ORIGINAL ARTICLE

High-mobility group box 1, an endogenous ligand of toll-like receptors 2 and 4, induces astroglial inflammation via nuclear factor kappa B pathway

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Background: Neuroinflammation has a definitive role in neurodegenerative diseases, such as Parkinson's and Alzheimer's disease. In addition to its pathogenic ligands, toll-like receptors (TLRs) can be activated by damaged endogenous molecules that induce inflammatory signalling pathways such as high-mobility group box 1 protein (HMGB1).
Materials and methods: Using an ex-vivo rat optic nerve (RON) model, we sought to determine the effects of lipopolysaccharides (LPS; TLR4 agonist), zymosan (TLR2 agonist) or HMGB1 — with or without TLR2/4 antagonists, on the expression of glial fibrillary acidic protein (GFAP) and nuclear factor kappa B (NF-κβ) for signalling pathway and astrocyte reactivity, using double immunohistochemistry; as well as on the modulation of the neurotoxicity. HMGB1-treated RON had significantly higher expression and co-localisation of GFAP and NF-κβ as compared to the untreated control, which was a similar result to those treated with LPS and zymosan.
Results: Moreover, the HMGB1-induced inflammation was blocked by TLR2/4 antagonists (p = 0.05). However, the HMGB1-induced cell death was unblocked by TLR antagonists. Overall, HMGB1 endogenously mediates the signalling mechanisms of neuroinflammation through TLR2/4.
Conclusions: Whereas, the neuronal death mechanism resulting from HMGB1 could be caused by a different signalling pathway. Gaining an understanding of these mechanisms may help researchers discover new therapeutic targets for neurodegenerative diseases. (Folia Morphol 2018; 78, 1: 10–16)

Key words: toll-like receptors, immunohistochemistry, signalling pathway, high-mobility group box 1

INTRODUCTION

From series studies of the literatures, strong evidence has been established regarding the contribution of an innate immune system in regulating neurological functions, whether in health or disease [4, 18, 21, 22, 24]. This system recognises invading microorganisms using pattern-recognition receptors (PRRs) [28]. Toll-like receptors (TLRs) are the most commonly studied family of PRRs. TLR expression has been shown in various particles found inside the central nervous system, including microglia [20], astrocytes [7], oligodendrocytes [25], neurons and

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neuronal progenitor cells [29]. These proteins induce the nuclear factor-kappa B (NF-$\kappa\beta$) signalling pathway [28]. Additionally, astrocytes and microglia induce MyD88 and TRIF, thereby creating NF-$\kappa\beta$ signalling pathways throughout the TLR4-mediated pro-inflammatory environment [11].

Nuclear factor-kappa B is a transcription factor that is translocated into the nucleus and that triggers the transcription of several genes implicated in immune responses, such as the releasing of pro-inflammatory cytokines, e.g. tumour necrosis alpha and interleukin-6 (Il-6) [5]. NF-$\kappa\beta$ is a centre for regulating inflammation in all cellular types within mammal's bodies, mediated by several stimulators, such as viruses, bacterial, matrix metalloproteinases, C-reactive protein, chemokines, cytokines and adhesion molecules [5]. Its stimulation plays a main role in immune responses and cell surviving [5]. The NF-$\kappa\beta$ is formed from five dissimilar subunits including NF-$\kappa\beta$1 (p50), RelA (p65), RelB, RelC, and NF-$\kappa\beta$2 (p52) [3]. The triggered NF-$\kappa\beta$ subunits will gather to form the complexes of the homo- or hetero-dimerised transcription factor. According to the formed dimer, the cellular function proceeds despite neuronal inflammation or neurotoxicity or in the presence of neuronal defences [27].

When no infection exists, TLRs interact with host-derived endogenous molecules and induce immune responses [2, 16]. These damage-associated molecular pattern molecules (DAMPs) include the high-mobility group box 1 protein (HMGB1) [31]. There is substantial evidence to support the theory that TLR stimulation by endogenous ligands can contribute to pathology of neurodegenerative diseases [9, 16]. For example, HMGB1 is an endogenous ligand of TLR4-induced stimulation of neuroinflammation that occurs after brain trauma [15]. HMGB1 is a DNA-binding nuclear protein that is widely distributed in all cells of the vertebrae. Extra-nuclear HMGB1 is produced from inflamed necrotic cells, such as macrophages, and it behaves like a pro-inflammatory cytokine [12]. It binds with TLR2 and TLR4 [31], as well as with the receptors for advanced glycation end product (RAGE) [23], thus inducing an NF-$\kappa\beta$ signalling pathway [5].

