The Activation of RAGE and NF-kappaB in Nerve Biopsies of Patients with Axonal and Vasculitic Neuropathy

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ABSTRACT

Introduction: The receptor for advanced glycation end products (RAGE) is a pattern recognition receptor expressed in tissues and cells which have role in the immunity. The activation of RAGE results with the translocation of nuclear factor kappa B (NF-kB) to nucleus for the expression of proinflammatory molecules. The role of the RAGE pathway in the pathogenesis of diabetic complications is well determined. We aimed to investigate the role of RAGE pathway in axonal and vasculitic neuropathy.

Method: We immunoreacted nerve biopsy samples from 17 axonal neuropathy (AN), 11 vasculitic neuropathy (VN) and as control group 12 hereditary neuropathy with liability to pressure palsy (HNPP) patients with antibodies to NF-kB and RAGE. Subsequently, we performed double staining with the antibodies to NF-kB and RAGE in AN, VN, AN and HNPP patients.

Results: The activation of RAGE results with the translocation of nuclear factor kappa B (NF-kB) to nucleus for the expression of proinflammatory molecules. The role of the RAGE pathway in the pathogenesis of diabetic complications is well determined. We aimed to investigate the role of RAGE pathway in axonal and vasculitic neuropathy.

Conclusion: The activation of RAGE pathway predominant in CD8 (+) T cells underscores its role in VN. In AN patients, the immune reactivity to NF-kB and RAGE in macrophages may support their role in axonal degeneration without inflammatory milieu.

Key words: Vasculitic neuropathy, axonal neuropathy, RAGE, NF-kB

Conflict of interest: The authors reported no conflict of interest related to this article.
Introduction

Advanced glycation end products (AGE) are nonenzymatic additions of glucose or other saccharides to proteins, lipids and nucleotides (1,2). The receptor for AGE named receptor of advanced glycation end products (RAGE) is a pattern recognition receptor (PRR), like Toll-like receptors (TLR) which is expressed in tissues and cells that are critical for immune surveillance such as lung, liver, endothelium, monocytes, dendritic cells and neurons (2). The binding AGE with RAGE leads the translocation of nuclear factor kappa B (NF-κB) to nucleus. It regulates target genes such as cytokines (Tumor necrosis factor-α, Interleukin- 1β and 6), adhesion molecules (ICAM and VCAM-1), prothrombotic and vasoconstructive gene products, RAGE and its inhibitor IkBα (3).

The RAGE pathway plays a key role in diabetic complications including diabetic neuropathy (4,5). The activation of this pathway has been demonstrated in inflammatory neuropathies such as vasculitic neuropathy (6,7). AGE, RAGE and NF-κB has been detected in lymphocytes and macrophages, especially near the vessels both in vasculitic and diabetic neuropathy. However the role of this pathway in axonal degeneration and demyelination is still not well determined.

In this immunohistochemical study, we aimed to investigate the role of the RAGE pathway in different neuropathies. We used the nerve biopsy specimens of the patients with axonal neuropathy (AN), systemic and non-systemic vasculitic neuropathy (NSVN), and as control group hereditary neuropathy with liability to pressure palsy (HNPP).

Methods

Patients

Nerve biopsies performed in our university Neurology Department Neuromuscular Disease Laboratory between 1999 and 2010 were analyzed. We included 17 axonal neuropathy (AN) patients, 11 vasculitic neuropathy (VN) patients and 12 hereditary neuropathy with liability to pressure palsy (HNPP) who had undergone sural or superficial peroneal nerve biopsy as part of the diagnostic work-up of their neuropathy and whose nerve biopsy specimens were suitable for the immunohistochemical analysis.

Axonal neuropathy was diagnosed by electrophysiology and nerve biopsy findings with active axonal degeneration, mild fiber loss and without inflammation. Diabetes mellitus or other endocrinopathies, hereditary neuropathies (HSMN type 2 and 4), mitochondrial neuropathies, chronic alcohol abuse, vitamin deﬁciencies, neoplasia, bacterial or viral infections, drugs, intoxication, vasculitic neuropathies and metabolic causes such as a-beta lipoproteinemia, Fabry disease, uremic neuropathy were excluded. VN was diagnosed according to published criteria (8). Systemic vasculitis was diagnosed by the Chapel Hill consensus criteria (9). The patients with HNPP were diagnosed by clinical, electrophysiological and nerve biopsy findings.

Twelve sural nerve biopsies with hereditary neuropathy with liability to pressure palsy (HNPP) were included in the study.

Approval by the local ethics committee was granted and patients participated after written informed consent (01.07.2010; B.30.2.HAC.020.05.04/352).

