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Analysis of serum amyloid A in sarcoidosis patients

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Summary
A crucial pathogenetic role of serum amyloid A (SAA) in granulomatous inflammation of sarcoidosis has recently been reported. In this study we analyzed SAA expression in detail, starting from proteomic analysis of serum of sarcoidosis patients. We also used the faster ELISA method that enabled us to examine a greater number of samples. Serum concentrations of SAA were significantly higher in sarcoidosis patients than controls ($p < 0.001$), inversely correlated with FEV_1 and significantly higher in patients with subacute onset requiring prolonged and multiple steroid treatments (class 6 SCAC) than in patients with subacute onset not requiring therapy (class 4 SCAC) ($p < 0.001$). Our results suggest that serum amyloid A could be a suitable marker of sarcoidosis: its serum concentrations are significantly higher in sarcoidosis patients than controls, the protein is only expressed in gels of sarcoidosis patients and not in healthy subjects, and the SAA1 isoforms could match the unidentified biomarker of sarcoidosis reported in a previous proteomic study by another group. The effectiveness of SAA as a clinical biomarker of sarcoidosis should now be investigated in a large prospective study.

Introduction

Sarcoidosis is a systemic inflammatory granulomatous lung disease with unknown etiology. Its clinical course is unpredictable: spontaneous remission is reported in about 50% of cases, while 10–30% of patients develop a chronic disorder with respiratory impairment.[1–4] The most widely studied biomarkers of the disease include: angiotensin converting enzyme (ACE), soluble interleukin-2 receptor (sIL-2R), chitotriosidase, lysozyme, neopterin, and other cytokines and chemokines.[5–10] However, no single biomarker has sufficient sensitivity and specificity to be recommended for clinical use.

The proteomic approach applied to the study of bronchoalveolar lavage (BAL) and serum of sarcoidosis patients revealed a protein pattern different to those of other interstitial lung diseases and healthy controls, indicating some

Abbreviations: BAL, bronchoalveolar lavage; ILD, interstitial lung diseases; SAA1, serum amyloid A1.

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proteins of interest as potential biomarkers.[11–16] At the beginning of our studies on proteomics we compared BAL and serum protein profiles in interstitial lung diseases[11,12] and we found increased expression of certain acute phase proteins, especially serum amyloid A, in serum of sarcoidosis.[14]

Serum amyloid A (SAA) is an acute phase protein released by the liver and the most abundant of its six isotypes is SAA1.[17] Its production is stimulated by IL1 and IL6. Serum amyloid A is an acute phase reactant that transiently binds high-density lipoproteins during inflammatory responses.[17] It may displace apo A-I, causing increased catabolism of high-density lipoproteins, or inhibit lecithin–cholesterol acyltransferase activity, leading to low levels of esterified serum cholesterol.[18,19] Serum amyloid A plays a variety of immunoinflammatory functions: it induces chemotaxis and adhesion molecule expression, it up-regulates metalloproteinases (in particular matrix-metalloproteinase-9 over-expressed in IPF and involved in lung fibrotic remodeling) and it regulates apolipoprotein metabolism.[17] There is great interest in SAA in sarcoidosis and the protein was recently proposed as a key regulator of granulomatous inflammation in this disease.[20] It induces production of several cytokines and regulates Th-1 immune responses through interaction with Toll-like receptor-2[20] but only a limited number of studies have considered its potential as a biomarker of disease severity.

SAA concentrations have been detected in serum of sarcoidosis patients together with other inflammatory and acute phase proteins (including C-reactive protein, sIL-2r and ACE). SAA levels proved significantly higher in sarcoidosis patients than controls,[10] particularly those with associated anomalies of lipid metabolism.[18]

Our interest in SAA1 sprang from our proteomic analysis (two-dimensional electrophoresis) of sarcoidosis patients.[14,21] Serum samples showed one or two spots of SAA, which were never found in gels of control serum. This prompted us to do detailed analysis of SAA1, also using the faster ELISA method in order to handle a greater number of samples.

Aims of the present study were: 1) to identify SAA1 by a proteomic approach in serum of patients with sarcoidosis and controls, 2) to determine concentrations of SAA1 by ELISA 3) to further investigate SAA patterns in serum of sarcoidosis patients.

