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Adhesive Properties and Inflammatory Potential of Citrullinated Myelin Basic Protein Peptide 45–89

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Abstract Deimination of arginyl residue of myelin basic protein (MBP) reduces cationicity of MBP and impedes the normal myelin membrane assembly. Less ordered structure of MBP is more susceptible to proteolytic attack that may lead to the release of highly immunogenic deiminated peptides into extracellular milieu. We have studied the association of peptides 45–89 derived from citrullinated MBP (C8 isomer) and phosphorylated MBP (C3 isomer) with the myelin lipids in a model membrane system using optical waveguide lightmode spectrometry. The analysis of association/dissociation kinetics to planar lipids under controlled hydrodynamic conditions has shown that MBP 45–89 peptide from citrullinated C8 isomer is less effectively adsorbed on the lipid membrane, than peptide from phosphorylated C3 isomer and packing densities for phosphorylated 45–89 MBP peptide is higher than for citrullinated forms. On the other hand, our results shown that continuous (24 h) exposure of mixed oligodendrocyte/microglial cells to peptides 45–89 from MBP-C8 induces apoptosis via mitochondrial pathway. In addition, peptides 45–89 stimulated the secretion of nitric oxide from microglial cells via induction of iNOS and decreased the level of the inhibitory protein IkB, indicating involvement of the transcription factor NF-kB in these processes. Our results suggest that some citrullinated peptides, initially released from oligodendrocytes, might activate microglia, which produces reactive nitrogen species and generates in turn fatal feedbacks that kill oligodendrocytes.

Keywords Myelin basic protein · Deimination · Citrullinated peptides · Myelin lipids · Primary Glial cells · Inflammation

Introduction

Activated microglia is significant component of the brain pathology during the chronic neuroinflammatory diseases. Among many endogenous or exogenous factors that regulate microglial activation and the brain inflammation, secretary products of neurons, oligodendrocytes and vascular endothelium have been suggested to play a key role in the regulatory processes [1]. Various proteases are released from apoptotic nerve cells during neuroinflammation and linked to pathologic conditions of the CNS, including ischemia, multiple sclerosis, Alzheimer's disease, and malignant glioma [2]. In additions to extracellular matrix macromolecules, these proteases can degrade myelin sheath proteins that are important in the pathogenesis of inflammatory demyelinating diseases.

The 18.5 kDa isoform of myelin basic protein (MBP) is one of the principal constituents of the mammalian myelin sheath and plays a structural role in maintaining myelin stability. MBP exhibits charge microheterogeneity as a result of post-translational modifications such as phosphorylation, deamidation, deimination, arginine methylation, and N-terminal acylation [3]. Alteration of MBP cationicity may represent a regulatory mechanism for

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normal myelin assembly or a degradative mechanism in demyelinating disorders. Deimination of arginyl residue by the action of peptidylarginine deiminase (EC 7.5-3.15) reduces cationicity of protein and generates citrulline, an important irreversible modification of MBP [4]. Citrullinated MBP is structurally less ordered and more susceptible to proteolytic attacks. The reduction in cationicity of citrullinated MBP impedes the membrane assembly and exposes an immunodominant epitope of the membrane-bound protein to proteases [5].

Immune response to citrullinated peptides is influenced by class II major histocompatibility complex antigen (MHC class II), which is abundantly expressed in microglia in various neuropathological conditions [6]. The cytokines, increased during neuroinflammation can induce microglia to express MHC-II [1] and accordingly to bind immunogenic peptides of MBP. This process may start a cascade of events that includes several feedback loops, setting in motion a self-sustaining cycle inflammatory interaction between activated microglia, oligodendrocytes and neurones.

Noncovalent, mainly electrostatic and hydrophobic, interactions are responsible for the multilamellar structure and stability of myelin. Myelin is enriched in acidic lipids, like glycosphingolipids, galactosylceramide, and its sulfated derivatives and their contribution in the formation and maintenance of myelin is significant [7]. Myelin lipids and proteins (mainly MBP) work synergistically to provide the adhesion, and excess or deficit of MBP or anionic lipids cause destruction of myelin structure [8, 9]. Thus, interaction of various cationic isoforms of MBP and their fragments with acidic lipids is important for electrostatic interactions and stabilization of multilamellar structure. Although the interaction of MBP and MBP related peptides with artificial membrane systems has been studied intensively [10, 11], the interaction and adhesion of MBP fragments with myelin natural lipids are not known. We hypothesized that deiminated i.e., citrullinated peptides of MBP have a low adsorption potential to the myelin lipids, they are rapidly released after myelin sheet breakdown and further increase the secretion of proinflammatory substances.

