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MMP-9 expression is increased in B lymphocytes during multiple sclerosis exacerbation and is regulated by microRNA-320a

Latt L. Aung, M.D.¹, M. Maral Mouradian, M.D.¹, Suhayl Dhib-Jalbut, M.D.¹, and Konstantin E. Balashov, M.D., Ph.D.^{1,*}

¹Department of Neurology, Rutgers-Robert Wood Johnson Medical School, 125 Paterson Street, New Brunswick, NJ 08901

Abstract

B cells are necessary to maintain disease activity in relapsing multiple sclerosis (MS) and produce matrix metalloproteinase-9 (MMP-9), which disrupts the blood-brain barrier.

MMP-9 protein expression was increased and expression of microRNA-320a (miR-320a), which targets MMP-9 mRNA, was significantly decreased in B lymphocytes of MS patients during a disease relapse compared to remission. Functional significance of these findings was demonstrated by transfecting human B lymphocytes with miR-320a inhibitor, which led to increased MMP-9 expression and secretion.

In summary, expression of miR-320a is decreased in B cells of MS patients and may contribute to increased blood-brain barrier permeability and neurological disability.

Keywords

Multiple Sclerosis; B lymphocytes; microRNA; MMP-9; miR-320a

1. Introduction

Multiple sclerosis (MS) is an immune-mediated chronic inflammatory disease of the central nervous system (CNS) of unknown etiology. The contribution of B lymphocytes to the pathogenesis of MS is well established. The elimination of B cells with monoclonal antibodies suppresses MS activity (Bar-Or et al., 2008, Hauser et al., 2008, Kappos et al., 2011b, Naismith et al., 2010, Sorensen et al., 2014) notably without reducing immunoglobulins in the cerebrospinal fluid (CSF) (Cross and Waubant, 2011). B cells migrate across the blood-brain barrier in MS patients (von Budingen et al., 2012). The

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*Corresponding author: Konstantin E. Balashov, MD, PhD, Department of Neurology, Rutgers-Robert Wood Johnson Medical School, 125 Paterson Street, Rm 6200, New Brunswick, NJ 08901, Phone: (732) 235-7335 (office), konstantin.balashov@rutgers.edu.

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precise mechanism of B cell traffic through the blood brain barrier in MS is not known; however, matrix metalloproteinase-9 (MMP-9 (aka gelatinase B)) produced by activated B cells (Trocme et al., 1998) has emerged as one of the key pathogenic molecules which disrupts the blood-brain barrier and degrades myelin basic protein (Asahi et al., 2001, Chandler et al., 1995). B cell accumulation in the CSF correlates with acute brain inflammation and intrathecal production of MMP-9 (Kuenz et al., 2008), while drugs used to treat MS decrease the expression of MMPs (Kurzepa et al., 2005, Leppert et al., 2001). Additionally, serum MMP-9 levels are increased in MS patients during clinical relapses and in patients with active lesions on brain MRI (Lee et al., 1999). To date, the expression level of MMP-9 in B cells during MS exacerbation has not been studied.

Regulation of MMP-9 expression in B cells is complicated and includes VLA-4-mediated pathway (Redondo-Munoz et al., 2006) targeted by disease-modifying treatments in MS (Kappos et al., 2011a). As recent studies suggest that the complex polygenic susceptibility described in MS is likely to be anchored in the regulatory elements outside of protein-coding genes (Maurano et al., 2012), we decided to explore whether microRNAs (miRNAs) impact MMP-9 expression in B cells of MS patients. MicroRNAs are small, endogenous noncoding RNAs that direct posttranscriptional regulation of protein-coding gene expression by binding to partially complementary sites of target messenger RNAs (mRNAs), resulting in translation repression or mRNA deadenylation and degradation (Ambros, 2004). Different lineages of cells have unique miRNA expression profiles (Davidson-Moncada et al., 2010). miRNAs are closely involved in B cell regulation (Davidson-Moncada, Papavasiliou, 2010, Lu and Liston, 2009, O'Connell et al., 2010, Sonkoly et al., 2008). Expression of select miRNAs has been found to be impaired in B cells of patients with stable MS (Sievers et al., 2012). However, the impact of miRNAs on B cell function relevant to maintenance of MS activity is not understood.

