

## Increased expression of receptor activator of NF- $\kappa$ B ligand (RANKL), its receptor RANK and its decoy receptor osteoprotegerin in the colon of Crohn's disease patients

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### SUMMARY

Crohn's disease (CD) is associated with low bone mass due to chronic inflammation and other factors. Receptor activator of NF- $\kappa$ B ligand (RANKL), its receptor RANK and its decoy receptor osteoprotegerin (OPG) are potentially involved in this process as they regulate osteoclastogenesis and are influenced by pro-inflammatory cytokines. The aim of this study was to determine the levels of soluble RANKL (sRANKL), RANK and OPG expression both in the serum and in the colon of CD patients. Levels of sRANKL and OPG were assessed in the serum and the supernatants of cultured colonic biopsies in patients with CD and controls by ELISA. RANK expression was explored by immunostaining and immunofluorescence of fixed colonic samples. OPG and sRANKL levels were higher in the serum of CD patients as compared to age- and sex-matched controls. Levels of sRANKL and OPG were significantly enhanced in cultured colonic biopsies from CD, and OPG levels correlated with histological inflammation, and pro- and anti-inflammatory cytokine levels. No significant correlation was found for sRANKL. RANK<sup>+</sup> cells were increased in the colon of CD, particularly in inflamed areas. These cells were positive for CD68 or S100 protein. We conclude that serum and local levels of sRANKL and OPG are increased in CD. Moreover, RANK is expressed in the colonic mucosa by subpopulations of activated macrophages or dendritic cells at higher levels in CD compared to normal colon.

**Keywords :** Crohn's disease ; osteoporosis ; inflammation ; osteoprotegerin ; RANKL.

### INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract. The aetiology of the disease is not fully understood but an abnormal immuno-inflammatory reaction takes place in the bowel wall [1]. This abnormal reaction is characterized by an increased number and an aberrant activation of T lymphocytes and monocytes-macrophages. A predominant production of Th1 cytokines and an excessive activation of NF- $\kappa$ B are observed consequently [2,3]. While CD is mainly an inflammatory bowel disease, it is frequently associated with extra-intestinal manifestations and in particular, with an increased risk of osteopenia, osteoporosis, and fractures [4-9]. Mechanisms responsible for bone loss in CD are various including calcium and vitamin D deficiency, steroid treatment, but also chronic inflammation [10] as a result of longstanding elevation of circulating pro-inflammatory cytokines.

The production of the Receptor Activator of NF- $\kappa$ B ligand (RANKL), notably by T lymphocytes or by osteoblasts has recently been identified as a potential mechanism underlying bone loss in systemic inflammatory diseases [11]. After binding to its receptor RANK, RANKL increases osteoclast development, osteoclast activity and survival [12]. Moreover, RANKL expression in osteoblasts is stimulated by pro-inflammatory cytokines [12]. In this case, RANKL may be produced in the bone microenvironment in response to high levels of circulating proinflammatory cytokines including tumour necrosis factor (TNF) and interleukin (IL)-1 [12]. Alternatively, RANKL could also be produced in the gut and act in an endocrine manner on bone metabolism. In physiological conditions, RANKL is not highly expressed in the intestine but RANKL expression in both T-cells and osteoblastic cells is known to be tightly regulated [13,14].

Cell response to RANKL depends on the level of expression of its receptor RANK but also on the presence of its decoy receptor OPG. By binding to RANKL, OPG prevents RANK activation and signalling as observed in bone remodeling where OPG acts as a potent inhibitor of osteoclastogenesis [15]. OPG is highly expressed in bone

marrow stromal cells but OPG transcripts are detected in the gastrointestinal tract of mouse embryo [14,15]. OPG synthesis is induced in B lymphocytes, dendritic cells (DC) and follicular DC, notably through CD40 - CD40 ligand interaction [16]. However, the exact role played by OPG in the immune system is presently not well understood.

If RANKL and OPG were produced in significant amount in the colon, it would be critical to know the level of RANK expression in the colon in the light of the prominent role played by RANKL-RANK interaction on T lymphocytes and DC [17,18]. While RANK mRNA is detected in many tissues in mice including the small and large intestines, RANK protein expression is mainly detectable on the surface of mature DC [19]. RANKL-RANK interaction results in an increase in DC survival and DC production of cytokines [18,19].

