Src-family tyrosine kinases are required for the development of autoantibody-induced inflammatory diseases

PhD Theses

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INTRODUCTION

The varoius autoimmune diseases are a great burden on society and the health care system affecting a considerable portion of human population and without a proper treatment for each and every case, which calls for the research of new therapeutic approaches.

Autoantibody production and immune complex deposition in the tissues are characteristic features of many autoimmune diseases and are the major underlying cause of tissue damage initiating immune responses which normally target invading pathogens. This improper activation leads to sterile inflammation, tissue damage, pain and other clinical symptoms of the disease.

The research on autoimmune diseases has been relying heavily on various in vivo animal models. K/BxN serum transfer arthritis and the autoantibody-dependent blistering skin disease model frequent human diseases like rheumatoid arthritis, bullous pemphigoid or epidermolysis bullosa acquisita. A common pathomechanistic feature is the abundance and activation of myeloid cells at lesion sites requiring Fcγ receptors for immune complex recognition and β_2 integrins for the leukocyte migration into the site of inflammation.

Src-family tyrosine kinases were first identified as cellular counterparts of viral oncogens leading to the discovery of protooncogens. Their best known and so far most important role is the regulation of cell survival and proliferation but they mediate cell surface integrin signaling and cell adhesion as well. Most important to us is their critical role in leukocyte β1 and β2 integrin signaling. Based on their role in tumor progression they have become prime targets of antitumor therapy research.

Src-family kinases being critical in β_2 integrin signaling and β_2 integrins being vital for the development of autoimmune inflammatory diseases, in my PhD work we set out to investigate the possible role of Src-family kinases in autoantibody-induced inflammatory disease models.

AIMS

In my PhD work we aimed to investigate the following questions:

1. Do Src-family kinases Hck, Fgr and Lyn have any role in the development autoantibody-induced arthritis?
2. Of Hck, Fgr and Lyn which is or which are important for the effect?
3. In which cell type are SFKs required?
4. What are the underlying pathophysiological features? Are SFKs required for
    a. the inflammatory leukocyte invasion to the leasion?
    b. the cell-autonomous endogenous migratory ability of myeloid cells?
    c. the development of a proper inflammatory milieu?
5. Which known signaling pathway is affected in the absence of SFKs during autoantibody-induced arthritis?

To investigate these questions, in vivo mouse models of human autoimmune diseases and genetically modified mouse strains were used.

MATERIALS AND METHODS

Mice

Genetically modified mouse strains. Hck^{tm1Hev} ($Hck^{-/-}$), Fgr^{tm1Hev} ($Fgr^{-/-}$), Lyn^{tm1Sor} ($Lyn^{-/-}$), $Itgb2^{tm2Bay}$ ($CD18^{-/-}$), $Itgal^{tm1Hogg}$ ($CD11a^{-/-}$), $Itgam^{tm1Myd}$ ($CD11b^{-/-}$), $Fcer1g^{tm1Rav}$ ($FcR\gamma^{-/-}$), Mcl-1^{flox}LysM-Cre (Mcl-$1^{\Delta myeloid}$) and Tg(TcraR28,TcrbR28)KRNDim (KRN) mouse strains were used on the C57Bl/6 genetic background. The strains were maintained almost exclusively in homozygous form. Allele-specific PCR was used for genotyping. C57Bl/6 mice were used as wild type control. All animal experiments were approved by the institutional ethics review board of Semmelweis University and the University of Pécs.

Bone marrow chimeras. Bone marrow chimeras were created by transplanting CD45.2-expressing whole donor bone marrow by intravenous injection into lethally irradiated CD45.1-expressing wild type recipients. Rate of repopulation and the efficacy of the bone marrow transfer was evaluated using flow cytometry. Mixed bone marrow chimeras were generated for *in vivo* competitive migration assays as described below.

K/B×N serum transfer arthritis

The K/BxN serum transfer arthritis model was triggered by intraperitoneal injection of autoantibody-containing or control serum (300-400 µl per mouse) into wild type and mutant mice followed by daily clinical scoring, ankle thickness measurement and assessment of the articular function by a wire hanging test.

Autoantibody-induced skin blistering model

The murine model of the human autoantibody-induced blistering skin disease epidermolysis bullosa acquisita was triggered by systemic administration of antibodies against collagen VII.

In vivo infiltration

Hematoxylin-eosin-stained ankle sections was used for histological analysis. For quantitative analysis 4 days after arthritis induction ankle and front paw areas of mice were flushed to collect synovial fluid. It was then stained with fluorescent antibodies and

analysed with flow cytometry to count $Ly6G^+$ neutrophils and $Ly6G^-F4/80^+$ or $Ly6G^-CD11b^+$ monocytes/macrophages.

