Influence of receptor activator of nuclear factor kappa B ligand, osteoprotegerin and interleukin-33 on bone metabolism in patients with long-standing ulcerative colitis


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Abstract

Background: Ulcerative colitis (UC) is a chronic disease with periods of remission and recurrences. Dysfunction of the local immune response leads to chronic inflammation within the large intestine which triggers morphological changes in the intestinal wall as well as induces the synthesis of numerous factors that have an adverse impact on the bone metabolism.

The aim of the study was to determine the expression of RANKL, OPG and IL-33 in mucosal biopsies of UC patients with long disease duration as well as serum level of these cytokines in the context of bone density and bone metabolism.

Materials and methods: The UC group consisted of 56 patients with average disease duration of 16 y. The control group comprised 37 healthy individuals. Local expression of cytokines was assessed in the biopsies of colonic mucosa by the real-time PCR and immunohistochemistry (IHC), and their serum concentration was measured by ELISA.

Results: The increased bone resorption observed in patients with UC was reflected by low bone density and high serum level of C-terminal telopeptide (CTX). Mucosal RANKL expression and serum concentration were similar in UC group and healthy subjects, however, UC patients had higher local expression of OPG and serum OPG concentration. Increased IL-33 gene expression was observed only in UC at the mRNA level. We propose that bone resorption in UC patients...
The balance of pro- and anti-inflammatory cytokines in the colonic mucosa is not only fundamental for normal gut homeostasis but also for the proper bone metabolism. Crohn’s disease (CD) and ulcerative colitis (UC) are the two major forms of inflammatory bowel disease (IBD) which are characterized by chronic inflammation of the colon (UC, CD) and/or small intestine (CD). Moreover, a significant reduction of bone mass during the course of disorder is observed.17,19,20,27

The receptor activator of nuclear factor-κB ligand (RANKL), its receptor — RANK, and its soluble receptor — osteoprotegerin (OPG) play a key role in osteoclast differentiation and bone metabolism. OPG acts as a receptor for RANKL that prevents it from binding to and activating RANK. RANKL–RANK interaction is crucial for osteoclast maturation. RANKL and OPG are produced not only by osteoblasts and bone marrow stromal cells, but also by many other cell types including the cells involved in immune responses such as T lymphocytes.7,26

Interleukin-33 (IL-33) formerly known as nuclear factor from high endothelial venules (NF-HEV) is a new member of the IL-1 cytokine family, and is expected to be essential for induction of Th2-type immune response, which is a characteristic feature of UC.13,14,23 IL-33 is a ligand for the IL-1 receptor-related protein (ST2), and is expressed in such cell types as endothelial cells, macrophages, and dendritic cells.15,23 In vitro, human IL-33 (30 kDa) can be cleaved by caspase-1 to create a mature form (20–22 kDa) of the protein.12

The aim of our study was to assess local expression of RANKL, OPG and IL-33 in the mucosal biopsies of UC patients including serum levels of these cytokines in relation to the parameters of bone metabolism.

2. Materials and Methods

2.1. Patients

Histological and endoscopic classifications of specimens were based on endoscopic, clinical, and histopathological outcomes. UC patients (n = 65) were divided into 2 groups according to Modified Truelove and Witts Severity Index (MTWSI).28 The first group consisted of 56 patients with moderate disease activity (30 patients with proctitis or left-sided colitis and 26 with pancolitis) and the second one comprised 9 UC patients in remission. The average duration of the disease was 16 y.

The control group included 37 healthy participants who underwent screening colonoscopy, showed normal colonic mucosa and had no history of immune-mediated diseases. All studies were confirmed by the local Ethics Committee and a voluntary written informed consent was obtained from all individuals involved in the study.

Patients with active UC were treated with aminosalicylates (sulfasalazine or mesalazine), corticosteroids or/and immunosuppressive drugs (azathioprine). Among UC patients, four of them underwent additional treatment with bisphosphonates (alendronate, ibandronate).