The first aim of our study was to determine the level of sRANKL and OPG proteins in the serum of CD patients. As serum levels were found to be increased in CD, we next looked at the local production of sRANKL and OPG in the colonic mucosa and we also tested the possibility of a local function for sRANKL and OPG by studying RANK colonic expression in CD patients.

## METHODS

### *Serum levels of sRANKL and OPG in CD*

Twenty-five patients with CD, prospectively seen at our outpatient clinic, were recruited for serum level measures. Diagnosis of CD was made on the basis of clinical, radiological and histological data, according to standard criteria [20]. Clinical characteristics of the patients were recorded during the medical visit and are shown in Table 1. Crohn's Disease Activity Index (CDAI) [21] were retrospectively calculated, based on the symptoms of the last week. Fifteen had active disease, defined by a CDAI >150 and 10 had inactive disease. Median CDAI was 283 (range 79-609) and median CRP was 22 mg/l (range 3-148 mg/l). These patients were compared to 18 age- and sex-matched controls, who were laboratory workers. Serum samples were collected and stored at -20°C before OPG and sRANKL measurements. Levels of OPG were measured using a sandwich enzyme-linked immunosorbent assay procedure (R & D Systems, Minneapolis, MN, USA). Levels of sRANKL were measured with an improved sensitivity ELISA according to the manufacturer's recommendations (Biomedica Gesellschaft mbH., Wien, Austria). Results were expressed in pg/ml for OPG and for sRANKL. Sensitivity of the test was 5 pg/ml and 1.6 pg/ml for OPG and RANKL, respectively.

### *Production of sRANKL, OPG, and correlation to pro- and anti-inflammatory cytokines by colonic mucosa*

*Intestinal biopsies.* Colonic biopsies were obtained in macroscopically and microscopically unaffected or affected areas from 23 consecutive CD patients undergoing colonoscopy for medical reason. Characteristics of the patients are given in Table 1. We were able to collect biopsies in both inflamed (I) and uninflamed (UN) areas for 6 of these patients (paired biopsies), we could collect biopsies only in uninflamed area in 8 patients and only in inflamed area in 9 patients. The control group included 7 patients without inflammatory bowel disease who underwent endoscopy for irritable bowel syndrome or polyp/cancer screening. All controls were free of colonic lesion. The protocol was approved by the Ethic Committee of Liège University Hospital.

*Tissue culture.* After collection, biopsy specimens were transferred to the laboratory and handled within a maximal lag of 3 h after biopsy. Tissues were gently washed three times in CMF-Hank's medium (Gibco, Invitrogen, Merelbeke, Belgium) supplemented with antibiotics, blotted carefully, weighted and individually placed in 24-well tissue culture plates in triplicates. Intestinal tissues were cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 2 mM L-glutamine (Gibco), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified 95% air/5% CO<sub>2</sub> as previously described [22]. After 18 h, culture media were collected, centrifuged and stored at -80°C until required for immunoassays. Lactate dehydrogenase levels were measured to control for the absence of necrosis at the end of the culture period and histological analyses were performed in parallel to demonstrate structural integrity [23].

*Immunoassays.* Levels of OPG and sRANKL produced in the culture medium of CD patients and controls were analysed using a sandwich enzyme-linked immunosorbent assay procedure as described above. Results were corrected for the weight of the biopsy and expressed as pg/ml of OPG or sRANKL per mg of tissue. TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10 and MMP-3 production were measured with specific immunoassays, according to manufacturer's instructions (EASIA, from Biosources Europe, Fleurus, Belgium). Results were corrected for the weight of the

biopsy and expressed as pg/ml per mg of tissue. The median weight of the biopsies was 13.7 mg (6.3 mg-27.2 mg). Sensitivity of the ELISA were 5 pg/ml, 1.6 pg/ml, 3 pg/ml, 2 pg/ml, 2 pg/ml, 1 pg/ml and 100 pg/ml, for OPG, sRANKL, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10 and MMP-3, respectively. For OPG, sRANKL, the cytokines and MMP-3, the final result corresponds to the mean of 3 individual tissue cultures obtained from the same patient. The mean coefficient of variation between the 3 individual biopsies was 27.2%  $\pm$  13.1 for OPG, 31.4%  $\pm$  15.4 for sRANKL, 33.6%  $\pm$  13.5 for TNF- $\alpha$ , 23.4%  $\pm$  12.7% for IL-1 $\beta$ , 42.1%  $\pm$  13.1 for IL-6, 34.5%  $\pm$  14.3 for IL-10, and 34.2%  $\pm$  11.7 for MMP-3.