Patients and methods

Subjects

Proteomic analysis of SAA was performed in serum of seven sarcoidosis patients and seven controls. Serum concentrations of SAA detected by ELISA were evaluated in 48 sarcoidosis patients and 17 controls. Sarcoidosis was diagnosed according to ATS/ERS/WASOG international criteria.[22] Detailed medical history, including professional exposure, smoking history and pharmacological history, was obtained at onset. All patients underwent lung function tests including single-breath carbon monoxide diffusion capacity according to ATS/ERS guidelines and reference standards.[23] Chest X-rays in posterior–anterior and lateral projections were performed and radiological staging was done by an experienced radiologist according to widely accepted criteria.[24]

Serum samples were obtained at the time of bronchoscopy. The patients were consecutive cases of sarcoidosis not on therapy (immunosuppressants or steroids) when serum samples were drawn and bronchoalveolar lavage carried out. The patients had been observed at the Sarcoidosis Regional Referral Centre (Siena) for at least 3 years. Bronchoalveolar lavage was performed for diagnostic and clinical purposes with informed consent of patients. Briefly, the first sample was kept separate from the others and was not used for immunological tests. Cells were separated by centrifuge and the fluid fraction was frozen for enzyme assays. Cell differential counts were performed. Lymphocyte phenotype was analyzed by flow cytometry (Facs-Calibur Becton & Dickinson) using anti-CD3, CD4 and CD8 monoclonal antibodies (Becton & Dickinson). Serum ACE concentrations, BAL differential cell count and BAL lymphocyte phenotype were also determined in all patients.

Patients included in the study were all sarcoidosis patients with subacute onset of the disease. Patients with acute Löfgren's syndrome (bihilar lymphadenopathy, erythema nodosum and arthritis) were excluded. Our population of patients was divided according to phenotype activity score SCAC.[25] They were assigned to one of three groups (corresponding to SCAC classes 4–6) according to their need for treatment with steroids after SAA1 assessment. One group consisted of 15 patients with subacute onset and no need for systemic steroid therapy (class 4 SCAC). Another group consisted of 17 sarcoidosis patients with subacute onset requiring one period of immunosuppressive treatment of 12 months or less, at onset or in the follow-up period (class 5 SCAC). The last group consisted of 16 patients with subacute onset who needed several periods of immunosuppressive treatment or long lasting treatment (more than 1 year) (class 6 SCAC).

Controls were all Caucasians with no history of asthma or allergy and were not on any type of therapy. They had been observed for 12 months without developing any disease. Their lung function parameters were within normal limits and bronchoscopy did not reveal any alterations. Patients and controls gave their written informed consent to the study, which was approved by the Local Ethics Committee.

Two-dimensional electrophoresis

Two-dimensional electrophoresis (2-DE) was performed as previously described.[26] The first dimension was run on a nonlinear wide-range immobilized pH gradient IPG (pH 3.5–10, 18 cm IPG strips; Amersham Biosciences, Uppsala, Sweden). The second dimension was run on 9–16% polyacrylamide linear gradient gels subsequently stained with ammoniacal silver nitrate. Electrophoretogram images were obtained with a computing densitometer (Molecular Dynamics 300S; Sunnyvale, CA, USA) and processed with the program Melanie. Spots were identified by gel matching with reference gels in a 2-D database (SWISS-2D PAGE) and by western blot, as previously reported.[27]

Serum amyloid A assay

Serum amyloid A concentrations were determined by a commercial quantitative sandwich immunoassay modified by us (ANOGEN, Canada). Serum samples of patients and controls were placed in appropriate microtiter wells, pre-coated with a monoclonal antibody specific for SAA, and incubated. SAA was bound and immobilized by the antibody pre-coated in the wells. To quantify SAA in the sample, the wells were washed and a standard preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody specific for SAA was added to each well to "sandwich" the SAA immobilized in the first incubation. The microtiter plate was incubated a second time. The wells were thoroughly washed to remove all unbound HRP-conjugated antibodies and TMB substrate solution was allowed to react over a short incubation period. The wells containing SAA and enzyme–substrate reaction changed color. The enzyme–substrate reaction was terminated by addition of sulphuric acid solution and the color change was measured spectrophotometrically at 450 nm. Serum concentrations of SAA were expressed in μg/ml.

