The RANKL/OPG system is activated in inflammatory bowel disease and relates to the state of bone loss

A R Moschen, A Kaser, B Enrich, O Ludwiczek, M Gabriel, P Obrist, A M Wolf, H Tilg

Background and aims: A substantial proportion of patients with inflammatory bowel disease (IBD) develops osteopenia and osteoporosis in the course of disease. Recent data from a mouse model of colitis suggest that the receptor activator of nuclear factor kappa B (RANKL)/osteoprotegerin (OPG) system may be responsible for bone loss.

Methods: We investigated the activation state of the RANKL/OPG system and its association with bone loss in human IBD. Plasma levels of OPG and RANKL were correlated with bone mineral density and current IBD therapy. Colonic secretion of OPG and RANKL and cell types responsible for such secretion were determined.

Results: OPG plasma levels were elevated 2.4-fold in Crohn’s disease (CD) and 1.9-fold in ulcerative colitis (UC) whereas soluble RANKL (sRANKL) levels were not significantly different in IBD patients compared with healthy controls. High levels of OPG were released from colonic explant cultures (CEC) derived from inflamed IBD specimens, and colonic macrophages and dendritic cells costained for OPG. sRANKL levels from CEC were low both in IBD patients and healthy controls. Interestingly, increased expression of RANKL was mainly confined to cells in the lamina muscularis. A significant negative correlation was found between OPG plasma levels and femoral neck/lumbar spine bone mineral density.

Conclusions: We have demonstrated that IBD is associated with alterations in the RANKL/OPG system. Applying results from a murine model of colitis associated bone loss, the constellation of OPG and sRANKL regulation observed in our study raises the possibility that RANKL/OPG may contribute to the development of bone loss in IBD.
Colonic explant cultures (CEC)
Involved and non-involved colonic biopsies were obtained from a group of 68 IBD patients, 40 with CD and 28 with UC. The colonic mucosa was first assessed by gross endoscopic appearance and further specified by histological evaluation of biopsies taken in parallel. Thirty patients undergoing screening colonoscopy served as the healthy control population. CEC were performed essentially as described previously with minor modifications. Briefly, colonic biopsy specimens were collected in phosphate buffered saline (4°C), washed, weighed, and cultured in duplicate for 24 hours in 48 well plates in 1 ml of RPMI 1640 (Schoeller Pharma, Vienna, Austria) supplemented with 10% heat inactivated (56°C, 30 minutes) fetal calf serum, 100 U/ml penicillin G, and streptomycin (all Gibco BRL, Vienna, Austria). Supernatants of CEC were determined by commercially available specific ELISAs according to the manufacturers’ protocols (DuoSet Development System, human OPG/ TNFRSF11B, R&D Systems, Minneapolis, Minnesota, USA; sRANKL ELISA, Biomedica GmbH, Vienna, Austria). The lower detection limit of the OPG assay was 62.5 pg/ml and that of sRANKL 1.6 pg/ml. Absorption was determined with an ELISA reader at 450 nm.

Biochemical parameters and bone mineral density (BMD) assessment
Testing for routine biochemical parameters was performed in the central laboratory of the University Hospital Innsbruck according to standard automated techniques. BMD was assessed with a Hologic QDR 4500W x-ray bone densitometer for PA lumbar spine and proximal femur (femoral neck) in a subpopulation (total 43 IBD patients) of those patients that participated in the plasma levels study. BMD was performed on clinical grounds (that is, where indicated on a periodic schedule as part of the routine management of our IBD patients). BMD, and T and Z scores were determined for each patient according to the WHO Study Group Recommendations for the Definition of Osteoporosis and Osteopenia: a T score of $-1.0$ to $-2.5$ was classified as osteopenia and a T score $<-2.5$ was classified as osteoporosis. Details of the patients assessed are presented in table 2.

