Osteoprotegerin as biomarker for inflammatory bowel diseases

Osteoprotegerin: a novel faecal biomarker in paediatric inflammatory bowel diseases

Abstract

Background: Recently, Osteoprotegerin (OPG) has been identified as a faecal biological marker reflecting intestinal inflammation in inflammatory bowel diseases (IBD). To maintain remission, it is important to prevent relapses, especially in paediatric IBD where failure to thrive is frequently seen. This study aims to identify the diagnostic and predictive value of faecal OPG in paediatric IBD management.

Methods: Stool samples, disease activity index scores and inflammatory markers were recorded from children diagnosed with CD or UC during regular visits every three months. An enzyme-linked immunoassay was used to measure faecal OPG levels in these children.

Introduction

Crohn's disease (CD) and ulcerative colitis (UC), both an inflammatory bowel disease (IBD), are severe, chronic diseases affecting the gastrointestinal tract. CD occurs throughout the whole gut but is commonly seen near the ileum, whereas UC is mainly restricted to the colon. IBD deteriorates the intestinal mucosa and causes barrier disruption of the gut leading to abdominal pain, diarrhoea and rectal bleeding [1, 2]. A worrisome increase in the world-wide IBD population, particularly in developed countries, has been seen over the past decades [10]. Therefore, early diagnosis and early treatment are important key factors in IBD management, especially in

children where IBD causes failure to thrive and impairs growth and pubertal development [13]. Etiologically, our understanding of the etiopathogenesis in IBD is still not completely elucidated but our best hypothesis poses that inflammation of the intestinal mucosa is induced by the intestinal flora causing a deregulated immune response in both the innate and the adaptive immune system often in patients with predisposed genetic factors [14-18].

Currently, colonoscopy, albeit unpleasant, invasive and expensive, delineates mucosal inflammation and is the " gold standard" in diagnosing and monitoring IBD [11, 12]. Consequently, many investigators conducted studies to inflammatory indicators trying to find less-invasive and more accessible ways of assessing gastrointestinal inflammation. Several indices have been developed and validated, however not any as sensitive and specific as colonoscopy [39, 40]. Also, inflammatory markers such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) have been studied but do not differentiate among several other inflammatory diseases. [37, 38]. Nowadays, non-invasive faecal markers are deemed promising in diagnosing and monitoring IBD since previous studies have shown non-invasive faecal markers to reflect intestinal inflammation and mucosal healing [42-45].

In intestinal inflammation, one of the inflammatory pathways is the Nuclear Factor (NF)- κB pathway controlling inflammatory response and modulated by (pro)-inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1 and osteoprotegerin (OPG) [19, 20]. OPG or TNFRSF11B is a protein and member of the Tumour Necrosis Factor Receptor (TNFR) superfamily. OPG was first described in bone metabolism where it decreases

bone-breakdown modulating the OPG/receptor activation of NF-κB (RANK)/ RANK ligand (RANKL) pathway. In bone, RANK, which is expressed on osteoclast progenitor cells, binds RANKL and thereby inducing osteoclastogenesis. OPG, expressed by osteoblasts and acting as a decoy receptor for RANK, shows competitive binding with RANKL subsequently preventing a RANK-RANKL ligation and bone breakdown [24, 25]. Since both RANKL and OPG are members of the TNFR-family and thus affecting several inflammatory mediators and cytokines (e. g. TNF-α, IL-1, IL-8 and interferon (IFN)- γ) the OPG/RANK/RANKL pathway also modulates inflammation. Moreover RANKL is synthesized by T-cells whereas OPG is produced by B-cells and dendritic cells (DC) indicating an even more evident role for both proteins in the immune system [26-29]. Although the exact role of OPG in inflammation is yet to be found, recently conducted studies clearly highlight a potential role for OPG as a non-invasive faecal marker in paediatric IBD.

Several studies postulate OPG as a promising non-invasive faecal marker since OPG correlates positively with inflammation markers (e. g. C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)) and IBD index scores [20, 30-32]. Moreover, OPG levels decrease significantly after IBD treatment indicating less inflammation [32-34]. In addition, increased OPG levels were not only found in serum but also in intestinal mucosa and stool indicating a distinct role for OPG in intestinal inflammation [20, 30-32, 34, 41]. The aim of this study is to describe levels of OPG with respect to disease state and whether OPG levels change over time while receiving treatment or on behalf of the relapse-remitting pattern of IBD. Furthermore we evaluate

the diagnostic and predictive value of OPG as a non-invasive biological marker in paediatric IBD.

Methods

Patients

All patients (<18 years of age)diagnosed with CD or UC consistent with endoscopic, histological and radiological criteria [35, 36] at the Christchurch City Hospital from July 2012 onwards were invited to participate in this prospective study. Informed consent was obtained from parents and where possible from the child prior to handing in their first stool sample. Approval for this study was obtained from the Multi-Region Ethics Committee, Wellington, New Zealand.

