Expression of epithelial growth factor receptor, tumor necrosis factor-α and nuclear factor κB in inflammatory bowel diseases

Ekspresja receptora dla naskórkowego czynnika wzrostu, czynnika martwicy nowotworów α i czynnika jądrowego κB w nieswoistych chorobach zapalnych jelit

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Słowa kluczowe: choroby zapalne jelit, receptor dla naskórkowego czynnika wzrostu, czynnik martwicy nowotworów α, czynnik jądrowy κB.

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Abstract

Introduction: The role of epithelial growth factor receptor (EGFR) in inflammatory bowel diseases (IBD) pathogenesis has not been fully elucidated. Overexpression of EGFR in colonic inflamed mucosa in patients with IBD has been observed by some authors. Moreover some studies confirm the role of EGFR in upregulating nuclear factor κB (NF-κB) and tumor necrosis factor-α (TNF-α) expression, which may lead to pronounced inflammatory response in IBD.
Aim: To evaluate the expression of EGFR, NF-κB and TNF-α in remission and active phase of Crohn disease (CD) and ulcerative colitis (UC).
Material and methods: Biopsy specimens from colonic mucosa were taken at colonoscopy from 62 patients with CD, 92 patients with UC and from 18 healthy subjects with normal colonic mucosa (control). The expression of EGFR, NF-κB and TNF-α was evaluated by immunohistochemical staining.
Results: The colonic mucosal immunoexpression of EGFR in active phase of IBD was significantly lower compared to the remission. The immunoexpression of NF-κB and TNF-α was significantly higher in the active disease phase of IBD than in remission. The results suggest that EGFR does not promote inflammation in IBD but most probably is involved in mucosal regeneration in IBD.
Conclusions: High expression of NF-κB and TNF-α in active phases of UC and CD confirm their proinflammatory role in these diseases.

Streszczenie

Wprowadzenie: Rola receptora dla naskórkowego czynnika wzrostu (*epithelial growth factor receptor* – EGFR) w patogenezie nieswoistych chorób zapalnych (NChZJ) jest słabo poznana. Część autorów stwierdziła wysoką ekspresję tego receptora w błonie śluzowej jelita grubego u osób z NChZJ. Ponadto wiele badań dowodzi stymulującego wpływu EGFR na wzrost ekspresji jądrowego czynnika wzrostu κB (*nuclear factor* κB – NFκB) i czynnika martwicy nowotworów α (*tumor necrosis factor*-α – TNF-α), co może skutkować nasileniem odpowiedzi zapalnej w NChZJ.
Cel: Ocena ekspresji EGFR, NF-κB i TNF-α w fazie aktywnej oraz w remisji choroby Leśniowskiego-Crohna (ChLC) i wrzodziejącego zapalenia jelita grubego (WZJG).
Materiał i metody: Badaniem objęto 62 osoby z ChLC, 92 chorych na WZJG oraz 18 zdrowych osób (grupa kontrolna). Badane osoby poddano kolonoskopii, podczas której pobrano wycinki do badania histopatologicznego. Ekspresja EGFR, NF-κB i TNF-α były oceniane przy wykorzystaniu metod immunohistochemicznych.
Wyniki: Immunoekspresja EGFR w nabłonku jelita grubego była niższa w aktywnej fazie NChZJ w porównaniu z fazą remisji. Z kolei immunoekspresja NF-κB i TNF-α okazały się wyższe w aktywnej fazie NChZJ niż w fazie remisji.
Wnioski: Uzyskane wyniki przeczą prozapalnej roli EGFR i sugerują udział tego receptora w procesach regeneracyjnych błony śluzowej w NChZJ. Wysoka ekspresja NF-κB i TNF-α w aktywnych postaciach ChLC i WZJG potwierdza ich prozapalne działanie w NChZJ.

Introduction

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine synthesized by monocytes, macrophages, lymphocytes and neutrophils. It activates TNF-R1 and TNF-R2 receptors on the cell membrane of immunocompetent cells (lymphocytes B and T) leading to induction of chemotaxis, phagocytosis, and induction of acute phase proteins secretion. Moreover, the TNF-α pathway activates the function of nuclear factor κB (NFκB) by TNF receptor associated factor-2 (TRAF2). This protein stimulates I B kinase which degrades the inhibitor of NFκB – the nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α (IκBα). This process promotes cell proliferation and activates the inflammatory response [1].