Nerve Biopsies

The biopsies were all performed for diagnostic purposes. Seven of eleven VN patients had superficial nerve biopsy with peroneus brevis muscle biopsy whereas four patients had sural nerve biopsy. All biopsies were snap-frozen within 5 minutes of surgical intervention and stored at-80 ºC until analysis. All frozen sections were stained with hematoxylin/eosin and modified Gomori trichrome stains. Semithin (1µm) plastic sections from nerve tissue were also prepared. Ten µm frozen sections were held from each tissue for immunohistochemistry.

Immunohistochemistry

Air-dried serial 10 µm frozen sections were blocked with Histostatin-plus kit (Zymed) for 20 minutes. Then the sections were incubated with rabbit polyclonal anti-NFκB (1:50; Santa Cruz Biotechnology, sc-109) or with rabbit polyclonal anti-RAGE (1:50; Santa Cruz Biotechnology, sc-5563) antibodies for 1 hour at 37 ºC. Thereafter, a biotinylated secondary antibody against mouse IgG and an avidin-biotinylated peroxidase complex were used. The peroxidase reaction product was developed with 3,3-diaminobenzidine H2O2 (6 mg diaminobenzidine, 10 cm3PBS, and 0.01 cm33% H2O2).

Semiquantitative Analysis

Semiquantitative analysis was used to evaluate RAGE and NFκB positive cells. The sections were analyzed by two authors independently. For each specimen, the mean value of the two authors’ assessment was calculated. The values of semiquantitative assessment were defined as follows: Negative 0 (without cell), scattered positive cells + (1-2 positive cells), few positive cells ++ (3-5 positive cells), many positive cells +++ (6-10) and dense positive cells ++++ (more than ten positive cells).

Co-localization Studies

To determine the immunoreactive cells, we performed double staining with the immunofluorescence methods. Three samples from each group were selected randomly. The samples were staining with rabbit polyclonal anti-NFκB or with rabbit polyclonal anti-RAGE and with mouse monoclonal anti-CD4 (1/50; Santa Cruz Biotechnology, Inc. Sc-65544) for CD4 (+) T cells, mouse monoclonal anti-CD8 (1/50; Santa Cruz Biotechnology, Inc. Sc-70794) for CD8 (+) T cells, mouse monoclonal anti-CD68 (1/50; Santa Cruz Biotechnology, Inc. Sc-20060) for macrophages or mouse monoclonal anti-S100 (1/50; Santa Cruz Biotechnology, Inc. Sc-71993) for Schwann cells. The sections were analyzed with confocal microscope (Zeiss, Oberkochen, Germany). First T cells, macrophages and Schwann cells were counted then NFκB and RAGE positive T cells, macrophages and Schwann cells were determined and the ratio was calculated for each section staining.

Results

Patients

The group of AN consisted of 11 men and 6 women with a median age of 62 years (range 21-83). The group of VN consisted of 4 men and 7 women with a median age of 56 years (range
7-78). The group of HNPP consisted of 6 men and 6 women with a median age of 26 years (range 17-40). The mean age was 56 (7-78) in patients with VN, 59 (21-83) in patients with AN whereas 26 (16-62) in patients with HNPP. The median age of HNPP patients was lower than VN and AN patients (p<0.05).

Six of the VN patients had NSVN whereas five had systemic vasculitis. Among the systemic VN patients, two had polyarteritis nodosa (PAN), two had rheumatoid arthritis (RA) and one had Churg-Straus Syndrome (CSS).

**Immunohistochemistry**

The staining patterns of NFκB and RAGE were similar to each other.

NFκB and RAGE immunoreactivity were observed in perivascular cuff in epineurial vessels in all nerve biopsies from patients with VN except one patient. There was not any difference in staining patterns between systemic and non-systemic vasculitic neuropathy patients. NFκB and RAGE immunoreactivity was higher in patients with VN compared with AN and HNPP patients (p<0.05) (Table 1) (Figure 1).

NFκB and RAGE immunoreactive endoneurial cells were more than 10 in each fascicle in 40% of patients with VN and the endoneurial immunoreactivity to NFκB and RAGE were higher in VN patients compared to AN and HNPP patients. Although there is not any significant difference between the groups, nerve biopsies from patients with AN showed higher NFκB and RAGE immunoreactivity than HNPP patients (Table 1) (Figure 2).

**Co-localization Studies**

In VN patients 70% of NFκB and RAGE positive cells were CD8 (+) T lymphocytes whereas 30% of positive cells were macrophages (Figure 3a).

In AN patients, all NFκB and RAGE positive cells were macrophages whereas all NFκB and RAGE positive cells were Schwann cells in HNPP patients (Figure 3b, c).