Here we isolated natural lipids from myelin and used for the characterization of adsorption properties of citrullinated and noncitrullinated forms of MBP 45–89 peptide. Because the planar geometry seems to be a better approximation to the low curvature of the myelin sheath than the small, highly curved vesicles, we have used OWLS technique for careful analysis of the association and dissociation kinetics. The planar geometry makes it easy to analyse the data with true heterogeneous kinetics, rather than the pseudohomogenous approximation which was used for the experiments with vesicles [5]. We have found that deiminated

isoforms has low adsorption properties and releases more rapidly from myelin natural lipids than corresponding phosphorylated MBP peptides. Besides, our results have shown that the released citrullinated peptides increase the inflammatory potential of microglial cells and could induce damage of oligodendrocytes.

Materials and Methods

Preparation of MBP and MBP 45–89 Peptides

MBP was isolated and purified from bovine brain white matter according to the method of Chou et al. [12] followed by HPLC. Briefly, the acid-soluble material was dissolved in urea-glycine buffer, pH 9.6 and applied on a CM-52 cellulose cation exchange column, equilibrated in urea-glycine buffer, pH 10.5. Following application of the sample, urea-glycine buffer (10.5) flow was continued until the first big peak had completely eluted. This unbound fraction is the least cationic isomer, i.e., MBP C8. The charge isomers C-5, C4, C3, C2 and C-1 were eluted from the column with a sodium chloride gradient (0–0.2 M) in the glycine-urea buffer, pH 10.6. The most cationic, least modified charge isomer was C-1. Further purification of C-8 was achieved by HPLC on a C-18 reverse phase column (Nova Pak, Waters) using a trifluoroacetic acid (0.05 %)-acetonitrile (0–60 %) solvent system [13]. For the cathepsin D digestion 0.5–0.7 mg MBP was suspended in 1 ml of 0.05 M ammonium acetate, pH 3.5. A solution of cathepsin D (enzyme:substrate ratios was 1:100) was added to the protein suspension and the mixture was incubated at 37 °C for 30 min. MBP 45–89 peptide from citrullinated MBP was purified by HPLC as described by Cao et al. [14].

Total Myelin Lipid Preparation

Density gradient-purified CNS myelin was extracted twice in 10 volumes of chloroform–methanol 2:1 (vol/vol) and centrifuged. The pellet of the preparation was re-extracted twice with the same solvent mixture. The supernatant was dried in a stream of nitrogen. Lipids were dissolved in hexane–ethanol 9:1 (vol/vol) at a final concentration of 20 mg/ml [7].

Optical Waveguide Light Mode Spectroscopy (OWLS)

The membrane-coated waveguides were assembled into a flow-through cuvette [5] and mounted in the measuring head of an IOS-1 integrated optics scanner (Artificial Sensing Instruments, Zürich). The effective refractive indices of the zeroth order TE and TM modes were

continuously monitored during the following three consecutive stages: (1) buffer flow; (2) MBP solution flow (over a range of bulk protein concentrations c_b from 2 to 100 $\mu g/cm^{-3}$); and (3) buffer flow. Flow was laminar at a wall shear rate of 22.4 s^{-1}, and controlled by a precision syringe pusher. The temperature was 25.0 ± 0.2 °C. The effective refractive indices were converted into numbers (v) of adsorbed MBP molecules (or total adsorbed mass $M = mv$, where m is the mass of one molecule of MBP) per unit area of membrane. Quantitative evaluation was performed by the general equation for association–dissociation kinetics at the solid–liquid interface as described early [15]. Since complete desorption would have required tens or hundreds of hours, we characterized it by fitting the empirical function $v(t) = (v_0 - v_\infty)e - Kdt + v_\infty$ to the measured portion, where v_0 is the value immediately before starting desorption (at $t = 0$) and v_∞ the "irreversible" residue, with v_∞ and kd as the fitting parameters. We then calculated the parameter of adhesion strength $D = 1 - v_\infty/v_0$ as a measure of desorbability.