Mucosal overexpression of TNF-α is observed in active phases of many autoimmune diseases (inflammatory bowel diseases (IBD), asthma, rheumatoid arthritis). Macrophage and T lymphocyte activation leads to the increase of synthesis of proinflammatory cytokines such as interleukin (IL)-1, IL-6 and interferon-γ (INF-γ) [2, 3].

Nuclear factor κ light chain enhancer of activated B cells (NF-κB) is a protein transcription factor present in many human cells, which in response to stimuli of cytokines, free radicals or many antigens, triggers transcription of relevant proteins in the cell nucleus. Nuclear factor κB is a key factor regulating the cell cycle, apoptosis and inflammation. Activation of NFκB may lead to overexpression of proinflammatory cytokines – TNF-α, IL-1, IL-6 and also IL-12 and IL-23, promoting a Th1-dependent lymphocyte immunological response [4].

Epithelial growth factor receptor (EGFR), a member of the ErbB receptor family, is a transmembrane glycoprotein presenting tyrosine kinase activity. It plays an important role in regulation of cell proliferation, migration and adhesion. Mutations causing its overexpression are one of the key factors leading to carcinogenesis.

Despite the association of the EGFR pathway with TNF-α and NFκB function, its role in the inflammatory response is not clear. Many authors suggest that the EGF receptor might promote mucosal regeneration in inflammatory tissue.

However, some authors have shown that activation of this receptor leads to NFκB overexpression, resulting in progression of inflammation. Moreover, *in vitro* studies on colonocytes have shown that EGF receptor stimulation by metalloproteinases triggers TNF-α secretion by the ERK1/2 MAPK pathway, resulting in an increase of mucosal permeability [5].

High expression of ErbB mucosal receptors (EGFR family member) was shown in active phases of inflammatory diseases – IBD [6–9], asthma [10], rheumatoid arthritis [11]. On the other hand, other authors suggest that EGFR inhibition might cause NFκB activation, resulting in an inflammatory response [12, 13].

Many investigations focus on the role of EGFR in mucosal regeneration in IBD. Yoo *et al*. have shown that high TNF-α expression induces EGFR activation in myofibroblasts, which induces healing processes [14]. Interestingly, EGF topical treatment in an animal model of colitis and also in ulcerative colitis in humans induced remission of the disease with almost complete mucosal healing. However, further investigations were halted due to potential carcinogenic capability of this growth factor.

Modern therapy of IBD focuses on achieving mucosal healing. Investigating the role of EGFR and its relations with TNF-α and NFκB pathways in IBD might be useful in further understanding the pathogenesis of these diseases.

Aim

The aim of the study was to evaluate EGFR, NFκB and TNF-α colonic mucosal expression in the active phase and remission of Crohn disease (CD) and ulcerative colitis (UC). In addition, the possible usefulness of these biomarkers in assessing the IBD severity index has been examined.

Material and methods

A group of 154 IBD patients (62 CD, 92 UC) who did not undergo anti-TNF-α therapy was examined. Biopsy specimens of colonic mucosa were taken at colonoscopy from the most inflammatory changed areas. As a control, biopsy specimens were taken from 18 individuals with no colonic organic disease. Obtained material was evaluated histopathologically in hematoxylin and eosin staining. The intensity of inflammation was described by the D'Haens scale in CD which included epithelial damage, architectural changes, infiltration of mononuclear and polymorphonuclear cells in the lamina propria and epithelium, presence of erosions and ulcers, presence of granulomas and number of biopsy specimens evaluated [15]. In UC histopathological lesions were evaluated by Morson's scale, which distinguishes an active phase (irregular surface of the mucosa, loss of epithelium integrity, infiltration of lymphocytes and plasmocytes in lamina propria, focal infiltration of granulocytes, crypt abscesses, hyperemia, decreased number of goblet cells) and remission (loss of regularity and branching of gland tubules, shortening and defragmentation of gland tubules, partial degradation of muscularis mucosae layer, Paneth cell metaplasia) [16, 17].

The expression of EGFR, NF-κB and TNF-α was evaluated by an immunohistochemical method. The following antibodies were used: EGFR (Novocastra, Leica Bio-

system, Newcastle, dilution 1 : 200), TNF-α (p65(C) IBL, Hamburg, dilution 1 : 250) and NFκB (ab6671, Abcam, Cambridge, dilution 1 : 300).

