Role of interleukin-24 in the pathomechanism of inflammatory bowel disease

PhD Thesis

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1. Introduction

The two main manifestations of inflammatory bowel disease (IBD) are Crohn's disease (CD) and ulcerative colitis (UC). The exact pathogenesis of IBD - as in the cases of chronic, lifelong diseases - is not known exactly, but our knowledge is constantly expanding, and we get to know more and more predisposing factors. It is considered a multifactorial disease, so genetic, immunological and environmental factors play a role in the development of the disease.While IBD was a rare disease in the early 20th century, its incidence has increased rapidly since the middle of the 20th century, and nowadays, these changes have become even stronger. The main symptoms are almost the same in children and adults. In children, the disease usually progresses more rapidly and it is associated with more extensive and severe intestinal involvement. Because of the longer course of the disease due to the young age and the more severe symptoms, the IBD in childhood cause a greater public health problem. In summary, studies of IBD in childhood, the research of additional factors contributing to its development, a more accurate understanding of the pathomechanism, and the provision of effective causal therapy are of paramount importance.

Under physiological conditions, bacteria and their products do not pass through the intestinal wall. However, various environmental and genetic factors impair the function of the intestinal mucosal barrier, loosen the tight gaps between epithelial cells, making it permeable to microbial antigens, and triggering chronic inflammation and tissue remodeling by activating lamina propria immune cells.

Fibroblasts play a prominent role in tissue remodelling caused by chronic inflammation.There are several theories about their origin in the intestinal tract, but the most likely origin is from dormant subepithelial fibroblasts, the activation of which is induced by various profibrotic (TGF-β, PDGF) factors.

Due to the increased ability of activated fibroblasts to proliferate and migrate, they multiply at the site of injury, producing large amounts of extracellular matrix (ECM) components in an attempt to restore the degraded tissue structure.The ECM is a dynamically changing structure in each organ, characterized by continuous, controlled reconstruction.
Its components undergo a constant change in quantity and quality in order to maintain their physiological tissue structure. During chronic inflammation, this physiological balance is disrupted, and scar tissue develops, leading to a decrease or loss of organ function. Scarring has serious complications such as thickening of the intestinal wall, narrowing of the lumen, intestinal obstruction, perforation, and fistula formation, which in most cases is an indication for surgery. More than 70% of people with Crohn's disease undergo surgery at least once in their lives. My research focuses on IL-24, a member of the IL-20 cytokine subfamily and the IL-10 cytokine family, which plays a significant role in the regulation of the natural immune response and the maintenance of epithelial cell integrity. The IL-20 subfamily includes IL-19, IL-20, IL-22, IL-24, and IL-26. The target cells and biological functions of the subfamily cytokines are similar, derived mainly from myeloid and lymphoid immune cells, and their receptors are predominantly expressed on epithelial cells. IL-24 can also bind to the IL-20RA / IL-20RB and IL-22RA1 / IL-20RB heterodimeric receptors. Increased IL-24 has been shown in association with inflammatory, tissue rearrangement disorders such as inflammatory bowel disease, psoriasis, or rheumatoid arthritis. However, little is known about the role of IL-24 in IBD. Previously, Andoh A. and Fonseca-Camarillo G. et al. showed an increased expression of IL-24 in intestinal biopsy samples from adult patients with IBD.According to studies by Andoh A. et al., IL-24 increases the production of mucin-1, -3, and -4 in colonic epithelial cells, suggesting an anti-inflammatory effect of IL-24. Adenoviral overexpression of IL-24 induces apoptosis in a number of tumor cell lines, overexpressing reduces the proliferation of keloid-derived fibroblasts, and increasing its expression in the skin during wound healing, suggesting that IL-24 is involved in tissue rearrangement during wound healing.Our research

team identified IL-24 as one of the most highly expressed molecules in an animal model of renal fibrosis.The role of IL-24 in tissue remodelling is highly hypothesized, but the exact mechanism is still unknown.

