Currently, gastrointestinal diseases are lifestyle diseases. The diet of modern man influences the development of inflammatory bowel disease (IBD), which includes, among others ulcerative colitis (UC) and Crohn’s disease (CD). Drug of first choice in these conditions is sulfasalazine (SAS) and its active metabolite 5-aminosalicylic acid (5-ASA). Despite intensive research conducted by many reputable research centers, the mechanism of action of these drugs is still not fully recognized. Sulfasalazine exhibits bacteriostatic, anti-inflammatory and immunosuppressive activity. The therapeutic effect of SAS results from the action of its pharmacologically active derivative - 5-aminosalicylic acid (5-ASA) are used in the treatment of inflammatory bowel disease. 5-ASA mechanism of action on the one hand, involves the inhibition of the cyclooxygenase and lipoxygenase activity, and thus decrease of synthesis of prostaglandins, leukotrienes and free radicals, on the other hand, the suppression of the immune response in the intestinal mucosa. Myofibroblasts, which are located just below the basement membrane, are important element of the mucosa. Due to its secretory activity they may interact with other cells, including epithelial cells. Examining SAS and 5-ASA cytotoxic properties on human normal, colon subepithelial myofibroblasts (CSEMF) it was found that the first of these compounds in a concentration of 1 mM significantly reduced the number of these cells as compared to the control, while the latter exhibited an action at the 5-fold higher concentration (5 mM). Moreover, SAS concentration greater than 0.25 mM reduced IL-8 secretion by CSEMF, and 5-ASA had no effect in the tested range of concentrations, i.e., up to 7.5 mM.

**Keywords:** sulfasalazine, 5-aminosalicylic acid, interleukin 8, colon subepithelial myofibroblasts

A chronic, relapsing bowel inflammation is accompanied by increased production of pro-inflammatory cytokines, increased permeability of the intestinal epithelium and changes in the synthesis of mucus. Epithelial cell functions such as proliferation, differentiation, cytokine secretion, motility and permeability can be regulated, among others, by myofibroblasts (6). These cells, participating in the regulation of local inflammatory processes, synthesizes many cytokines. Not only do they secrete pro-
inflammatory cytokines such as IL-1, IL-6, IL-8, RANTES (regulated on activation, normal T-cell expressed and secreted), monocyte chemotactic protein-1 (MCP-1), but also anti-inflammatory cytokines such as IL-10 (7, 8).

The aims of study was to investigate the influence of SAS and 5-ASA on colon subepithelial myofibroblasts (CSEMF) viability and the secretion of IL-8 by these cells stimulated by TNF-α.

EXPERIMENTAL

Cell cultures
Normal human colon myofibroblasts CCD-18Co were obtained from American Type Culture Collection. The cells were cultured in minimum essential medium (MEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma), 100 IU/mL penicillin G, 100 mg/mL streptomycin and 10 mM HEPES (Gibco). The cell cultures were maintained at 37°C in 5% CO₂ atmosphere.

Cytotoxicity assay
The XTT (In Vitro Toxicology Assay Kit XTT Based, TOX-2, Sigma) assay was used to assess cell viability. The method is based on the ability of mitochondrial dehydrogenases of viable cells to cleave the tetrazolium ring of XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt), yielding orange formazan crystals, which are soluble in aqueous solutions. Myofibroblasts were dispensed at a density of 1000 cells / 0.2 mL into 96-well plates and were cultured in MEM supplemented with 10% FBS for 72 h. After this time, the cells were washed three times with RPMI (without phenol red dye), then 150 µL of XTT with 0.1, 0.25, 0.5, 1 mM SAS and 0.5, 1, 2.5, 5 mM 5-ASA solution was added into each well for 4 h. The absorbance of samples was measured at 450 nm (reference 690 nm) using a plate reader (MRX Revelation, Dynex). The absorbance was directly proportional to the amount of the living cells.

IL-8 assay
Myofibroblasts were dispensed at a density of 5000 cells / 0.2 mL into 96-well plates. The cells were grown 4 days in MEM supplemented with 10% FBS. Twenty four hours before initiation of the proper experiment, the medium was replaced with a medium containing 1% FBS and then the cells were cultured with 0.1, 0.25, 0.5, 1 mM SAS and 1, 2.5, 5, 7.5 mM 5-ASA for 24 h in present of 37.5 ng/mL TNF-α. After this time, the supernatants of the cells were frozen in -70°C and the amount of living cells was evaluated with the XTT test.

The concentrations of IL-8 in the supernatants (diluted 1 : 10) were determined by ELISA MAX™ according to the instructions of the manufacturer (Biolegend). The absorbance at 450 nm (570 nm references) was measured with the plate reader (MRX Revelation, Dynex). Quantity IL-8 [ng] was determined from a standard curve and the resulting values were calculated per 10⁶ of living cells.