Membrane preparation

Dissolved myelin lipids were spread on a laboratory-built Langmuir trough with which lipid bilayers were assembled on smooth planar optical waveguides as described by Ramsden [15]. The waveguides had the composition $Si_{0.62}Ti_{0.38}O_2$ and a surface roughness (determined using atomic force microscopy) of less than 0.09 nm, and were obtained from Artificial Sensing Instruments, Zürich, Switzerland (type 2400). These have a thin waveguiding layer optimized for detecting changes in the transverse electric (TE) and transverse magnetic (TM) guided modes with the highest possible sensitivity. A layer of water ca. 2–3 nm thick between the waveguide and the lipid ensures that the membrane has fluidity comparable to that of the physiological state.

Cell Cultures

Primary mixed glial cell cultures containing astrocytes, microglia and oligodendrocytes were prepared from whole brains of 2-day-old Wistar rats. The initial mixture of dissociated glial cells was seeded in 75 cm^2 flasks (2–3 brains/flask) coated with poly-D-lysine. The cultures were maintained in DMEM with 10 % fetal calf serum and cultured for 48 h. This step provides a means of separating adherent cells, such as microglia and astrocytes, from nonadherent cells, such as oligodendrocytes. The nonadherent oligodendrocyte fraction was plated into poly-D-lysine-coated 25 cm^2 flasks. For isolation of microglial cells, confluent cultures between days 12 and 14 were agitated on a rotary shaker at 37 °C at 220 rpm for 1 h. The

floating cells were collected and incubated in tissue culture plates. After 1 h the cultures were washed. The adherent cells were identified as microglia. The astrocytes, i.e., the cells remaining adherent after the removal of microglia, were removed from the flasks by incubation with 0.25 % trypsin, for 10 min.

Incubation of Cells with MBP 45–89 Citrullinated Peptide

Cells were incubated for 24 h in the absence and presence of 0.5 μM MBP 45–89 citrullinated peptide in serum-free DMEM, at 37 °C in a humidified atmosphere of 95 % air/5 % CO_2.

Cytotoxicity Assays

To assess cell viability, a modified 3-(4,5-dimethylthiasol-2yl)-2,5 diphenyltetrazolium bromide (MTT) assay, a sensitive first indicator of oxidative damage, was carried out as described by Liu et al. [16]. Briefly, 10^6 glial cells were treated with 0.5 mg ml^{-1} MTT followed by incubation at 37° C for 2 h in a CO_2 incubator. After brief centrifugation, supernatants were carefully removed and the formazan reaction product was dissolved in acidic isopropanol. The formazan product was quantified spectrophotometrically by its absorbance at 570 nm. Data are presented as a percentage of the absorbance obtained in untreated cultures, after subtraction of blank values obtained by incubation of MTT in medium alone. Trypan blue exclusion assays were carried out after incubation (5 min before counting) of cells with 0.04 % trypan blue (Sigma). Dead cells were stained blue. Three fields per coverslip were counted for each experimental condition, and between 100 and 200 cells were counted on average per field.

Assay for NO

Synthesis of NO was determined by assaying the culture supernatants for nitrite [17]. Briefly, 400 µl of culture supernatant was allowed to react with 200 µl of Griess reagent [18] and incubated at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 548 nm. Fresh culture medium served as the blank in all experiments.

Western Blot Analysis

Following 24 h of incubation in the presence or absence of MBP 45–89 peptides (0.5 μM), cells were removed from the dishes, washed with PBS and homogenised in ice-cold lysis buffer containing 1 % v/v Nonidet P-40, 100 mM

NaCl, 20 mM Tris (pH 7.4), 10 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl chloride, 1 µg ml^{-1} leupeptin, 1 µg ml^{-1} antipain, 1 µg ml^{-1} aprotinin and 1 µg ml^{-1} pepstatin A. Lysates were incubated at 4 °C for 15 min followed by centrifugation at 13,000 g for 15 min. The supernatant was used to study cytochrome c, iNOS and IkB by immune blotting. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane. Membranes were treated with primary and horseradish per-oxidase-conjugated secondary antibodies (Santa-Cruz Bio-technology, USA). Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL kit; Amersham, UK) in accordance with the manufacturer's instructions. The following primary antibodies (Santa-Cruz Biotechnology, USA) were used: against IkB, against iNOS and against cytochrome c. Protein concentrations were determined using a BCA protein assay kit (Pierce).