The present study shows that MS patients during disease exacerbation have up-regulated expression of MMP-9 associated with decreased miRNA miR-320a (aka miR-320) levels in B cells. These findings implicate dysregulation of this particular miRNA in increasing MMP-9 levels leading to disruption of the blood-brain barrier.

2. Material and Methods

2.1. Human Subjects

Healthy subjects and MS patients with either relapsing-remitting MS (RRMS) (Polman et al., 2011) or clinically isolated syndrome (CIS) and evidence of demyelination on brain magnetic resonance imaging (MRI) consistent with MS (Jacobs et al., 2000) of African, European and Hispanic descent were enrolled in the study. Thirteen healthy donors (11 females and 2 males) and 21 MS patients (15 females and 6 males) participated. Human subjects were 18- to 55-year-old. Mean age of the control group was 36.2 ± 2.7 years and that of the MS patients was 35.9 ± 2.1 years. The patients were not taking disease-modifying drugs. Most patients were clinically active and had at least one clinical relapse confirmed by the presence of symptomatic contrast-enhancing lesion(s) on brain or spine MRI (except optic neuritis) within the year preceding enrollment. A clinical relapse/exacerbation was defined as described in detail by Correale et al (Correale et al., 2006) and required the

development of a new symptom, or worsening of a pre-existing symptom, confirmed on neurologic examination, lasting at least 48 hours and preceded by stability or improvement lasting at least 30 days. Blood samples from patients in relapse (aka clinical exacerbation) were taken within 1-60 days of relapse onset. Clinical remission was defined as the relapse-free period starting six months after the onset of the previous clinical relapse and is not associated with increased neurological deterioration. Supplementary table S1 summarizes the main clinical characteristics of human subjects. Exclusion criteria included any immunomodulatory treatment in the previous 3 months or steroid treatment in the month prior to the blood draw; presence of other disorders that may be associated with abnormal immune response, abnormal neurological exam or demyelinating lesions on brain MRI; pregnancy; baseline Expanded Disability Status Scale score greater than 5 (Kurtzke, 1983); and clinical signs or history suggesting infection within 2 weeks prior to the blood draw.

2.2. Study Approval

The study was approved by the Institutional Review Board and all subjects gave written informed consent.

2.3. Cell separation

Peripheral blood mononuclear cells were isolated (Balashov et al., 2010) followed by negative immunomagnetic separation of B cells (Miltenyi Biotec, kit#130-091-151) and monocytes (kit#130-091-153). Fresh B cells were used for functional assays or transfection experiments. Aliquots of B cells were resuspended in RNA cell protect solution (Qiagen) for gene expression analysis or frozen at -80°C for Western blot experiments. More than 95% of separated B cells were CD19-positive. Due to a variation in B cell separation output, not all patients provided the number of B cells necessary for all experiments presented in the manuscript. Supplementary Table S1 indicates which samples were used in each analysis.

2.4. RNA isolation

Total RNA was isolated from 1×10^6 B cells using miRCURY RNA isolation kit (Cat#300110, Exiqon) with DNase treatment (Qiagen, Valencia, CA).

2.5. mRNA quantification by RT-qPCR

cDNA was obtained after reverse transcription according to the manufacture's protocol (Cat#4387406, Applied Biosystems). PCR amplification was performed on the Applied Biosystems 7500 Real-Time PCR. The primers and probe assays (Hs00234579_m1 for MMP-9 and Hs99999909_m1 for HPRT1) were purchased from Applied Biosystems. Data analysis was performed using the formula 2^{-C_t} and normalized against HPRT1.

2.6. Western Blot

The procedure was performed as described (Balashov, Aung, 2010). Membranes were probed with antibodies specific to MMP-9 (Cat#AB19016 Millipore) and beta-actin (Cat#A5441 Sigma-Aldrich), followed by probing with secondary antibodies (anti-rabbit and anti-mouse IgG conjugated to HRP (Cat#A6154 and A4416, Sigma-Aldrich). Endogenous beta-actin was used for protein loading normalization.