**Table 1.** Characteristics of the Crohn's disease (CD) patients ( $n = 57$ )

	CD patients included in the serum level measures ( $n = 25$ )	CD patients included in the colonic biopsy supernatant measures ( $n = 23$ )	CD patients included in the immunohisto- chemistry study ( $n = 9$ )
Age (years)†	35 (20-64)	37 (18-51)	29 (22-57)
Disease duration (months)†	108 (10-289)	94 (7-317)	75 (5-405)
Gender (F/M)	15/10	16/7	6/3
Disease location (Vienna classification)			
L1	1	3	0
L2	11	12	4
L3	12	8	5
L4	1	0	0
Treatment			
Glucocorticoids	19	10	5
Mesalamine	15	11	4
Immunosupp.	15	7	3
Antibiotics	8	5	1

†median (range)

### ***Immunostaining and immunofluorescence for RANK expression in the colon***

We used surgically resected colonic specimens from 5 controls and 9 CD patients (9 and 5 tissue samples, in inflamed (I) and uninfamed (UN) areas, respectively) for immunostaining and immunofluorescence. The 5 controls had underwent surgery for colorectal cancer and tissue samples were taken at distance from the cancer. The 9 CD patients had underwent surgery for either stricturing complication ( $n = 4$ ), fistulizing complication ( $n = 4$ ), or disease refractory to medical treatment ( $n = 1$ ). Characteristics of the patients are shown in Table 1. Tissue samples had been fixed in formalin and embedded in paraffin according to routine procedures and were stored at the pathology department of Liège University Hospital.

Briefly, five-micron sections of the biopsy specimens were deparaffinized and incubated with H<sub>2</sub>O<sub>2</sub> (3% in methanol) for 10 min. Antigen retrieval was then performed by treating the slides for 5 min in boiling heat cooker in EDTA buffer (1 mM; pH 9.0). After washing in distilled water, sections were permeabilized using Tween (0.015%; pH 7.2) in PBS buffer and incubated at room temperature with an anti-RANK monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at a dilution of 1/500 for immunoperoxidase and for 60 min at a dilution of 1/250 for immunofluorescence. The slides were then washed and incubated at room temperature for 30 min with goat antirabbit immunoglobulins conjugated to peroxydase (Envision + ; DAKO A/S, Glostrup, Denmark) or for 60 min with swine antirabbit FITC-antibody (1/40; DAKO A/S). The first antibody (anti-RANK) was omitted as a negative control. Fluorescent positive cells were visualized by using a fluorescent microscope (Leica DMLB microscope, Heidelberg GmbH, Germany) equipped with a 40x objective whereas immunoperoxidase staining was evaluated by light microscopy after chromogenic revelation with diaminobenzidine (0.5 mg/ml). To compare RANK expression between CD and control tissues, the density of immunofluorescent cells was precisely determined by selecting 5 fields in the most positive regions of each section and by counting the number of positive cells per field at 400x magnification. The mean value was then calculated and recorded for each section [24].

In order to identify RANK-expressing cells, serial sections were analysed by using the same immunostaining protocol with the following antibodies: CD3 polyclonal rabbit antibody (1/40; DAKO), CD1a monoclonal mouse antibody (1/10; NOVOCAS TRA, Newcastle, UK), CD20 monoclonal mouse antibody (1/2000; DAKO), CD68 monoclonal mouse antibody (1/3500; DAKO), MAC3S7 monoclonal mouse antibody (1/1600; DAKO), S100

polyclonal rabbit antibody (1/2000; DAKO).