In vivo migration

Bone marrow from CD45.2 allele-expressing wild type, $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$, $CD18^{-/-}$ or $FcR\gamma^{-/-}$ donors was mixed with bone marrow from CD45.1 allele-expressing wild types at varying ratio and trasnplanted into lethally irradiated wild type recipients. To assess cell-autonomous in vivo migratory capacity flow cytometry was used to determine the ratio of CD45.2-positive myeloid cells in the peripheral blood and synovium of the mixed chimeras 4 days after arthritis induction.

In vitro analysis of neutrophils and monocytes/macrophages

Isolation. Primary bone marrow neutrophils were isolated with PerColl gradient centrifugation and monocytes with magnetically labeled antibodies. The cell were used immediately.

In vitro cell responses. ELISA plates were coated with human serum albumin,(HSA), blocked and treated with anti-HSA IgG to prepare immune complex surface. Neutrophils were incubated on this surface for 1 or 6 hours at 37°C and cell-free supernatant was subjected to ELISA analysis of inflammatory mediators. Superoxide production of neutrophils on immune complex surface was measured using photometry. Area under the curve was used for statistical analysis. Transwell system was used to measure in vitro migration of neutrophils and monocytes. After 1 hour of incubation the amount of transmigrated cells was measured using microscopy and an acidic phosphatase assay. Neutrophil adhesion to immune complex or ICAM-1 surface was determined after 1 hour incubation at 37°C by phase-contrast microscopy and an acidic phosphatase assay.

Analysis of inflammatory mediators

Cell free supernatants of synovial fluid and in vitro stimulated neutrophils were subjected to IL-1β, KC, MCP-1, MIP-1α, MIP-2, and LTB_4 ELISA measurements using commercially available kits.

Presentation of the data and statistical analysis

Experiments were performed the indicated number of times. Quantitative graphs show mean and SEM from all independent in vitro experiments or from all individual mice

from the indicated number of experiments. In case of kinetic assays, the area under the curve (AUC) calculated after subtraction of the zero time point values has been used for further calculations and analyses. For the determination of statistical significance, the response of each genotype was calculated by subtracting the control values from that of the stimulated samples, followed by normalization to the WT response within each experiment. A two-tailed Student's t test with unequal variance was then performed on all normalized samples to determine whether the response in a given mutant strain was statistically different from that in the WT samples (indicated by p-values in the text). P-values <0.05 were considered statistically significant.

RESULTS

Hck, Fgr, and/or Lyn are required for autoantibody-induced arthritis

First, we investigated whether Src-family kinases Hck, Fgr and Lyn have any role in the development of autoantibody-induced arthritis. Autoantibody administration triggered a robust arthritis in wild type mice with classical clinical signs, ankle thickening and loss of articular function. Genetic deficiency of Hck, Fgr, and Lyn, however, completely protected mice from all signs arthritis. Bone marrow chimeric mice lacking Src-family kinases only in their hematopoietic system were also completely protected. On the other hand, chimeras generated by transplanting wild type cells into $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ recipients showed a normal disease course similar to wild type controls.

Overlapping role of Hck, Fgr, and Lyn during autoantibody-induced arthritis

Hck, Fgr, or Lyn single deficiency did not affect arthritis development in our model. $Hck^{-/-}Fgr^{-/-}$ and $Fgr^{-/-}Lyn^{-/-}$ double knockout chimeras showed no protection either. The $Hck^{-/-}Lyn^{-/-}$ double mutation caused substantial but incomplete reduction of arthritis development; however, the poor overall health status of these chimeras may have affected their response in our model. Importantly, complete protection from arthritis development was only seen in the absence of all three kinases, indicating significant functional overlap between Hck, Fgr, and Lyn during autoantibody-induced inflammation.

Neutrophil-exclusive lack of Hck, Fgr and Lyn is sufficient for complete protection against arthritis

As we expected, neutrophil-deficient $Mcl\text{-}1^{\Delta myeloid}$ mice were completely protected against arthritis. The presence of wild type hematopoietic tissues in addition to neutrophil-deficient bone marrow restored arthritis development. The presence of $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ cells, however, did not; indicating a critical role of Src-family kinases in neutrophils during arthritis development.

Myeloid cells fail to accumulate at the site of inflammation

Autoantibody-induced arthritis triggered robust leukocytic infiltration of the synovial area of wild type but not $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ mice. Flow cytometric analysis revealed a dramatic increase of neutrophil numbers in the synovial tissue of arthritic serum-treated

wild type mice that was entirely dependent on CD18. Importantly, the $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ mutation also abrogated neutrophil infiltration at the synovial area. Analysis of a skin blistering model epidermolysis bullosa acquisita yielded similar results, myeloid infiltration of the skin was observed in wild type but not in Src family kinase-deficient animals.