Epithelial growth factor receptor and nuclear factor B immunostaining was present in colonocyte cytoplasm. The index of immunoreactivity was measured by a semiquantitative method (0 points – none, 3 points – highest reactivity). Nuclear factor B was expressed in the colonocyte nucleus. The number of immunoreactive cells on the surface area of 1 mm^2 was calculated (labeling index – LI).

Correlation of the results of histological activity scales and the expression of examined biomarkers was evaluated. The project received the approval of the Bioethics Committee of the Medical University of Lodz (RNN/44/09/KE).

Statistical analysis

For statistical analysis the nonparametric test ANOVA rank Kruskal Wallis and multiple comparison test were used.

Results

Mucosal EGFR expression in remission of CD was significantly higher than in the active phase of the disease: 1.6 ±0.66 vs. 0.8 ±0.75 (p = 0.003). In UC a similar tendency was observed, but the results were not statistically significant: 0.95 ±0.59 vs. 0.85 ±0.77 (p = 0.07).

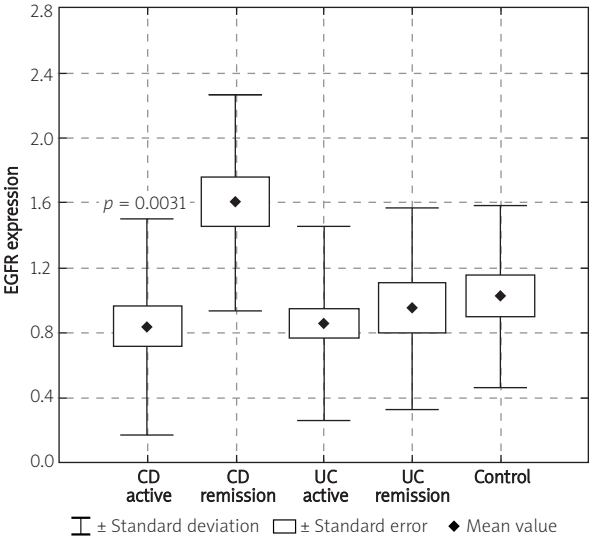

Fig. 1. EGFR expression in active phase and remission of CD and UC. Statistical significance was observed for CD active phase and remission (p = 0.0031)

Ryc. 1. *Ekspresja EGFR w aktywnej fazie i w remisji ChLC i WZJG. Znamienność statystyczną wykazano dla porównania ChLC aktywnej i ChLC remisji (p = 0,0031)*

Additionally, there was no difference between EGFR expression in control and other groups (p > 0.05). The immunoreactivity of EGFR in colonic mucosa of CD and UC was similar: 1.05 ±0.75 vs. 0.98 ±0.67 (p > 0.05) (Figure 1).

The expression of NFκB in colonic mucosa was significantly higher in the active phase of CD compared to the remission phase of the disease: 331 ±193 vs. 134 ±117 immunoreactive cells/1 mm^2 (p = 0.0031). Immunoexpression of NFκB in the active phase of CD was higher than in the control (331 ±193 vs. 35 ±12; p = 0.0001), but in the remission phase no such differences were observed (134 ±117 vs. 35 ±12; p > 0.05). The analysis of mucosal expression of NFκB in UC showed higher immunoreactivity for this transcription factor in the active phase of the disease than in remission: 443 ±193 vs. 89 ±57 (p = 0.0001). Immunoexpression of NFκB in the active phase of UC was also higher than in the control: 443 ±193 vs. 35 ±12 (p = 0.0001). However, no difference between NFκB expression in remission and control groups was observed: 89 ±57 vs. 35 ±12 (p > 0.05). Mucosal immunoreactivity of NFκB in CD and UC was similar: 255 ±187 vs. 323 ±227 (p > 0.05) (Figure 2).

The semiquantitative method of TNF-α expression in colonocyte cytoplasm showed higher expression of this cytokine in the active phase of CD than in remission of CD: 2.03 ±0.41 vs. 1.37 ±0.73 (p = 0.014). The immunoreactivity of TNF-α in those groups was higher than in healthy mucosa: respectively 2.03 ±0.41 vs. 0.45 ±0.13; 1.37 ±0.73 vs. 0.45 ±0.18; (p < 0.05). Similarly, expression of TNF-α in the active phase of UC was higher than in the remission phase of the disease: 1.85 ±0.53 vs. 0.71 ±1.01 (p = 0.001). Immunoreactivity of this cytokine was higher in the active phase of UC than in the control: 1.85 ±0.53 vs. 0.45 ±0.18 (p = 0.001). However, no differen-ces were observed in TNF-α expression between remission of UC and control groups: 0.71 ±1.01 vs. 0.45 ±0.18 (p > 0.05). Additionally, no differences were shown between immunoexpression of TNF-α in CD and UC: 1.83 ±0.52 vs. 1.72 ±0.68 (p > 0.05) (Figure 3).

