INFLUENCE OF TROGLITAZONE, SODIUM BUTYRATE, 5-AMINOSALICYLIC ACID AND BAY 11-7082 ON THE CHEMOKINE ENA-78/CXCL5 SECRETION IN THE INTESTINAL SUBEPITHELIAL MYOFIBROBLASTS

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Crohn’s disease (CD) and ulcerative colitis (UC) are inflammatory bowel diseases (IBD) with a chronic relapsing and remitting course. Both of them have a similar etiology, but differ in location and degree of intestinal injury (1). The pathogenesis of inflammatory bowel disease is complex, involving environmental, genetic, microbial, immune factors and mucosal permeability abnormalities (2).

It has been demonstrated that nuclear factor κB (NF-κB) plays a central role in the regulation of intracellular inflammatory signaling pathways in the large gut. NF-κB activates the expression of many genes associated with immune function in the gut, e.g., IL-8, IL-6, tumor necrosis factor-α (TNF-α), granulocyte/macrophage colony stimulating factor (GM-CSF), and epithelial neutrophil-activating peptide-78 (ENA-78/CXCL5). Chemokines IL-8/CXCL8 and ENA-78/CXCL5 play complementary and sequential roles in neutrophil recruitment in the inflamed tissue in ulcerative colitis patients (3). NF-κB levels are increased in the intestinal lamina propria of patients with IBD. It was reported that a specific p65 antisense oligonucleotide can inhibit p65 expression and pro-inflammatory cytokine production by lamina propria macrophages in patients with active CD and UC. Although activation of NF-κB does not occur in all patients with IBD, its perpetuated activation makes it a very attractive target for the therapeutic intervention in this disease (4).

5-Aminosalicylic acid (5-ASA) is one of the oldest anti-inflammatory agents used in the treatment of IBD, but the mechanism underlying its intestinal effects remains unknown. Recent data showing peroxisome proliferator-activated receptor gamma (PPAR-γ), is the major functional receptor mediating the common aminosalicylate activities in IBD have also reinforced the role of this receptor in the control of intestinal inflammation (5). PPAR-γ ligands inhibit NF-κB signaling pathway. High levels of PPAR-γ expression have been reported in colonic tissues in epithelial cells (3). PPAR-γ binds several different groups of drugs such as thiazolidinediones, also known as glitazones. Troglitazone (Tro) was the first glitazone developed for therapeutic use in patients with diabetes (6). Activation of PPAR-γ provides the protection against experimental IBD (7).

Short-chain fatty acid enriched diet is recommended for IBD patients. One of these acids – butyric acid has been recognized as histone deacetylase inhibitor. According to Quivy et al. (8) deacetylation and acetylation processes are implicated in the regulation of NF-κB transcriptional activity.

Fundamental biological processes such as cell motility, proliferation, differentiation, apoptosis, morphogenesis, tissue repair, inflammation, and the immune response in gut are controlled by myofibroblasts. Intestinal subepithelial myofibroblasts (ISEMF) are located in the lamina propria under the epithelial cell layer (9). The present study was aimed at evaluating ENA-78/CXCL5 secretion by human normal colon myofibroblasts CCD-18Co treated...
Influence of troglitazone, sodium butyrate, 5-aminosalicylic acid and BAY 11-7082 on the chemokine...

EXPERIMENTAL

Cell cultures

Human normal colon myofibroblasts CCD-18Co were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% bovine fetal serum (FBS), 100 IU/mL penicillin G (sodium salt) and 100 mg/mL streptomycin (antibiotic solution, Gibco) and 10 mM HEPES (Gibco). The cell cultures were maintained at 37°C in 5% CO₂ atmosphere.