2. Objectives

The exact biological role of IL-24 in IBD and in tissue remodelling is still largely unknown, so we aimed to investigate it in vivo and in vitro.
We were looking for answers to the following questions:

- How does the expression of IL-24 cytokine change in the colonic mucosa of children in untreated inflammatory bowel disease and in DSS-induced colitis compared to control groups?
- On which cells of the IL-24 receptor, IL-20RB, is expressed in human and mouse colonic mucosa?
- What effect do inflammatory factors known in the pathomechanism of IBD have on the expression of IL-24 on immune cells?
- What effect does IL-24 have on the expression of a profibrotic molecule in intestinal epithelial cells?
- What effect does the IL-24 molecule have on the migration of intestinal fibroblast cells and the expression of extracellular components?
- What is the effect of direct IL-24 treatment on the expression of mouse colonic mucosal profibrotic molecule and extracellular matrix components?
- How does the lack of Il20rb affect the symptoms and the amount of extracellular matrix deposited during DSS-induced colitis?

3. Methods

3.1. IBD patient population and control group

The children included in the study were newly diagnosed, untreated patients. In the case of control children we performed an endoscopic examination as part of their gastroenterological examination due to chronic abdominal pain, diarrhea, or suspicion of polyposis. The biopsy samples were negative in each control case.

3.1.2. Colon biopsy samples

Samples used in Western blot analysis were obtained from the colonic mucosa. We collected 10 control samples. Biopsies were taken from macroscopically non-inflamed (CD non-inflamed, n = 7) and inflamed (CD inflamed, n = 7) mucosa in children with Crohn's disease, and from inflamed mucosa (UC, n = 7) in ulcerative colitis.

3.1.3. Serum samples

Blood samples were taken from 15 control children and 12 children with Crohn's disease for the ELISA examination.

3.2. Animal model and treatment protocols

The experiments were performed on 7-8 week old male wild-type (WT) C57B1 / 6J and IL20rb knockout (KO) mice, respectively.

3.2.1. Intracolonal IL-24 injection

The procedure described by Boni et al. was used to inject recombinant IL-24 into the colon wall on the antimesenterial side 30 mm from the anus. Control animals were injected only with vehicle. 6-6 mice were included in both treatment groups.

3.2.2. DSS-induced colitis in a mouse model

During DSS (dextran sodium sulfate) -induced colitis, both WT (n = 6) and *Il20rb* KO mice (n = 6) were allowed to drink 2.5% (w / v) DSS water until day 7 of the experiment, then they were given clean water for 12 days.

The control groups (WT, n = 6; Il20rb KO, n = 6) received only pure drinking water for 19 days. Disease activity index (DAI) and body weight of the animals were determined during DSS treatment.

3.3. Cell lines

In our experiments, we examined HT-29 colon epithelial, HK-2 renal tubule epithelial cells, CCD-18Co fibroblast cell line, circulating peripheral blood mononuclear cells, and lamina propria mononuclear cells.

3.3.1. HT-29 colon and HK-2 proximal tubule epithelial cell culture and treatments

. In our experiments, the HT-29 colon epithelial cell line and human proximal tubular epithelial HK-2 cells were treated with recombinant human IL-24 and control cells with vehicle. The duration of treatment was 24 hours, then annexin / PI staining was performed, and RNA was isolated from the cells.

3.3.2. CCD-18Co colon fibroblast cell culture and their treatment

CCD-18Co human colon fibroblast cells were treated with recombinant IL-24, TGF-β1 and PDGF-B (n = 6 / group) for 24 hours. Control cells were treated only with vehicle. Cells treated as described above, then annexin / PI staining, RT-PCR, MTT and SiriusRed assays were performed.

3.3.2. Peripheral circulating mononuclear cells (PBMC)

From the peripheral blood, mononuclear cells were obtained from a healthy 5-year-old boy, who was examined in our department. Each group (n = 6 / group) was treated with recombinant IL-1β, LPS, recombinant TNF-α, recombinant TGF-β1, recombinant IL-17, and H2O2 (hydrogen peroxide). Control cells were treated with vehicle. From cells treated as described above mRNA expression was measured.