2.7. miRNA profiling by microarrays

Total RNA was processed by miRCURY™ LNA Array microRNA Profiling Services (<http://www.exiqon.com/microRNA-array-profiling-services>) to assess 904 human miRNAs listed in miRBASE version 14.0 at the Sanger Institute (<http://www.mirbase.org>). Following quality control, samples were divided and labeled using either miRCURY™ Hy3 or Hy5 and hybridized on the miRCURY™ LNA Array (Exiqon, version 5th Generation arrays). The quantified signals (background corrected) were normalized, and two filtering criteria were used. A criterion for eliminating a particular miRNA from further analysis is that 3 or more of the 4 replicate measures of this miRNA were flagged by the image analysis software due to very low or extremely high signal intensity. Second, miRNAs with both Hy3 and Hy5 signals lower than 1.5X of the median signal intensity of the given slide were excluded. A list of miRNAs which were detected in at least 7 out of 10 subjects was generated.

2.8. miRNA detection by RT-qPCR

cDNA was obtained after reverse transcription according to the manufacture's protocol (Cat#203300, Exiqon). RT-qPCR amplification was performed with miRCURY LNA SYBR Green master mix (Cat#203450, Exiqon) and specific microRNA LNA™ PCR primer sets (Exiqon) using iCycler iQ Real-Time detection System (Bio-Rad Laboratories). Control reference miRNAs (hsa-miR-101, hsa-miR-19b, and hsa-miR-720) were selected based on their stable expression in miRNA arrays by NormFinder software. All RT-qPCR experiments were done in triplicates, and data analysis was performed using the formula 2^{-C_t} and normalized against the mean of three control reference miRNAs.

2.9. miR-320a detection in B cells by in-situ hybridization

miRNA in-situ hybridization was done using B cells from a healthy donor and QuantiGene ViewRNA miRNA ISH Cell Assay (Cat# QVCM0001, Affymetrix) according to the manufacturer's instructions. A probe set for miR-320a (VM1-10346-01) was designed by Affymetrix. As a negative control, human scramble-miR (Cat# VM1-10338-01, Affymetrix) was used. After in-situ hybridization, cells were blocked with 10% goat serum for 30 minutes at room temperature and incubated with rabbit anti-human CD20 Ab (IgG) (Cat#PA5-16701, Thermo Fisher) or isotype control rabbit IgG (Cat# SC-2027, Santa Cruz Biotechnology) overnight at 4°C. After washing with PBS three times, cells were incubated with Alexa Fluor 488 conjugated with Goat anti-Rabbit IgG (Cat# A11008, Life Technologies) for one hour at room temperature. Cells were washed with PBS and mounted on glass slide. Images were acquired using Leica TCS SP5 confocal microscope.

2.10. Cell transfection/modulation of miRNA expression

B cells were transfected with 100 nM of anti-miR-320a (Cat#MH11621, Ambion) or negative control anti-miR miRNA inhibitors #1 (Cat#4464076) using TransIT-TKO transfection reagent (Cat#MIR2154, Mirus Bio) for 48 hours according to manufacturer's instructions. Cellular MMP-9 expression was assessed by Western blotting. The concentration of MMP-9 and IL-6 in culture supernatants was measured by ELISA (cat#DY911 and DY206, respectively, R&D systems). Transfection efficiency was

evaluated using Dy547-labeled miRIDIAN microRNA mimic transfection control (cat#CP-004500-01-05, Thermo Scientific). Flow cytometry analysis showed that 85% of B cells were transfected.

2.11. Statistical analysis

Two tailed unpaired *t* tests (Figures 1 and 2) or paired *t* tests (Figure 3) were applied using GraphPad Prism 4 software package. Data are presented as Means $\pm$ SEM. Not all assessments were obtained on all the samples.

3. Results

First, we studied MMP-9 mRNA and protein expression in B cells of study subjects. There was no significant difference in MMP-9 mRNA expression between MS patients during a clinical relapse (0.0549 ± 0.0194 , *n*=6) and patients in remission (0.0610 ± 0.0266 , *n*=7). However, MMP-9 protein expression was significantly higher in MS patients during a clinical relapse (0.4354 ± 0.0843 , *n*=6) compared to patients in remission (0.2295 ± 0.0347 , *n*=7), *p* < 0.05 (Figure 1, Supplementary Figure S1). Compared to healthy subjects (0.0025 ± 0.0011 , *n*=7), MMP-9 mRNA expression in a group of MS patients (combining those during relapse and those in remission) was higher (0.0581 ± 0.0163 , *n*=13), *p* < 0.05. In addition, MMP-9 protein expression was significantly higher in MS patients during a clinical relapse (0.4354 ± 0.0843 , *n*=6) compared to healthy subjects (0.1919 ± 0.0398 , *n*=6), *p* < 0.05.