Drugs

Tro and BAY 11-7082 were dissolved in dimethyl sulfoxide (DMSO), NaB was dissolved in phosphate buffer saline (PBS) and 5-ASA solution was made in 2 M NaOH (Sigma).
Cytotoxicity assay

The XTT (In Vitro Toxicology Assay Kit XTT Based, TOX-2, Sigma) 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 solution. Myofibroblasts were harvested and dispensed at a density of 8000 cells/mL into 96-well plates. They were incubated with various concentrations of Tro (1, 10, 20, 50, 100 µM), BAY (0.01, 0.1, 1, 10, 20 µM), NaB (0.5, 1, 5, 10, 20 mM) and 5-ASA (0.1, 0.5, 1, 2, 5 mM) under standard conditions for 72 h. In parallel wells, control cells were cultured in the absence of drugs. Cells were incubated with XTT solution for 4 h. The absorbance was measured at 450 nm with plate reader (Triad LT Multimode Detector, Dynex Technologies). Cell survival was expressed as a percentage of inhibition calculated with the use of the following formula: cell viability = (mean sample absorbance – mean control sample absorbance) × 100%.

ENA-78/CXCL5 assay

ENA-78/CXCL5 secretion by TNF-α-stimulated myofibroblasts was evaluated using the enzyme-linked immunosorbent assay (ELISA) kit. CCD-18Co cells were grown in culture to 90% confluence in the medium with 10% FBS in 96-well plates for 24 h before initiation of the experiment. Then, the medium was replaced with medium containing 1% FBS. The culture supernatant (200 µL) was removed after 24 h, and evaluated for ENA-78/CXCL5.

Statistics

Statistical comparisons were made by the analysis of variance (ANOVA; Tukey’s post hoc test). A value of p < 0.05 was considered statistically significant. The results are expressed as the means ± SD of indicated number of experiments.

RESULTS

Cell viability

The toxicity of the studied compounds were characterized by the parameter EC50 – the concentration that causes 50% of inhibition of cells proliferation. As shown in Figure 1, PPAR-γ agonist Tro, NF-κB inhibitor BAY 11–7082, NaB and 5-ASA inhibited the proliferation of CCD-18Co cells in a dose-dependent manner. The EC50 values were 28.5 µmol/L, 2.78 µmol/L, 7.48 mmol/L and 6.02 mmol/L for Tro, BAY NaB and 5-ASA, respectively. An increase of the proliferation was observed with concentrations of 0.01 µM BAY and 0.1 mM 5-ASA. The solvent itself showed no influence on cell proliferation.
The influence of Tro, 5-ASA, BAY and NaB on ENA-78/CXCL5 secretion by CCD-18Co cells

As shown in Figure 2A, TNF-\(\alpha\)-stimulated CCD-18Co cells increased the secretion of ENA-78/CXCL5 in a dose-dependent manner. Maximal secretion was detected at 50 ng/mL TNF-\(\alpha\). ENA-78/CXCL5 release from myofibroblasts was increasing with time (4–12 h) of TNF-\(\alpha\)-stimulation (50 ng/mL) (Fig. 2B). The influence of Tro, BAY, 5-ASA and NaB on ENA-78/CXCL5 secretion in CCD-18Co cells is shown in Figure 3. NaB, BAY and 5-ASA inhibited ENA-78/CXCL5 release in a dose-dependent manner (Fig. 3B, 3C and 3D). The effect of Tro was confirmed in three independent experiments. Tro at the concentration of 10–30 µM increased ENA-78/CXCL5 secretion compared with the control (Fig. 3A). The solvent itself showed no influence on ENA-78/CXCL5 secretion.

DISCUSSION AND CONCLUSION

Dysregulated cytokine production and signaling mechanisms in the epithelial cells, mucosal lymphocytes and macrophages have been implicated in the pathogenesis of both CD and UC, the two major forms of IBD (4).

Normal human colon fibroblasts play significant role in regulation of immune and inflammatory responses in the gut. These cells are specialized mesenchymal cells that exhibit the ultrastructural features of both fibroblasts and smooth muscle cells and can be characterized by positive immunoreac-

