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Reduced expression of membrane-bound (m)RAGE is a biomarker of multiple sclerosis disease progression

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ABSTRACT

Objectives: This study is one in series measuring RAGE axis (receptor for advanced glycation end products, its isoforms, and ligands) as a biomarker in multiple sclerosis (MS). We identified and quantified membrane-bound RAGE (mRAGE) expression levels on freshly isolated PBMCs and its subpopulation (monocytes and T cells), and determined the relationship between mRAGE expression levels and MS disease severity.
Materials and methods: mRAGE expression was determined for 28 MS patients and 16HCs, by flow cytometry, using fluorochrome unconjugated primary RAGE monoclonal antibody and a polyclonal secondary antibody conjugated to R-Phycoerythrin (PE).
Results: After adjusting for multiple comparisons and correcting for group differences in age and gender, MS patients showed higher percentages of mRAGE-positive on PBMCs (12.4 ± 2.1 vs. 4.08 ± 0.8, $P = 0.02$), monocytes (37.4 ± 5.8 vs. 20.1 ± 5.0, $P = 0.08$) and T cells (4.1 ± 1.2 vs. 2.1 ± 0.3, $P = 0.05$). SPMS patients' showed lower percentages of RAGE-positive monocytes (13.7 ± 5.5 vs. 49.5 ± 6.6, $P = 0.0006$) and RAGE-positive T cells (4.1 ± 1.8 vs. 6.6 ± 1.5, $P = 0.04$) than RRMS patients. We observed a negative relationship between the percentages of mRAGE-positive PBMCs and MS severity scale (MSSS) ($r = -0.39$, $P = 0.04$), monocytes and EDSS ($r = -0.48$, $P = 0.01$), monocytes and MSSS ($r = -0.58$, $P = 0.001$), and T cells and MSSS ($r = -0.40$, $P = 0.04$). Monocytes expression of mRAGE showed 0.811 area under the curve (95% CI: 0.64–0.98) sensitivity/specificity for MSSS.
Conclusion: The reduced mRAGE expression on PBMCs in general, and on monocytes in particular, can be used as biomarker of MS disease severity and progression.

1. Introduction

Multiple sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system and the most common neurological disorder of young adults. The disease is present initially with a relapsing remitting (RR) course which can progress to a secondary progressive (SP) in the majority of patients (SP) (Wingerchuk and Weinshenker, 2000). The course of the disease is unpredictable, involving multiple pathologies. As a result, more than a single biomarker is necessary to accurately diagnose the rate of disease progression.

The membrane bound receptor for advanced glycation end products (mRAGE), its many soluble isoforms, and its multiple ligands present a suitable set of inflammatory/neurodegenerative-related biomarkers (Santilli et al., 2009) with a potential to be used in MS.

mRAGE is a signal transduction transmembrane protein belonging to the immunoglobulin superfamily of the cell surface receptors (Neeper et al., 1992). The mRAGE ectodomain is constituted by one V-type followed by two C-type domains, and the N-terminal V-domain seems to be involved in ligand recognition (Yan et al., 2000). Although it was initially recognized as the receptor for advanced glycation end products (AGEs) (Kislinger et al., 1999), it is known to also bind the high-mobility group box-1 (HMGB1) (Scaffidi et al., 2002), the beta$_2$-integrin Mac-1 (Chavakis et al.,

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2003), S100/calgranulins (Jin et al., 2011), and the beta amyloid (Deane et al., 2003).

RAGE signaling leads to the activation of the proinflammatory NF-B transcription factor, and subsequent production of an array of inflammatory mediators, as well as RAGE itself. The increase in RAGE fuels further the release of RAGE ligands and denovo synthesis of NF-B, with the potential for undermining endogenous negative-feedback mechanisms (Bierhaus et al., 2001). Signaling through RAGE has been shown to play a role in chronic inflammatory disease such as diabetic arteriosclerosis (Yamamoto and Yamamoto, 2013), autoimmune disease such as rheumatoid arthritis (Sunahori et al., 2006), and neurodegenerative disease such as Alzheimer disease (Matrone et al., 2015).

We have recently shown that RAGE axis is also involved in MS. The RAGE ligands, carboxyethyllysine (CEL) (Sternberg et al., 2010) and pentosidine (Sternberg et al., 2011a), both of which belong to the class of AGEs, and HMGB1 (Sternberg et al., 2015) are upregulated in MS patients, and correlate with disease activity and/or severity.

Among the most studied RAGE isoforms are the soluble RAGE (sRAGE), the byproduct of the proteolytic cleavage and shedding of the transmembrane RAGE, and the endogenous secretory RAGE (esRAGE), which is produced by the alternative splicing of the full-length RAGE mRNA. These soluble forms are thought to regulate the activity of the mRAGE by competitive inhibition or by its displacement (Raucci et al., 2008; Kalea et al., 2009).

