Ph.D. thesis

THERAPEUTIC OPPORTUNITIES IN THE RATS INFLAMMATORY MODEL

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List of abbreviations

CD Crohn-disease

ECM extracellular matrix

ELISA enzyme linked immunosorbent assay

IBD inflammatory bowel disease

I.C. intra colonic

IL-6 Interleukin 6

JNJ 10191584 H4 receptor antagonist, (5-chlorobenzimidazolyl N-methylpiperazine carboxamide)

JNJ 26993135 LTA_4H inhibitor (1-[4-(benzothiazol-2-yloxy)-benzyl]-piperidine-4-carboxil acid

LTA_4H leukotriene A_4 hidrolase

LTB_4 leukotriene B_4

MMP matrix metalloproteinase

MMP-9 matrix metalloproteinase 9

MPO myeloperoxidase activity

TIMP tissue inhibitor of metalloproteinases

TIMP-1 tissue inhibitor of metalloproteinases 1

TNBS 2,4,6-trinitrobenzenesulfonic acid

TNF-α Tumor necrosis factor-alpha

UC Ulcerative Colitis

Keywords

Crohn disease, TNBS model, H4 histamine receptor antagonist, leukotriene B4 inhibitor, MMP-9, TIMP-1

Introduction

Inflammatory bowel diseases (IBDs) characterized by relapsing-remitting phases inflammation can affect any part of the gastrointestinal tract. Numerous environmental and genetic factors are contribute to the pathogenesis of IBDs/diseases. Furthermore, several molecular interactions and immunological mechanisms are also involved. The major types of IBDs are Crohn's disease (CD) and ulcerative colitis (UC). CD is a multifactorial disorder with chronic inflammation involving any segment of the digestive tract and all layers of the gut wall. It occurs most often in the distal part of the ileum and in the colon. UC is accompanied by chronic inflammation of mucosal and submucosal area localizated in the colon.

For symptomatic treatment, several types of drugs can be used: These are *derivatives of salicylic acid*, which affect locally on the mucosa. *Glucocorticosteroids* are used in severe cases, but the side effects are not negligible. The local effects of these drugs are similar to the salicylic acid derivatives. *Immunosuppressive and immunomessive drugs* are used in the case of steroid resistance. *Antibiotics* through their antibacterial effect can reduce the recurrence of the disease. The *biologically active substances* are used to inhibit the expression of pro-inflammatory cytokines, to increase the number of regulator T cells. Owing to this fact, the extent of the inflammed areas and the degree of inflammation decrease. Their disadvantage is that the usage is extremely expensive.

MMPs are zinc and calcium-dependent proteolytic enzymes which participate in dynamic formation and degradation of the extracellular matrix (ECM), as well as in the intestinal inflammation. MMP-9 is the most common type of matrix metalloproteinases (MMPs) expressed in the colon of CD patients, and therefore it is considered to be as biomarker which evaluate the severity and activity of chronic gut inflammation. Under physiological conditions, a tissue inhibitor of MMP (TIMP) regulates the function of MMPs, but this effect is not completed in several cases.

Histamine H4 blockers inhibit the neutrofil infiltration and therefore they could be beneficial in several inflammatory diseases.

Leukotrienes and their derivatives have pro-inflammatory effects, thus their inhibitors seem to be potential targets of pharmacology.

Based on these data, oral administration of *other candidates of anti-inflammatory substances*, such as selective histamine H4 receptor antagonists or LTA_4H selective inhibitors, may be promising therapeutic approach.

Aims

In the first part of my study, we aimed to evaluate the role of different regulator proteins in the development of srictures in **chronic** inflammatory bowel disease:

I. Are there any alterations in the expression and activity of ECM regulators, MMP-9 and TIMP-1 proteins in IBD?

In the second part of this study, our goal was to investigate:

II. How do the inflammatory markers change using histamine H4 receptor blocker in **acute** TNBS model?

III. How does the inhibition of LTB_4 affect the CD in **acute** TNBS model?

Methods

Modelling of Inflammatory Bowel Diseases

The 2, 4, 6-trinitrobenzene sulphonic acid (TNBS) model is used in our experiments as a chemically-induced animal model of gut inflammation. A single dose of TNBS can induce acute intestinal inflammation. Repeated TNBS-treatments cause the symptoms of CD accompanied by chronic gut inlammation.

I. Chronic studies

a. *Chronic TNBS modell:* The rats were randomised before commencement of the study and housed in groups, control, 1x-, 2x-, 3x TNBS. The rats were treated once, twice or three times with TNBS (10 mg). 2 weeks passed between the treatments. The rats were sacrificed 90 or 120 day after the last TNBS administrations removing the distal 8 cm portion of the colon (measured from the rectum).

b. *Determination of the markers of inflammation:* The rats were weighed every 2 weeks. The extent of macroscopically apparent damage and haemorrhagic necrosis in the 8 cm segment was determined in a randomised manner from the colour images via computerised planimetry. The area of macroscopically visible mucosal damage was calculated and expressed as the percentage of the total colonic segment area under study.

