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Evaluation of circulating levels of inflammatory and bone formation markers in axial spondyloarthritis

Kenia Rodrigues de Andrade [a,*], Gláucio Ricardo Werner de Castro [b,1], Geison Vicente [c,2], Julia Salvan da Rosa [c,2], Marina Nader [c,2], Ivanio Alves Pereira [a,3], Tânia Silvia Fröde [c,2]

[a] Rheumatology Service, Professor Polydoro Ernani São Thiago University Hospital, Federal University of Santa Catarina (UFSC), Campus Universitário, Trindade, Florianópolis, Santa Catarina 88040-970, Brazil
[b] Rheumatology Division, Governador Celso Ramos Hospital, Irmã Benwarda street, 297, Florianópolis, Santa Catarina 88015-270, Brazil
[c] Department of Clinical Analysis, Center of Health Science, Federal University of Santa Catarina (UFSC), Campus Universitário, Trindade, Florianópolis, Santa Catarina 88040-970, Brazil

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ABSTRACT

Studies have demonstrated the important role of bone remodelling and osteoimmunology in the progression of inflammatory lesions in axial spondyloarthritis (SpA) disease. This study was conducted to evaluate the inflammatory response by analysis of the serum levels of pro-inflammatory and new bone formation markers in patients with axial SpA who were treated or not treated with anti-tumour necrosis factor-α (anti-TNF-α) or non-steroidal drugs (NSAIDs) and to identify whether these drugs modify the activity and severity of the disease. The serum levels of myeloperoxidase (MPO), adenosine deaminase (ADA), nitric oxide metabolites (NO_x), bone alkaline phosphatase (BAP), Dickkopf-1 (DKK-1), and osteoprotegerin (OP) were measured in 52 SpA patients who were treated or not with anti-TNF-α or NSAIDs and in 26 healthy controls using colourimetric and enzyme immunoassay tests. The activity and the severity of illness in patients with SpA were assessed using questionnaires (Bath Ankylosing Spondylitis Metrology Index (BASMI), Bath Ankylosing Spondylitis Functional Index (BASFI), and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI)). A significant difference between the controls and the patients without medication was observed in relation to NO_x, BAP, and OP ($p < 0.01$). When the patients were compared with regard to their treatment, there were no clinically significant differences between the groups ($p > 0.05$). In conclusion, The NOx, BAP, and OP are emerging as important inflammatory pathways in axial SpA. Also the anti-TNF-α or non-steroidal drugs reduce the inflammation and destructions, however these treatments do not modify the serum levels of these biomarkers.

1. Introduction

The spondyloarthritis (SpA) is a group of rheumatic diseases, with an inflammatory component, which affects mainly the spine, but also entheses and peripheral joints [1,2]. The ankylosing spondylitis (AS) is the prototype of this group. It has an important association with the human leucocyte antigen (HLA) B27 [3].

It is well known that inflammatory response causes bone erosion and new bone formation, that promotes spinal ankylosis [4,5]. The real SpA pathogenesis has not been fully clarified yet so, the study of markers related to the inflammation and new bone formation could help physicians to better understand about this disease and to identify the possible therapeutic targets.

The tumour necrosis factor-alpha (TNF-α) seems to play the most important role in the inflammatory response to SpA [4,6,7], and the use of anti-TNF-α medication contributes significantly in improving the inflammatory condition, thus promoting a better quality of life and increasing the likelihood of survival for these patients [6,8]. Furthermore, several molecules appear to contribute to new bone formation in patients with AS, such as bone-specific alkaline phosphatase (BAP)

Abbreviations: ADA, deaminase; Anti-TNF-α, anti-tumour necrosis factor-α; AS, ankylosing spondylitis; ASAS, Spondyloarthritis International Society; BAP, bone alkaline phosphatase; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index; DKK-1, Dickkopf-1; HLA, human leucocyte antigen; iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; NO_x, nitric oxide metabolites; NSAIDs, non-steroidal anti-inflammatory drugs; OP, osteoprotegerin; RANKL, ligand of transcription factor of NF-kappa beta; SpA, spondyloarthritis; TNF-α, tumour necrosis factor-alpha; Wnt, Wingless.