A double RANK/S100 and RANK/CD68 immunostaining was also carried out. After RANK immunodetection performed as already described, sections were rinsed in PBS Tween (0.015% pH 7,2), then incubated at room temperature with either S100 polyclonal rabbit antibody (1/2000) or with CD68 monoclonal mouse antibody (1/3500). The sections were then washed and incubated for 30 min at room temperature with anti mouse/anti-rabbit antibody coupled to alkaline phosphatase (DAKO). After washing, the chromogenic revelation was performed with fast red (DAKO).

### Statistical analysis

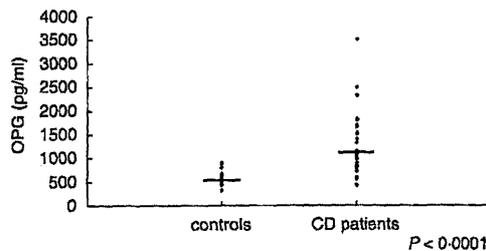
Levels of OPG and sRANKL in the serum and in the supernatants of cultured colonic biopsies were expressed as median (interquartile range) and were compared by the Mann-Whitney test. Levels of OPG and sRANKL in the supernatants of paired cultured biopsies were compared by the Wilcoxon test. Correlations between OPG or sRANKL levels in the serum and CDAI or CRP as well as between OPG and sRANKL in the culture supernatants and pro- or anti-inflammatory cytokines were studied by the Spearman test. The number of RANK<sup>+</sup> labelled cells/field in the colon of controls, I and UN CD were expressed as median (range) and were compared by the Mann-Whitney test. For all these tests, the differences were considered as significant at  $P < 0.05$ .

## RESULTS

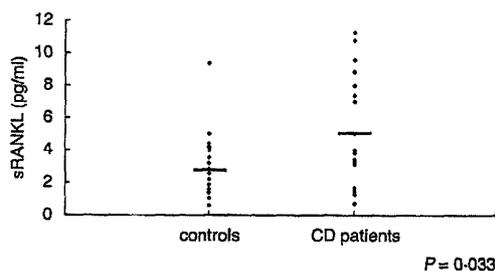
### Serum levels of sRANKL and OPG in CD

OPG and sRANKL serum levels were significantly higher in CD patients than in controls (Figs 1 and 2). OPG and sRANKL serum levels were not significantly higher in clinically active (CDAI >150), than in clinically inactive CD (OPG: 1405 pg/ml (800-1695) and 1075 pg/ml (830-1655), respectively; sRANKL: 4.0 pg/ml (range 1.4-8.86 pg/ml) and 5.0 pg/ml (range 1.7-7.4 pg/ml) respectively). There was no significant correlation between CDAI and sRANKL or OPG serum concentrations. However, CRP levels in the serum correlated to those of OPG ( $r = 0.48$ ;  $P = 0.05$ ) but not to those of RANKL.

**Fig. 1.** Serum levels of OPG in Crohn's disease (CD) and controls. Levels of OPG in the serum of 25 patients with CD and 18 sex- and age- matched normal subjects (controls) measured by ELISA. Results are expressed in pg/ml. Horizontal bars correspond to the medians. CD and controls were compared with a Mann-Whitney test. Median levels and interquartile ranges were 540-5 pg/ml (range 479-634) and 1150 pg/ml (range 830-1690), in controls and CD, respectively ( $P < 0.0001$ ).



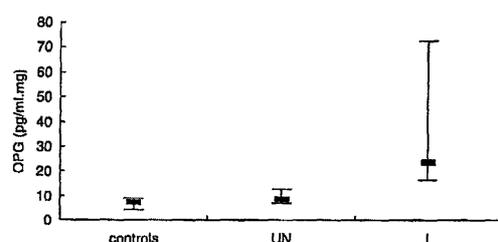
**Fig. 2.** Serum levels of sRANKL in Crohn's disease (CD) and controls. Levels of sRANKL in the serum of 25 patients with CD and 18 sex- and age- matched normal subjects (controls) measured by ELISA. Results are expressed in pg/ml. Horizontal bars correspond to the medians. CD and controls were compared with a Mann-Whitney test. Median levels and interquartile ranges were 2.71 pg/ml (1.6-4.14) and 5.0 pg/ml (3.26-7.4), in controls and CD, respectively ( $P = 0.033$ ).