We hypothesised that HMGB1 produced by the damaged neurons may play a key role in the neuroinflammation and neurodegeneration through activation of the NF-$\kappa\beta$ signalling pathway, specifically through RelA (p65). Using an ex-vivo rat optic nerve (RON) model, we sought to determine the effects of TLR2- and TLR4-bacterial ligands as well as HMGB1, an

endogenous ligand, on stimulation of NF-$\kappa\beta$ signalling from reactive astrocytes. We also aimed to study their neurotoxicity on RON and determine if such effects can be blocked by their antagonists.

MATERIALS AND METHODS

Animals

Wistar male rats weighing 250–300 g were sacrificed following the guidelines for animal handling as set out by the Biomedical Ethics Research Committee (Reference No. 335 l6). An ex-vivo model of RONs was performed as described in previously published studies [1, 26]. After dissection, RONs were immediately washed with an oxygen-bubbled artificial cerebrospinal fluid (aCSF) (126 mM NaCl, 3 mM KCl, 2 mM MgCl2, 2 mM NaH2PO4, 26 mM NaHCO3, 10 mM glucose, 2 mM CaCl2·4H2O). Then, each nerve was immediately placed onto 6-well tissue culture plates (SPL Life Sciences) filled with 5 mL of aCSF solution per well.

Optic nerve treatments with TLR2 and TLR4 agonists with/without their antagonist

After optic nerve dissection and isolation, the RONs were separately treated by 1 μg/mL of lipopolysaccharide (LPS) from *Escherichia coli* (TLR4 ligand) (InvivoGen, catalogue number: 13I06-MM), 10 μg/ /mL of zymosan from *Saccharomyces cerevisiae* (TLR2 ligand) (InvivoGen, catalogue number: 14L17-MM) or 1 μg/mL of HMGB1, LPS-free (an endogenous TLR2/4 ligand) (HMGBiotech, catalogue number: HM-120) in a 5 mL well of aCSF media or with aCSF alone as a control.

For TLR blocking, nerves were blocked by 10 μg/mL of LPS from *Rhodobacter sphaeroides* (TLR4 antagonist) (InvivoGen, catalogue number: 15L17-MM) or 1 μg/mL of anti-TLR2-monoclonal IgG (TLR2 antagonist) (InvivoGen, catalogue number: 10C05-MM) for at least 10 min before adding the TLR agonists (LPS, zymosan or HMGB1).

All treated RONs were incubated in an oxygenated incubator of 95% O2 and 5% CO2 at 37°C for 60 and 120 min. Immediately after the experiment, RONs were fixed by 4% paraformaldehyde/0.1 M phosphate buffer solution (PBS) for 1 h at room temperature and then rapidly frozen in Tissue Tec (Sakura) and kept at –80°C.

Immunohistochemistry

Rat optic nerves were sliced by cryostat at 15 μm thickness. Then, slides were blocked by 0.1 PBS,

10% goat serum and 0.5% Triton (PBST) for 1 h at room temperature. Primary antibodies, namely: anti-glial fibrillary acidic protein (GFAP) (BioLegend, clone number: MCA-5C10) and anti-NF-κβ p65 (BioLegend, clone number: poly6226) were added at a concentration of 1:100 for both antibodies and kept overnight at 4°C. After several washes with PBST, a mix of secondary antibodies (Alexa Fluor 488-Goat anti-mouse IgG [Abcam, clone number: ab150117] and Alexa Fluor 594-Goat anti-rabbit IgG [Abcam, clone number: ab150080]) at a concentration of 1:1000 were added for 2 h. After incubation, immunostained slides were washed several times in PBST and 0.1 M PBS, and finally placed in mounting media and covered by a coverslip. Images were captured using a fluorescence microscope (Olympus BX51, Japan), three to five randomly selected area on each immunostained slide from RONs (treated or un-treated; n = 6) were used for analysis with a double-blind producer.