Dual-energy X-ray absorptiometry (DXA) was used to measure bone mineral density (BMD) of the femoral neck and lumbar vertebrae L1–L4. T and Z scores were determined for 32 patients with UC and 7 individuals with UC in remission according to the WHO definition of osteoporosis and osteopenia (T-score of −1 to −2.5 was classified as osteopenia and T-score ≤−2.5 was classified as osteoporosis). The clinical characteristics of patients are shown in Table 1.

2.2. Tissue Harvest and Human Serum Collection

Biopsies from patient and control groups to determine mRNA and protein expression were immediately placed in liquid nitrogen, and stored at −80 °C until processed. Sera were obtained from clotted blood after centrifugation at 2500 rpm for 15 min were stored at −80 °C until use.

2.3. Total RNA Extraction and Reverse Transcription

Biopsies were lysed in a tissue homogenizer (MagNA Lyser, Roche, Basel, Switzerland) and RNA was extracted using the commercially available Total RNA kit (ABE Biotechnology, Gdynia, Poland) based on the phenol–chloroform–isoamyl alcohol and silica membrane technique, according to the manufacturer’s instructions. Isolated RNA was stored at −80 °C. Total RNA was reverse-transcribed to complementary DNA (cDNA) using M-MuLV Reverse Transcriptase (Fermentas, Inc., Hanover, MD, USA) and oligo-dT18 primer (Sigma-Aldrich, St. Louis, MO, USA). Samples were stored at −20 °C until processed.

2.4. Real-time PCR Analysis

The specific primers for RANKL, OPG, IL-33 and β-actin were designed using primer design tools: Primer3Plus and Vector NTI. For RANKL, forward primer was 5′-GGAGACAATGGGATGTCG-3′, reverse primer was 5′-GGAAACAGATGGGATGTCG-3′. For OPG, forward primer was 5′-AAAGACACCTGTAGAAAACCA-3′, reverse primer was 5′-GTTGCGGTTTATCCTCCTCTA-3′. For IL-33, forward primer was 5′-GGGGAACAGACACGGC-3′, reverse primer was 5′-AAGCAGAGATGGCGCTCGAG-3′. For β-actin, forward primer was 5′-TGTGCCCATCTACGAGGGGTATGC-3′, reverse primer was 5′-GGTACATGGTGGGTTCTGAC-3′. The quantitative PCR analyses were performed in a fluorescent temperature cycler (iQ Cycler, Bio-Rad Laboratories, Inc., Hercules, CA, USA) using SYBR Green
Supermix (Bio-Rad, USA) under the following parameters: 3 min for 95 °C and 40 cycles of 94 °C for 15 s; 56 °C (RANKL), 52 °C (OPG), 63 °C (IL-33) and 60 °C (β-actin) for 20 s; 72 °C for 20 s and 77 °C for 5 s. Amplification of specific fragments was confirmed both by examination of melting peaks and by agarose gel electrophoresis of the PCR products. RANKL, OPG and IL-33 expression ratios were determined by the comparative Ct (threshold cycle) method\textsuperscript{16} in relation to the mean rate of housekeeping gene ACTB.

2.5. ELISA for sRANKL, OPG, IL-33 and CTX

Serum levels of sRANKL, OPG, IL-33 and C-terminal cross-linked telopeptide of collagen type I (CTX) were measured by commercially available ELISAs (sRANKL: Immunodiagnostik AG, Bensheim, Germany; OPG: BioVendor, Brno, Czech Republic; IL-33: BioLegend, Inc., San Diego, CA, USA; CTX: Immunodiagnostic Systems Ltd., Boldon, United Kingdom) according to the manufacturer's instructions. The detection limits of sRANKL, OPG, IL-33 and CTX were 1.56 pg/ml, 12 pg/ml, 4.14 pg/ml and 0.02 ng/ml, respectively.