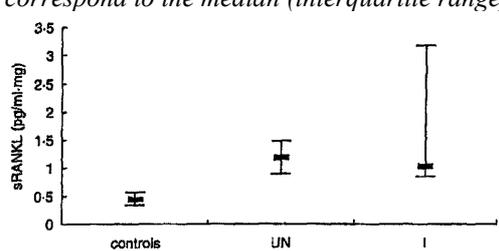
### Production of sRANKL and OPG by colonic mucosa. Correlation with pro- and anti-inflammatory cytokines

We first confirmed by RT-PCR that RANKL, RANK and OPG transcripts were present in human colonic biopsies (data not shown). We next studied protein levels of sRANKL and OPG by immunoassay. Levels of OPG in supernatants of cultured colonic biopsies were significantly higher in CD than in controls and were higher in I than UN areas (6.6 pg/mg ml (range 4.2-6.8 pg/ml), 9.0 pg/mg ml (range 8.6-12.5 pg/ml), and 23.7 pg/mg ml (range 15.6-79.2 pg/ml), in controls, UN and I, respectively;  $P = 0.0016$  between controls and UN;  $P < 0.0001$  between UN and I areas of (CD) (Fig. 3). When comparing I and UN areas in paired biopsies, OPG levels were also significantly higher in I areas of CD patients ( $P = 0.003$ ). Levels of sRANKL were also higher in supernatants of UN CD biopsies than in controls (1.2 pg/mg.ml (0.89-1.49) and 0.46 pg/mg.ml (0.34-0.57), respectively;  $P = 0.0066$ ), but there was no further significant increase in I CD (1.03 pg/mg.ml (0.85-3.18);  $P = 0.58$ ) (Fig. 4). When comparing paired biopsies in I and UN areas of CD patients, there was no significant difference either. Correlations between OPG, sRANKL and pro- (TNF, IL-1, IL-6) or anti-inflammatory (IL-10) cytokines in CD are shown in Table 2. OPG was significantly correlated to all pro- and anti-inflammatory cytokines tested, while sRANKL did not significantly correlate to anti-inflammatory cytokines. MMP-3 was chosen as a marker of tissue destruction [25]. OPG, but not RANKL, was significantly correlated to this protease [Table 2].

**Fig. 3.** Production of OPG by colonic mucosa. Levels of OPG in supernatants of cultured colonic biopsies obtained from 23 CD patients in macroscopically and microscopically uninflamed (UN,  $n = 14$ ) or inflamed (I,  $n = 15$ ) areas and 7 patients free of intestinal disease (Controls) during colonoscopy (UN CD versus controls:  $P = 0.0016$ ; UN CD versus I CD:  $P < 0.0001$ ).



**Fig. 4.** Production of sRANKL by colonic mucosa. Levels of sRANKL in supernatants of cultured colonic biopsies obtained from 23 CD patients in macroscopically and microscopically uninflamed (UN,  $n = 14$ ) or inflamed (I,  $n = 15$ ) areas and 7 patients free of intestinal disease (Controls) during colonoscopy (UN CD versus. controls:  $P = 0.0066$ ; UN CD versus. I CD:  $P = 0.58$ ). After collection, biopsy specimens were washed before weighing and cultured in triplicate as indicated in the methods. OPG and sRANKL levels in the supernatant were measured by ELISA. Results are expressed in pg/ml per mg of tissue (pg/mlmg) and correspond to the median (interquartile range).



**Table 2.** Correlation between cytokines, MMP-3 and OPG or sRANKL in the supernatant of cultured colonic biopsies (Spearman test).

