Role of interleukin-24 in the pathomechanism of coeliac disease

PhD Thesis

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1. Introduction

Coeliac disease (CD) is a chronic, autoimmune enteropathy caused by exposure to dietary gluten in genetically susceptible individuals. Due to multiple and variable extraintestinal symptoms and to specific autoantibodies, CD can define as systemic disorder also. CD is highly prevalent, it affects approximately 1% of the population worldwide. Currently, the therapy of CD is limited to the strict life-long gluten free diet (GFD), which results in the termination of pathological processes in the duodenal mucosa and in complete remission of the symptoms. However, the high number of patients with non-responsive CD and the significant therapeutic burden of GFD necessitate the better understanding the pathomechanism of the disease.

Through the function of the gastrointestinal tract, luminal epithelial cells are constantly exposed to mechanical, biological or chemical stimuli, thus tissue damage and regeneration are persistent and parallel processes in the duodenum. The architecture of the small intestinal mucosa is maintained by a precisely controlled balance among enterocyte proliferation in the crypts, migration along the villi and shedding of the senescent epithelial cells at the tip of the villi into the intestinal lumen. This fine-tuned process requires tight cooperation between enterocytes and the underlying subepithelial myofibroblasts. In CD, direct cytotoxicity of gluten and the chronic mucosal inflammation disturbs this fine-tuned process leading to villous atrophy and crypt hyperplasia.

The interleukin (IL)-20 subfamily of cytokines comprises five related molecules, including IL-19, IL-20, IL-22, IL-24 and IL-26. Within the subfamily, IL-19, IL-20 and IL-24 form a separate group, as they use the common IL-20RA/IL-20RB and IL-22RA/IL-20RB receptor heterodimers. The cytokines are mainly produced by immune cells, however, other cell types, such as epithelial cells or myofibroblasts can

also release them. Their most well-known functions are enhancing host defence mechanisms, regulation of immune response and tissue repair processes. Recently, the role of IL-20 cytokine subfamily has been proposed in diseases with chronic inflammation and tissue remodeling. Increased expression of other IL-20 family members has been described in psoriasis and rheumatoid arthritis, and our research group reported increased expression of IL-24 in biological samples of patients with inflammatory bowel diseases and chronic kidney disease.

2. Objectives

In the present work, the pathomechanism of coeliac disease was investigated, focusing to the role of IL-20 cytokine subfamily members in duodenal mucosal tissue remodeling.

Our objectives were as follows:

- To investigate the presence of markers of tissue remodeling in the duodenal mucosa.
- To investigate the gene expression and protein amount of IL-19, IL-20, IL-24 cytokines and of their receptor in healthy and coeliac pediatric duodenal samples.
- To define the cell types responsible for the production of IL-19, IL-20 and IL-24 in the gut.
- To determine the effect of coeliac related inflammatory cytokines on the expression of IL-19, IL-20, IL-24 and IL-20RA, IL-20RB and IL-22RA receptor subunits.
- To investigate the role of IL-24 in duodenal mucosal remodeling through examining its effect to duodenal epithelial cells and myofibroblasts.

3. Methods

3.1. Duodenal biopsies

Amount of *IL19*, *IL20*, *IL24*, *IL20RB* mRNA and of IL-24, IL-20RB, α-smooth muscle actin (α-SMA) and fibronectin (FN) proteins were investigated in duodenal biopsies derived from pediatric therapy naive CD patients and controls. CD was diagnosed based on the criteria of the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. Controls were referred with chronic abdominal pain, growth retardation and diarrhoea and an upper gastrointestinal endoscopy was part of their diagnostic procedure.

3.2. Cell cultures and treatments

Our *in vitro* experiments were performed on FHs74Int duodenal epithelial cell line, on primary myofibroblasts (pdMF) derived from the duodenal mucosa of control children and on mononuclear cells (PBMC) isolated from the peripheral blood of control and coeliac children.

Expression of *IL19*, *IL20*, *IL24*, *IL20RA*, *IL20RB* and *IL22RA* mRNA and of IL-24 protein were investigated in untreated cells and after IL-1ß, TNF-α, TGF-ß or IL-17 treatment.

Role of IL-24 in mucosal tissue remodeling was investigated in FHs74Int cells after oxidative stress induction by H_2O_2 treatment, and in pdMFs activated by PDGF-B or TGF-ß. After the treatment with 0.1 ng/ml recombinant IL-24 for 24 hours, cell viability, morphology, collagen production, and changes in mRNA expression were measured.

3.3. Immunofluorescence staining

Presence and localisation of IL-24, IL-20RB, α-SMA and actin was investigated by immunofluorescence staining on tissue samples, FHs74Int cells and primary myofibroblasts. Nuclei were counterstained

with Hoechst 33342. Samples were analyzed with a Nikon C2 confocal or an Olympus IX81 fluorescence microscope.
α-SMA stress fiber orientation of primer myofibroblasts was graphically assessed using ImageJ software.

3.4. mRNA expression measurement

Gene expression measurements were performed after RNA isolation, complementer DNA synthesis and real-time PCR. Relative mRNA expression was determined by comparison with *RPLP0* as internal control and presented as the ratio of control group.