Image J software was used to calculate the mean fluorescence intensity (MFI) of GFAP conjugated to Alexa Fluor 488 (green) and NF-κβ conjugated to Alexa Fluor 594 (red). Co-localisation analysis was conducted for both GFAP and NF-κβ images using Coloc 2 plugins for Image J. The rate of co-expression was interpreted using the Costes method and Pearson's correlation coefficient.

Dead cell counts

Ethidium bromide is a fluorometric labelling of deformation in cell biology that stains the nucleic acid of dead cells but is unable to stain the undamaged living cells [14, 26]. The sections of RONs, which were treated by TLR2 and TLR4 agonists and antagonists were stained by ethidium bromide (LOBA Chemie, catalogue number: 03715) with a dilution of 1:500, were prepared from 10 mg/mL stock solution and incubated at room temperature for 10 min. Then, these slides were rinsed with PBS 3 times, mounted and enclosed with a coverslip. Images were captured by a fluorescence microscope (Olympus BX51, Japan).

Image J was used blindly to count the number of dead cells (NDC) for all nerves. To calculate the ratio (%) of dead cells, the NDC of the RONs sections treated with LPS, zymosan, HMGB1, LPS with TLR4 antagonist, zymosan with TLR2 antagonist, HMGB1 with TLR4 or TLR2 antagonists were divided over the NDC of the untreated nerve.

Statistical analysis

Graph-Pad Prism software version 7.0 was used to analyse the data of MFI for GFAP and NF-κβ, as well as for the ratio (%) of dead cells. One-way analysis of variance (ANOVA; Bonferroni test) was utilised to compare the variances between the untreated and the treated RONs with different TLR agonists and antagonists. P values < 0.05 were considered significantly different.

RESULTS

### HMGB1 induces astrocytes reactivity

We aimed to determine the effects of TLR ligands on the expression levels of GFAP and NF-κβ for astrocyte reactivity and whether the inflammation was specifically co-localised into astrocytes (Fig. 1). Immunohistochemical analysis revealed that the rate of the co-localisation was higher with LPS (TLR4 agonist, 46%) and did not differ with the zymosan (TLR2 agonist, 33%) as compared to the untreated RON (33%). Surprisingly, the rate of co-localisation was highest after HMGB1 stimulation (91%) and was reversed by TLR2 (39%) and TLR4 (41%) antagonists. All images demonstrated a significant correlation with a Costes test of p value = 1. Moreover, the Pearson's image correlation coefficient was r > 0.5 for all nerves treated with TLR ligands.

HMGB1 augments the inflammation via TLR signalling

Although the ratio of co-localisation demonstrated an astrocytic reaction after TLR ligand treatment, we asked if there is a difference between TLR agonists and their antagonists. After RON was dissected and isolated, the nerves were exposed to LPS, zymosan, HMGB1, LPS with TLR4 antagonists, zymosan with TLR2 antagonist, HMGB1 either with TLR4 or TLR2 antagonists, or with aCSF alone as an untreated control, and they were incubated for 60 or 120 min. Then, the immunofluorescence intensities for GFAB and NF-κβ were measured for untreated and TLR ligand-treated nerves, as shown in Figure 2.