* Corresponding author at: Rheumatology Service, Professor Polydoro Ernani São Thiago University Hospital, Federal University of Santa Catarina (UFSC), Campus Universitário, Trindade, Florianópolis, Santa Catarina 88040-970, Brazil. Tel.: +55 48 3721 9150, +55 48 3721 0234.
E-mail addresses: keniarod@gmail.com (K.R. de Andrade), castrogrwc@gmail.com (G.R.W. de Castro), geisonvicente@grad.ufsc.br (G. Vicente), julinha_sr@hotmail.com (J.S. da Rosa), marinanaderr@gmail.com (M. Nader), ivanio.pereira@ufsc.br (I.A. Pereira), Tania.frode@ufsc.br, taniafrode@zipmail.com.br (T.S. Fröde).
[1] Tel.: +55 48 32517000.
[2] Tel.: +55 48 99614846.
[3] Tel.: +55 48 3721 0234.

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and osteocalcin [9–12]. BAP is an important bone growth biomarker. The classical pathway of the protein "Wingless" (Wnt) signals the translocation of β-catenin to the nucleus, leading to the stimulation of osteoblastogenesis and the inhibition of osteoclastogenesis. Studies have demonstrated that Dickkopf-1 (DKK-1) is capable of inhibiting the Wnt pathway. Osteoprotegerin (OP) can inhibit bone resorption by inhibiting osteoclast activation, blocking the effects of ligand of transcription factor of NF-kappa beta (RANKL) [13–16]. However, the actual function of these markers and their correlation with SpA disease activity are not elucidated yet.

Many studies have demonstrated that pro-inflammatory cytokines enhance the release of pro-inflammatory enzymes, such as myeloperoxidase (MPO), adenosine deaminase (ADA), and inducible nitric oxide synthase (iNOS), from leucocytes and/or endothelium at the injury site. These inflammatory markers promote the activation of neutrophils and lymphocytes in chronic inflammatory diseases, including SpA [17–19]. The quantification of the activity of ADA in the serum has been used as a predictive marker of disease activity in patients with rheumatoid arthritis [20].

Nitric oxide (NO) and its metabolites (NO_x) play an important role in the pathologic process that contributes to the inflammatory-related tissue degradation [18,19]. Additionally, MPO and ADA are involved in leucocyte activation and in the chemotaxis of these cells at the inflammatory response site, including chronic inflammatory diseases [18,19]. Because the inflammatory response plays a key role in injury in SpA, the study of these inflammatory markers could help to better understand SpA pathogenesis and to identify the possible new therapeutic targets. The objectives of this study were 1) to investigate the role of the inflammatory parameters MPO, ADA, NO_x and the bone metabolism biomarkers BAP, DKK-1, and OP in patients with SpA; 2) to identify whether the anti-TNF-α and non-steroidal anti-inflammatory drug (NSAID) treatments can modify these parameters; and 3) to identify whether the anti-TNF-α and non-steroidal anti-inflammatory drug (NSAID) treatments can modify the activity and severity of the disease using questionnaires: Bath Ankylosing Spondylitis Metrology Index (BASMI), Bath Ankylosing Spondylitis Functional Index (BASFI), and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI).

2. Patients and methods

2.1. Study design

This study was conducted in the ambulatory spondyloarthritis unit in the rheumatology division at the Professor Polydoro Ernani São Thiago University Hospital at Federal University of Santa Catarina (UFSC), Florianopolis, Brazil, from May 2012 to January 2013. This study was approved by the Human Research Ethics Committee at the Federal University of Santa Catarina (protocol number CAAE-00883312.1.0000.0121-05/2012). Free and informed consent forms were signed by all of the participants. This study was conducted according to the principles of the Declaration of Helsinki, 2000 (Declaration of Helsinki, 2012) [21].

2.2. Subjects

A total of 52 patients with SpA fulfilled the modified New York criteria, 1984 [22] or the new criteria for axial spondyloarthritis proposed by the development of Assessment of Spondyloarthritis International Society (ASAS) group [23] and 26 healthy volunteers (controls) paired to age and sex to SpA patients were invited to participate in the study.

