The role of the Syk tyrosine-kinase in immune complex-mediated autoimmune inflammation

PhD thesis-book

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1. Introduction

Rheumatoid arthritis is a chronic and systemic autoimmune disesase, which has around 1 % global prevalence. The clinical symptoms in most patients start with pain, oedema and swelling of the joints on the hands and feet, which can develop into symmetric polyarthritis. During the disease the synovial membrane, which includes few layers of cells becomes thick and the synovial fluid can be increased in the synovial cavity. In the inflamed joints, increased immune cell (e.g. neutrophil, synovial fibroblast) recruitment and high concentration of pro-inflammatory cytokines and chemokines (CXCL2, CCL3) can be detected.

There are many therapeutic options in the treatment of the disease, like conventional synthetic disease-modifying antirheumatic drugs, glucocorticoids, biological disease-modifying antirheumatic therapies or Janus kinase inhibitors. In a significant proportion (approximately 5-20 %) of patients sustained remission is not achievable, so there is a need to investigate the pathogenesis in more details and to find new drugs and drug targets.

Many immune and non-immune cell types can take part in the development and maintenance of the disease. Synovial fibroblasts, which are located in the synovial membrane can release matrix metalloproteinases, inflammatory mediators that

can damage the extracellular matrix or they can stimulate the immune cell recruitment in the site of inflammation.

Besides synovial fibroblasts, neutrophils can also take part in the initiation and in the maintenance of the autoimmune inflammation. In rheumatoid arthritis, an increased number of neutrophils can be detected in the synovial fluid under inflammatory conditions. On their cell surface, they have many receptors, like Fc receptors, integrins or chemokine and cytokine receptors, which can sense the inflammatory signals. Triggered by the stimulus, neutrophils are able to leave the peripheral blood and reach the affected tissue, where they can exert their effector responses (like pro-inflammatory mediator production, respiratory burst or neutrophil extracellular trap formation). Neutrophil extracellular trap components can directly damage the endothelium or as an autoantigen, it can increase the autoantibody production. Under inflammatory conditions, immune complexes can stimulate neutrophil degranulation by their Fcγ receptors.

In autoimmune arthritis, cell surface molecules (like Fcγ receptors and β2-integrins) are important mediators. During the disease, Fc receptors can make interactions with immune complexes, which trigger the inflammatory responses. In previous studies, it has been described, that the absence of the

Fc receptor γ-chain, resulted in a total protection from the development of autoantibody-induced experimental arthritis. Moreover, the lack of Fc receptor γ-chain from neutrophils prevented the mice from arthritis. In line with these results, the lack of Fcγ receptor III showed a moderate protection from the development of joint inflammation. In an experimental model of arthritis the absence of Fcγ receptor IV showed a protection from arthritis. These results with further observations indicate that Fcγ receptors have an important role in autoimmune arthritis.

β2-integrins are also critical cell surface molecules on neutrophils, which can take part in autoimmune arthritis. In rheumatoid arthritis, an increased expression of β2-integrin ligands on synovial endothelial cells can be detected, which can trigger the influx of immune cells in the inflamed tissues. It has been shown, that the absence of the β2-integrin subunit CD18, the arthritis was not observed and also in CD11a-deficiency mice the severity of oedema significantly decreased.

A shared point between the Fcγ receptors and β2-integrins that both of them use the Syk tyrosine-kinase for their signal transduction. It has been described that the absence of Syk from the hematopoietic system resulted in a total protection from the development of experimental autoimmune arthritis. Moreover,

the neutrophil-specific Syk deletion massively protected mice from the development of autoimmune arthritis. These mentioned results indicate that neutrophils, FcγRs, β2-integrins and Syk play a critical role in autoimmune arthritis.

According to these findings we wanted to test what is the effect of selective Syk inhibition on the development of experimental arthritis. Entospletinib is a second generation Syk-selective inhibitor, which is an orally available blocker with a tolerable safety profile according to hematological clinical trials in patients with chronic lymphoid leukemia. In our experiments we investigated the effect of the Syk-selective entospletinib in the experimental K/BxN serum transfer arthritis model, where neutrophils, Fc receptors and and β2-integrins are crucial.

2. Objectives

During our experiments, we were interested in the following questions:

1) How pharmacological inhibition of Syk influences the development of autoantibody-induced experimental arthritis?

2) How entospletinib affects the leukocyte infiltration to the joints and the in vivo pro-inflammatory mediator production?

3) What is the effect of Syk inhibition on Fcγ receptor- and β2-integrin-mediated effector cell responses of mouse neutrophils?

4) How Syk-selective inhibitors act on the immune complex-triggered cell functions of human neutrophils?