3.5. Protein analysis

The protein level of IL-24, α-SMA and FN in duodenal biopsy samples was measured by Western blot, and that of IL-24 in the supernatant of FHs74Int cells and pdMFs was measured by ELISA. Evaluating Western blot results, the relative protein levels were determined by comparison with GAPDH as internal control and presented as the ratio of control group. In the case of ELISA, protein concentration was determined using a standard calibration curve.

3.6. Viability measurements

The viability of FHs74Int cells and pdMFs after various treatments was investigated using MTT cell viability, LDH cytotoxicity and Annexin V apoptosis assays.

3.7. Sirius Red assay

Collagen production of pdMFs was measured by Sirius Red assay.

3.8. Statistical analysis

After testing normality with Kolmogorow-Smirnov test, data were analyzed using t-test or Mann-Whitney U-test for two groups and ANOVA or Kruskal-Wallis test for more than two groups. Multiple comparisons of row data derived from assay-like measurements were performed using multiple t-test and ordinary two-way ANOVA with Dunnett correction. To determine the correlation between the relative mRNA expressions in biopsy samples and the patient's clinical parameters, Pearson correlation analysis was performed.

4. Results

4.1. Alteration of IL-19, IL-20, IL-24, IL-20RB, α-SMA and FN in the duodenal mucosa of children with CD

The mRNA expression of *IL24* was increased in the duodenal mucosa of children with CD compared to that of controls. There was no difference in the mRNA expression of *IL19* between the groups, and the expression of *IL20* was undetectable. There was no correlation between *IL24* expression and clinical parameters of coeliac patients. Protein amounts of IL-24, FN and α-SMA were elevated in the duodenal mucosa of children with CD compared to controls. Strong IL-24 and IL-20RB immunoreactivity was observed in the duodenal crypt enterocytes of children with CD.

4.2. Expression of IL-19, IL-20, IL-24 in PBMCs

In the PBMCs of children with CD, significantly higher *IL19* and *IL24* expression was observed compared to controls. *IL20* mRNA was not detectable either in the control or CD groups.

4.3. Effect of IL-1β, TNF-α, TGF-β and IL-17 treatment on the expression of *IL19*, *IL20*, *IL24* and *IL20RA*, *IL20RB*, *IL22RA*

Results are summarized on Figure 1. Although, all factors influenced the production of IL-19, IL20 and IL24, the effect of IL-1β was the most significant. IL-1β treatment increased the expression of all three cytokines in FHs74Int cells, pdMFs and PBMCs also. The only exception was *IL20* expression in PBMCs, which remained undetectable after IL-1β or any other treatment. The effect of TNF-α was cell type dependent. While TNF-α treatment increased the expression of *IL19*, *IL20* and *IL24* in the pdMFs, it decreased their expression in the FHs74Int epithelial cells and had no effect in the PBMCs. IL-24 protein amount was also

elevated after IL-1ß and TNF-α treatments. Majority of the treatments increased the expression of *IL20RB* and decreased that of *IL20RA* and *IL22RB*.

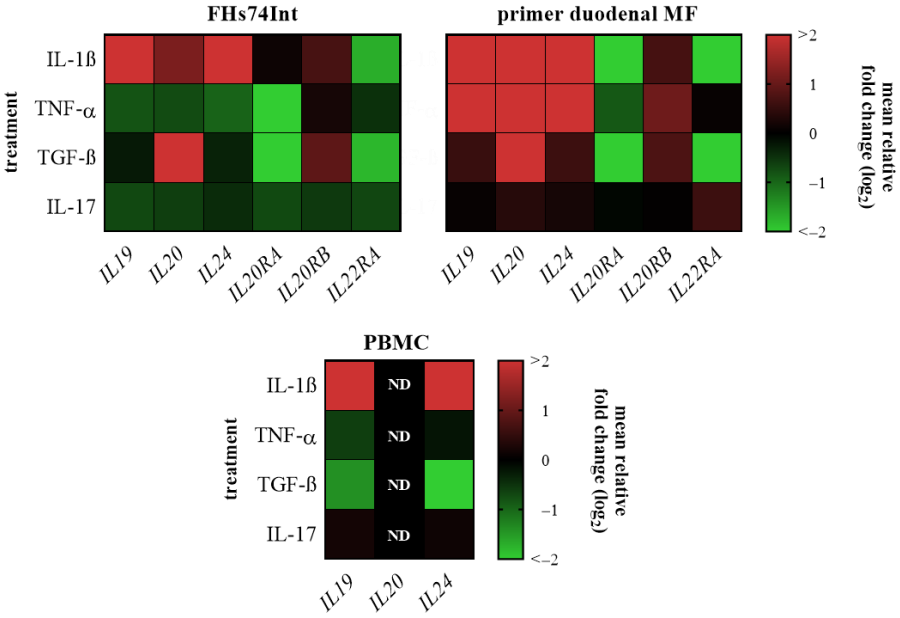

Figure 1. Effect of IL-1ß, TNF-α, TGF-ß and IL-17 treatment on the expression of *IL19, IL20, IL24* and *IL20RA, IL20RB, IL22RA*

4.4. Effects of IL-24 on duodenal epithelial cells

In our experiments, oxidative stress of FHs74Int cells was induced by H_2O_2 and effect of IL-24 was examined afterward.