We have recently shown that MS patients have lower than normal sRAGE serum levels. In these patients, sRAGE serum levels inversely correlated with the expanded disability status scale (EDSS) and with the rate of clinical relapse (Sternberg et al., 2008). Similarly, esRAGE serum levels correlated with multiple sclerosis severity scale (MSSS) and biannual rate of clinical relapse. (Sternberg et al., 2014). Furthermore, MS patients treated with disease-modifying drugs (DMDs) showed significantly higher esRAGE serum levels, as compared to DMD-naïve patients (Sternberg et al., 2014), suggesting that DMDs may exert clinical effects, in part, through modulating esRAGE serum levels.

The current study used flow cytometry technique to identify and quantify mRAGE on freshly isolated PBMCs and their subpopulations (monocytes and T cells), comparing mRAGE cell surface expression between MS patients and healthy controls (HCs). In addition, we determined the relationship between mRAGE cell surface expression levels and indicators of MS disease severity.

2. Methods

2.1. Population

For the purposes of the study, a total of 28 clinically stable MS patients (7 males), with a mean age of 53.8 ± 9.8 years, EDSS of 4.03 ± 2.0, and disease duration of 16.8 ± 12 years, who were either naïve to DMDs (Interferon-beta, Glatiramer acetate, Natalizumab) or were not taking these drugs three months prior to the time of blood draw, were recruited from the Baird MS Center, Department of Neurology, Jacobs Neurological Institute, Buffalo, NY, USA.

Patients were excluded from the study if they had other autoimmune and/or inflammatory conditions, including rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, diabetes mellitus, coronary artery disease, and renal dysfunction. In addition, patients taking statins or corticosteroids were excluded since these classes of drugs are known to exert anti-inflammatory effects (Cuccurullo et al., 2006; Coutinho and Chapman, 2011). Pregnant patients were also excluded due to the fact that hormonal changes may affect RAGE expression (Germanova et al., 2010). Patients' demographic information is summarized in Table 1.

Table 1
Subjects' demographics.

Demographics	HC
Subjects (*n*)	16 (6 M)
Age (Year)	45.6 ± 12 (24–63)
	MS
Subjects (*n*)	28 (7 M)
Age (Year)	53.8 ± 9.8 (32–66)
RRMS	20
SPMS	8
EDSS	4.03 ± 2.0 (1–9)
Disease duration	16.8 ± 12 (1–42)
Rate of relapse (biannual)	0.9 ± 2.0 (0–10)

Patients were compared with 16 (6 males) healthy controls (HCs), with a mean age of 45.6 ± 12 years, who were recruited among hospital staff and nurses, with no documentation of autoimmune or cardiovascular diseases.

2.2. PBMCs isolation

Patients and HCs' PBMC were obtained by Ficoll gradient (Histopaque®-1077, Sigma–Aldrich) and washed with phosphate buffered saline (PBS), which included 0.5% BSA and 0.2% NaN_3, pH 7.4. The cell pellet was resuspended in 10 mL of the same buffer and counted using Trypan Blue dye exclusion technique. The cell number was adjusted to 2.0×10^7 cells/mL.

2.3. PBMCs immunophenotyping

Flow cytometry was used to quantify mRAGE expression on freshly isolated peripheral PBMCs and its subpopulation monocytes and T cells, using flurochrome-conjugated CD45, CD14, and CD3 antibodies respectively. Table 2A describes the antibodies that were used for the immunophenotyping study. A directly-conjugated anti-RAGE antibody was not commercially available; therefore an indirect staining procedure was adopted to quantify PBMC cell surface mRAGE expression. Therein, cells were first labeled with an unconjugated primary anti-RAGE antibody and subsequently detected with a phycoerythrin (PE)-labeled polyclonal secondary antibody preparation that recognized several different epitopes on the primary anti-RAGE antibody. Consequently, this modification resulted in amplification of the RAGE detection signal, which is expressed on leukocytes at relatively low levels.

To establish proper detector voltage settings and to perform spectral compensation calculations, a series of control samples were acquired by flow cytometry in addition to the antibody-labeled test articles, which are described in Table 2B. These controls included the following tubes (1) autofluorescence, consisting of only unlabeled PBMCs; (2) Single-color compensation control tubes containing: (2a) FITC-conjugated anti-human CD3 antibody; (2b) PE-conjugated anti-human CD8 antibody; (2c) PerCP-conjugated anti-human CD14 antibody; (2d) APC-conjugated anti-human CD45 antibody; and (3) a secondary antibody control tube containing cells stained only with secondary antibody to determine the amount of non-specific binding associated with the detection reagent.

For the purpose of the study, 50 μL of the volume-adjusted PBMC suspension (1×10^6 total cells) was aliquoted into 5-mL polystyrene round bottom tubes. Each 50-microliter aliquot of PBMCs was incubated with 25 μL of the HEPES-BSA diluted human IgG Fc receptor-blocking agent for 10–20 min on ice. Antibodies for immunophenotyping were then added directly to Fc-blocked cells, mixed by vortexing, and incubated for at least 30 additional minutes on ice while protected from light. When labeling samples for the determination of RAGE expression, the primary mouse