Statistics
To evaluate the influence of SAS and 5-ASA on the secretion of IL-8 by myofibroblasts, the arithmetic mean as a measure of the average and standard deviation as a measure of dispersion were used.

Differences in IL-8 secretion were analyzed for statistical significance using analysis of variance (ANOVA) and Tukey test. The results of SAS and 5-ASA cytotoxicity test were analyzed by Kruskal-Wallis and U-Mann-Whitney test. Normality was verified by Shapiro-Wilk test, and homogeneity of variance by Brown-Forsythe test. A p-value < 0.05 was considered significant. The analysis was performed using Statistica 10.0 software (StatSoft, Poland).

RESULTS AND DISCUSSION
The SAS mechanism of action is still under discussion. Putative anti-inflammatory action of 5-ASA includes the modulation of cytokines secretion, the inhibition of macrophage activation, induction of apoptosis, reduction of the transcriptional activity of the nuclear transcription factor (NF-κB), inhibition of prostaglandins and leukotrienes biosynthesis, the interaction with the peroxisome proliferator-γ activated receptor (PPAR-γ), inhibition of neutrophil chemotaxis and the influence on reactive oxygen species (ROS) (9, 10).

Sulfasalazine also inhibits the synthesis of a number of pro-inflammatory cytokines, i.e., interleukin-1, -2, -12 (IL-1, IL-2, IL-12), tumor necrosis factor (TNF-α), interferon gamma (IFN-γ), reduces the number of B cells and decreases the antibodies synthesis. It reduces the metabolism of granulocytes (11). SAS influences NK (natural killer) cells, epithelium and endothelium cells, neutrophils, T cells and macrophages (10). Colon subepithelial myofibroblasts (CSEMF) regulate local inflammatory processes. They secrete both pro- and anti-inflammatory cytokines, chemokines, growth factors, mediators of the inflammatory response and are the source of reactive oxygen species (12, 13). Di
Sabatino et al. (14) found in patients suffering IBD the increased levels of TNF-α, which interfering with the target cell receptor leads to activation of the NF-κB pathway. Since TNF-α and IL-1 can induce NF-κB forming a positive autocrine loop, it may be supposed that NF-κB is crucial factor in the pathogenesis of chronic inflammation (15, 16). SAS inhibit PMA-, TNF-α-, or LPS-induced activation of NF-κB (11), that induces apoptosis, probably by preventing the expression of anti-apoptotic genes.
IBD is associated with extremely low level of cells apoptosis at the site of inflammation. In a healthy organism anergy and apoptosis in T cells occurs. In intestinal mucosa apoptosis is intense, while during the CD this process is reduced, which is associated with excessive production of anti-apoptotic cytokines IL-2, IL-6, IL-15, IL-17 or IL-18 (17).

In this paper, colon myofibroblasts survival in the presence of SAS and 5-ASA was shown as a percentage of control. The study showed that SAS at a concentration of 1 mM significantly reduces the CSEM number as compared to the control (p < 0.0353, Kruskal-Wallis test) (Fig. 1A), while 5-ASA exhibits cytotoxicity at 5-fold higher concentration.
(5 mM) (p < 0.0158, Kruskal-Wallis test) (Fig. 1B). Myofibroblast proliferation is enhanced at the edges of ulcers of IBD patients, helping in wound healing. Thus, observed cytotoxicity of the investigated compounds has probably negative impact on this process.

The study assessed the effect of the SAS and 5-ASA on the secretion of IL-8 by TNF-α-stimulated human CSEMF. Statistically significant reduction of this cytokine secretion was observed at a concentration equal to or higher than 0.25 mM of SAS, while 5-ASA did not show such an effect at tested concentrations (up to 7.5 mM). 5-ASA affects intestinal inflammation by inhibition the secretion of IL-8 by macrophages and monocytes (18). There is no constitutive secretion of IL-8 by human CSEMF, however, when IFN-γ treated, they start to secrete this cytokine. IL-8 is also secreted by LPS stimulated CSEMF (19). Our findings showed that similar effect is observed when these cells are stimulated by TNF-α. IBD patients IL-8 increased secretion by the ISEMF is due to the increased IL-17 secretion by T cells, predominantly IL-23-dependent Th17 subpopulation. This interleukin is composed of two protein subunits p40 and p19, which is found in myofibroblasts (20). In the mucosa of IBD patients increased secretion of IL-22 is also observed, that results in the enhanced expression of IL-8 by the target cells.

CONCLUSION

SAS up to concentration of 0.5 mM had no influence on CSEMF viability, simultaneously decreasing TNF-α-dependent IL-8 secretion. There is no effect of 5-ASA on IL-8 secretion by CSEMF at investigated concentrations of this compound. Therefore, low doses of SAS may support the IBD treatment with 5-ASA.

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REFERENCES


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