Discussion

The results obtained in this study have shown the highest expression of EGFR in remission of IBD. The results of recent publications on colonic mucosal EGFR activity in IBD bring discrepant conclusions. Galandiuk and Trzcinski observed high expression of EGFR in colonic mucosa in a murine model of colitis, which according to the authors suggested a role of this receptor in promoting the inflammatory response [6–9].

Yoo *et al*. conducted *in vitro* studies on human colonic fibroblasts obtained from IBD patients, showing high expression of EGFR with concomitant activation of

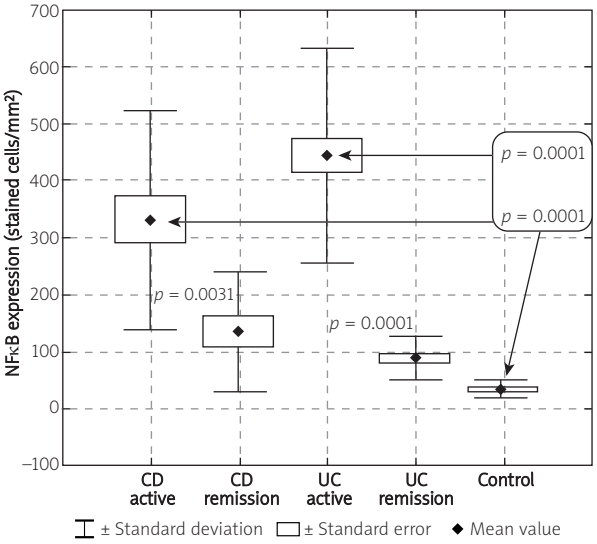

Fig. 2. NFκB expression in active chase and remission of CD and UC. Statistical significance was observed for the following: CD active phase vs. CD remission (p = 0.0031); UC active phase vs. UC remission (p = 0.0001); control group vs. CD active phase (p = 0.0001); control group vs. UC active phase (p = 0.0001)

Ryc. 2. *Ekspresja NF-κB w aktywnej fazie i w remisji ChLC i WZJG. Znamienność statystyczną uzyskano dla porównań: ChLC faza aktywna vs ChLC remisja (p = 0,0031), WZJG faza aktywna vs WZJG remisja (p = 0,0001), grupa porównawcza vs ChLC faza aktywna (p = 0,0001), grupa porównawcza vs WZJG faza aktywna (p = 0,0001)*

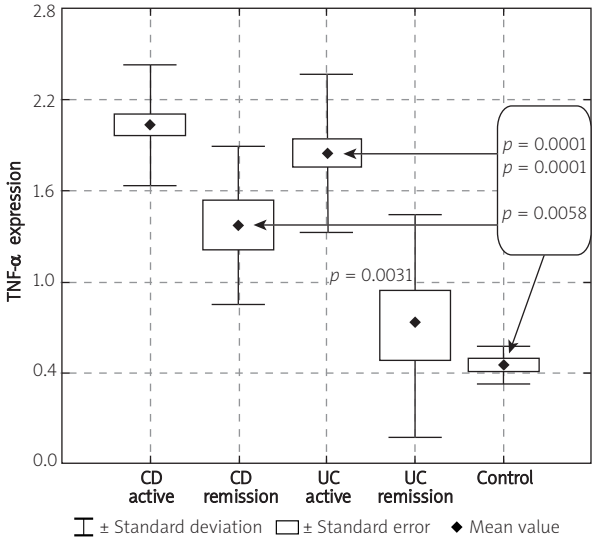

Fig. 3. TNF-α expression in active phase and remission of CD and UC. Statistical significance was observed for the following: CD active phase vs. CD remission (p = 0.0139); UC active phase vs. UC remission (p = 0.0001); control group vs. CD active phase (p = 0.0001); control group vs. CD remission (p = 0.0058); control group vs. UC active phase (p = 0.0001)

Ryc. 3. *Ekspresja TNF-α w aktywnej fazie i w remisji ChLC i WZJG. Uzyskano znamienność statystyczną dla porównań: ChLC faza aktywna vs ChLC faza remisji (p = 0,0139), WZJG faza aktywna vs WZJG faza remisji (p = 0,0001), grupa porównawcza vs ChLC faza aktywna (p = 0,0001), grupa porównawcza vs ChLC faza remisji (p = 0,0058), grupa porównawcza vs WZJG faza aktywna (p = 0,0001)*

ERK kinase and high cyclooxygenase 2 activity, which explained the potential role of EGF receptor both in inflammation and increased risk of carcinogenesis in IBD [14].