Immunohistochemistry
Sections of colonic tissue specimens (4 μm) were prepared from formalin fixed paraffin embedded specimens of colonic biopsies. Additionally, several historical surgical resection specimens obtained from the Institute of Pathology were also included in this analysis. Sections were deparaffinised in xylene and rehydrated in graded alcohols. A high temperature antigen retrieval technique by autoclaving slides in antigen unmasking solution (Vector Laboratories, Burlingame, California, USA) was used for OPG detection. For RANKL detection, antigen retrieval was performed by proteolytic treatment with proteinase K (DakoCytomation, Glostrup, Denmark). Endogenous peroxidase activity was quenched with 3% H₂O₂ in phosphate buffered saline for 10 minutes. Endogenous alkaline phosphatase activity was blocked with levamisole solution. Non-specific binding sites were blocked with serum free protein block (DakoCytomation). For OPG mouse antihuman OPG monoclonal antibody (mAb) was purchased from Imgenex (San Diego, California, USA). RANKL was detected by a mouse antihuman RANKL mAb from R&D Systems. Appropriate control antibodies for mouse IgG₁ and mouse IgG₂b were purchased from DakoCytomation. Slides were incubated with primary anti-RANKL (1:25; 20 μg/ml), anti-OPG (1:50; 10 μg/ml), and control Abs, diluted in Dako antibody diluent at 4°C overnight. After washing (3×5 minutes in phosphate buffered saline) OPG was visualised with a Dako LSAB+ Kit (DakoCytomation) with AEC (3-amino-9-ethylcarbazol) substrate and RANKL using a Vectastain ABC alkaline phosphatase kit with Vector Red alkaline phosphatase substrate (Vector Laboratories). Finally, sections were counterstained with haematoxylin QS (Vector Laboratories). OPG slides were aqueously mounted in Dako Faramount Mounting Medium and RANKL slides were dehydrated and mounted permanently in Eukitt (O Kindler GmbH, Freiburg, Germany).

Double immunofluorescence labelling
Colonic tissue specimens were embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, Netherlands). Sections (5 and 10 μm) were prepared, dried overnight, and fixed in 4°C acetone. For all staining experiments, isotype matched control antibodies were included to exclude

### Table 1 Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Crohn’s disease</th>
<th>Ulcerative colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>75</td>
<td>111</td>
<td>69</td>
</tr>
<tr>
<td>Age (y)*</td>
<td>36.8 (14.5)</td>
<td>33.4 (13.6)</td>
<td>36.4 (12.4)</td>
</tr>
<tr>
<td>Disease duration (y)*</td>
<td>35/42</td>
<td>0/66</td>
<td>36/33</td>
</tr>
<tr>
<td>Activity score (mean (SD))*</td>
<td>3.12 (4.93)</td>
<td>4.69 (6.8)</td>
<td>[0–23.3]</td>
</tr>
<tr>
<td>Medication*</td>
<td>None</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Aminosalicylate</td>
<td>46</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Steroid steroid</td>
<td>30</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Azathioprine</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Antibiotic</td>
<td>12</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Ciclosporine</td>
<td>11</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Values are mean (SD) [range].
†Crohn’s disease activity index (CDAI).κ
‡Activity assessed according to Rachmilewitz.κ
§Some patients had combined therapy.

### Table 2 Bone mineral density in the inflammatory bowel disease study population

<table>
<thead>
<tr>
<th>Patient characteristic</th>
<th>n</th>
<th>Controls</th>
<th>Crohn’s disease</th>
<th>Ulcerative colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD (g/cm²) lumbar vertebrae</td>
<td>0.912 (0.15)</td>
<td>0.925 (0.15)</td>
<td>0.918 (0.15)</td>
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<tr>
<td>T score (L1-L4)</td>
<td>1.48 (1.48)</td>
<td>1.46 (1.46)</td>
<td>1.48 (1.48)</td>
<td></td>
</tr>
<tr>
<td>Z score (L1-L4)</td>
<td>-1.25 (1.46)</td>
<td>-1.25 (1.46)</td>
<td>-1.25 (1.46)</td>
<td></td>
</tr>
<tr>
<td>BMD (g/cm²) femoral neck</td>
<td>0.713 (0.12)</td>
<td>0.713 (0.12)</td>
<td>0.713 (0.12)</td>
<td></td>
</tr>
<tr>
<td>T score (femoral neck)</td>
<td>-1.5 (1.13)</td>
<td>-1.5 (1.13)</td>
<td>-1.5 (1.13)</td>
<td></td>
</tr>
<tr>
<td>Z score (femoral neck)</td>
<td>-1.2 (1.17)</td>
<td>-1.2 (1.17)</td>
<td>-1.2 (1.17)</td>
<td></td>
</tr>
</tbody>
</table>

*Values are mean (SD) [range].
OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa B; sRANKL, soluble receptor activator of nuclear factor kappa B; BMD, bone mineral density.
RESULTS

OPG plasma levels are increased in IBD

We assessed OPG and sRANKL plasma levels in 112 patients with IBD and 45 control subjects. As depicted in fig 1A, plasma levels of OPG were significantly increased in CD as well as in UC. Highest OPG levels were detected in CD (CD 2111 (83) pg/ml, control 867 (48) pg/ml; p<0.001) (fig 1A). In IBD patients, OPG plasma levels were weakly positively correlated with leucocyte and platelet counts, while a negative correlation was found for erythrocyte counts (table 3).