3. Methods

Experimental animals

T cell-receptor heterozygous transgene (KRN) animals were mated with wild type (C57BL/6) mice, while non obese diabetic (NOD) mice were purchased from Jackson Laboratory. Mice were kept in individually ventilated cages in conventional animal facility. All animal experiments were approved by the Animal Experimentation Review Board of Semmelweis University.

K/BxN serum transfer arthritis

KRN mice were mated with NOD mice to perform transgene-positive (arthritic) K/BxN and transgene-negative (non-arthritic) BxN serum. Transgene positivity tested with the help of allele-specific PCR. Arthritic and non-arthritic serum prepared by a retro-orbital blood collection. Arthritis was induced by a single intraperitoneal injection of 300 μl K/BxN arthritic serum. BxN serum was used as control. The severity of joint inflammation measured by ankle thickness measurement and clinical scoring.

Oral administration of vehicle and the inhibitor

The selective Syk inhibitor entospletinib was diluted in mucilage and water, while vehicle contained only mucilage and water. The mice were administered orally twice a day and the

experiment begun with a pre-treatment one day before the arthritis induction.

Flow cytometry analysis

In the vehicle- or entospletinib-treated mice the circulating neutrophil and monocyte numbers were identified with the help of Ly-6G-PE and anti-CD11b antibodies on the basis of forward and side scatter characteristics by flow cytometry. Ly-6G- and CD11b-positive cells were determined as neutrophils, while Ly-6G-negative CD11b-positive cells were monocytes.

The infiltrated local neutrophil and macrophage numbers were also identified by their Ly-6G and CD11b expression pattern. During our flow cytometric analysis, the isolated bone marrow neutrophils were identified by an anti-Ly-6G-PE antibody, while their Fcγ receptor expression was followed by anti-Fcγ receptor II/III and anti-Fcγ receptor IV antibodies. The expression of some critical β2-integrin components on neutrophils were detected by anti-CD18, anti-CD11a, anti-CD11b antibodies. Antibodies were visualized by PerCP-Streptavidin if they were biotin-conjugated or in case of anti-Fcγ receptor II/III a secondary FITC-conjugated antibody was used.

In vitro measurement of synovial fibroblast numbers and activation

Arthritic limbs were digested (with Liberase) and in the digested limbs, the local synovial fibroblast numbers were identified with the help of a specific cell surface marker (by an anti-CD90.2-PE antibody). The activation status of the cells were detected by an anti-MHC Class-II-FITC antibody, while the intracellular tyrosine- phosphorylation was followed by an anti-phosphotyrosine antibody after fixation and permeabilization steps with flow cytometry.

In vitro analysis of neutrophil cell responses

Mouse neutrophils were isolated from femurs and tibias, then the red blood cells were lysed with a hypotonic buffer. Neutrophils were isolated by Percoll gradient centrifugation. Human neutrophils were prepared from peripheral blood from healthy volunteers, plasma was separated and neutrophils were isolated by Ficoll centrifugation. Red blood cells were lysed by a hypotonic buffer.

To investigate the in vitro neutrophil cell responses, we blocked Nunc MaxiSorp F96 plates with immobilized immune complexes or integrin ligands, while the functionality of mouse neutrophils was tested by a Protein kinase C activator, by Phorbol 12-myristate 13-acetate (PMA). After an incubation step, we measured the in vitro neutrophil cell responses in the

presence of the vehicle or the Syk selective inhibitors (entospletinib or lanraplenib). We tested the superoxide production with a cytochrome c-reduction assay, the cell spreading by phase contrast microscopy, while the cytokine production was followed by commercial ELISA kits.

Quantification of pro-inflammatory mediator levels in the joint

Joints were washed out with PBS supplemented 10 mM EDTA and 20 mM HEPES and the pro-inflammatory mediator concentrations were measured by commercial ELISA kits.

Statistical analysis

Experiments were repeated for the indicated number of times. We used STATISTICA program for statistical analysis. Two- or three way ANOVA was used (where the inhibitor, the arthritic serum-treatment or the in vitro stimulation ± time were the independent variables). Diagrams and curves show mean and SEMs. For kinetic measurements we used area under the curve dimension. P-values below 0.05 were statistically significant.

4. Results

The Syk-selective inhibitor entospletinib decreased the macroscopic signs of autoantibody-induced experimental arthritis

The oral administration of the lower (50 mg/kg) dose of entospletinib moderately reduced the macroscopic signs of experimental joint inflammation, while the higher (100 mg/kg) dose of the inhibitor significantly reduced the severity of the oedema. While the inhibitor could dose-dependetly reduce the autoantibody-induced experimental arthritis, the cell counts in the bone marrow, in the peripherial blood or in the synovial area did not decrease under non-inflammatory conditions (Ref. I.).