High-mobility group box 1 protein significantly increased expression of both GFAB and NF-$κβ$ (p = 0.05), like LPS and zymosan (p = 0.01), as compared to the untreated control. Furthermore, this augmentation was blocked by both TLR2 and TLR4 antagonists (p = 0.05). These data confirm that HMGB1 augment the inflammation from astrocytes through TLR signalling.
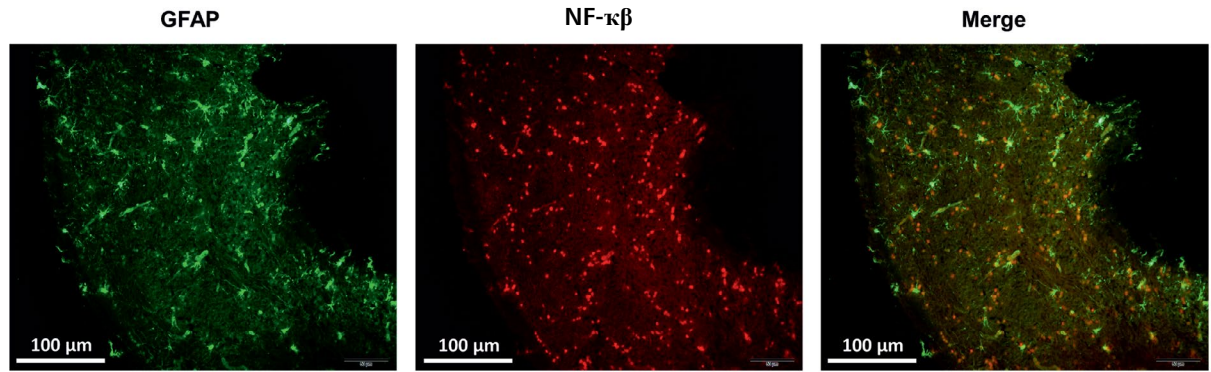
Treatments	Untreated	LPS	LPS + TLR4 antagonist	Zymosan	Zymosan + TLR2 antagonist	HMGB1	HMGB1 + TLR4 antagonist	HMGB1 + TLR4 antagonist
Rate of colocalisation	33%	46%	34%	33%	32%	91%	41%	39%
	r = 0.34	r = 0.79	r = 0.75	r = 0.83	r = 0.59	r = 0.83	r = 0.61	p = 0.89

Figure 1. Co-localisation of reactive astrocytes from rat optic nerve (RON). Representative images of double immunostaining by glial fibrillary acidic protein (GFAP; green) and nuclear factor kappa B (NF-κB; red) for RON treated with toll-like receptor agonist. Image J was used for co-localisation (merge, magenta right panels) analysis and was determined by the Costes method, for which a P value of 1 indicates 100% statistical confidence. Pearson's image correlation coefficient (r) is also represented; LPS — lipopolysacharide; TLR — toll-like receptor; HMGB1 — high-mobility group box 1
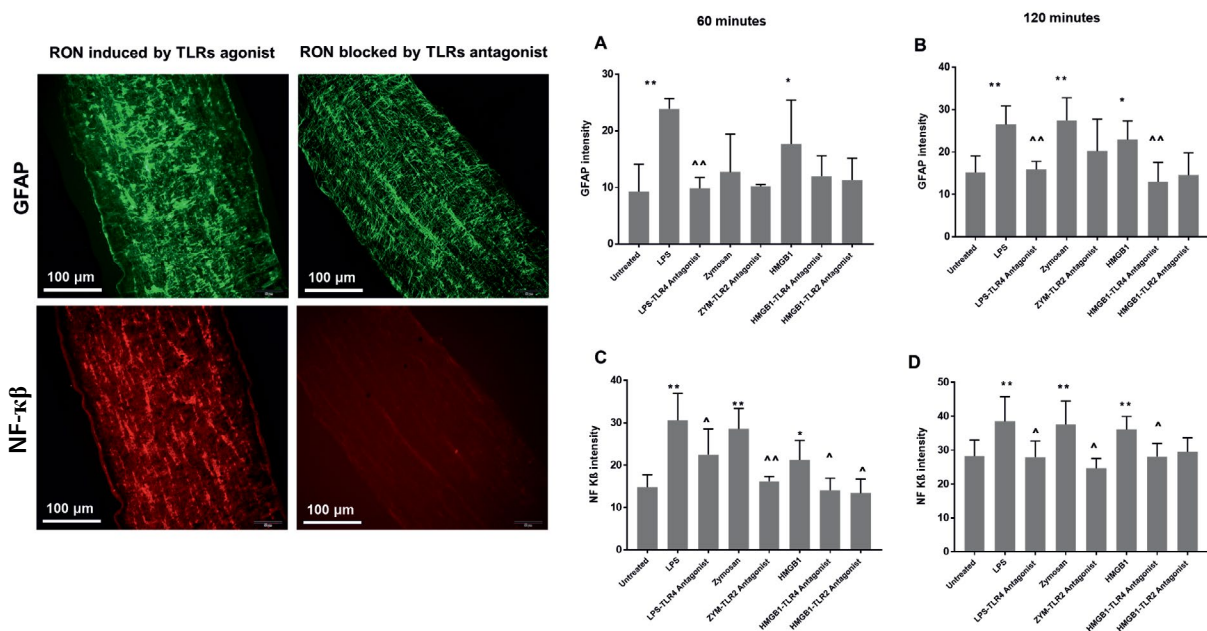
Figure 2. High-mobility group box 1 protein (HMGB1) induces the expression of inflammation. Representative images of double immunostaining by glial fibrillary acidic protein (GFAP; green) and nuclear factor kappa B (NF-κB; red) for rat optic nerve (RON) induced by toll-like receptors (TLRs) agonist or blocked by TLRs antagonist; **A, B.** Fluorescence intensity levels of GFAP; **C, D.** Fluorescence intensity levels of NF-κβ, expressed by RONs in response to lipopolysaccharides (LPS; TLR4 agonist), zymosan (ZYM) (TLR2 agonist) and HMGB1 with or without TLR2 and TLR4 antagonists, either after 60 or 120 min of incubation. Comparisons were assessed between untreated versus treated RON: *p < 0.05; **p < 0.01; or agonist versus antagonists: ^p < 0.05, ^^p < 0.01. Values were shown as mean ± SEM. Statistical significance was determined by ANOVA (n = 6).