In contrast, sRANKL plasma levels were not significantly different in IBD patients compared with healthy control subjects, and there were no differences between CD and UC (fig 1B). It should be noted that our detection system primarily detects free unbound sRANKL, thus not complexed with OPG and therefore presumably biological active.

To gain further insight into the activation state of the OPG/RANKL system, we calculated the OPG/sRANKL ratio based on the plasma level data. As outlined in fig 1C, the OPG/sRANKL ratio was significantly higher in CD and UC compared with control subjects, with CD showing the highest OPG/sRANKL ratio (CD 286.2 (16.6); control 98.1 (8.3); p<0.001).

Osteoprotegerin plasma levels are inversely correlated with BMD

Next, we assessed the relationship between OPG plasma levels and BMD, particularly in IBD patients. A significant negative correlation was found for erythrocyte counts (table 3)}
negative correlation between OPG and femoral neck (fig 2A) and lumbar spine (fig 3A) BMD was observed, although it should be noted that the correlation was not particularly strong. No such correlation was found between sRANKL plasma levels and BMD (fig 2B, 3B). Subgroup analysis in IBD patients with normal BMD (T score $>2.1$), with osteopenia (2.5 $\leq$ T score $< -1.0$), and with osteoporosis (T score $\leq -2.5$) revealed a gradual increase in OPG plasma levels, with a statistically significant increase observed in osteoporotic patients (fig 2A, 3A). Again, no such association was found for sRANKL (fig 2B, 3B). Indeed, sRANKL levels tended to decrease with the level of bone loss although the decline did not reach statistical significance (fig 2B, 3B).

As might be inferred from the aforementioned data, analysis based on OPG/sRANKL levels showed a gradual increase from controls to osteopenic to osteoporotic IBD patients, as demonstrated for femoral neck (fig 2C) and lumbar spine (fig 3C) BMD.

**Colonic explant cultures from inflamed IBD regions release large amounts of OPG**

To gain insight into whether the intestine might indeed be a source of sRANKL and OPG and therefore could in

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**Figure 2** Osteoprotegerin (OPG) and soluble receptor activator of nuclear factor kappa B (sRANKL) plasma levels and bone mineral density (BMD) in inflammatory bowel disease (IBD) patients. Left panels show the correlation of OPG levels (A), sRANKL plasma levels (B), and the OPG/sRANKL ratio (C) with femoral neck BMD. Right panels show analysis of OPG, sRANKL plasma levels, and the OPG/sRANKL ratio in IBD patients classified as normal, osteopenic, and osteoporotic according to their BMD.
principal account for the increased plasma OPG levels observed, we obtained CEC. CEC are a useful method of determining cytokine secretion in tissue as ex vivo cultured colonic specimens are likely to reflect the in vivo situation and provide this information without the bias introduced by studying only a particular cell population or by in vitro stimulation. As shown in fig 4A, a 3.4-fold increase in OPG release was noted in specimens derived from inflamed tissue of CD patients compared with healthy controls while OPG release from non-involved CD specimens was essentially identical to healthy controls (fig 4A). With regard to UC, OPG release from involved specimens was on average increased 3.8-fold (showing wide variation in the levels detected) while non-involved regions showed OPG levels comparable with healthy controls as well as non-involved CD (fig 4A). Low amounts of sRANKL were released irrespective of disease state in CD, UC, and healthy controls (fig 4B).

**Relationship between OPG and sRANKL levels and IBD therapy**

We next asked whether there is an association between therapy and OPG and sRANKL levels. While no such association was found for OPG (fig 5A), IBD medication had a