Statistical Analysis

Data shown in this study are expressed as mean ± SEM. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Tukey test with the significance level set at $p < 0.05$.

Results

Adhesion Properties of 45–89 Peptides Isolated from Citrullinated (C8) and Phosphorylated (C3) MBP Isomers

Decreases of positive charge of MBP in the citrullinated isomers affect the ability of the protein to interact with lipid bilayer. Deimination of MBP alters the secondary and tertiary structures of MBP, producing a more unfolded, open conformation that increases its susceptibility to extracellular proteases. However, MBP peptide 45–89, which contains an immunodominant and encephalitogenic epitopes in MBP, was shown to be more stable for further degradation than other MBP peptides released by proteases and accumulated in extracellular fluids [14]. There are two serine (Ser 54, Ser 56) and three arginine (Arg 49, Arg 54 and Arg 65) residues in the bovine MBP which are able to undergo modifications among this sequence [19]. There-fore, we examined the adhesion properties of peptide 45–89 isolated from citrullinated MBP (C8 isomer) and compared it with peptide 45–89 isolated from phosphory-lated MBP (C3 isomer). Figure 1 shows the representative adsorption/desorption curves and the calculated associa-tion/dissociation parameters are collected in Table 1.

Figure 1 shows two representative adsorption/desorp-tion curves. For both modified forms of MBP peptides, the
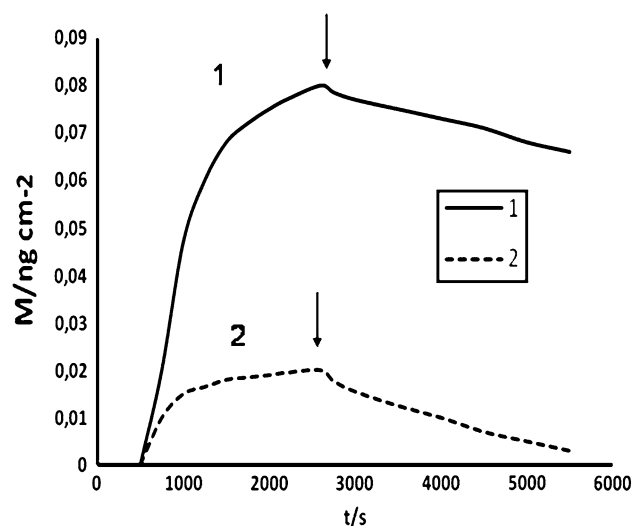

Fig. 1 Typical adsorption/desorption kinetics (plots of adsorbed amount of protein versus time) for phosphorylated (*1*) and citrulli-nated (*2*) MBP peptides. The absorption/desorption kinetics of phosphorylated (*1*) and citrullinated (*2*) MBP peptides (both a bulk concentrations c_b, 20 µg ml^{-1}) were monitored by optical waveguide lightmode spectrometry as described in "Materials and Methods". The *arrows mark* when the bulk concentration c_b was set to zero. The *curve* is a representative of three independent experiments

amount adsorbed gradually approached a plateau, i.e., dM/dt approaching zero, indicative of the jamming of the surface by the binding of at most a protein monolayer to the membrane. Parameter M_∞, which represents an amount of adsorbed protein, shows that incorporation of 45–89 MBP peptide from phosphorylated isoform into lipids is higher (86 ± 9 ng/cm^2), whereas M_∞ for citrullinated 45–89 MBP peptide was equal only to 22 ± 8 ng/cm^2. However, parameter a, which represents an area occupied by one molecule of peptide and describes the amount of peptide packing in the lipid bilayer, is lower for phosphorylated one. Area occupied per molecule (a) of phosphorylated 45–89 MBP peptide upon adsorption at the membrane is only 17.5 nm^2, whereas the same parameters for citrulli-nated peptide was higher and equal to 52 nm^2. These results suggest that packing densities for phosphorylated 45–89 MBP peptide is higher than for deiminated one. The association rate coefficient (ka) is derived from the adsorption energy barrier; thus the higher long-range pro-tein-membrane repulsion is because of the smaller ka. Furthermore, we have found that ka for citrullinated 45–89 MBP peptide is smaller than ka for phosphorylated iso-form, but the desorbability (D) i.e., a coefficient charac-terizing the dissociation rate of protein from lipids is the same for both peptides. Thus, comparing the adsorptive properties of different modified isoforms could be con-cluded that phosphorylated 45–89 MBP peptides more efficiently adsorbs on the lipid membrane than citrullinated

