paw inflammation were suggested to be centrally mediated as the authors did not detect proinflammatory mediators in the bloodstream during hind paw inflammation (Huber et al., 2006). It should be mentioned, though, that a separate study was able to detect increased serum levels of IL-6 after hind paw inflammation and suggested that the circulating IL-6 is upstream of cox-2 induction in spinal cord and brain endothelial cells during hind paw inflammation (Oka et al., 2007). Currently, the investigation of pro-inflammatory mediators in bloodstream during hind paw inflammation has been limited to a few molecules. A more extensive analysis of the effect of peripheral tissue inflammation on humoral factors is needed to better understand the role of the immune system on CNS changes that occur during peripheral tissue inflammation.

Summary

The present study demonstrates that focal inflammation in the periphery can cause robust cellular and molecular changes in the choroid plexus, including a program of gene activation that is notable for the sharp increases in SCYA2/MCP-1, cytokines and E-selectin. Elucidating the role(s) of the SCYA2/MCP-1-expressing cells may provide insight into the behavioral and physiological significance of these choroid plexus changes during peripheral tissue inflammation and suggest that this tissue too plays a role in the general mobilization of the innate immune system during conditions of nociception, inflammation and tissue damage that signal a threat to the integrity of the organism (Yang et al., 2007, Mitchell et al., 2008).

Acknowledgements

This research was supported by the Intramural Research Program, NIDCR, NIH, DHHS.

Abbreviations

accum	accumbens
Adam 17	A disintegrin and metalloproteinase domain 17
CCR2	C-C chemokine receptor type 2
CCR4	C-C chemokine receptor type 4
CCR7	C-C chemokine receptor type 7
cho plexus	choroid plexus
BBB	blood brain barrier
BCSFB	blood–CSF barrier
CNS	central nervous system
cox-2	cyclooxygenase-2

CSF	cerebrospinal fluid
E-selectin	Endothelial leukocyte adhesion molecule 1
f cortex	frontal cortex
hypoth	hypothalamus
ICAM	Intercellular adhesion molecule
IL-6	interleukin-6
IL-1β	interleukin-beta
IK$\beta$$\alpha$	inhibitor of kappa B alpha
IL-1R2	Interleukin-1 receptor type II
LPS	lipopolysaccharide
MADCAM	Mucosal addressin cell adhesion molecule
MCP-1	monocytes chemoattractant protein-1
NF-κB	nuclear factor-kappa B
PAG	periaqueductal gray
PECAM	Platelet endothelial cell adhesion molecule
SCYA2	small inducible cytokine A2
sub nigra	substantia nigra
TJP	tight junction protein
TLR4	toll-like receptor 4
TNF-α	tumor necrosis factor-alpha

TNF-R

tumor necrosis factor-receptor

VCAM

vascular cell adhesion protein

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Fig. 1. Distribution of SCYA2 mRNA in choroid plexus and in selected brain regions. Total RNA preparations from tissues were subjected to semi quantitative RT-PCR analysis using specific primers for SCYA2. Tissues were taken from non-perfused animals (A) or animals that were perfused with saline (B) to flush blood leukocytes out of the vascular tree. The levels of SCYA2 mRNA were normalized to a housekeeping gene (see Materials and Methods). All graphs are presented as mean $\pm$ SEM.

Fig. 2.

Examination of the effects of hind paw inflammation on SCYA2 mRNA levels in choroid plexus and in selected brain regions. Tissues were taken from non-perfused animals (A) or animals that were perfused with saline (B) to flush blood leukocytes out of the vascular tree. Samples are from control and hind paw inflamed animals as indicated in the figures. The levels of SCYA2 transcripts in hind paw inflamed versus control animals were compared for each tissue. For each tissue, SCYA2 expression from control animals was set to a value of one (control expression/control expression). Graphed values for SCYA2 expression in 24h hind paw inflamed animals were determined by dividing average expression following

inflammation by the average obtained in control animals. Graphs are presented as mean $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$ as determined by Student's t -test.

Fig. 3.

Effects of hind paw inflammation on SCYA2/MCP-1 levels in rat choroid plexus. (A) Time course of SCYA2 expression as determined by RT-PCR in choroid plexus of hind paw inflamed animals. Mean $\pm$ SEM are presented. *** $P < 0.001$ as determined by a one-way ANOVA followed by a Bonferroni correction. (B) Representative gel images showing bands corresponding to SCYA2 and GAPDH. At 1 h, no differences could be detected in SCYA2 levels as compared to control samples. A marked increase could be detected by 24 h. (C) Increase in MCP-1 protein 24 h after hind paw inflammation. Supernatant was obtained from homogenized choroid plexus taken from saline perfused animals. MCP-1 levels were determined by ELISA which was normalized to total protein. Graph is presented as mean $\pm$ SEM. * $P < 0.05$ as determined by Student's *t*-test.

Fig. 4.