2.6. Immunohistochemistry

Frozen sections (10 μm) were prepared, dried at room temperature for 30 min, and fixed in ice cold acetone. Samples were blocked for endogenous peroxidase activity by using 0.3% hydrogen peroxide in methanol for 30 min. Sections were then incubated with 5% normal serum (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA) to block nonspecific binding of immunoglobulin. Immunohistochemical (IHC) staining was performed using mouse monoclonal anti-human RANKL IgG (25 μg/ml) (R&D Systems, Inc., Minneapolis, MN, USA) or mouse monoclonal anti-human OPG IgG (10 μg/ml) (Imgenex, San Diego, CA, USA) or rabbit polyclonal anti-human IL-33 IgG (0.9 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA). After incubation for 2 h with primary antibodies at room temperature, slides were washed in PBS and incubated with an appropriate biotinylated secondary antibody (Vectastain ABC Kit, Vector Laboratories, Inc., USA) for 45 min. Slides were rinsed in PBS and incubated with biotin-avidin HRP complex (Vectastain ABC Kit, Vector Laboratories, Inc., USA) for 30 min. Immunoreactive cells were visualized by addition of a diaminobenzidine (DAB) solution (Vectastain ABC Kit, Vector Laboratories, Inc., USA) and counterstained with hematoxylin. Sections were then dehydrated, mounted in DPX mounting medium and viewed under a Nikon Eclipse E800 light microscope. The specificity of the IHC staining was determined by a negative control, which was prepared under the same conditions as mentioned, replacing primary antibodies with 5% normal serum.

2.7. Statistical Analyses

Statistical analyses were performed using Statistica version 8.0 software (StatSoft, Inc., Tulsa, OK, USA). The results were presented as mean ± standard deviation (SD). Differences resulting in \( p < 0.05 \) were considered to be statistically significant. Data were analyzed using nonparametric tests: Mann–Whitney \( U \) test and Wilcoxon signed-rank test. The relationships between RANKL, OPG, IL-33 and other parameters were determined using the Spearman correlation technique.

3. Results

3.1. Expression Level of RANKL mRNA in the Mucosa of UC Patients was Comparable to Healthy Control

RANKL mRNA relative expression levels were analyzed in 43 UC patients, 7 patients with UC in remission and 31 healthy patients.
individuals from the control group. No statistical differences were found between UC patients, individuals with UC in remission and the control group (Fig. 1A). There were no statistically significant differences between inflamed and noninflamed colon biopsies in patients with UC (Fig. 1B).

### 3.2. Increased Expression of OPG mRNA in UC

The mean level of OPG expression in 35 UC patients was significantly higher than in the control group ($p < 0.0001$) and patients with UC in remission ($p < 0.01$) (Fig. 2A). OPG expression in noninflamed tissues in UC patients was also higher than in the control group ($p < 0.001$) and patients with remission of the disease ($p < 0.01$) (data not shown). There was statistical significance ($p = 0.039$) between inflamed and noninflamed tissues in patients with UC (Fig. 2B).

### 3.3. Overexpression of IL-33 mRNA in UC

The highest expression of IL-33 was noted in UC patients ($n = 40$) in comparison to the control group ($p < 0.0001$) and individuals with UC in remission ($p < 0.05$) (Fig. 3A). As

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**Figure 1**  
A — Analysis of RANKL mRNA relative expression levels in UC patients (inflamed tissue, $n = 43$), UC in remission (noninflamed tissue, $n = 7$) and control groups (noninflamed tissue, $n = 31$).  
B — Analysis of RANKL mRNA relative expression levels in inflamed and noninflamed tissues in UC patients ($n = 30$). Data were calculated with Mann–Whitney U test (A) and Wilcoxon signed-rank test (B).

**Figure 2**  
A — Analysis of OPG mRNA relative expression levels in UC patients (inflamed tissue, $n = 35$), UC in remission (noninflamed tissue, $n = 7$) and control groups (noninflamed tissue, $n = 28$), *$p < 0.0001$ in comparison to the control group, **$p < 0.01$ in comparison to UC in remission group (Mann–Whitney U test).  
B — Analysis of OPG mRNA relative expression levels in inflamed and noninflamed tissues in UC patients ($n = 26$), *$p = 0.039$ (Wilcoxon signed-rank test).
shown in Fig. 3B, IL-33 expression level in inflamed tissues was significantly elevated than in noninflamed tissues in patients with UC (p = 0.0001).