According to MTT, LDH and Annexin V apoptosis assays, H_2O_2 induced vigorous cell death, which was significantly reduced by IL-24 treatment. IL-24 treatment decreased the H_2O_2 induced mRNA expression of *IL1A, IL6* and *TNF* as well.

4.5. Effects of IL-24 on duodenal myofibroblasts

Effect of IL-24 on the proliferation, morphology and stress fiber orientation, and ECM production of pdMFs was investigated.

Based on MTT and LDH assays, IL-24 treatment decreased the endogenous and the PDGF-B induced proliferation of pdMFs also. Accordingly, IL-24 treatment decreased the PDGF-B induced expression of proliferation markers, including *PCNA* and of *KI67*.

Effect of IL-24 on cell morphology and stress fiber orientation was investigated by microscopy after immunofluorescent staining. In untreated pdMFs, elongated morphology with highly parallel α-SMA immunopositive stress fibers were observed. However, the angles between the stress fibers and the longitudinal axis of the cells increased, and circular fiber displacement and sheet-like cell shape were experienced after IL-24 treatment. Accordingly, IL-24 treatment increased the mRNA expression of cytoskeletal structural components (α-SMA, ß-actin, vimentin) and cell morphology regulators (Snail and Slug).

According to the results of SiriusRed assay and gene expression measurements, IL-24 did not affect endogenous and TGF-ß induced ECM expression of pdMFs.

5. Conclusions

In this work the role of IL-20 cytokine subfamily in coeliac disease was investigated. Based on our results our statements are as follows:

- Elevated amount of α-SMA and FN in the biopsy samples of coeliac children indicate tissue remodeling in the duodenal mucosa.
- Expression of IL-24 is elevated in the duodenal mucosa and in PBMCs of children with coeliac disease compared to controls.
- Epithelial cells, myofibroblasts and immune cells may be responsible for the expression of IL-19, IL-20 and IL-24 in the duodenum.
- Coeliac disease related inflammatory factors, especially IL-1β alter significantly the expression of IL-19, IL-20, IL-24 and their receptors *in vitro*.
- Under *in vitro* conditions, IL-24 reduces epithelial cell death and expression of inflammatory cytokine induced by oxidative stress.
- IL-24 decreases the endogenous and PDGF-B induced proliferation of myofibroblasts *in vitro*.
- IL-24 causes significant alteration in morphology and stress fiber displacement of myofibroblast *in vitro*.

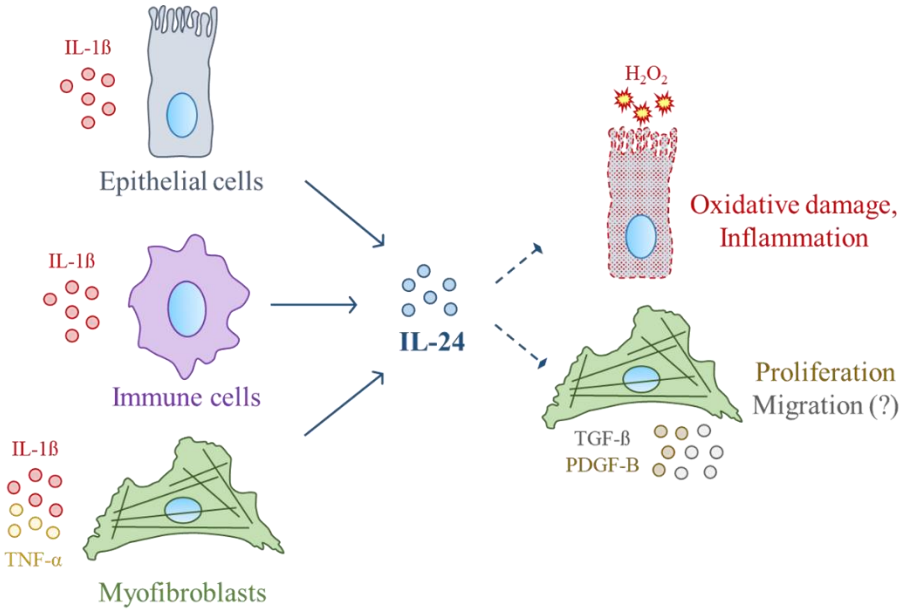

Figure 2. Hypothetic role of IL-24 in mucosal remodeling in coeliac disease. In the inflamed mucosa, IL-1ß and TNF-α increase the expression of IL-24 in epithelial cells, myofibroblasts and immune cells. Then, IL-24 promote the protection of epithelial cells against oxidative damage, decreases the proliferation of myofibroblasts and causes altered cytoskeletal organization characteristic to myofibroblasts with decreased motility.

6. Bibliography of the candidate's publications

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

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