<table>
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<tr>
<th>Table 1. RAGE and NFκB immunoreactivity in patients</th>
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<tr>
<td><strong>Localization</strong></td>
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<tr>
<td><strong>Staining intensity</strong></td>
</tr>
<tr>
<td>Epineural vessels</td>
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AN: Axonal neuropathy, HNPP: Hereditary neuropathy with liability to pressure palsy, NF-κB: Nuclear factor kappa B, RAGE: Receptor advanced glycation end products, VN: Vasculitic neuropathy)

* RAGE immunoreactivity in epineurial vessels was higher in VN patients compared with AN and HNPP patients (p<0.05)
‡ NFκB immunoreactivity in epineurial vessels was higher in VN patients compared with AN and HNPP patients (p<0.05)
§ RAGE immunoreactivity in endoneurial cells was higher in VN patients compared with AN and HNPP patients (p<0.05)
** NFκB immunoreactivity in endoneurial cells was higher in VN patients compared with AN and HNPP patients (p<0.05)
Discussion

RAGE pathway has been extensively studied in the context of diabetic complications and has been shown to constitute a link between hyperglycemia and microvascular damage (5). Furthermore, RAGE serves as an important pro-inflammatory receptor in vasculitis (10). The RAGE pathway has been demonstrated as a mediator in the pathogenesis of a number of inflammatory neuropathies (3,4,6,7). Thus RAGE may be a therapeutic target and its activation may have a prognostic value (11). It is therefore important to know more about the expression of NFκB and RAGE in different neuropathies.

In this study we aimed to compare vasculitic neuropathy and axonal neuropathy without an identifiable cause as both of the groups show axonal degeneration in their pathology and a demyelinating hereditary neuropathy, HNPP as control group.

We observed that NFκB and RAGE immunoreactivity were higher around epineurial vessels and endoneurial cells in nerve biopsies from patients with VN compared with AN and HNPP patients and immunoreactive cells were usually CD8 (+) T cells. Previously, Kissel et al. showed that 70% of epineurial inflammatory cells were T cells and 2/3 of them were CD8 (+) T cells in 22 VN patients (12). The increased NFκB and RAGE immunoreactivity in CD8 (+) T cells confirm the prominent role of CD8 (+) T cells. Similarly, we have recently showed that AGE and RAGE were increased in dermal endothelial cells and T-cells of NSVN and DN patients compared to controls (13). Earlier, Haslbeck et al. reported increased NFκB and RAGE immunopositivity in 70%-100% in macrophages whereas 40%-70% in CD8 (+) T cells in VN patients (7). Although we observed NFκB and RAGE immunoreactive macrophages, they were fewer compared with CD8 (+) T cells. This difference could be related to the interaction of NFκB with several transcription factors such as CBP300 which hide NFκB from immunohistochemical studies (14). The down-regulation of RAGE due to high AGE concentration might cause decreased RAGE immunoreactivity in macrophages.

We also observed that although there is not any significant difference between the groups, nerve biopsies from patients with AN showed higher endoneurial NFκB and RAGE immunoreactivity than HNPP patients and the immunoreactive cells were macrophages in AN patients. The hematoxylin -eosin sections of the nerve samples with AN did not show inflammation but macrophages which are responsible for the axonal degeneration showed NFκB and RAGE activity. This observation is not sufficient to speculate a prominent role of RAGE pathway in axonal degeneration but supports that RAGE pathways is one of the activated pathways in macrophages without need of inflammatory milieu.

Previous studies on inflammatory neuropathies and RAGE pathway used the nerve samples of patients with Charcot-Marie Tooth neuropathies (6,7). In our study we have chosen the nerve samples of HNPP patients because of reversible clinical symptoms and distinct pathological findings with the focal enlargement of myelin sheet called “tomaculae” and scattered onion-bulb structures and Schwann cells proliferation (15). We observed that NFκB and RAGE immunoreactivity were low in HNPP patients compared with VN and AN patients and the immunoreactive cells were Schwann cells. The RAGE pathway activation could be related with remyelination and Schwann cell proliferation in HNPP patients. In Schwann cells, the increase of cAMP and phosphorylation of Ser 276 of p65 subunit of NFκB A start the myelin formation (16). Moreover, the suppression of peripheral nerve regeneration with sRAGE (an inhibitor of RAGE) has been shown in mouse with sciatic nerve injury (17). Our study confirms these findings as the activation of RAGE pathway has been shown in Schwann cells of HNPP patients.

Our study has some limitations. The number of subjects was small and the immunoreactivity in patients could not be correlated with the treatment response as the samples were obtained from our tissue archive and the clinical follow-up of some patients are missing as they were only evaluated for biopsy. Our findings underscore the role of RAGE pathway in vasculitic neuropathy and show its activation in axonal degeneration and give the idea for new treatment studies.

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References