c. *Histological examinations:* The colonic tissue samples were taken from each animal area of the stricture. Colonic samples of age-matched controls were also collected. The histology blocks were used to prepare semithin (1 µm) sections, which were stained with toluidine blue solution for the light-microscopic study.

d. *The expression and activity of MMP-9 and TIMP-1 proteins:* At the end of the experimental period (120 day), the expression and activity of MMP-9 and TIMP-1 were measured from tissue homogenates originated from

the circular and longitudinal smooth muscle layers of the colon. The expression and activity was determined int x mm^2. To determine the protein content we used Bradford assay.

e. *Statistical evaluation:* Statistical analysis of the results was performed by using one-way ANOVA and the Newman-Keuls test, and a probability P < 0.05 p<0,05 ; p<0,01 ; p<0,001 was set as the level of significance. The results were expressed as mean ± SEM(standard error of mean).

II. Acute studies

a. *Acute TNBS model:* The rats were administered intra colonic TNBS (10 mg). At the end of the experiment, 72 hours after TNBS administration (i.e., on the morning of day 3), the distal colon was exposed and the terminal 8 cm dissected.

b. *Study of H4 histamine antagonist*

i. Use of treatments at different doses (4 days), the doses of JNJ 10191584 were 10, 30 and 100 mg/kg, p.o., twice a day.

ii. *Determination of the markers of inflammation:* the body weight of the animals was determined each day of the study.

The extent of macroscopically apparent damage and haemorrhagic necrosis in the 8 cm segment was determined in a randomised manner from the colour images via computerised planimetry. The area of macroscopically visible mucosal damage was calculated and expressed as the percentage of the total colonic segment area under study. In addition to the quantitative measurement of area of damage, the degree of colonic damage was also assessed in a randomised blinded fashion using a Damage Score, utilizing a 1–10 scale than has been adapted from that used previously.

The tissue was subsequently cut into longitudinal strips, each strip being thus 8 cm long and included the whole of the zone of injury. This tissue was weighed, processed and the resulting supernatant stored at for the subsequent determination.

The myeloperoxidase activity was determined in 8 cm longitudinal strips of the colon. Myeloperoxidase activity was expressed as mU/mg protein.

The TNF-α levels were determined with quantitative TNF-α Enzyme Linked ImmunoSorbent Assay (ELISA). The TNF-α values were expressed as pg/mg protein.

To determine the protein content we used Bradford assay.

iii. *Histological examinations:* For light microscopy, the sections were stained with hematoxylin and eosin or with toluidine blue using standard procedures for histological evaluation of colon damage. The depth of the mucosa and submucosa were measured in several sections from each tissue sample and expressed in µm, determination of the number of neutrofils.

c. *LTA_4H inhibitor study*

i. Use of treatments at different doses (4 days), the doses of JNJ 26993135 used were 5, 15 and 30mg/kg p.o., twice a day.

ii. *The determination of the markers of inflammation* were determined by the same method as h4 histamine receptor antagonists methods (II. b.).

The IL-6, LTB4 expression levels were determined with quantitative ELISA. The values were expressed as pg/mg protein.

d. *Statistical evaluation:* Results shown in the figures are expressed as mean ± S.E.M. For statistical comparisons, the two-tailed Student's t-test and the analysis of variance with the Bonferoni test were used where appropriate. $p<0.05$; $p<0.01$; $p<0.001$ was taken as significant.

Results

I. Chronic experiments

a. In the chronic phase of the inflammation, the expression of MMP-9 protein was significantly increased comparing to the control group at day 90, while it was slightly elevated at day 120.

b. Activity of MMP-9 was also significantly elevated comparing to the control animals (at the 90th day).

c. TIMP-1 was not detectable in the present experiment.

II. Acute experiments

a. Oral administration of H4 histamine antagonist was associated with significant and dose-dependent reduction of inflammatory parameters (lesion magnitude, shitchiness of lesions, degree of tissue inflammation, number of neutrofil granulocytes, weight of animals, degree of colon edema, increase in MPO activity, proinflammatory cytokines (TNF-α) expression.

b. Oral administration of LTA_4H inhibitor also reduced the inflammatory parameters significantly in dose-dependent way (lesion magnitude, lesions, decrease in body weight, degree of colon edema, increase in MPO activity, proinflammatory cytokines (IL-6, TNF-α) and LTB_4 expression).

Conclusion

I. In **chronic TNBS model**, the disease-associated complications worsened, and strictures developed.

a. Significant MMP-9 protein expression and activity was demonstrated in the lamina propria. The MMP-9 is suitable as a biomarker in this model.

b. TIMP-1 expression was not detectable, which based on literature data, suggesting that it does not affect the level and activity of MMPs during this inflammatory process.

II. In the **acute TNBS model**, the treatment of histamine H4 receptor antagonist significantly reduced the inflammatory parameters, depending on the applied dose.

III. In the **acute TNBS model**, treatment with LTA_4H inhibitor was also reduced the inflammatory parameters in a dose-dependent may and significantly reduced the inflammatory parameters.

IV. Based on these findings, the histamine H4 receptor antagonists and LTA_4H inhibitors may be crucial targets for drug development and therapy of gut inflammation.

List of publications

MTMT code : 10027758

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