The exclusion criteria for SpA patients and controls were as follows: systemic inflammatory diseases, active infections, neoplasms, metabolic bone disease, pregnancy, current use of corticosteroids, immunosuppressive agents (including sulfasalazine, methotrexate, and leflunomide), bisphosphonates, teriparatide, strontium ranelate, or denosumab.

The patients with SpA were subdivided into three groups according to their treatment status: 1) patients without treatment (N = 14); 2) patients using NSAIDs continuously for at least 4 weeks (N = 12); and 3) patients using anti-TNF-α medication for at least 12 weeks (N = 26).

2.3. Biochemical assessment

The fasting blood samples were collected from patients with SpA and from controls to measure the serum levels of bone formation markers, such as BAP (Mybiosource, San Diego, CA, USA), OP (Abcam, Cambridge, UK), and DKK-1 (Abcam Inc, Cambridge, UK), and NO metabolite (NO_x) levels of MPO and ADA activities.

2.3.1. Quantification of nitric oxide metabolites (NO_X) levels

The quantification of the nitric oxide products (nitrite (NO_2^-) and nitrate (NO_3^-)) was performed according to the methodology described for the Griess method [24]. The serum samples from patients with SpA and controls (100 μL) were collected, and vanadium chloride 0.05 M (150 μL) in HCl 1.0 M (50 μL) was added to reduce nitrate to nitrite. Immediately, the Griess reagent (150 μL of naphthylethylenediamide dihydrochloride (0.004 M) in H_2O and sulphanilamide 0.06 M (150 μL) in H_3PO_4 0.03 M, vol. 1:1) was added. After incubating at 37 °C for 45 min, the reaction was transferred to an ELISA microplate. The nitrite/nitrate concentrations were determined by means of a colourimetric measurement at 540 nm and by interpolation from a standard curve of sodium nitrite (0–150 μM) on an enzyme-linked immunosorbent assay (ELISA) plate reader (Organon Teknika, Roseland, NJ, USA).

2.3.2. Quantification of myeloperoxidase (MPO)

Standard samples with differing concentrations of myeloperoxidase (from human neutrophils, Sigma: M6908, St. Louis, MO, USA) were prepared to obtain a standard curve in the range of 0.07–140 mU/mL. Samples of serum from patients with SpA and controls (40 μL) and the standards were transferred to cuvettes, and a reaction was initiated by the addition of 360 μL of assay buffer (0.167 mg/mL of o-dianisidine 2HCl and 0.0005% H_2O_2). The reaction was stopped with 1% of sodium azide. Afterward, the samples were centrifuged at 50 ×*g* for 5 min, the supernatants were separated, and the rates of changes were determined. The activity of MPO was determined by means of colourimetric measurement at 450 nm and by interpolation from the standard curve on an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) [25].

2.3.3. Quantification of activity of adenosine-deaminase

Initially, standard samples with different concentrations of $NaH_2PO_4 \cdot H_2O$ (35 mM), $Na_2HPO_4 \cdot H_2O$ (15 mM) and NH_3SO_4 (15 mM) were prepared to obtain a standard curve in the range of 10–50 U/L. Samples of serum from patients with SpA and controls (20 μL) were transferred to cuvettes, and a reaction was initiated by the addition of adenosine phosphate buffered solution (pH 6.5, 500 μL, composition: $NaH_2PO_4 \cdot H_2O$ (35 mM), $Na_2HPO_4 \cdot 12H_2O$ (15 mM) and adenosine (0.5 mM)). After incubating for 1 h at 37 °C, the reaction was stopped by the addition of a solution (1000 μL) of phenol (1 mM), nitroprussiate (0.17 mM) and alkaline buffer (1000 μL: NaOCl: 11 mM). This solution (final volume 2000 μL) was also added to the cuvettes with the different standard samples. The activity of ADA was determined by means of a colourimetric measurement at 620 nm and by interpolation from a standard curve on an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) [26].

2.3.4. Quantification of Dickkopf-1 (DKK-1) levels

For the analysis of the Dickkopf-1 (DKK-1) levels, the serum samples from patients with SpA and controls (100 μL) were collected and immediately prepared to quantify DKK-1 levels. Commercially available kit was used with monoclonal specific antibody specific for human DKK-1 (human DKK-1 (Dickkopf homolog 1), Cat. No ELH-

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