3.4. Correlations Between RANKL/OPG and IL-33 mRNA Expression Levels in Inflamed Tissues From UC Patients

RANKL mRNA expression levels showed an inverse correlation with IL-33 mRNA expression levels in inflamed tissues in UC patients (r = −0.38; p < 0.05) (Fig. 4A). Significant correlation in opposite direction was noted between OPG mRNA and IL-33 mRNA expression levels in inflamed areas (r = 0.43; p < 0.05) (Fig. 4B). No statistically significant correlation was found between RANKL mRNA/OPG mRNA and IL-33 mRNA expression levels in noninflamed tissues from healthy individuals (data not shown).

3.5. Serum Level of sRANKL in Patients With UC was Comparable to the Control Group

There were no significant differences in serum sRANKL levels between UC patients (n = 44) and healthy participants (n = 24) (Fig. 5A).

3.6. Serum Level of OPG was Elevated in UC

The mean serum OPG level (523 ± 255 pg/ml) in UC patients (n = 46) increased significantly (p < 0.05) in comparison with the control group mean (362 ± 170 pg/ml) (Fig. 5B).
Figure 5  A — Analysis of sRANKL serum levels in UC patients (n = 44), UC in remission (n = 7) and control groups (n = 24). B — Analysis of OPG serum levels in UC patients (n = 46), UC in remission (n = 7) and control groups (n = 23). C — Analysis of CTX serum levels in UC patients (n = 46), UC in remission (n = 7) and control groups (n = 24). *p < 0.05; **p < 0.0005 in comparison to the control group (Mann–Whitney U test).
3.7. Serum Levels of IL-33 were Below the Detection Limit

As to the IL-33 (20–22 kDa) serum level, its concentration was below the detection limit (4.14 pg/ml) in each study group (data not shown).

3.8. Increased Serum CTX Concentration in Patients With UC

In order to analyze changes in bone turnover the serum level of CTX — bone resorption marker was assessed.

The mean CTX concentration in all healthy individuals (n = 24) was 0.191 ± 0.175 ng/ml (Fig. 5C) and this result was similar to reference value for healthy adults based on the manufacturer's instructions (0.29 ng/ml, n = 351).

The mean CTX concentration was 3-fold and 2.83-fold higher in UC (p < 0.0005) and in patients with UC in remission (p < 0.05), respectively, compared with the control group (Fig. 5C).

3.9. Correlations Between Serum Levels of OPG and CTX Serum Levels/Patient’s Age in UC

OPG serum levels showed positive correlation with CTX serum levels in UC patients (r = 0.31; p < 0.05) (Fig. 6A). Significant correlation was also found between serum levels of OPG and patient’s age (r = 0.66; p < 0.05) (Fig. 6B). No relationships were noted between mentioned values in the control group (data not shown).

3.10. Immunohistochemical Staining of RANKL, OPG and IL-33 in Colonic Mucosa

Immunostaining showed the presence of RANKL⁺, OPG⁺ and IL-33⁺ cells between crypts in inflamed mucosal layer of the intestine. Immunopositive cells were mostly lamina propria mononuclear cells (Fig. 7A–D). There were a large number of RANKL⁺ and OPG⁺ cells in inflamed areas of UC patients (Fig. 7A–C) compared with IL-33⁺ cells (Fig. 7D). RANKL immunostaining was also presented in the cytoplasm of crypt epithelial cells (Fig. 7A–B). Only few immunoreactive cells were visualized in noninflamed tissues in the control mucosa (data not shown).