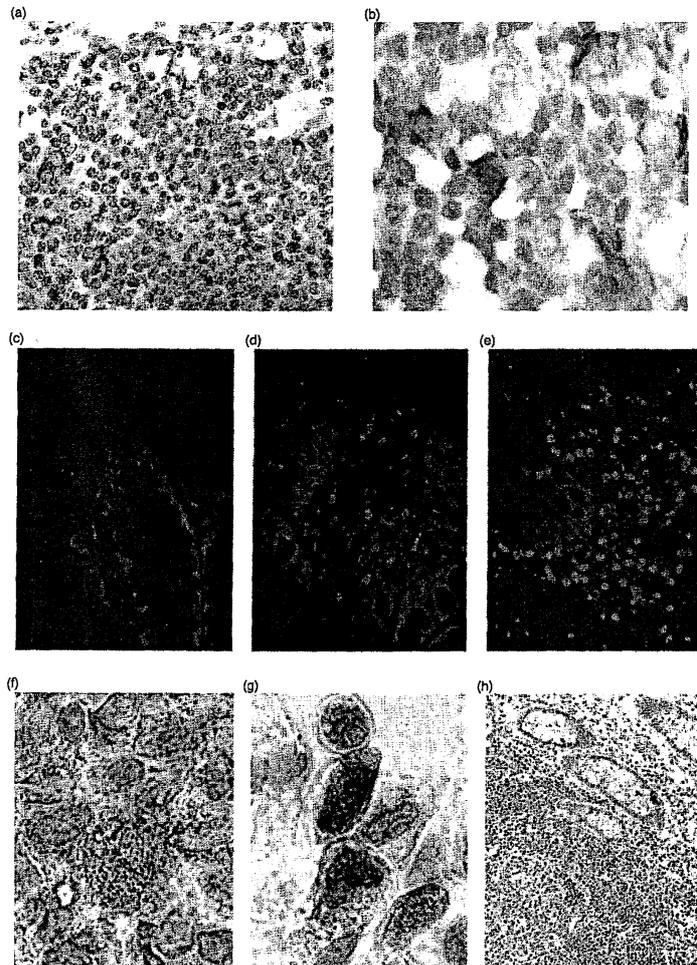
	TNF $\alpha$	IL1 $\beta$	IL-6	IL-10	MMP3
OPG	$P = 0.016$ ; $r = 0.53$	$P = 0.002$ ; $r = 0.65$	$P < 0.0001$ ; $r = 0.80$	$P = 0.002$ ; $r = 0.65$	$P < 0.0001$ ; $r = 0.79$
sRANKL	$P = 0.87$	$P = 0.71$	$P = 0.84$	$P = 0.95$	$P = 0.59$

### ***RANK expression in the colon by immunofluorescence and immunostaining***

Immunoperoxidase demonstrated the presence of RANK-positive cells in the mucosal layer of the intestine and, at a lesser extent, in the submucosa. No or very little RANK immunoreactivity was detected in the muscularis propria and in the subserosa. This was mainly seen in CD and less visible in controls where only isolated lymphoid follicles showed a focal staining in the germinal centres. Positive cells were essentially large mononuclear cells (Fig. 5a,b). After immunofluorescence, the different densities of positive cells between controls and CD became more evident (Fig. 5c-e). The density of RANK<sup>+</sup> cells was significantly increased in inflamed areas of CD patients as compared to controls (16.8 (2.2-41.6) *versus* 4.2 (0-10), respectively;  $P = 0.048$ ), but there was no significant difference with uninflamed area, either in positive cell density (12.5 (0.2-24.6)) or in staining distribution.

As RANK<sup>+</sup> cells exhibited morphological features of inflammatory mononuclear cells, serial sections were used to compare the immunostaining patterns of RANK and of markers of lymphocytes (CD3, CD20), macrophages (MAC387, CD68) and dendritic cells (CD1a, S100). There was a close correlation between RANK and CD68 or S100 immunostaining patterns. A double RANK/S100 and RANK/CD68 immunostaining was then carried out to further identify the nature of RANK<sup>+</sup> cells. As shown in Fig. 5f,g, many cells exhibited the double staining, demonstrating that RANK<sup>+</sup> cells likely belong to subpopulations of activated macrophages or dendritic cells.