We hypothesized that increased MMP-9 protein expression in B cells of patients during a clinical relapse might be linked to its regulation by miRNAs. Several miRNAs directly target MMP-9 mRNA and regulate its expression. These miRNAs include miR-212 and miR-132 expressed in mammary stroma (Ucar et al., 2010), miR-491-5p expressed in glioma cells (Yan et al., 2011) and miR-320a (aka miR-320) expressed in fibroblasts (Bronisz et al., 2012). To study whether these miRNAs are expressed in human B cells in healthy subjects and MS patients, we employed miRNAs microarrays and tested B cells isolated from five untreated clinically active MS patients and five control healthy donors. Out of 904 miRNAs assessed, only 109 miRNAs including miR-320a were detected based on filtering criteria described in Materials and Methods (Supplementary Table S2). MiR-212, miR-132 and miR-491-5p were not detected in B cells. As additional confirmation, we applied in situ hybridization which revealed that miR-320a probe was co-localized in CD20-positive B cells (Supplementary Figure S2).

To determine whether the expression of miR-320a is dysregulated during disease activity, we compared MS patients in clinical relapse and in remission using RT-qPCR as described in Material and Methods. On average, relative expression of miR-320a was decreased 10 fold in B cells of patients with MS during a clinical relapse (0.0191 ± 0.0080 , *n*=6) compared to those in remission (0.1922 ± 0.0720 , *n*=7), *p* < 0.05 (Figure 2). In addition, miR-320a expression was significantly decreased in MS patients during a clinical relapse (0.0191 ± 0.0080 , *n*=6) compared to healthy subjects (0.1387 ± 0.0332 , *n*=8), *p* < 0.05.

To demonstrate the physiological significance of miR-320a in human B cells, we studied whether decreased expression of endogenous miR-320a is mechanistically linked to increased expression of its pro-inflammatory target molecule MMP-9. B cells from healthy donors were transfected with specific inhibitor to miR-320a (anti-miR-320a) or negative control miRNA inhibitor (anti-miR control) followed by analysis of MMP-9 protein expression in cells and its secretion in the culture medium. Treatment with anti-miR-320a led to significant upregulation of cellular MMP-9 protein in B cells (Figure 3A) as well as increased spontaneous secretion (Figure 3B). The secretion of the control cytokine reflecting B cell activation, IL-6, was not affected by anti-miR-320a (Figure 3C).

4. Discussion

The contribution of B cells and their products to the pathogenesis of MS is well established. B cells migrate across the blood-brain barrier in patients with MS (von Budingen, Kuo, 2012), expand clonally, produce oligoclonal bands, and populate the cerebrospinal fluid (CSF), brain parenchyma and meningeal lymphoid follicles (Baranzini et al., 1999, Kuenz, Lutterotti, 2008, Lovato et al., 2011, Obermeier et al., 2011, Qin et al., 1998). Activated B cells serve as antigen-presenting cells, regulate T cell activation in MS, and produce inflammatory cytokines (reviewed in (Racke, 2008)). The elimination of B cells with anti-CD20 monoclonal antibodies suppresses disease activity in patients with RRMS (Bar-Or, Calabresi, 2008, Hauser, Waubant, 2008, Kappos, Li, 2011b, Naismith, Piccio, 2010, Sorensen, Lisby, 2014). However, the mechanisms of increased B cell traffic to the CNS and regulation of disease activity by B cells in MS are poorly understood.

MMP-9 is expressed in B cells of MS patients (Bar-Or et al., 2003) and is a validated target of miR-320a/miR-320 (Bronisz, Godlewski, 2012). MMP-9 is able to disrupt the blood-brain barrier and to digest myelin basic protein (Asahi, Wang, 2001, Chandler, Coates, 1995). Its concentration is increased in serum and CSF of MS patients, especially in those with active disease (Fainardi et al., 2006, Kuenz, Lutterotti, 2008), while drugs used to treat MS decrease the expression of MMPs (Kurzepa, Bartosik-Psujek, 2005, Leppert, Lindberg, 2001). MMPs exist in both membrane-anchored and secreted forms (Bauvois, 2012). Therefore, increased expression and secretion of MMP-9 by B cells may contribute to increased blood-brain barrier permeability, B cell traffic to the CNS, myelin destruction and neurological disability.