Table 1 Association and dissociation parameters, for the bulk concentration $c_b = 20$ µg ml^{-1} of phosphorylated and citrullinated MBP 45–89 peptides

Type of peptides	M_∞ (ng/cm^2)	a (nm^2)	Ka (cm s^{-1} 10^{-5})	D
MBP 45–89 peptide from phosphorylated isoform	86 ± 9	17.5	1.4 ± 0.15	$5.9 \times 10^{-4} \pm 1.3 \times 10^{-4}$
MBP 45–89 peptide from deiminated isoform	$22 \pm 8^*$	52	$0.21 \pm 0.01^*$	$4.4 \times 10^{-4} \pm 2.8 \times 10^{-4}$

Association and dissociation parameters were analyzed by optical waveguide lightmode spectroscopy as described in "Materials and Methods". a represent an area occupied by one molecule of peptide; ka is association rate coefficient; M_∞ is total areas occupied by bulk concentration of peptide; D is the parameter of adhesion strength. The data were normalized to control values from untreated cells. Values represent the mean ± SEM of three independent determinations performed in duplicate

* $P < 0.05$ versus 45–89 MBP peptides from phosphorylated isoform

Citrullinated MBP 45–89-Induced Cell Death in Mixed Primary Oligodendrocyte/Microglial Cell Cultures

Since, citrullinated MBP 45–89 peptide fragment could rapidly dissociate from the myelin sheath into the extracellular fluids and may interact with the various membrane receptors, in the next series of experiments, the activity of this peptide was examined. We have found that after continuous 24 h exposure of mixed oligodendrocyte/microglial cells to 0.5 µM citrullinated MBP 45–89 only about 65 % of the cells were still alive (Table 2). Interestingly, the decrease of the viability of cells takes place only in mixed oligodendrocyte/microglial cultures, but not in pure cultures of astrocytes, oligodendrocytes or microglia separately. To examine whether the citrullinated MBP 45–89-induced cell death has apoptotic features, the release of mitochondrial cytochrome c into the cytosol was determined. We isolated the cytosolic fractions from oligodendrocyte/microglial cells treated with citrullinated MBP 45–89 peptide and detected the released cytochrome c by Western blot analysis. The exposure of oligodendrocyte/microglial cells to 0.5 µM citrullinated MBP 45–89 peptide resulted in the release cytochrome c from mitochondria (Fig. 2A). Thus, citrullinated MBP 45–89 peptide appears to induce cell death mainly through mitochondrial pathway.

Citrullinated MBP 45–89 Induces Production of NO and Activation of NF-kB in Microglial Cell Cultures

Nitric oxide, produced by inducible NO synthase (iNOS), may play a central role in inflammatory demyelinating disease of the central nervous system. Nitric oxide (NO) derived from the activation of iNOS in astrocytes and microglia under the influence of proinflammatory cytokines is presumed to contribute to oligodendrocyte degeneration in demyelinating diseases [17, 20]. Because microglia rather than astrocytes are implicated in demyelinating pathology [21] in the next series of experiments we examined the effect of citrullinated MBP 45–89 peptide on the production of NO and activation of NF-kB in microglial cell culture. Figure 2C show that continuous exposure for 24 h of primary microglial cells to MBP 45–89 peptide markedly increased production of NO. To understand the mechanism of this stimulation, we examined the effect of citrullinated MBP 45–89 on the level of iNOS. Western blot analysis with antibodies against iNOS clearly shows that MBP 45–89-mediated nitrite production is engendered by induction of iNOS (Fig. 2A, line 4). Since the activation of NF-kB is known to be necessary for the transcription of iNOS gene in glial cells [22], we examined the effect of

Table 2 Viability of astrocytes, oligodendrocytes, microglial cells and mixed oligodendrocytes/microglial cells after incubation with citrullinated MBP 45–89 peptides