3.3.4. Lamina propria mononuclear cells (LPMC)

Lamina propria mononuclear cells were obtained from control, wild-type mice and then each group (n = 6 / group) were treated with recombinant IL-1β, LPS, recombinant TNF-α, recombinant TGF-β1, recombinant IL-17, and H2O2, respectively. Control cells were treated with vehicle. From cells treated as described above mRNA expression was measured.

3.4. Annexin V / propidium iodide (PI) staining

The extent of apoptosis was examined with FITC Annexin V Apoptosis Detection Kit I. Viable (Annexin V and PI negative), early apoptotic (Annexin V positive), late apoptotic and dead cells (Annexin V and PI positive) were identified by their staining.

3.5. Fibroblast migration assay

CCD-18 Co cells were plated in a 96-well plate containing a non-toxic agarose gel barrier and then treated with recombinant IL-24 or vehicle.The size of the remaining cell-free areas in the middle of each well was determined.

3.6. MTT cell proliferation assay

Cell Proliferation Kit I was used for our MTT cell proliferation assay.
The absorbance of the dissolved dye was measured at 570 nm using a Hidex Chameleon Microplate Reader (Triathler, Plate Chameleon, 300SL Lablogic Systems, Inc. Brandon, FL, USA) using MikroWin software.

3.7. SiriusRed kollagén detekciós eljárás
SiriusRed collagen detection procedure

CCD-18Co cells were fixed, washed, and then incubated in Sirius Red dye solution. Unbound dye was removed. An amount of bound dye proportional to the collagen produced by the cells was eluted, and its absorbance was measured using a Hidex Chameleon Microplate Reader with MikroWin software.

3.8. Lactate dehydrogenase (LDH) cytotoxicity assay

Lactate dehydrogenase is a cytoplasmic enzyme found in all cells that enters the intercellular space following toxic effects on the cells. By measuring the enzyme activity of LDH, the extent of the toxic effect on the cells can be estimated.

3.9. Immunofluorescent staining

The tissue and intracellular (HT-29, HK-2, CCD-18Co) localization of α-SMA and IL-20RB were examined by immunofluorescence staining. Hoechst 33342 dye was used for DNA detection (seed staining). Staining was examined with a Nikon C2 confocal laser scanning microscope.

3.10. RNA isolation, cDNA synthesis and PCR

During the measurements, RNA was isolated from the tissues and cells, and complementary DNA (cDNA) was synthesized from the RNA. Real-time RT-PCR measurements were performed on a LightCycler 480 automaton. Primers specific for the genes to be tested were designed based on the sequences in the NCBI (National Center for Biotechnology Information) nucleotide database. The mRNA expression of the different target molecules was determined as the quotient of the glycerol – aldehyde – 3 – phosphate dehydrogenase (GAPDH) household gene according to the formula $x = 2\text{-}\Delta Cp$.

3.11. Enzyme-linked antibody test (ELISA)

Human serum IL-24 levels were determined by sandwich ELISA.

3.12.Flow cytometry (FACS)

HT -29 and HK-2 cells were permeabilized and incubated with anti-TGF-β1 and anti-PDGF-B primary antibody, followed by secondary antibody. Samples used as negative controls were labeled with secondary antibody only. Analysis was performed on a BD FACSAriaTM. Living cells were delimited by standard deviation in size and granularity. Results were analyzed using BD FACSDiva Software.