**Fig. 5.** *RANK expression in the colon by immunohistology. Representative examples of RANK immunostaining in CD and control tissues. (a,b). Immunoperoxidase staining. Detection of many RANK-positive cells in the mucosa of a CD biopsy specimen (magnification x400) (a); RANK immunostaining of mononuclear macrophage-like cells (magnification x1000) (b); (c-e). Immunofluorescence detection of RANK-positive cells in normal colon tissue of a non CD (c) or CD (d) patient and in inflammatory colon tissue of a CD patient (e). (f,g). Double immunostaining demonstrating the presence of RANK (brown)/CD68 (red)-positive cells (f) and of RANK (brown)/S100 (red) positive cells (g) (magnification x1000). (h). No immunostaining in the mucosa of a CD biopsy specimen when the primary antibody is omitted (negative control).*



## DISCUSSION

In this study, we have shown increased levels of OPG and sRANKL in the serum of patients with CD. We also demonstrated an increased production of OPG and sRANKL by the colonic mucosa in CD. Moreover, RANK was expressed in the human colonic wall, mainly in the mucosa and submucosa by mononuclear cells positive for CD68 and/or s100. The number of RANK-positive cells appeared to be increased in the colon of CD.

RANKL is a critical factor for osteoclast differentiation and activation [14]. In the bone microenvironment, RANKL is produced by cells from the osteoblast lineage and its expression is induced after treatment with pro-inflammatory cytokines. RANKL expression on T-cells is considered to play a major role in bone loss observed in rheumatoid arthritis, an inflammatory disease associated with joint destruction [11]. In CD, which is characterized by chronic systemic inflammation and decreased bone mass, expression of RANKL and production of sRANKL had never been studied until very recently [26]. In this recent study, serum levels of sRANKL were measured in a cohort of patients before and during glucocorticoid treatment. No significant change was observed although there was a significant decrease in BMD after 12 weeks [26]. Yet, sRANKL levels were not compared to levels in control subjects. Our data show that there is a significant increase in sRANKL serum levels in CD patients. Currently, available kits for sRANKL measurement only detect free sRANKL [27]. Therefore, the observed increase in sRANKL levels could be the result of a decrease in RANKL's decoy receptor OPG. This does not seem to be the case in our study as we found a concomitant increase in OPG levels. Functionally, the increase in OPG may counter-balance the effect of sRANKL on bone metabolism [15]. It is however, impossible to draw conclusions on the biological consequences of both OPG and sRANKL increase since their effect has to be considered in the bone, where the local concentration may be affected differently.

Understanding the source of sRANKL and OPG protein levels in the serum is crucial. As already established, these mediators are synthesized by bone stromal cells and osteoblasts [14,15]. They may also be produced by activated DC and lymphocytes, mainly T lymphocytes for RANKL and B lymphocytes for OPG [11,13,14,16-19]. As a dramatic increase in activated T and B lymphocytes is present in the colonic mucosa and submucosa in CD [1], we looked at the production of OPG and sRANKL in the colon. After confirming the presence of both RANKL and OPG transcripts in the human colon, we observed a significant increase in both OPG and sRANKL levels in the culture media of colonic biopsies obtained from CD as compared to controls. OPG levels in the culture supernatant of colonic biopsies were significantly correlated to pro-inflammatory cytokines, suggesting that OPG is up-regulated in the colon as a result of chronic inflammation. In accordance with this observation, OPG is up-regulated in DC and primary B cells by activation through CD40 [16]. In osteoblasts, OPG synthesis is also enhanced by several cytokines including TNF- $\alpha$  [28]. Both CD40 expression and TNF- $\alpha$  production are increased in CD, potentially contributing to the observed increase in OPG protein levels [1,29].

While sRANKL was significantly increased in the culture supernatant of CD colonic biopsies as compared to controls, there was no further increase when comparing I and UN area of the colon in CD patients. Again, like in the serum, this may be due to a significant amount of sRANKL bound to OPG in the supernatant and therefore undetectable using our ELISA. Alternatively, RANKL may be differently affected by inflammation. However, this hypothesis is unlikely as pro-inflammatory cytokines are known to induce both RANKL and OPG expression [14]. RANKL mRNA is constitutively expressed in memory, but not naïve T-cells [13,19] and RANKL-expression in T cells is induced by antigen-receptor engagement [11,30]. The increase in sRANKL production described here in the colonic mucosa of CD may correspond to an increased proportion of memory activated T cells.