The effect of entospletinib on the local cell numbers

In the pathogenesis of autoimmune arthritis, many cell types are involved. We tested the effect of entospletinib on cell infiltration or proliferation in the synovium. Entospletinib significantly reduced the local neutrophil recruitment in both entospletinib concentrations, while the local macrophage and synovial fibroblast numbers did not reduce under inflammatory conditions. We also measured the activation status of the cells. In the inflammatory microenvironment, the synovial fibroblast MHC II expression increased, while a higher dose of entospletinib significantly decreased it. However, when we tested the effect of the inhibitor on the intracellular tyrosine

phosphorylation, the inhibitor did not reduce it compared to the vehicle-treated samples.

To sum it up, we saw that entospletinib did not reduce the accumulation of local macrophages or the proliferation of synovial fibroblasts, while the local neutrophil counts significantly decreased in the arthritic environment. Our results suggest that mainly the neutrophil-dependent mechanisms can stand behind the macroscopic phenotype (Ref. I.).

Pro-inflammatory cytokine levels in the synovial area

Pro-inflammatory cytokines are important mediators of autoimmune arthritis. In our experiments, we tested the synovial concentrations of CCL3, CXCL2 and IL-1β. We saw that entospletinib-treatment significantly decreased the synovial levels of these mediators compared to the vehicle-treated samples (Ref. I.).

Immune complex-mediated cell responses of neutrophils

In the next step, we wanted to validate our in vivo observations, so we detected the Fcγ receptor-mediated neutrophil cell responses in the presence of entospletinib. We found that entospletinib did not reduce the expression of Fcγ receptor II/III and Fcγ receptor IV. Meanwhile, the inhibitor reduced the immune complex-triggered neutrophil superoxide release, cell spreading and cytokine production in a dose-

dependent manner compared to the vehicle-treatment (without affecting neutrophil functionality) (Ref. I. and Ref. II.).

Integrin-dependent neutrophil cell responses in the presence of entospletinib

As we previously described, integrins are important in the development of autoimmune arthritis, therefore we tested how entospletinib affected the cell surface expression of integrin α and β chains on mouse neutrophils. We saw that the inhibitor did not reduce the expression of these components. Meanwhile, on a fibrinogen surface in the presence of TNF-α, the investigated superoxide production, cell spreading and cytokine release were reduced in the presence of entospletinib. These results support the theory that entospletinib may be able to decrease the β2-integrin-mediated effector functions under in vivo conditions (Ref. I.).

We think that entospletinib achieves the inhibitory effect, when neutrophils are stimulated through their Fcγ receptors or β2-integrins at the site of inflammation.

The effect of Syk-selective inhibitors on human neutrophil cell responses

We wanted to strengthen the translational aspects of our experiments, therefore we followed how entospletinib influenced the cell responses of human neutrophils. During our

in vitro experiments, the immune complex activated human neutrophils released high amounts of superoxide and spread over the surface, while entospletinib-treatment significantly decreased it (Ref. II.).

Besides entospletinib, we also tested the effect of another Syk-selective inhibitor, lanraplenib. We found that lanraplenib reduced the immune complex-triggered superoxide production and cell spreading of human granulocytes compared to vehicle-treatment (Ref. II.).

These in vitro results with our in vivo observations raised the possibility that Syk could be a potential therapeutic target in the treatment of autoimmune arthritis and both Syk-selective blockers (entospletinib or lanraplenib) could be therapeutic options.

5. Conclusions

Our new findings are the followings:

1) The oral administration of the Syk-selective inhibitor entospletinib effectively reduced the severity of a neutrophil-, Fc receptor- and integrin-dependent autoantibody-mediated experimental arthritis.

2) We also noted that entospletinib effectively reduced the recruitment of neutrophils to the joints and also lowered the levels of pro-inflammatory mediators at the site of inflammation upon arthritis induction.

3) We also found that under in vitro conditions, the selective Syk inhibition dose-dependently decreased the investigated cell responses of isolated mouse neutrophils, while the maturation, the Fcγ receptor and the β2-integrin expression was not altered.

4) Selective Syk-inhibition by entospletinib and lanraplenib decreased the immune complex-mediated human neutrophil superoxide release and cell spreading.

6. Bibliography of the candidate's publications

The results were based on the following publications:[1]

I. **Káposztás E**, Balogh L, Mócsai A, Kemecsei É, Jakus Z, Németh T. The selective inhibition of the Syk tyrosine kinase ameliorates experimental autoimmune arthritis. Front. Immunol. 2023;14:1279155, Impact factor: 5.7

II. Németh T, Balogh L, **Káposztás E**, Szilveszter KP, Mócsai A. Neutrophil-Specific Syk Expression Is Crucial for Skin Disease in Experimental Epidermolysis Bullosa Acquisita. J Invest. Dermatol. 2023;143(7):1147-56, Impact factor: 5.9

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[1] *Káposztás Eszter is the maiden name of Kálmán Eszter.*