Neuroscience Letters xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Plenary Article

Perivascular iron deposits are associated with protein nitration in cerebral experimental autoimmune encephalomyelitis

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HIGHLIGHTS

- Perivascular iron deposits are colocalized with nitrotyrosine in mice with cEAE.
- Iron-catalyzed nitration of proteins is a plausible mechanism of tissue damage in cEAE and possibly MS.
- Extravasation of albumin may be a protective mechanism to limit perivascular tissue damage.

ARTICLE INFO

Article history:
Received 24 March 2014
Received in revised form 1 May 2014
Accepted 2 May 2014
Available online xxx

Keywords:
Blood–brain barrier leakage
Endothelial nitric oxide synthase
Hemorrhage
Inducible nitric oxide synthase
Nitrotyrosine
Multiple sclerosis

ABSTRACT

Nitration of proteins, which is thought to be mediated by peroxynitrite, is a mechanism of tissue damage in multiple sclerosis (MS). However, protein nitration can also be catalyzed by iron, heme or heme-associated molecules independent of peroxynitrite. Since microhemorrhages and perivascular iron deposits are present in the CNS of MS patients, we sought to determine if iron is associated with protein nitration. A cerebral model of experimental autoimmune encephalomyelitis (cEAE) was utilized since this model has been shown to have perivascular iron deposits similar to those present in MS. Histochemical staining for iron was used together with immunohistochemistry for nitrotyrosine, eNOS, or iNOS on cerebral sections. Leakage of the blood–brain barrier (BBB) was studied by albumin immunohistochemistry. Iron deposits were colocalized with nitrotyrosine staining around vessels in cEAE mice while control animals revealed minimal staining. This finding supports the likelihood that nitrotyrosine formation was catalyzed by iron or iron containing molecules. Examples of iron deposits were also observed in association with eNOS and iNOS, which could be one source of substrates for this reaction. Extravasation of albumin was present in cEAE mice, but not in control animals. Extravasated albumin may act to limit tissue injury by binding iron and/or heme as well as being a target of nitration, but the protection is incomplete. In summary, iron-catalyzed nitration of proteins is a likely mechanism of tissue damage in MS.

1. Introduction

Abnormal iron deposition occurs in the CNS of patients with multiple sclerosis (MS), but the role that these deposits have relative to pathogenesis is not fully understood. Iron deposits have been observed within the cerebrum in perivascular locations, around the edges of plaques, and in deep gray matter structures such as the caudate, putamen, globus pallidus, thalamus, etc. [1,2]. Experimental autoimmune encephalomyelitis (EAE) in rodents can be a useful model of MS to help advance the understanding of pathogenic mechanisms; however, the predominance of pathology is often present in the spinal cord and hindbrain [3,4] thereby limiting investigations on the role of iron deposits in the development of cerebral pathology. For instance, the amount of abnormal iron deposits was roughly comparable between the cerebrum and spinal cord of SJL mice with EAE despite a several-fold greater area for the former structure indicating a relatively low density of pathology in the cerebrum [5]. To counter this limitation, we developed a cerebral model of EAE (cEAE) [6]. This model resulted in perivascular iron deposits in cerebral structures similar to that observed in MS [6]. In cEAE and MS, perivascular iron deposits are thought to be

Abbreviations: BBB, blood–brain barrier; cEAE, cerebral experimental autoimmune encephalomyelitis; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NGS, normal goat serum; MS, multiple sclerosis; PBS, phosphate buffered saline; PLP, proteolipid protein; TX, Triton-X 100.

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http://dx.doi.org/10.1016/j.neulet.2014.05.004
0304-3940/

due, at least in part, to microhemorrhaging [6–10]. As red blood cells degenerate, hemoglobin is liberated and hemin, the oxidized version of heme, can be released [11].

Iron and hemin may contribute to pathogenesis in EAE and MS via a variety of mechanisms. For instance, both can catalyze the formation of hydroxyl radical, which can promote oxidative damage to an array of targets, e.g., proteins, lipids, RNA, etc. [11–13]. A recent study on MS brain specimens demonstrated an association of iron deposits with oxidative damage to phospholipids [14], which would be consistent with iron's role in catalyzing hydroxyl radical formation [12,13]. In addition to oxidative damage, heme, and to a lesser extent free iron, can catalyze the nitration of proteins independent of hydroxyl radical or peroxynitrite formation [15–17]. In the present study, we investigated whether perivascular iron deposits are associated with protein nitration using the cEAE model.

2. Materials and methods

2.1. cEAE model

All studies involving the use of animals were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Female SJL mice aged 5–6 weeks (Jackson Laboratory, Bar Harbor, ME) were used for cEAE induction as previously described [6]. Animals were divided into two groups: (1) cEAE (Freund's adjuvant with *Mycobacterium tuberculosis* and proteolipid protein (PLP) peptide; pertussis toxin injections; intracerebral injection of cytokines) as previously described [6], and (2) controls (in place of the encephalitogen, no emulsion was given or an emulsion containing Freund's with *M. tuberculosis* but no PLP peptide, which gave similar results to no emulsion; pertussis toxin injections; intracerebral injection of saline).