In situ hybridization of SCYA2 transcript expression in choroid plexus after hind paw inflammation. Representative microphotographs showing SCYA2 expression in the choroid plexus in a control animal (A) or in a hind paw inflamed animal (B). Higher magnification of SCYA2-expressing cells demonstrates that they are not epithelial cells (black arrow) or cells located on the apical side (*) of the epithelium such as epiplexus cells. In contrast, the SCYA2 expressing cells appear on the basolateral side (white arrow) of the epithelium where they could be in the stroma.

Fig. 5.

Effects of hind paw inflammation on cytokine transcript levels in rat choroid plexus. Total RNA preparations from tissues were subjected to semi quantitative RT-PCR analysis using specific primers for TNF- α (A), IL-1 β (B), caspase 1 (C), IL-1R2 (D), I κ B α (E) and Adam 17 (F). Tissues were taken from animals that were perfused with saline. Graphs shows relative levels of mRNAs and are presented as mean $\pm$ SEM. * P < 0.05, ** P < 0.01, and *** P < 0.001 as determined by a one-way ANOVA followed by a Bonferroni correction.

Fig. 6.

Effects of hind paw inflammation on cell adhesion molecule transcript levels in rat choroid plexus. Total RNA preparations from tissues were subjected to semi quantitative RT-PCR analysis using specific primers for various cell adhesion molecules (A). Tissues were taken from animals that were perfused with saline. Graphs shows relative levels of mRNAs and are presented as mean $\pm$ SEM. *** $P < 0.001$ as determined by Student's *t*-test. Insert shows gel image of E-selectin in choroid plexus from control and 24 h hind paw inflamed animals. (B) Time course of E-selectin transcript induction following hind paw inflammation. Tissues were taken from animals that were perfused with saline. Graphs shows relative levels of E-selectin mRNAs and are presented as mean $\pm$ SEM. * $P < 0.05$ as determined by a one-way ANOVA followed by a Bonferroni correction.

Fig. 7.

Effects of hind paw inflammation on tight junction protein transcript levels in rat choroid plexus. Total RNA preparations from tissues were subjected to semi quantitative RT-PCR analysis using specific primers for TJP2 and claudin 5 (A), TJP1 and occludin (B). Tissues were taken from animals that were perfused with saline. Graphs shows relative levels of mRNAs and are presented as mean $\pm$ SEM. Graphs are presented as mean $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$, and *** $P < 0.001$ as determined by a one-way ANOVA followed by a Bonferroni correction.

Table 1

RT-PCR primer pairs and length of PCR products

Gene	Primer pairs	Product (bp)	Gene	Primer pairs	Product (bp)
GAPDH	ACCAAGTCCATGCCATCAC TCCACCACCCCTGTGTGTA	452	β -Actin	TGTTGGCATAGAGGTCTTTACGG TGAGAGGGAATCGTGCCTG	278
SCYA2	CCAGAAACAGCCAACTCTC CCGACTCATTTGGATCATCT	192	CCR2	CTTGTGGCCCTTATTTTCCA GAATTCCTTGGAAAGGTGTCA	248
CCR4	GAGCACTGGTGGGTAGAAA GGTCCAGGATGGCTAAAGT	249	CCR7	GTGTGCTTCTGCCAAGATGA AGGACTTGGCTTCGCTGTAG	304
TNF- α	AGATGTGGAACTGGCAGAGG GGGCTTGTCACCTCGAGTTT	250	Caspase 1	GTGTTCCCTCAAGTTTTGC TGCAGCAGCAACCTTCATTC	193
IL-1 β	TGAAGCAGCTATGGCAACTG TGCCTTCCCTGAAGCTCTTGT	199	IL-1R1	GACGAAAGAGGGACAGACC GGCTTTTGACCTCTGAAACG	253
IL-1R2	CATGGGAGATGCAGGCTATT TACCAGTTCCCAGGAACACC	203	TNF-R1	CAGGCTGTACATCGCTCTCA GAATGACGCTCTGGAAGC	302
TNF-R2	AACCTCTCCCAACAACACTG CAGTCCCTGAGATTCCCTGA	298	Adam17	ACGTAAATTGAGCGGTTTTGG AACGGCTTGATAATGCGAAC	250
IkB α	AATGGCTTGGCGAAGTTCTA CAATAGAAATGCTCGGGCTA	251	E-Selectin	TGCCAAGAACAGGAATACCC CTCCAGGATTTGAGGAACA	248
ICAM-1	CAGGTGCTTTCTCTCAAAAG GGGCATGAGACTCCATTGTT	249	VCAM-1	CATTCCCTGAAGACCAGAA CGAGGCAAAACAAGAGCTTC	249
PECAM-1	TCCCTCCTGCCCTTGTAAATG CAGTATTTGACGGCAGCAGA	247	MADCAM-1	GTGGAGTCTGAAGCTTTCAG TGGAGTCCCTCAGTCTCTT	248
TJP1	CCAGAGTCTCGGAAAAGTGC TACCTGGGCTGACAGGTAG	250	TJP2	CAGAACAGGACTGGCAACAA TGGAGAAAAGATCGGTGG	250
Claudin 5	TCTCACAGAGAGGGGTCGTT CAGCTGCCCTTTCAGGTTAG	241	Ocludin	TTGTCTGGGGTTTCATGATT CTTTGCCGTTGGAGGAGTAG	252