Disease assessment

Assessment of patients' disease activity was measured using the Paediatric Crohn's Disease Activity Index (PCDAI) for CD patients or the Paediatric Ulcerative Colitis Activity Index (PUCAI) for patients diagnosed with UC. PCDAI scores comprise symptoms (e. g. abdominal pain), physical examination (e. g. peri-rectal disease) and blood results (haematocrit, ESR and albumin) whereas PUCAI scores are only based on subjective symptoms characterizing UC [7-9]. However, previous studies have indicated PCDAI as a poor indicator of intestinal inflammation since it is not correlating well with faecal biomarkers such as calprotectin, lactoferrin and S100A12 [3-5]. Therefore a modified PCDAI was developed and validated based on merely blood parameters (haematocrit, ESR and albumin) [6]. Eventually, both the

PCDAI and the modified PCDAI were used for disease assessment in CD patients.

CD or UC patients were classified as in remission/inactive, mild, moderate or severe disease state. When scoring a PCDAI <10, a modified PCDAI <7. 5 or a PUCAI <10 the disease was classified as in remission/inactive. A PCDAI between 10 and 27. 5 or a PUCAI between 10 and 30 was identified as mild, whereas a PCDAI between 30 and 37. 5 or a PUCAI between 35 and 60 delineate a moderate disease. A PCDAI score over 40 or modified PCDAI score over12. 5/17. 5[x1]or PUCAI score over 65 was classified as a severe disease [6-9]. Since the modified PCDAI only differentiates between an in remission/inactive and a severe form of CD, scores between 7. 5 and 12. 5/17. 5 accounted for a mild/moderate disease state [6]. Subsequently, relapses were defined when patients changed from an inactive disease state to a mild, moderate or severe state or showed a ≥12. 5 point / ≥40 point increase in PCDAI score [9] or PUCAI score [7, 8]respectively. On other hand, improvement was defined as a decrease in PCDAI score of ≥12. 5 points [9] or a decrease in PUCAI score of ≥35 points [7].[x2]

Sample collection

Eight stool samples per patient were collected over a period of 30 months as follows : stool samples were obtained every three months for the first year and then every 6 months for one and a half year. Stool samples were collected and immediately stored at 4°C. After transporting the samples to the laboratory they were aliquoted and stored in a -80°C freezer.

Moreover, inflammation parameters (e. g. CRP, ESR, albumin, haematocrit, and platelet count), weight, length and Body Mass Index (BMI) were recorded during regular visits. As part of patients' monitoring these visits took place every three months corresponding with collecting stool samples.

Faecal extraction

After removing stool samples from the freezer, a weighted amount of stool between 250 mg and 400 mg was added to an Eppendorf tube. Then, the same volume (between 250 µL and 400 µL) of buffered saline (PBS) containing 11 µg/mL aprotinin (Sigma), 2. 5 µg/mL leupeptin hemisulfate (Sigma) and 0. 5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (Sigma) was added creating a 1: 1 ratio weight/volume. Next, samples were agitated on a vortex machine (Global Science, Auckland, NZ) for 30 seconds and homogenized on a suspension mixer (Gyrotory shaker model G2, New Brunswick Scientific Co, Edison, NJ, US) for 30 minutes. After centrifuging at 13, 500g for 10 minutes on 22°C supernatant was transferred to an Eppendorf tube and stored at -20°C until analysis.

ELISA

OPG levels in stool samples were measured using a human OPG/TNFRSF11B ELISA-kit (R&D Systems) following the manufactures' instructions. We used this kit and protocol since it was successfully utilized by Nahidi et al [34]. First, 100 µL per well of capture antibody (mouse anti-human OPG with a working concentration of 2. 0 µg/mL in a PBS-dilution) was added to a 96-well plate (Falcon, Corning NY, US) incubating overnight at room temperature. Then, the plate was washed three times with wash buffer (0.

05% Tween 20 in PBS pH 7. 2-7. 4) and tapped dry on paper towel. Next, the plate was blocked by adding 300 µL per well of reagent diluent (1% bovine serum albumin (BSA) in PBS pH 7. 2-7. 4) incubating for 1 hour at room temperature. Meanwhile, 120 µL of faecal extraction and 120 µL of reagent diluent were added to an Eppendorf tube creating a final 1: 2 working dilution of sample. After washing the plate, as aforementioned, 100 µL per well of diluted samples and standards (recombinant human OPG) were added in duplicates incubating for 2 hours at room temperature. Next, the plate was washed and 100 µL per well of detection antibody (biotinylated goat anti-human OPG with a working concentration of 200ng/mL diluted in reagent diluent with 2% heat inactivated normal goat serum) was added incubating for two hours at room temperature. Following, after washing, 100 µL per well of Streptavidin-Horseradish peroxidase (HRP) was added for 20 minutes at room temperature and protected against light using tin foil. After washing, 100 µL per well of substrate (H_2O_2 and Tetramethylbenzidine in a 1: 1 dilution) was added for 20 minutes and protected against light. Hereafter 50 µL per well of Stop Solution ($2M\ H_2SO_4$) was added and optical density was immediately analysed using a 450 nm microplate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA, USA). The lower detection limit of this assay was 250 pg/mL.

Statistical analysis

The obtained data from the microplate reader was calculated using Softmax Pro (version 5. 3, October 1998; Molecular Devices, Sunnyvale, CA, USA).

[x1]Either 12. 5 or 17. 5. Differs between articles

[x2]Not sure if this is right but found this in other articles