Figure 3. Effect of Tro (A), BAY (B), NaB (C) and 5-ASA (D) on ENA-78/CXCL5 secretion by CCD-18Co cells. Cells were incubated with TNF-\(\alpha\) (50 ng/mL). The medium contained 1% FBS. The results represent the mean ± SD (n = 5); * p < 0.05 (ANOVA) compared with the control K.
tivity for both smooth muscle α-actin and vimentin (9, 10). The present study showed that CCD-18Co cells were stimulated by TNF-α to release ENA-78/CXCL5. Its secretion was observed between 4 and 12 h. Enterocytes are the primary source of chemokine ENA-78/CXCL5 in the normal colon and in ulcerative colitis. Caco-2 and T84 human intestinal epithelial cell lines produced ENA-78/CXCL5 after stimulation with IL-1β and TNF-α. Caco-2 cells showed increased ENA-78/CXCL5 release at 8 h. Human colonic cells from patients with UC showed elevated levels of ENA-78/CXCL5 mRNA (24-fold increase) and protein (4-fold increase) compared with normal tissue. The kinetics of ENA-78/CXCL5 induction is delayed and prolonged compared to IL-8 (11). Keates et al. (11) proposed that ENA-78/CXCL5 and IL-8 play complementary and sequential roles in neutrophil recruitment in ulcerative colitis. Thus, we suggest that myofibroblasts located in the lamina propria under the epithelial cells can participate in the initial phases of recruitment, i.e., activation of neutrophils within the intestinal microcirculation, leading to neutrophil diapedesis, transmigration into the lamina propria along a chemotactic gradient.

PPAR-γ ligands were shown to inhibit IL-8 in TNF-α-stimulated colonic epithelial cells (12). In the present study, ENA-78/CXCL5 secretion by CCD-18Co cells was suppressed by the use of inhibitor BAY 11–7082 of the NF-κB signaling pathways, which suggests that TNF-α-stimulated ENA-78/CXCL5 secretion depends on NF-κB activation. At 1 μM BAY, which had no effect on cells proliferation (data not shown), a twofold decrease in the ENA-78/CXCL5 secretion was observed in the present study.

In the present study, 5-ASA and NaB also inhibited ENA-78/CXCL5 secretion in colon myofibroblasts. The putative anti-inflammatory actions of 5-ASA include modulation of inflammatory cytokine production, a decrease of transcriptional activity of NF-κB by modulating RelA/p65 phosphorylation, and inhibition of the biosynthesis of prostaglandins and leukotrienes. 5-ASA was also able to bind to PPAR-γ. The therapeutic effect of 5-ASA depends on the direct contact of the molecule with the epithelium of the colon. The concentrations of 5-ASA are in the feces median order of 30 mM (5). Lower concentrations of 5-ASA (1–5 mM) were able to inhibit ENA-78/CXCL5 secretion and the proliferation of human colon myofibroblasts, as observed in our study.

Butyrate, a short-chain fatty acid, is generated by anaerobic fermentation of fibers in the colon. It is the major luminal source of energy for colonocyte. Clinical trials suggest that short-chain fatty acids ameliorate inflammation in UC. The anti-inflammatory effects of butyrate may be mediated by an inhibition of IκBα dependent activation of NF-κB (13). In the present study, NaB inhibited the ENA-78/CXCL5 secretion at all concentration used and its concentrations of 0.5–5 mM had no effect on the myofibroblasts proliferation. Thus, NaB similarly like 5-ASA could inhibit the neutrophils activation.

On the contrary to the above results, troglitazone showed different effect on the CCD-18Co cells. We observed that the concentrations of 10–30 μM of troglitazone increased the ENA-78/CXCL5 secretion by CCD-18Co cells compared with the untreated cells. Maximum increase of the ENA-78/CXCL5 secretion was observed at 25 μM Tro (data not shown). Because therapeutic plasma concentration range for troglitazone is 5–15 μM (14), it can be involved in the activation of neutrophils within the intestinal microcirculation. Several studies suggested that PPAR-γ has no anti-inflammatory activity, or might indeed exert a pro-inflammatory response (15–17).

The chronic inflammatory process leads to the disruption of the epithelial barrier and formation of epithelial ulceration. Resolution of inflammatory activity is associated with repair processes that facilitate tissue remodeling, which restores normal intestinal architecture. Maintenance and reconstitution of basement membrane is a key process in mucosal repair. ISEMF play an important role in these processes via their growth and secretion of various factors such as collagens and growth factors. The transient appearance of activated myofibroblasts is a feature of normal wound healing, but the persistence of these cells is associated with excessive collagen deposition and fibrosis (18). All the studied compounds decreased the proliferation in a dose-dependent manner. Thus, decreases in both proliferative responses of ISEMF and secretion of growth factors may contribute to the reduction of fibrotic changes in CD patients.

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REFERENCES

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