No cell-autonomous migration defect in Hck−/−Fgr−/−Lyn−/− myeloid cells

The previous results suggest that the lack of Hck, Fgr, and Lyn lead to β_2 integrin-dependent migration defect. To reveal whether the defective leukocyte recruitment in $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ mice is due to such a cell-autonomous migration defect, we directly compared the accumulation of wild type and mutant cells within the same animal. To this end, we generated mixed bone marrow chimeras and subjected them to arthritis. As expected, CD18-deficient neutrophils and monocytes/macrophages failed to enter the synovium. Surprisingly $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ myeloid cells could migrate to the lesion site as well as wild types. *In vitro* migration of neutrophils and monocytes toward major proinflammatory chemoattractants does not depend on Hck, Fgr, or Lyn irrespective of the requirement for β_2 integrins in the given assay

Defective generation of the inflammatory environment

The apparent contradiction between the lack of a myeloid accumulation *in vivo* but normal cell-autonomous migratory capacity of myeloid cells both *in vivo* and *in vitro* in $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ knockouts could be explained by a role for myeloid Src family kinases in the generation of the inflammatory environment. In wild type mice high levels of IL-1β, KC, MCP-1, MIP-1α, MIP-2, and LTB_4 were found which were absent in Src family kinase-deficient animals.

Src-family kinases are required for neutrophils to respond to immune complexes

Immune complex recognition is a vital step for *in vivo* arthritis development, therefore we tested the ability of neutrophils to respond to them *in vitro*. Wild type neutrophils produce superoxide and various inflammatory mediators (the very same that were found *in vivo* in the inflammatory environment) on immune complex surface, Src-family kinase-deficient neutrophils, however, do not. Adhesion to immune complex and ICAM-1 surface was also blocked by Src-family kinase deficiency.

FcRγ deficiency recapitulates the phenotypes of Hck−/−Fgr−/−Lyn−/− mutants

Similarly to $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ mice, Fc-receptor-γ-chain knockout ($FcR\gamma^{-/-}$) mice (which lack all activating Fcγ receptors) are completely protected from autoantibody-induced arthritis and have a complete defect of myeloid cell accumulation at lesion sites. Cell-autonomous migratory capacity of myeloid cells however is normal both *in vivo* and *in vitro*. Like Src-family kinases FcRγ-chain is also required for the generation of a proper inflammatory environment *in vivo*. *In vitro* $FcR\gamma^{-/-}$ neutrophils fail to produce superoxide and inflammatory mediators. The presence of wild type hematopoietic tissues in addition to $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ restored arthritis development, whereas the presence of $FcR\gamma^{-/-}$ hematopoietic tissues did not.

LFA-1 is the relevant β_2 integrin *in vivo* and Mac-1 *in vitro*

In agreement with prior reports $CD18^{-/-}$ mice were protected from arthritis. In $Mac\text{-}1^{-/-}$ animals the arthritis is somewhat more severe than in wild types. $LFA\text{-}1^{-/-}$ mice are greatly but (contrary to previous reports) not completely protected. Cell-autonomous migratory capacity of $Mac\text{-}1^{-/-}$ myeloid cells is even higher than wild types, LFA-1–/– myeloid cells failed to enter the synovium. In in vitro neutrophil responses on immune complex surface, however, Mac-1 is the dominant β2 integrin. Lack of CD18 or Mac-1 decreased superoxide and inflammatory mediator production but the lack of LFA-1 did not.

CONCLUSIONS

1. Hematopoietic expression of the Src-family kinases Hck, Fgr, and Lyn is required for the development of autoantibody-induced arthritis.
2. Hck, Fgr and Lyn have an overlapping role in arthritis development.
3. Neutrophil-exclusive lack of Hck, Fgr and Lyn is sufficient for complete protection against arthritis.
4. Src-family kinases are required for the myeloid cells to accumulate at the site of inflammation.
5. Cell-autonomous endogenous migratory capacity of $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ myeloid cells is normal both *in vivo* and *in vitro*.
6. In the absence of Hck, Fgr and Lyn the generation of the inflammatory environment is severely impaired.
7. Src-family kinases are required for the in vitro neutrophil superoxide production, adhesion and inflammatory mediator production on immune complex surface.
8. FcRγ deficiency recapitulates the phenotypes of $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ mutants. $FcR\gamma^{-/-}$ mice are protected from arthritis, lack tissue accumulation of myeloid cells in spite of their endogenous migratory capacity being normal. Similar to Src-family kinases FcRγ-chain is critical for the generation of a proper inflammatory environment *in vivo* and the immune complex-dependent neutrophil responses *in vitro*.
9. $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ and $FcR\gamma^{-/-}$ myeloid cells cannot compensate each other in arthritis development *in vivo*.
10. *In vivo* phenotypes of the loss of β_2 integrins are dependent on LFA-1, *in vitro* phenotypes on Mac-1.

PUBLICATIONS

Publications relevant to the theses

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