3.13. Protein isolation and Western blot

A vizsgált humán és egér colon biopsziás mintákat homogenizáltuk, centrifugáltuk, majd a felülúszó összfehérje koncentrációját spektrofotometriás módszer segítségével határoztuk meg. The human and mouse colon biopsy samples were homogenized, centrifuged, and the total protein concentration of the supernatant was determined spectrophotometrically. Protein samples were denatured, electrophoresed, and the separated proteins were blotted onto a nitrocellulose membrane. Nonspecific binding sites and interactions were inhibited, and membranes were incubated with IL-24, α-SMA, fibronectin, pro-collagen-1α2, and GAPDH-specific primary antibodies, followed by secondary antibodies. GAPDH was used as an internal control. Immunoreactive bands were visualized using the chemiluminescent Western blot detection protocol, and the resulting images were visualized with VersaDoc 5000MP and analyzed using Quantity One v4.6.9 and ImageJ 1.48v software. Finally, the results were expressed as relative optical densities.

3.14. Statistical analysis

Data were analyzed using GraphPad Prism 6.01 software.

The normal distribution of the data sets was determined using the Kolmogorow-Smirnov test. In the case of a normal distribution, the difference between the groups was determined by a 2-sample unpaired t-test for two groups and by a one-way ANOVA test for more than two groups. If the condition of the normal distribution was not met, the Mann-Whitney-U- test was used for two groups and the Kruskall-Wallis test for several groups. Multiple comparisons of data from MTT, LDH cytotoxicity, migration assay, and SiriusRed staining were performed using two-way ANOVA and multiple-t test. Significant differences were indicated by different symbols at the probability of $p < 0.05$.

4. Results

4.1.Investigation of IL-24 and its receptor in children with IBD

Elevated IL-24 protein levels were found in biopsy specimens obtained from inflamed colon areas in both ulcerative colitis and Crohn's disease patients compared to control specimens. IL-24 levels in samples from the macroscopically non-inflamed colon area of Crohn's patients were unchanged from the control. Serum IL-24 levels in children with Crohn's disease were elevated compared to serum levels in control children. The biological activity of IL-24 can be exerted when the target cell expresses the IL-20RB subunit of IL-24 heterodimer receptors. IL-20RB immunopositivity is present in both the control and patients 'colon biopsy specimens in the lamina propria layer, the basal region of epithelial cells, and subepithelial fibroblasts.

4.2. Investigation of IL-24 and its receptor in DSS-induced colitis

In our studies, we found that DSS treatment increased IL-24 mRNA expression and protein levels. IL-20RB immunopositivity is present in the colonic mucosa of both DSS-induced and control mice in the lamina propria layer, the basal region of epithelial cells, and subepithelial fibroblasts.

4.3. Effect of inflammatory factors on IL-24 synthesis in mononuclear cells (PBMC and LPMC)

We investigated the effect of IBD related inflammatory factors on IL-24 expression in mononuclear cells (PBMC, LPMC). While IL-1β, LPS, and H_2O_2 treatment increased IL-24 mRNA expression in both PBMCs and LPMCs, TNF-α decreased IL-24 expression in PBMCs compared to vehicle-treated cells.Treatment with TGF-β1 and IL-17 had no effect on IL-24 expression in PBMC and LPMC cells, respectively.

4.4. Investigation of the effect of IL-24 on HT-29 colon and HK-2 renal epithelial cells

Immunofluorescent staining revealed that both the HT-29 colon epithelial cells and the HK-2 renal proximal tubule epithelial cell line express the IL-20RB subunit. In the next step, the effect of IL-24 treatment on HT-29 colon and on HK-2 renal proximal tubule epithelial cells was investigated. IL-24 treatment did not affect the viability of HT-29 colon epithelial cells. However, IL-24 treatment increased the production of TGF-β1 and PDGF-B by HT-29 and HK-2 cells and the proportion of TGF-β1 + and PDGF-B + cells.

4.5. Investigation of IL-24 effect on CCD-18Co colon fibroblast cells

As in previous studies, the presence of IL-20RB was also confirmed on CCD-18Co fibroblast cells. IL-24 treatment had no effect on fibroblast cell viability, proliferation, or resulting extracellular matrix production. IL-24 treatment increased the mRNA expression of cells *COL3A1* and *FN1* and *MMP2, MMP9, TIMP1* and *TIMP2* compared to the control group. Furthermore, IL-24 treatment increased the cell migration activity.