4. Discussion

The present study demonstrated an elevated bone turnover as indicated by increased serum CTX concentration and low bone mineral density (BMD) in patients with chronic inflammatory disorder such as ulcerative colitis (UC). The data are similar to those seen in previous reports about altered bone metabolism in IBD.17,27

In our study, we observed that the main sources of RANKL, OPG and IL-33 were inflammatory lamina propria mononuclear cells infiltrated in colonic mucosa of patients with UC. We found that local expression of RANKL mRNA and serum level of sRANKL in individuals with UC were comparable with the control group. Similar studies were done by Moschen A. R. and his associates,9 but they analyzed RANKL and OPG only at protein level. They didn’t notice any change in sRANKL plasma levels and sRANKL synthesis in colonic explants cultures (CEC) in IBD. Their results support ours in that they indicate that RANKL isn’t responsible for enhanced osteoclastogenesis and bone resorption in UC. On the other hand, we showed that patients with UC had higher local expression of OPG mRNA and serum level than healthy participants. This unexpected finding is in agreement with Moschen et al.,9 who have found increased plasma level of OPG in UC. In addition, the authors found a significant inverse correlation between OPG plasma level and femoral neck and lumbar spine BMD.9 These data support our results, which showed that UC patients with higher OPG serum level have increased bone resorption reflected by elevated serum CTX concentration. Moreover, other authors have reported that the RANKL/OPG imbalance wasn’t only characteristic of ulcerative colitis, but was also typical of some other inflammatory diseases e.g. primary biliary cirrhosis (PBC).25 In addition, OPG serum levels showed positive correlation with CTX serum levels in PBC individuals25 and the same relationship was observed in our patients.
We also proved that the highest expression of IL-33 mRNA was seen in inflamed tissues of UC patients in comparison to healthy participants and individuals with UC in remission. These data are supported by Kobori et al.,3 Pastorelli et al.15 and Seidelin et al.24 The researchers confirmed that IL-33 mRNA expression was significantly increased in active lesions of UC patients.3,15,24 In contrast to local overexpression of IL-33 mRNA in UC, a mature form (20–22 kDa) of the protein wasn’t detectable in serum in all our groups. Nevertheless, several investigators have reported that serum IL-33 (20–22 kDa) levels were higher in IBD patients than in the control group.1,15 Further studies should be carried out to elucidate the findings.

The effect of IL-33 on osteoclast differentiation still needs to be determined. Mun et al.11 found that IL-33 induces RANKL-independent formation of functional osteoclasts from human monocytes in vitro. Otherwise, Schulze et al.22 reported that IL-33 inhibits osteoclastogenesis in vitro. Finally, Saidi et al.18

Figure 7  Immunohistochemistry of RANKL, OPG and IL-33 in inflamed areas of UC. A and B — Presence of RANKL-positive cells between crypts and in the crypt epithelium in the mucosa (400× and 1000×, respectively). C — Detection of many OPG+ cells in inflamed mucosa (1000×). D — Moderate IL-33 immunoreactivity in inflamed tissue (1000×). E (400×) and F (1000×) — No immunostaining in the mucosa (negative control).
demonstrated a lack of direct effect of IL-33 on bone remodeling. There could also be an indirect mechanism of increased bone turnover, which is induced by local overexpression of IL-33 in inflamed colonic mucosa in UC. The investigators showed that IL-33 can stimulate mast cells to release of proinflammatory cytokine — TNF-α, 5,6,8,19 Kudo et al. 5 found that TNF-α, in the presence of M-CSF, directly stimulated human osteoclast formation from peripheral blood mononuclear cells (PBMCs) in the way independent of RANKL. Additionally, Fuller et al. 2 confirmed osteoclastogenic potential of TNF-α leading to RANKL-independent bone resorption.

In conclusion, our results suggest that RANKL isn’t a major factor for increased bone resorption observed in UC and overexpression of OPG could be a compensatory mechanism. We propose an alternative pathway of altered bone metabolism wherein local expression of IL-33 induces production of TNF-α, which can activate RANKL-independent osteoclastogenesis in UC.

**Conflict of Interest**

All authors declare that they have no conflict of interest.

**Acknowledgment**

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