HMGB1 facilitates neurodegeneration

We also explored if the nerves were inflamed by HMGB1 and if other TLR agonists had some neuro-degenerative tendency. Particularly, a histology of the HMGB1-treated nerve demonstrated extensive atrophic and necrotic changes (Fig. 3). Thus, we examined the number of dead cells after staining with ethidium bromide. The ratio of dead cells was signifi-
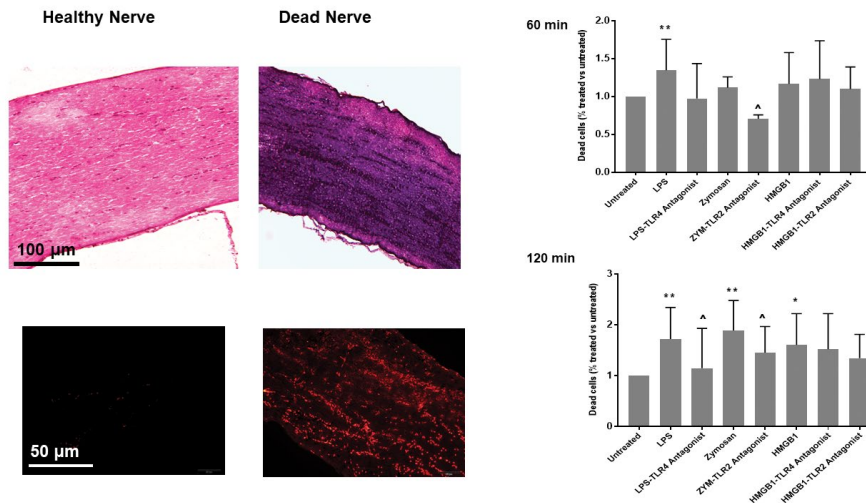
Figure 3. High-mobility group box 1 protein (HMGB1) modulates nerve death and atrophy. Microscopic histological features of rat optic nerves (H&E stain) of a healthy untreated nerve and a dead nerve treated with HMGB1 (top panels). Ethidium bromide staining to detect the number of dead cells (bottom panels). Graphic representation of the dead cells ratio (%) of lipopolysaccharides (LPS), zymosan (ZYM), HMGB1, LPS with TLR4 antagonist, zymosan with TLR2 antagonist, HMGB1 with TLR4/TLR2 antagonist. Comparisons were assessed between untreated versus treated rat optic nerve: *p < 0.05, **p < 0.01; or agonist versus antagonists: ˆp < 0.05. Values were demonstrated as mean ± SEM. Statistical significance was determined ANOVA (n = 6); TLR — toll-like receptor.

cantly higher with LPS (p = 0.01), zymosan (p = 0.01) and HMGB1 (p = 0.05) treatment when compared to untreated healthy nerves; after 120 min, but not after 60 min except for LPS (p = 0.01). Although, the TLR antagonists inhibited the LPS- and zymosan-induced nerve death, the HMGB1-induced nerve death was not reversed by TLR antagonists.

