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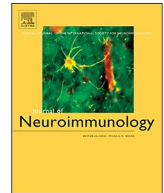

Short Communication

Rivaroxaban ameliorates disease course in an animal model of multiple sclerosis

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ABSTRACT

Recent studies have implicated an important role for coagulation factors in neuroinflammatory disorders like multiple sclerosis (MS). Here, we investigate the role of factor X (FX) in neuroinflammation by using rivaroxaban the selective inhibitor of activated FX (FXa) in experimental autoimmune encephalomyelitis (EAE, an animal model of MS). Rivaroxaban-treated rats were less susceptible to EAE compared to the untreated control group. This finding was accompanied by reduced T-cell infiltration and microglia activation. Our study identifies FX as a possible target in neuroinflammatory diseases. As FXa inhibitors are approved for other disorders, FXa blockade could serve as a fast available medication.

1. Background

Inflammation and demyelination are pathophysiological hallmarks of neuroinflammatory disorders like multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE).

While the triggers of MS have not yet been identified, recent studies using animal models indicate a narrow connection between the coagulation cascade and inflammatory processes in MS (Göbel et al. 2016a,b; Ryu et al. 2015). In particular, depositions of plasmatic coagulation factors such as fibrinogen and factor XII (FXII) have been described in MS lesions (Ryu et al. 2015; Göbel et al. 2016b). Another important player in the coagulation cascade is factor X (FX), which is also known to be involved in inflammatory disorders like inflammatory bowel disease. Moreover, individuals suffering from MS show enhanced levels of prothrombin and factor X (FX) in plasma (Göbel et al. 2016a).

However, the extent to which modulation of FX activity can serve as a therapeutic strategy in MS has not been examined so far. Within this study, we wanted to clarify if the inhibition of FX might play a role in autoimmunity in the central nervous system (CNS). Rivaroxaban is an oral, direct and highly selective inhibitor of activated FX (FXa) that is used for the prevention and treatment of thromboembolic disorders. Therefore, we treated myelin basic protein (MBP)-immunized Lewis rats with rivaroxaban and investigated its effects on the disease course and inflammatory processes.

2. Materials and methods

2.1. Induction and evaluation of EAE

All animal experiments were approved by and performed in accordance with the German laws and local regulations for animal welfare of North Rhine-Westphalia (84-02.04.2013.A142). EAE was induced in 10–12 week old female Lewis rats with 80 μg MBP peptide 68-86 (AnaSpec, Inc., USA) emulsified in 100 μl complete Freund's adjuvant (Sigma-Aldrich, Germany) containing 100 μg *Mycobacterium tuberculosis* (strain H37 Ra; Becton, Dickinson and Company, USA). Pharmacological modulation was achieved using rivaroxaban (Bayer Pharma AG, Germany). Rats were subjected to a rivaroxaban-containing diet (2.1 mg/kg body weight) starting 7 days prior to immunization. Control (Ctrl) animals received regular food. All animals were kept under standard conditions and had access to water and food ad libitum. The clinical course of EAE was monitored daily by blinded observers using the following scoring system: 0 no signs; 1 partial loss of tail tonus; 2 complete loss of tail tonus; 3 moderate paraparesis; 4 progressed paraparesis; 5 complete paraparesis; 6 paraplegia of one hind limb; 7 complete paraplegia; 8 tetraplegia; 9 moribund; 10 death.

2.2. Histology and immunohistochemistry

At disease maximum (d_{max}), spinal cords were removed, embedded

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in Tissue-Tek OCT compound (Sakura, Germany), and lumbar regions were cut into 10-μm-thin sections. Hematoxylin and eosin (HE) staining was performed according to standard procedures (Göbel et al. 2016b). For immunohistochemistry, sections were blocked for at least 1 h with PBS containing 10% bovine serum albumin (BSA) and 5% normal goat or rabbit serum and incubated with primary antibody at 4 °C overnight. Primary antibodies were used for cluster of differentiation (CD) 3 (clone SP7, Abcam, UK), ionized calcium-binding adapter molecule 1 (Iba-1, Wako Chemicals GmbH, Germany) and fibrinogen (Abcam, UK). The following secondary antibodies were used: biotinylated goat anti-rabbit immunoglobulin G (IgG; Jackson Immuno Research, USA) and biotinylated rabbit anti-sheep IgG (Vector Laboratories, Inc., USA). 3,3′-Diaminobenzidine (DAB) staining was performed afterwards using a standard protocol (Cerina et al. 2017). Stainings were examined by microscopy (Keyence BZ-9000, Japan) in a blinded fashion and analyzed with the AxioVision LE software (Zeiss, Germany). Inflammatory areas (HE) and numbers of CD3 cells or DAB positive pixels were determined using ImageJ software on three randomly selected complete spinal cord cross sections from the lumbar level of six to nine animals per group.

2.3. Flow cytometry

For detection of cell-surface markers and intranuclear staining by flow cytometry, single-cell suspensions from lymph nodes and spleens of Ctrl and rivaroxaban-treated animals were prepared 10 days (d_{10}) after immunization. Cells were stained for 30 min at 4 °C with appropriate combinations of indicated fluorescently labeled monoclonal antibodies in PBS containing 0.1% NaN_3 and 0.1% BSA. The following antibodies were used for the detection of cell-surface markers: CD3 (clone 1F4, Biolegend, USA), CD8a (clone G28, Biolegend), CD4 (clone W3/25, Biolegend), CD11b/c (clone Ox-42, Biolegend) and CD25 (clone OX-39, BD Pharmingen, USA). For intranuclear forkhead box P3 (Foxp3; clone FJK-16s, eBioscience, USA) staining, cells were fixed/permeabilized after cell-specific surface staining with the Foxp3 Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Stained cell suspensions were assayed on a multi-color flow cytometer (Gallios, Beckman Coulter, Germany) using the Kaluza software (Beckman Coulter).