Conditions	% of viability			
	Astrocytes	Oligodendrocytes	Microglia	Mixed oligodendrocytes and microglia
Cells + MBP 45–89 peptide	96.7 ± 4.5	87.4 ± 7.2	91.9 ± 7.3	$16.4 \pm 8.2^*$

Viability of astrocytes, oligodendrocytes, microglial cells and mixed oligodendrocytes/microglial cells after exposure to 0.5 µM citrullinated MBP 45–89 for 24 h were assessed by the MTT test as described in "Materials and Methods". The effect of peptide on growth inhibition was assessed as percentage of cell viability where saline-treated cells were taken as 100 viable. Data are presented as the means ± SEM of three independent experiments
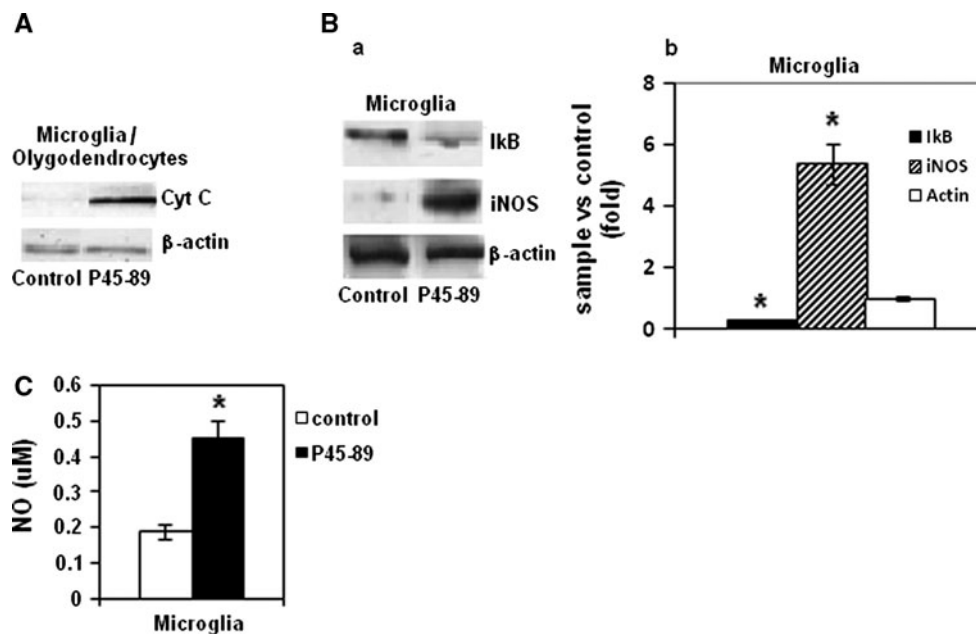
* $P < 0.05$ versus control values (saline-treated cells)

Fig. 2 Citrullinated MBP 45-89 peptide stimulates release of cyto-chrome *c* from mitochondria in mixed oligodendrocyte/microglial cells, induces the expression of iNOS through IkB degradation in microglial cells and increases the production of NO. **A** Citrullinated MBP 45–89 peptide (P 45–89) stimulates release of cytochrome *c* from mitochondria in mixed microglia/oligodendrocyte/cells; western blots, β-actin was used as loading control. **B** Citrullinated MBP 45–89 peptide (P 45–89) stimulates degradation of IkB and induction of iNOS in microglial primary cell cultures; β-actin was used as loading control; *a* western blots. Blot is representative of four similar experiments. *b* Immunoreactive bands were scanned and the

results are expressed as fold changes versus controls. The average densitometric quantification of the results of four independent experiments. Results is presented as mean ± SEM. *P < 0.05, compared with controls (saline-treated cells) by one-way ANOVA followed by the Tukey test. **C** Citrullinated MBP 45–89 stimulates production of nitric oxide in primary microglia cells. Microglial cells were treated with 0.5 μM citrullinated MBP 45–89 (P 45–89) for 24 h and then the yield of nitrite was measured in the supernatant as described in "Materials and Methods". Data are means ± SEM of four independent experiments. *P < 0.05, compared with controls (saline-treated cells) by one-way ANOVA followed by the Tukey test

MBP 45–89 on the activation of NF-kB. For this purpose, we determined the cytoplasmic levels of the inhibitory IkB protein after treatment of microglial cells with MBP 45–89. Western blot analysis with antibody against IkB showed that MBP 45–89 decreased the level of inhibitory protein (Fig 2A, line 3). Thus, it could be supposed that secreted from damaged myelin sheath citrullinated MBP 45–89 induces in the microglial cells degradation of IkB, subsequent nuclear translocation of NF-kB and induction of the iNOS gene. The resulting elevated concentration of nitric oxide causes the death of oligodendrocytes.