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**Figure 3** Osteoprotegerin (OPG) and soluble receptor activator of nuclear factor kappa B (sRANKL) plasma levels and bone mineral density (BMD) in inflammatory bowel disease (IBD) patients. Left panels show the correlation of OPG levels (A), sRANKL plasma levels (B), and the OPG/sRANKL ratio (C) with lumbar spine BMD. Right panels show analysis of OPG, sRANKL plasma levels, and the OPG/sRANKL ratio in IBD patients classified as normal, osteopenic, and osteoporotic according to their BMD.
significant influence on sRANKL levels (fig 5B). Patients treated with corticosteroids or combined azathioprine and corticosteroids showed significantly lower sRANKL levels compared with IBD patients who received no specific therapy (no medication: 12.7 (1.8) pg/ml; steroid: 7.2 (0.3) pg/ml, p < 0.01; azathioprine + steroid: 7.4 (0.6) pg/ml, p < 0.05). sRANKL levels of patients treated with 5-aminosalicylate or azathioprine alone were lower but this difference did not reach statistical significance (fig 5B). As active and inactive IBD do not show differences in sRANKL plasma levels (data not shown), it is unlikely that differences in clinical activity scores between the various treatment groups could explain the lower sRANKL plasma levels in steroid treated patients. No significant changes associated with medication were found for the OPG/sRANKL ratio (fig 5C).

Immunohistochemical localisation of OPG and RANKL in the mucosa

While few OPG+ cells were found in control mucosa, we observed abundant numbers of OPG expressing cells in colonic tissue sections derived from CD and UC patients (fig 6). While those few OPG+ cells in control subjects showed relatively faint staining, OPG staining in CD and UC samples was particularly bright in various cell types, including epithelial cells (fig 6). OPG+ cells were especially present in lymphoid aggregates, and notably in the lamina muscularis, the latter being completely absent in control subjects (data not shown). In control subjects, RANKL+ cells were

Figure 4  Osteoprotegerin (OPG) (A) and soluble receptor activator of nuclear factor kappa B (sRANKL) (B) release from colonic explant cultures. Colonic biopsies from Crohn’s disease (CD), ulcerative colitis (UC), and control subjects were cultured for 24 hours and supernatants assayed for OPG and sRANKL. Data are expressed as pg/ml normalised to the weight of the individual biopsies.

Figure 5  Association of inflammatory bowel disease (IBD) medication with osteoprotegerin (OPG) (A) and soluble receptor activator of nuclear factor kappa B (sRANKL) (B) plasma levels. Plasma levels of OPG, sRANKL, and the OPG/sRANKL ratio (C) were analysed with respect to current IBD associated medication.

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predominantly found in the submucosa while they were also present in between crypts in CD and UC (fig 7). Furthermore, RANKL+ cells were found in the lamina muscularis in CD whereas this finding was less pronounced in UC and absent in healthy controls (fig 7).

**Identification of cellular sources of OPG and RANKL in the colonic mucosa**

To gain insight into the cellular sources of OPG and RANKL in IBD mucosa, we performed immunofluorescence double staining for CD3, MHC II, CD163, and DC-LAMP. As depicted in fig 8 (A, B, C), OPG predominantly colocalised with MHC IIbright, CD163, and DC-LAMP, suggesting macrophages and dendritic cells as primary sources. In contrast, RANKL primarily colocalised with CD3, suggesting T lymphocytes as an important source, but was also detected on some MHC II+ cells (fig 8D, E, F).

**DISCUSSION**

In this study we have described alterations in the RANKL/OPG system in IBD and its relationship to decreased BMD in these patients. We demonstrated increased plasma levels of OPG as well as increased release from the inflamed colon in IBD, suggesting the colonic mucosa as a source of circulating OPG in IBD. The relative contribution of mucosa derived OPG and sRANKL to their corresponding serum levels was difficult to estimate in this human study. Furthermore, OPG plasma levels were inversely correlated with BMD (that is,
osteoporotic IBD patients showed substantial elevation in circulating OPG. On the other hand, we did not observe any change in sRANKL plasma levels or sRANKL release from CEC in IBD, although we noted increased numbers of RANK+ cells, particularly in the lamina muscularis of IBD patients.

At first glance, an increased level of the decoy receptor (OPG) with virtually no change in the osteoclastogenic agonist (sRANKL) in IBD strongly argues against a role for the RANKL system in the development of bone loss and osteoporosis in IBD. Indeed, the opposite could be true. This view is supported by the recent demonstration of mechanisms of bone loss via the RANK/RANKL system in the IL-2 deficient mouse model of colitis, which may be a valuable model for bone loss in IBD.10 Colitic IL-2−/− mice develop substantial osteopenia with increasing age, with bone loss detectable at the onset of colitis at the age of four weeks.10 Development of bone loss in this model is clearly dependent on sRANKL release from activated T cells.10 Notably, elevated sRANKL serum levels are only observed at an early stage at the onset of bone loss and colitis, and decline rapidly to normal levels by week 9.10 In contrast, OPG levels start to substantially increase later, without ever reaching a plateau until the end of the observation period.10 This may explain why we “only” observed normal levels of sRANKL and an increase in OPG levels in our human study, which could well be interpreted as an indication of the involvement of this system in IBD associated bone loss.