4.6. Effect of IL-24 on various factors involved in tissue remodelling in the colonic mucosa of wild-type mice

To investigate the effect of IL-24 on tissue remodelling in vivo, IL-24 was injected into the colon of the mice in our experiments. IL-24 treatment increased the mRNA expression of *Tgfb1, Pdgfb, Col1a1, Col3a1, Fn1, Acta2, Mmp2, Mmp9, Timp1*, and *Timp2* compared to vehicle-treated controls. IL-24 treatment increased the amount of FN1 and pro-COL1A2 protein compared to the control group.

4.7. Changes in body weight and disease activity index of wild-type and *Il20rb* KO mice with DSS treatment

The next step in our experiments was to investigate the effect of IL20rb deficiency on DSS-induced colitis in mice. Changes in disease activity index and body weight of mice in both WT and *Il20rb* KO DSS-treated mice were observed. DSS treatment increased disease activity index and

decreased body weight in mice in both groups. However, in the mice of the *Il20rb* KO group, mild symptoms were observed for several days after the start of DSS treatment and during the treatment period, and the *Il20rb* KO animals lost less weight.

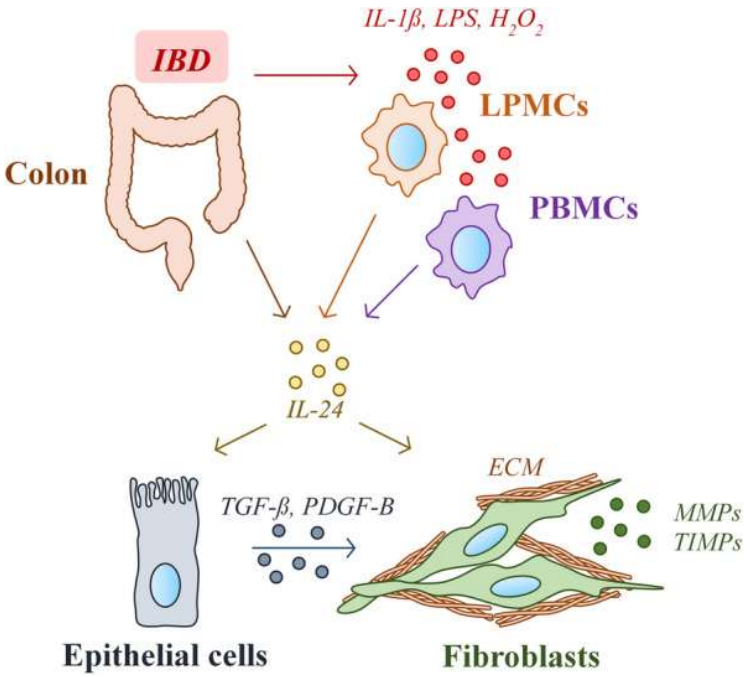
4.8. Investigation of factors involved in tissue remodeling in the colonic mucosa of DSS-treated WT and *Il20rb* KO mice

In the colon of WT and *Il20rb* KO mice, we examined the molecular biological changes induced by DSS treatment in the synthesis of factors involved in tissue rearrangement. In the WT group, DSS treatment increased the expression of Tgfb1, Col1a1, Col3a1, Mmp2, Mmp9, and Timp1, while Acta2 and Timp2 expression decreased compared to the untreated group. In the absence of the IL-20 receptor, there was less increase in *Tgfb1, Pdgfb, Col1a1, Col3a1, Fn1, Acta2, Mmp2, Timp1, and Timp2* mRNA expression during DSS treatment compared to the WT group. Similar to the changes in mRNA levels, we found that the amount of FN1 and αSMA protein were increased in the WT group with DSS treatment compared to the control WT group. The levels of these proteins in the *Il20rb* KO group showed no significant difference between the control and DSS groups.