On the other hand, many data suggest the important role of EGFR in regulation of mucosal healing processes in IBD. Koon *et al*. have shown in *in vitro* studies on human colonocytes that stimulation of EGF receptor by the anti-inflammatory cytokine tumor growth factor-β (TGF-β) promotes proliferation of colonic epithelial cells [18].

McCole *et al*. examined the role of stimulation of EGFR by its ligand EGF in a murine model of colitis, showing that it restores proper ion transport in colonocytes, which leads to resolution of diarrhea and regeneration of mucosa [19].

Interesting results of EGF use in the topical treatment of UC in humans were reported by Sinha *et al*. A randomized, double controlled study with placebo was conducted on 24 patients with UC. After 2 weeks of EGF solution enema application remission was reached in 83% of patients and in the placebo group only in 8% (p < 0.05) [20].

Our data confirm the hypothesis that EGF receptor is one of the factors responsible for mucosal healing in those diseases. The lack of difference in EGFR mucosal expression in the active phase of IBD and healthy individuals does not support the role of this receptor in promoting inflammation.

The results of our study show that NF-κB expression is higher in the active phase of CD and UC than in remission of these diseases. Literature data confirm the proinflammatory role of NFκB in CD and UC. Schreiber *et al*. analyzed colonic bioptates of 74 patients with IBD by Western blot, showing high expression of NFκB both in epithelium and inflammatory infiltrate in the active phase of the disease [21]. Similar results were obtained by Rogler *et al*., who used immunohistochemical and

immunofluorescent methods in evaluating NFκB expression in bioptates of human colonic mucosa in IBD [22].

Aronica *et al*. conducted studies on an animal model of colitis in transgenic mice with inactivated B/Rel proteins – members of NFκB cell signaling pathway in T lymphocytes. Serum concentration of proinflammatory cytokines (IFN-γ, IL-2, IL-4) was evaluated by ELISA. The obtained cytokine profile showed that blocking NFκB expression in colitis leads to inhibition of the Th2 response and simultaneously activation of Th1 lymphocyte activation [23].

The mechanism of many drugs used in IBD is focused on the NFκB pathway. Wahl and Adler found in *in vitro* studies that sulfasalazine blocks phosphorylation of IκBα protein – a subunit of NFκB, which inhibits development of inflammation [24]. Schreiber *et al*. suggest that glucocorticosteroids suppress nuclear factor κB by blocking IκBα and inhibiting the response to bacterial lipopolysaccharides [21].

The results of this study are consistent with literature data on NFκB's role in promoting inflammation. The high mucosal immunoreactivity of this transcription factor in the active phase of IBD and lack of differences between its expression in remission of IBD and healthy individuals additionally confirm the function of NFκB in activating inflammatory processes.

High expression of TNF-α in the active phase of IBD and also higher immunoreactivity of this cytokine in colonic mucosa in remission of CD than in healthy individuals were observed in this study. Available literature data confirm the proinflammatory role of TNF-α. Murch *et al.* and Lilia *et al*. evaluated immunohistochemical expression of this cytokine in post-surgical colon specimens of IBD patients, showing its high mucosal concentration both in CD and UC. The expression of TNF-α in CD was increased in both epithelium and inflammatory infiltrate of all layers of the colonic wall, whereas in UC TNF-α overexpression was present only in the mucosal and submucosal layer [25]. These data correspond to histological studies on the inflammatory response in CD and UC [26].

Reinecker *et al*. isolated inflammatory infiltrative cells from human colonic biopsy specimens and by using radioimmunological methods revealed significantly higher expression of TNF-α in active phases of CD and UC than in remission of these diseases [27].

Our data are consistent with cited publications. This confirms the important role of TNF-α in pathogenesis of IBD and may also suggest greater intensity of inflammation in CD than in UC.

High expression of NFκB and TNF-α in active phases of UC and CD and their positive correlation with the results of histological scales of disease severity may suggest their value as possible IBD activity markers.

High expression of EGFR in remission of CD may suggest the role of this receptor and its ligand, EGF, in colonic mucosal regeneration in this disease.

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