Statistical analysis

Data was expressed as mean ± standard deviation (M ± SD). Comparisons between groups were performed with the Mann–Whitney non-parametric *U* test ($p < 0.05$). The different variables were cross-correlated using the Spearman R coefficient of correlation ($p < 0.05$). Statistical analysis was performed using Stat Soft 7.0 software (Statistical 7.0 Stat Soft, Inc, 1984–2004, Tulsa, USA) and graphic representation of data was done with GraphPad Prism Version 4.0 software for Windows (GraphPad Software 4, Inc., 1992–2004, La Jolla, USA).

Results

Two-dimensional electrophoresis analysis of SAA was performed in serum of seven sarcoidosis patients (3 male, mean age 48.1 ± 22.79 years; M ± SD) and seven controls (4 male, mean age 44.6 ± 13.5). Serum concentrations of SAA (ELISA) were evaluated in 48 sarcoidosis patients (15 male, mean age 53 ± 19.1 years) and 17 controls (6 male; 49.5 ± 12.4 years). Table 1 shows the clinical characteristics of sarcoidosis patients and controls. Lung function parameters (including DLCO), radiological stages and BAL findings of sarcoidosis patients are reported together with functional and immunological parameters of healthy controls in Table 2. Globally sarcoidosis patients had normal lung function test parameters and elevated BAL lymphocyte count and CD4/CD8 ratio (Table 2).

Table 1 Personal data of sarcoidosis patients and controls.

	Sarcoidosis	Controls
Subjects	48	17
Ages (M ± SD)	53 ± 19.1	49.5 ± 12.4
Sex (M/F)	15/33	6/11
Race		
Caucasian	46	17
African	2	0
Smokers (%)		
Current	20.8	20
Ex	20.8	0
Never	58.3	80

Two-dimensional electrophoresis analysis

Two-dimensional electrophoresis analysis of SAA in serum of our sarcoidosis patients revealed that the protein resolved into two spots with isoelectric point/molecular weights of 5.69/11000 kDa (spot A) and 6.15/11000 kDa (spot B). These values are in line with those predicted for the primary gene product and the des-Arg modification of the SAA1-alpha locus. In 2-D gels, serum amyloid A1 was identified by matching with a reference gel from SWISS-2D PAGE and confirmed by immunoblot that revealed these two isoforms of the protein. The isoforms of SAA1 in the gel of a sarcoidosis patient are shown in Fig. 1. Relative volumes of spots A and B in serum of sarcoidosis patients and controls were calculated and expressed as percentage volumes. SAA1 spots were never observed in silver stained gels of serum of controls, whereas in sarcoidosis patients the mean percentage volumes of spots A and B were 0.05 ± 0.07 %vol (M ± SD) and 0.06 ± 0.08 %vol (M ± SD), respectively (Table 3). Interestingly, the highest percentage volumes of spots A and B were observed in two patients with active sarcoidosis experiencing a flare-up of respiratory symptoms accompanied by rapid deterioration of radiological and functional parameters. These patients also had ocular and cutaneous extrapulmonary localizations. They both required prolonged and repeated treatment with steroids.

ELISA assay

Serum concentrations of SAA analyzed by ELISA were significantly higher in sarcoidosis patients (4.07 ± 1.32 μg/ml)

Table 2 Lung function parameters, radiological stages and BAL findings in sarcoidosis patients and controls.

	Controls	Sarcoidosis	$p < 0.05$
FEV_1, % predicted	98.6 ± 20.5	96.3 ± 18.5	No
FVC, % predicted	99.5 ± 10.2	98 ± 16.6	No
DLCO, % predicted	98.8 ± 17.7	91 ± 18.4	No
Radiological stages			
0	–	29%	–
I	–	29%	–
II	–	22.9%	–
III	–	16.6%	–
IV	–	2.08%	–
BAL cell count			
Macrophages %	88.1 ± 20.5	63 ± 21.3	Yes
Lymphocytes %	7.5 ± 14.04	33.3 ± 20.8	Yes
Neutrophils %	1.1 ± 12.6	1.0 ± 0.3	No
Eosinophils %	0.7 ± 9.1	1.0 ± 1.1	No
CD4/CD8	1.9 ± 1.1	4.9 ± 3.2	Yes
ACE (IU/min/ml)	–	52.8 ± 13.3	–