5. Conclusions

The aim of my PhD work is to investigate the role of the interleukin-24 molecule in the pathomechanism of inflammatory bowel disease using human biopsy and serum samples, in vivo mouse model of DSS-induced colitis and *in vitro* cellular experiments. Based on our results, we can determine:

1. We found an increased level of IL-24 in the colonic mucosa and serum of untreated children with IBD compared to controls. The receptor of the IL-24 (IL-20RB) is present in both IBD and control human biopsies.
2. IL-24 is overexpressed in the inflamed colonic mucosa of DSS-treated mice compared to the control, untreated group, and the receptor of the IL-24 (IL-20RB) is present in the colonic mucosa of both the DSS-treated and control groups.
3. IL-1β, LPS, H_2O_2 and TNF-α, which are IBD-related inflammatory factors, influence the expression of IL-24 in mononuclear cells (PBMC, LPMC).
4. IL-20RB is present on HT-29 colon epithelial cells, and IL-24 treatment enhances the production of profibrotic factors (TGF-β and PDGF-B) of the epithelial cells.
5. IL-20RB is present on CCD-18Co fibroblast cells, IL-24 treatment enhances cell migration and synthesis of collagens, gelatinases and their inhibitors.
6. Direct IL-24 treatment enhances the production of profibrotic factors and various extracellular matrix components in vivo, in the mouse colonic mucosa.
7. We observed lower levels of profibrotic factors and extracellular matrix components in DSS-induced colitis in *Il20rb* KO mice than in WT mice.



1. ábra: IL-24 produced by inflammatory immune cells (LPMC, PBMC) enhances the deposition of collagen-rich ECM in inflammatory bowel disease directly through the production of profibrotic factors in epithelial cells and by stimulating the activation of fibroblasts.

6. Bibliography of the candidate's publications

6.1. Research articles related to the theme of the PhD thesis

- Ónody A, Veres-Székely A, Pap D, Rokonay R, Szebeni B, Sziksz E, Oswald F, Veres G, Cseh Á, Szabó AJ. (2021) Interleukin-24 regulates mucosal remodeling in inflammatory bowel diseases. Journal of translational medicine, 19: 1-16
- Pap D, Veres-Székely A, Szebeni B, Rokonay R, Ónody A, Lippai R, Takács IM, Tislér A, Kardos M, Oswald F, Fekete A, Szabó AJ, Vannay Á. (2020) Characterization of IL-19, -20, and -24 in acute and chronic kidney diseases reveals a pro-fibrotic role of IL-24. J Transl Med, 18: 172

6.2. Other publications

- Voros P, Sziksz E, Himer L, Onody A, Pap D, Frivolt K, Szebeni B, Lippai R, Gyorffy H, Fekete A, Brandt F, Molnar K, Veres G, Arato A, Tulassay T, Vannay A. (2013) Expression of PARK7 is increased in celiac disease. Virchows Arch, 463: 401-408

- Sziksz E, Molnár K, Lippai R, Pap D, Onody A, Veres-Székely A, Vörös P, Szabó D, Győrffy H, Veres G, Tulassay T, Vannay A, Arató A. (2014) Peroxisome proliferator-activated receptor-γ and thymic stromal lymphopoietin are involved in the pathophysiology of childhood coeliac disease. Virchows Arch., 465(4):385-93

- Sziksz E, Veres G, Vannay A, Prókai A, Gál K, Onody A, Korponay-Szabó IR, Reusz G, Szabó A, Tulassay T, Arató A, Szebeni B. (2010) Increased heat shock protein 72 expression in celiac disease. J Pediatr Gastroenterol Nutr, 51: 573-578

- Vannay A, Sziksz E, Prokai A, Veres G, Molnar K, Szakal DN, Onody A, Korponay-Szabo IR, Szabo A, Tulassay T, Arato A, Szebeni B. (2010) Increased expression of hypoxia-inducible factor 1alpha in coeliac disease. Pediatr Res, 68: 118-122