Discussion

MBP is a major component of the myelin sheath which interacts with opposed cytoplasmic surfaces of the myelin membrane and plays an important role in the formation and maintenance of myelin [10]. Modulated phosphorylation [23] and citrullination [24, 25] of MBP have been clearly correlated in demyelinating diseases. The formation of an abnormal myelin sheath would appear to be an ineluctable consequence of these modulated modifications. MBP

occurs normally as a number of charge isomers (C1 to C8), due to phosphorylation, deamidation and deimination. All of these modifications decrease the net positive charge of the protein and its ability to cause adhesion of the cyto-plasmic surfaces of myelin. Reduction in cationicity by deimination of arginine residues in MBP decreases myelin compaction and initiates a process that makes myelin susceptible to degradation. The extent and nature of post-translational modifications may be related to the extent of demyelination and disease heterogeneity in multiple scle-rosis (MS) as suggested by studies of citrullinated MBP. Kim et al. [23] demonstrated that MS MBP was less phosphorylated, more methylated, and more deiminated than MBP from normal tissue. They suggest that multiple sclerosis may represent a new class of diseases called "post-translational" diseases. Our results agree with this suggestion because we have found that heavy citrullination will tend to produce demyelination [5]. Exposure of an immunodominant epitope in the membrane-bound MBP to proteases and release of citrullinated encephalitogenic peptides from protein could induce sensitization of innate immune-derived cells of the CNS and peripheral T cells [26]. Thus, electrostatic and hydrophobic interactions

between MBP and myelin lipids have been shown to be critical for the formation and stability of the multilamellar myelin sheath [27–29].

The C-8 component from normal tissue showed increased citrullination in the arginine residues compared with the more cationic (phosphorylated) C1-C4 forms. Deimination in C-8 was found at 43, 49, 54, 65, 97, 122, 130, and 162 arginine residues, three of which (R49, R54, R65) are localized in peptide 45-89 [23]. These modifications change the α-helix structure of several peptides of MBP, including peptide 45–89 and their molar ellipticity is lower than molar ellipticity of peptides from more cationic isoforms of MBP [14]. Myelin is enriched in acidic lipids, like glycosphingolipids, galactosylceramide, and its sulfated derivatives. Thus, interaction of various cationic isomers of MBP and their fragments with acidic lipids is important for electrostatic interactions and stabilization of multilamellar structure [27]. We have analyzed the adsorption properties of peptide 45–89 with natural myelin lipids and have found that citrullinated MBP 45–89 peptide has low adsorption properties than the same peptide from more cationic (phosphorylated) MBP isoform C3. These data suggest that citrullinated MBP 45–89 peptides could release and accumulate in the extracellular milieu during myelin sheath degradation in a higher extent than phosphorylated peptides.

MBP and MBP derived peptides are apparently recognized by a different receptor systems. A major histocompatibility complex class II molecule, which is abundantly expressed in microglia in various neuropathological conditions, is contributed in response to citrullinated peptides [6]. Besides, these peptides bound with $\alpha_M\beta_2$ integrin [30], bFGF and ganglioside GM1 receptors [31], cannabinoid binding sites [32] and membrane actins [33–35]. Our data shown that citrullinated MBP 45–89 peptide induces NF-kB activation and iNOS gene expression in microglial cells, after which the secreted NO could cause the death of oligodendrocytes.

It has been proposed that some myelin components, initially released from oligodendrocytes, might activate microglia, which through proinflammatory cytokines, produce reactive oxygen species and generate in turn fatal feedbacks that kill oligodendrocytes [36]. We conclude that stable immunodominant peptide 45–89 from citrullinated MBP-C8, through microglial NF-kB activation, *iNOS* gene expression and NO production, could induce degradation of myelin sheet.

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