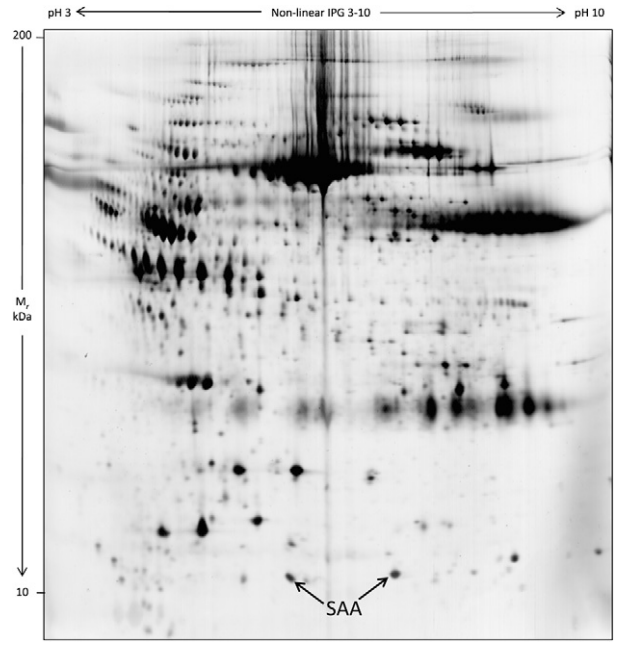

Figure 1 The two isoforms of SAA1 (spot A and spot B) in a two-dimensional electrophoresis gel of a sarcoidosis patient.

than controls (2.32 ± 1.63 μg/ml) ($p = 0.002$) (Fig. 2). Concentrations of this protein in serum were inversely correlated with FEV_1 percentages in sarcoidosis patients ($r = -0.3$; $p = 0.03$). No significant correlations were found between SAA concentrations and FVC percentages ($r = -0.1$; $p = 0.19$) or DLCO values ($r = -0.2$; $p = 0.12$) or radiological stages ($r = 0.1$; $p = 0.4$) or serum ACE levels ($r = 0.08$; $p = 0.6$). Serum concentrations of SAA were evaluated in the three SCAC classes (into which our cohort of patients with subacute onset was differentiated). SAA levels in SCAC classes 4, 5 and 6 were reported in Fig. 3. Concentrations of this acute phase protein were significantly higher in the class of patients requiring prolonged and repeated treatment with steroids (more than 12 months) at onset or during follow-up (SCAC group 6) than in patients who did not require any

Table 3 Spot A and Spot B percentage volumes (%vol) analyzed by proteomic approach in serum of seven sarcoidosis patients with short description of clinical manifestations.

	Spot A	Spot B
Patient 1 (minimal pulmonary sarcoidosis)	—	—
Patient 2 (severe pulmonary and ocular sarcoidosis)	188	209
Patient 3 (minimal pulmonary, lympho node and cutaneous sarcoidosis)	28	39
Patient 4 (severe pulmonary and cutaneous sarcoidosis)	77	46
Patient 5 (minimal pulmonary involvement)	5	3
Patient 6 (pulmonary and spleen involvement)	24	34
Patient 7 (minimal pulmonary and lympho node disease)	4	—

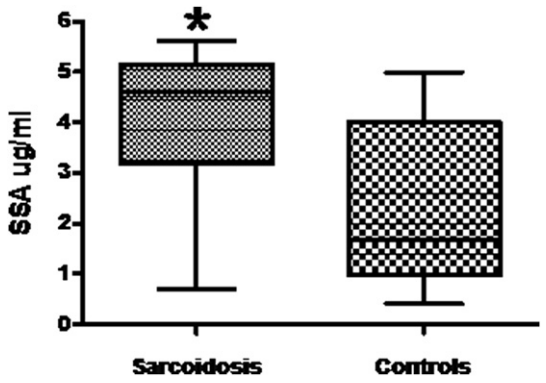

Figure 2 Serum concentrations of SAA (μg/ml) in sarcoidosis patients and controls ($p < 0.01$) (ELISA). The box extends from 25th to 75th percentile, with a line at the median (50th percentile).

therapy (SCAC group 4) ($p < 0.001$) or requiring less than 1 year treatment (SCAC 5; $p < 0.05$).

Discussion

Serum amyloid A is an acute phase protein involved in inflammation and lipid metabolism.[28,29] In the present study we identified serum amyloid A1 (the major isotype of SAA) in sarcoidosis patients by two-dimensional electrophoresis and quantified its percentage volumes in patients and controls. SAA1 consisted of two isoforms in gels of sarcoidosis patients but was not found in gels of healthy controls. Potential involvement of this protein in the immunoinflammatory pathogenesis of sarcoidosis was also suggested by evidence of significantly higher serum concentrations of SAA1 in sarcoidosis patients than controls detected by ELISA in a larger population. In our study, serum concentrations of SAA were inversely correlated with FEV_1 values.