As in the mouse colitis model, increased OPG levels may represent a continuing homeostatic response, attempting to reverse established osteopenia and RANKL driven osteoclasis, thus maintaining normal bone mass. This view is supported by the gradual increase in OPG levels in osteopenic and osteoporotic IBD patients, as shown in our study. However, longitudinal studies of OPG/RANKL and BMD are required to validate this model and to assess the relative contribution of the various factors that lead to bone loss in IBD. Of particular note, glucocorticoid total dose could be one factor indirectly associating inflammation with bone loss in IBD. Of particular note, glucocorticoid total dose was inversely associated with BMD in many studies of IBD,21 22 but not all.23 Current glucocorticoid use has been reviewed by Bernstein and Leslie.9 Although it is virtually impossible in the human system to precisely determine the relative contribution of the various factors that lead to bone loss in IBD, our human data interpreted in the context of the IL-2−/− animal model suggest that the RANKL/OPG system could be one factor indirectly associating inflammation with bone loss in IBD. Of particular note, glucocorticoid total dose was inversely associated with BMD in many studies of IBD,21 22 but not all.23 Current glucocorticoid use has been reported to show both no effect on BMD24 and diminished BMD.25Against common belief, it is thus still uncertain if an apparent glucocorticoid effect results from the medication itself or is simply a marker of more severe IBD.6 In this regard, it is interesting to note that we observed decreased levels of sRANKL in steroid treated IBD patients while there was no obvious alteration in OPG plasma levels in such patients.

In conclusion, we have provided evidence that the RANKL/OPG system is altered in IBD and further studies on its pharmacological manipulation in IBD is warranted.

Figure 8  Cellular localisation of osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B (RANKL) in colonic mucosa. OPG (A, B, C, red) and RANKL (D, E, F; red) expression in colonic tissue specimens was analysed by double immunofluorescent microscopy to identify cell types secreting these molecules. Anti-MHC II (DC, macrophages, B cells), anti-CD163 (macrophages), anti-DC-LAMP (activated DC), and anti-CD3 (T lymphocytes) are shown in green. Double staining is represented by yellow (green+red). Cell nuclei are shown in blue. Specificity of staining was confirmed by isotype matched irrelevant monoclonal antibodies for all antibodies used (data not shown). OPG primarily colocalised with MHC II, CD163, and CD-LAMP. RANKL predominantly colocalised with CD3, and in smaller numbers with MHC II. Magnification: A, E, 400×; B, F, 630×; C, D, 200×.

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Conflict of interest: None declared.

EDITOR’S QUIZ: GI SNAPSHOT

Answer

Abdominal computed tomography revealed gastric pneumatosis with pneumoperitoneum and complete thrombosis of the coeliac trunk. Upper endoscopy showed necrosis of the lesser curvature reaching the duodenum, diffuse erythema and swelling of the remaining gastric mucosa, and a prepyloric 1 cm wide gastric ulcer. Purulent sterile liquid surrounded the stomach at laparotomy. The great tuberosity of the stomach was necrotic with diffuse surface necrosis of the remaining stomach and the right and sigmoid colon. The small intestine was also ischaemic with diffuse petechiae on its surface. Extended thrombosis of the left femoral artery precluded clearing of the obstruction with a Fogarty catheter. At this point, no procedure was deemed possible and the patient died 12 hours later.

Gastric pneumatosis is not a disease but a finding that should be investigated based on the patient’s clinical situation, and on his past and presenting history. These situations include: (1) gastric emphysema (gastric outlet syndrome, gastric carcinoma, intestinal volvulus, small bowel obstruction, forceful vomiting, and gall stones); (2) emphysematous gastritis (Escherichia coli, Enterobacter, Clostridium species, Pseudomonas aeruginosa, Bacillus species, Aerobacter aerogenes, Staphylococcus aureus, caustic or corrosive substances, alcohol intoxication, ischaemia, infarction, previous gastrointestinal surgery, strongyloidosis, diabetes, and immunosuppression); and (3) pneumatosis cystoides intestinalis (neoplastic, infectious, chemical, and mechanical).

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