2.2. Nitrotyrosine immunohistochemistry and double labeling with iron histochemistry

Mice were anesthetized with isoflurane and perfused with phosphate buffered saline (PBS) followed by 10% buffered formalin (Fisher Scientific, Waltham, MA) and brains processed as described previously [6]. Sections were placed in 98% Superblock (Pierce, Rockford, IL) plus 2% normal goat serum (Vector Laboratories, Inc., Burlingame, CA) (blocking solution) for 1 h at room temperature; incubated with 1:400 rabbit anti-nitrotyrosine IgG antibody (Alpha Diagnostics Intl Inc, San Antonio, TX) in 10% blocking solution in PBS for 1 h, PBS 3 × 5 min, 1:500 biotinylated goat anti-rabbit IgG (Vector) in 10% blocking solution in PBS for 1 h, PBS 3 × 5 min, Vectastain Elite ABC (Vector) for 30 min, PBS 3 × 5 min, SG Peroxidase Substrate Kit (Vector) for 10 min, rinsed in H_2O, mounted on slides with gelatin, dehydrated through xylenes, and coverslipped.

Double staining began with iron histochemistry as previously described [6,18]. Although this method is generally thought to reveal non-heme, ferric iron, it can stain heme or ferrous iron under certain conditions [19], and it has been our experience that red blood cells can be stained by this method. Following iron histochemistry, the sections were stained for nitrotyrosine, with the only difference being a 1:250 dilution of the nitrotyrosine antibody.

Nitrotyrosine + iron stained sections between Bregma −0.70 and −2.00 mm were analyzed using a 10× objective by an evaluator blinded to the animal group. Although staining could occur in vessels throughout the section, only the somatosensory 1 barrel field/secondary somatosensory cortex on both hemispheres on one section per animal was evaluated for nitrotyrosine and iron staining around cortical vessels. The number of nitrotyrosine + iron positively stained vessels and the number of nitrotyrosine only stained vessels were counted.

2.3. eNOS and iNOS immunohistochemistry and double labeling with iron histochemistry

Similar to the findings by Yousef et al. [20], permeabilization of the tissue improved the detection of eNOS in vessels. Sections were processed through graded alcohols to xylenes and then rehydrated, incubated in 3% H_2O_2 in PBS for 15 min (optional since there was no clear effect) followed by incubation in Superblock (Pierce) containing 0.25% Triton-X 100 (TX) and 10% normal goat serum (NGS), a 1:100 dilution of rabbit anti-eNOS polyclonal antibody (BD610298; BD Transduction Laboratories, San Jose, CA) in 0.25% TX and 1% NGS for 1 h, PBS 3 × 5 min, 1:200 dilution of biotinylated goat anti-rabbit IgG (Vector) containing 1% NGS and 0.1% TX in PBS for 1 h, PBS 3 × 5 min, Vectastain Elite ABC (Vector) containing 0.1% TX and 1% NGS for 30 min, PBS 3 × 5 min, and SG Peroxidase Substrate Kit (Vector) for 12 min.

Staining for iNOS was performed as described for eNOS immunohistochemistry but without passing sections through the alcohols and xylenes, using a 1:2000 dilution of the rabbit anti-iNOS polyclonal antibody (N7782; Sigma, St. Louis, MO) and SG Peroxidase Substrate Kit (Vector) for 8 min.

Double staining began with sections processed through graded alcohols to xylenes and then rehydrated for eNOS immunohistochemistry. Sections were washed three times in PBS followed by iron histochemistry as previously described [6,18]. The sections were then stained for eNOS or iNOS as described above.

2.4. Albumin immunohistochemistry with or without double labeling with iron histochemistry

Brain sections were placed in blocking solution for 1 h, 1:250 dilution of HRP-conjugated, goat anti-mouse albumin antibody (Bethyl Laboratories, Inc, Montgomery, TX) in 10% blocking solution in PBS for 1 h, PBS 3 × 5 min, and SG Peroxidase Substrate Kit (Vector) for 10 min.

For each animal, an image at 4× power of one coronal section between Bregma −0.70 and −2.00 was captured using a Nikon 80i microscope and analyzed by an evaluator blinded to the animal group. Analysis of each section used Image J (NIH) histogram function in which each pixel represented a color that corresponded with the density of staining. The colors were categorized into bins and the number of pixels representing staining was summed.

Double staining began with iron histochemistry as previously described [6,18] and then stained for albumin as described above.

2.5. Statistics

Extravasated albumin (see Section 2.4) as well as cortical vessels stained for both nitrotyrosine and iron deposits (see Section 2.2) were evaluated using the Wilcoxon two sample test with $p \leq 0.05$ as significant. Control and cEAE groups each had an $n \geq 4$ for each time point.

3. Results

3.1. Nitrotyrosine is associated with iron deposits around cerebral vessels in cEAE mice

Since iron deposits were observed in a perivascular location in cEAE mice [6], and iron can be a catalyst of nitration [15–17], we examined whether nitrotyrosine was associated with vessels in cEAE mice. Nitrotyosine labeling was present around vessels in cEAE mice (Fig. 1A), similar to that seen for iron deposits [6].

Please cite this article in press as: S.A. Sands, et al., Perivascular iron deposits are associated with protein nitration in cerebral experimental autoimmune encephalomyelitis, Neurosci. Lett. (2014), http://dx.doi.org/10.1016/j.neulet.2014.05.004