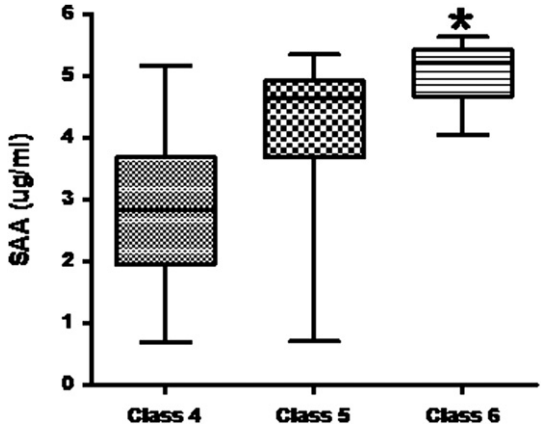

Figure 3 Serum concentrations of SAA (μg/ml) in our population of patients divided into three groups according to phenotype activity score (SCAC). Class 4 SCAC = patients with subacute onset and no need for systemic steroid therapy. Class 5 SCAC = patients with subacute onset requiring one period of immunosuppressive treatment, lasting 12 months or less. Class 6 SCAC = patients with subacute onset requiring several periods of immunosuppressive treatment or protracted treatment (more than 1 yr).

Increased serum levels of SAA have been reported in patients with active sarcoidosis (defined as progressive deterioration of radiological and functional features). In our study we observed significantly higher concentrations of this protein in patients requiring prolonged steroid treatment (more than a year) at onset or during follow-up, than in patients who did not require immunosuppressive therapy.[19] Salazar suggested a potential role of serum amyloid A and high-density lipoprotein cholesterol as biomarkers of sarcoidosis activity.[18] More recently, Miyoshi et al. evaluated serum concentrations of SAA, IL-2 receptor, ACE, lysozyme and KL6 in 43 sarcoidosis patients at onset, seeking a correlation with lymphocytic alveolitis.[30] The study concluded that although serum levels of SAA may be useful to monitor sarcoidosis activity (especially in follow-up), they did not reflect BAL alveolar lymphocytes as well as the biomarkers KL-6, sIL-2r and lysozyme.[30]

With regard to the literature, Bons and coworkers recently applied surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) to the study of serum from sarcoidosis patients and controls in an endeavor to identify new protein biomarkers.[31] They reported two unidentified serum proteins that were up-regulated in sarcoidosis patients. The molecular weights of these two proteins corresponded to those of the two isoforms of SAA1 (spot A and B) identified by us. These authors showed that the proteins with m/z values of 11,955 and 11,734 were up-regulated in serum of sarcoidosis patients and had much higher sensitivity and specificity than ACE and sIL-2R: 86% sensitivity and 63% specificity for the one with $m/z = 11{,}955$ and sensitivity 74% and specificity 71% for the one with $m/z = 11{,}734$.[31] The correspondence between the molecular weight of the proteins of Bons et al. and our isoforms of SAA prompts us to suggest that SAA is the unidentified biomarker of Bons's proteomic study, over-expressed in sarcoidosis and characterized by very high sensitivity and specificity.[31] This observation has particular relevance because a recent paper reported a crucial pathogenetic role of SAA in granulomatous inflammation of sarcoidosis.[20] The authors demonstrated that serum amyloid A is a component of the innate immune response regulating granulomatous inflammation in sarcoidosis through Toll-like receptor-2. Th1/Th2 balance and granuloma formation in sarcoidosis is influenced by this protein that could be targeted by new treatments.[20]

In conclusion, our results suggest that serum amyloid A could be a useful marker of sarcoidosis, since its serum concentrations are significantly higher in sarcoidosis patients than controls (especially patients with reduced FEV_1 requiring prolonged and repeated steroid treatment), SAA1 spots are only found in 2-DE gels of sarcoidosis patients and not in healthy subjects and they could match the unidentified biomarker of sarcoidosis reported in a previous proteomic study by another research group.[31] A large prospective study is now needed to verify its clinical utility.

Conflict of interest

All the authors declare that they have no conflict of interest related to the work described in this manuscript.

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