Here we demonstrate that B cells express MMP-9 and miR-320a in both healthy donors and MS patients. Importantly, we observe down-regulation of miR-320/320a and reciprocal up-regulation of its target molecule, MMP-9, in B cells of patients with MS during a disease relapse. In our study, MMP-9 expression at the mRNA level was not significantly different in B cells of patients during a relapse vs. those in remission. However, MMP-9 protein expression was increased in B cells of MS patients during a clinical relapse compared to patients in remission. This differential regulation of MMP-9 at the mRNA and protein levels is consistent with the notion that miRNAs may cause translation repression and altered protein levels without affecting degradation of select target mRNAs (Ambros, 2004). Studies on miRNAs and MMP-9 expression in B cells of MS patients are limited and have not addressed the expression of miR-320a or MMP-9 in patients during a clinical relapse

(Bar-Or, Nuttall, 2003, Lindberg et al., 2010, Sievers, Meira, 2012). It should be noted that MMP-9 is not the only MMP able to disrupt the blood-brain barrier. Other leukocytes, e.g., T cells and macrophages are able to produce MMP-9 (Oviedo-Orta et al., 2008).

The physiological relevance of our findings were validated by demonstrating that down-regulating endogenous miR-320a expression in B cells using a specific inhibitor leads to up-regulation of MMP-9 protein levels in cells as well as its increased spontaneous secretion. In contrast, the secretion of IL-6, which is a cytokine reflecting B cell activation but is not specifically targeted by miR-320a, was not affected. The factors that affect miR-320a expression during a disease relapse remain to be understood.

In summary, our findings indicate that B cells of MS patients during a disease relapse have decreased expression of miR-320/320a that leads to increased MMP-9 expression and secretion. This may contribute to increased B cell traffic to the CNS, myelin destruction and neurological disability during disease exacerbation. Therefore, miR-320a and the molecules it regulates can be novel biomarkers of disease activity and targets for therapeutic interventions in MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

CIS	clinically isolated syndrome
CNS	central nervous system
CSF	cerebrospinal fluid
miRNA	microRNA
MMP-9	matrix metalloproteinase-9
MS	multiple sclerosis
RRMS	relapsing-remitting multiple sclerosis

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Highlights

We examined MMP-9 expression in B cells of patients with multiple sclerosis (MS).

MMP-9 protein expression is increased in B cells during a disease relapse.

Expression of microRNA-320a is decreased in B cells during a disease relapse.

Endogenous microRNA-320a inhibits MMP-9 protein expression and MMP-9 secretion.

MicroRNA-320a may regulate disease activity in MS patients.

Figure 1. MMP-9 protein expression is increased in MS patients during a clinical relapse
 B cells were separated from MS patients during a clinical relapse (MS Relapse, n=6, mean age = 37.7 ± 5.0 years, five females and one male) and remission (MS Remission, n=7, mean age = 38.4 ± 4.3 years, six females and one male). Relative MMP-9 protein expression in cells was determined by Western blot. MMP-9 protein expression was increased in MS patients during a clinical relapse (0.4354 ± 0.0843) compared to patients in remission (0.2295 ± 0.0347), $p < 0.05$.

Figure 2. miR-320a expression is decreased in MS patients during a clinical relapse

B cells were separated from MS patients during a clinical relapse (MS Relapse, n=6, mean age 33.2 ± 2.8 years, four females and two males) and remission (MS Remission, n=7, mean age = 34.4 ± 3.3 years, four females and three males). The relative expression of miR-320a (aka 320) was determined by qRT-PCR. miR-320a was significantly less abundant in B cells of patients with MS during a clinical relapse (0.0191 ± 0.0080) compared to those in remission (0.1922 ± 0.0719), $p < 0.05$.

Figure 3. MiR-320a regulates MMP-9 expression

B cells from healthy donors were transfected with miRNA inhibitor specific to miR-320a (anti-miR-320a) or negative control miRNA inhibitor (control). A: Intracellular MMP-9 protein in transfected cells was determined by Western blot, and the results from three experiments with B cells from different subjects were averaged. B: MMP-9 secretion was determined by ELISA in the supernatants of transfected cells, and the results from six experiments with B cell media from different subjects were averaged. C: IL-6 secretion was determined by ELISA in the supernatants of transfected B cells, and the results from four experiments with B cell media from different subjects were averaged.