2.4. Statistical analysis

EAE data were analyzed by the two-way analysis of variance (ANOVA). For histological data as well as flow cytometric analysis the Mann-Whitney rank-sum test was used. Statistical analyses were performed using the GraphPad Prism 6 software (GraphPad, USA).

3. Results

3.1. Rivaroxaban protects against neuroinflammation

To evaluate the possible effect of rivaroxaban, we used $MBP_{68–86}$-immunized EAE rats. Untreated Lewis rats developed monophasic EAE mimicking a spontaneously remitting MS relapse in individuals suffering from relapsing-remitting MS (Fig. 1a). Treatment with rivaroxaban was associated with a reduced maximum disease severity (Ctrl: score 6.38 ± 0.50 vs. rivaroxaban: 3.86 ± 0.99; $p < 0.05$), while disease onset (Ctrl: day 10.91 ± 0.20 vs. rivaroxaban: day 11.86 ± 0.30; Fig. 1b) and body weight development (weight change in % at d_{max}: Ctrl: 96.04 ± 2.076 vs. rivaroxaban: 101.7 ± 4.962; Fig. 1c) were unaltered.

3.2. Rivaroxaban leads to reduced immune-cell infiltration into the CNS

Next, we evaluated the amount of immune cells that infiltrated the CNS of EAE animals at d_{max}. Histopathological results from lumbar spinal cord sections of EAE animals revealed significant effects on CNS inflammatory processes. HE staining showed reduced cell infiltration in rivaroxaban-treated animals compared to Ctrl (0.70% ± 0.07 vs. 1.2% ± 0.17; $p < 0.05$; Fig. 1d). In a next set of experiments, we characterized immune-cell subtypes. Interestingly, besides reduced $CD3^+$ T-cell infiltration (Ctrl: 185.20 ± 21.68 vs. rivaroxaban: 87.24 ± 15.50; $p < 0.05$; Fig. 1d), the number of activated microglia (Ctrl: 13.15 ± 1.70 vs. rivaroxaban: 5.47 ± 0.26; $p < 0.05$; Fig. 1d) was also significantly reduced.

As activation of FX usually leads to fibrin formation, we analyzed the extent of fibrin(-ogen) depositions in the CNS of EAE rats. As expected, the amount of fibrin(-ogen) was significantly decreased in rivaroxaban-treated animals compared to Ctrl (5.23 ± 1.07 vs. 10.38 ± 1.20; $p < 0.05$; Fig. 1d).

3.3. Rivaroxaban has no influence on immune-cell distribution in the course of EAE

Lastly, we examined the distribution of $CD4^+$ T cells, $CD8^+$ T cells (Fig. 1e), activated $CD4^+$ T cells ($CD4^+CD3^+CD25^+$) and regulatory $CD4^+$ T cells ($CD4^+CD3^+FoxP3^+$) at d_{10} in draining lymph nodes of control and rivaroxaban-treated rats by flow cytometry (Fig. 1f) to analyze whether functional alterations in the peripheral immune compartment could contribute to the EAE-protective phenotype. However, analysis revealed virtually no difference between T-cell subsets in both groups of animals.

4. Discussion

In this study, we could show that FXa inhibition is a potential therapeutic strategy to avoid inflammatory cell invasion during autoimmune CNS disease. This finding was accompanied by reduced microglial activation and fibrin(-ogen) deposition in vivo.

Experimental and clinical evidence suggests that inflammation and coagulation are intimately connected processes in neuroinflammation (Göbel et al. 2016b; Ryu et al. 2015). While the participation of immune cells in coagulation has been recognized as a major pathogenic risk factor in neurovascular diseases like stroke (Kleinschnitz et al. 2013), recent experimental findings have also identified a direct role of clotting proteases in intracellular signal transduction and modulation of inflammatory cell responses (Ryu et al. 2015; Göbel et al. 2016b). In the CNS, depositions of fibrinogen or FXII fostered inflammation and subsequent plaque formation (Adams et al. 2007; Göbel et al. 2016b). This concept of an important role of coagulation factors in neuroinflammation was further reinforced by the recent identification of increased coagulation levels in plasma of individuals suffering from MS (Göbel et al. 2016a). In this context, besides fibrinogen, prothrombin and FXII, FX has also gained interest as significantly higher levels could be revealed in relapsing-remitting and secondary-progressive MS patients (Göbel et al. 2016a).

Apart from its function as a protease, it is known that FXa can activate PAR2, a G-protein coupled receptor. A critical role of FXa signaling through PAR2 was described earlier in other autoimmune diseases like inflammatory bowel disease (Borensztajn et al. 2009) or in local edema formation (Cirino et al. 1997). FXa induces different pro-inflammatory responses and can lead to the release of pro-inflammatory cytokines by lymphocytes (Scaldaferri et al. 2011).

Interestingly, from a translational perspective, blocking FXa in rats with rivaroxaban resulted in a beneficial effect on disease severity. However, since FXa inhibition has anticoagulant properties and interferes with hemostasis, there is a considerable risk of inducing bleeding complications when using this compound. Notably, we never observed any major hemorrhages in the organs of rivaroxaban-treated animals subjected to EAE (not shown).

Taken together, our study identifies FXa as a potential target in MS and EAE and might represent a novel therapeutic approach to combat

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