DISCUSSION

The presence of HMGB1 is considered to be a risk factor for memory impairment, chronic neurodegeneration and advanced progression of neuroinflammation [6, 9, 13, 16, 19]. Additionally, the DAMP molecule for TLR4 is known to induce sterile neuroinflammation [6, 16]. Nevertheless, no study has been done to investigate the astroglial inflammatory mechanisms resulting from the ligation of HMGB1 to TLR4 and TLR2 via mediating signalling pathways, such as the NF-κβ [27]. The results of the current study demonstrated that ex-vivo nerves treated with HMGB1 have higher expression and co-localisation of GFAP and NF-κβ as compared to the untreated control. Moreover, the HMGB1-induced inflammation was inhibited by blocking TLR activity.

Previously, a Georgia team used different research methodologies, including an animal model, human CSF and tissue cultures from patients after being exposed to traumatic brain injury (TBI), to clarify the multifaceted role of HMGB1-TLR4 ligation in pro-

moting neurovascular impairment [15]. They found that TLR4 and HMGB1 were highly co-expressed post-trauma in a patient's CSF and exuberated neuroinflammatory responses through releasing IL-6 and activation of the aquaporin-4, an astrocytic water channel [15]. Another study supporting this notion demonstrated that elevated levels of HMGB1 in the brain induce memory impairment and that this could be mediated by TLR4 on a knockout RAGE model [19]. Unfortunately, despite their utilisation of animal and human tissue cultures, they were not able to identify the exact intracellular signalling pathways that lead to releasing IL-6 such as NF-κβ. Our functional study of an ex-vivo model shows that HMGB1 could activate astrocytes via TLR4 through NF-κβ signalling, supporting previous reports that suggest the co-localisation and correlation of both TLR4 and HMGB1.

However, the results of the present study showed that the HMGB1-induced cell death was not reversed by TLR2 or TLR4 antagonists. There is considerable evidence to support the theory that trauma of the brain and spinal cord may cause abundant HMGB1 accumulation [10, 13, 15]. HMGB1, an endogenous ligand for TLR4, may cause stimulation of innate immunity after trauma [15], but another mechanism, such as RAGE, may lead to neuronal damage and death [10]. This concept is consistent with a report which demonstrated that post-trauma, HMGB1 and RAGE are highly co-expressed in both rat and human

brains [10]. Thus, the accumulated traumatic HMGB1 could contribute to the pathology of neurodegenerative diseases through different mechanism other than TLR signalling.

Furthermore, the NF-κβ dimer has diversity in its function that could promote neuroprotection, neuroinflammation or neurotoxicity [27]. The inhibition of NF-κβ to astrocytes reduces the chemokine expression and leukocyte infiltration into the injured spinal cord [8]. Altogether, this means stimulation of NF-κβ p65 through TLR4 will induce inflammation while another diversion via RAGE may lead to neuronal degeneration and/or differentiation [30]. Further exploration of these signalling pathways will clarify the therapeutic approaches that might act to block HMGB1. Actually, a recent study reported that HMGB1 inhibition in the in-vivo model of TBI showed plummeting of cerebral leukocyte recruitments and brain oedema, similar to effects of enoxaparin on neurologic recovery [17]. However, this study has a major limitation in that the scientists did not demonstrate which signalling pathway was used by enoxaparin as a target against HMGB1.

CONCLUSIONS

The result of the current study showed that HMGB1 activated NF-κβ signalling into reactive astrocytes, and this was inhibited by TLR4 and TLR2 antagonists, a result similar to LPS and zymosan ligands of TLR4 and TLR2, respectively. However, further work is needed to study the cause of the neuronal degeneration resulting from HMGB1. How HMGB1 modulates neuronal atrophy and death and whether this damage is mediated by the RAGE receptor or other receptors remain to be determined. Overall, HMGB1 is a dangerous molecule for astrocytes that will lead to their activation and inflammation and subsequent neurotoxicity. Gaining an understanding of these cellular functions may help researchers discover therapeutic targets for neurodegenerative diseases.

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