Hence, we have shown that the colon may be an important source of RANKL and OPG in CD and we suggest that these locally produced mediators may spill out into the blood and possibly reach the bones where they can influence bone remodeling. Beyond this, the production of OPG and sRANKL in the colonic mucosa is intriguing since these mediators play a prominent role in lymphocytes and DC activation and differentiation and could therefore act locally by influencing the mucosal immune regulation. Nevertheless, the implication of sRANKL or RANKL in the regulation of the immune response in the bowel wall would require the local presence of its receptor RANK. Low levels of RANK mRNA have been described in the murine colon [14,15] but significant levels of the protein have been identified on mature DC [31]. We confirmed the presence of RANK transcripts in the normal human colon and RANK protein expression in the colon by immunostaining. RANK-expressing cells were mainly localized in the colon mucosa and had morphological features of large mononuclear cells suggestive of the monocyte-macrophage and/or dendritic cell lineages. Immunofluorescence data clearly showed increased densities of RANK<sup>+</sup> cells in CD patients in uninvolved colon tissues with a further increase in involved areas. Double immunostaining experiments demonstrated that RANK<sup>+</sup> cells likely belong to subpopulations of activated macrophages or dendritic cells. However the precise characterization of these

RANK<sup>+</sup> cells will require further experiments using other more specific markers on frozen biopsy specimens and other techniques including confocal microscopy.

Beyond its potential implication in bone remodeling, overexpression of RANK, RANKL and OPG in colonic mucosa in CD may be relevant to CD pathophysiology. CD is characterized by an increased amount and activation of mononuclear cells in the bowel wall [1]. Particularly, T cells are abnormally resistant to apoptosis [32,33] and are thought to be sensitized against luminal material [1]. DCs play an important role in this abnormal T cells activation in CD although only few data are currently available on the specific phenotype and the activation of intestinal DCs in CD [34,35]. The intensity of an immune response is amplified by direct cell-cell contact between antigen-presenting cells and T-cells, through costimulatory molecules. For instance, CD40-CD40L costimulatory proteins are most likely involved in CD as T-cells and DCs overexpress CD40L and CD40, respectively [28]. Interestingly, RANK is induced on DCs by CD40L stimulation [13]. While CD40-CD40L interaction is mainly involved in early T/B cells priming and activation, RANK-RANKL binding may rather control later stage of activation and have a role in memory T cells responses [17]. As other TNFR family members, RANK can activate NF- $\kappa$ B in response to RANKL-expressing T lymphocytes [13]. Our data suggest that RANK-RANKL interaction on DCs and lymphocytes could contribute to the increased immuno-inflammatory reaction characterizing CD. This is in agreement with recent data on the mechanisms of colitis in IL2-deficient mice. In these mice, both colitis and osteopenia were caused by increased production of RANKL which, by acting via its receptor RANK, increases bone turnover but also promotes intestinal DC survival *in vivo* [36]. Locally, OPG may serve as a negative retro-control mediator on the differentiation, activation and increased survival of T cells and DCs in response to RANK-RANKL interaction leading to NF- $\kappa$ B activation. This is again in agreement with recent data on IL2-deficient mice. In this model, the treatment with exogenous recombinant OPG reversed skeletal abnormalities and reduced colitis by decreasing colonic DC number [36].

Another potentially relevant implication of OPG is its binding to tumour necrosis factor- $\alpha$ -related apoptosis-inducing ligand (TRAIL), another member of the TNF superfamily [37]. TRAIL induces apoptosis of transformed and non transformed cells and high doses of OPG inhibit this process [37,38]. Excess of OPG could therefore lead to a lack of apoptosis in CD as observed in other systems [38]. However the affinity of OPG to TRAIL is lower than for RANKL and the *in vivo* relevance of TRAIL-OPG interaction has still to be established.

In conclusion, our data show for the first time increased expression of RANK, RANKL and OPG in the colon of CD patients. These mediators and receptors may not only have a prominent role in bone loss associated to CD but also could have a considerable importance in the abnormal immune activation characterizing CD. Further functional studies will be necessary to clarify their respective role in CD. The study of other acute and chronic inflammatory disorders of the colon, including ulcerative colitis, will also be important to assess the specificity of the modifications described in this study.

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