Chapter 7

Decreased redox state in red blood cells from patients with sarcoidosis

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Abstract

Background

The glutathione system has a key role in the defense against oxidative stress. To function properly, this system needs NADPH to maintain glutathione (GSH) in its reduced form. We hypothesized that the clinical problems associated with sarcoidosis might be related to a decreased anti-oxidant defense and we therefore measured the activity of the NADPH-generating enzyme glucose-6-phosphate dehydrogenase (G6PD), the GSH-regenerating enzyme glutathione reductase (GR) and indirectly the level of NADPH in red blood cells from patients with sarcoidosis.

Methods

In a population of sarcoidosis (n = 88) patients, G6PD, GR and GR activity after incubation with chromate (GR-Cr) were measured in erythrocytes. A decreased concentration of NADPH was revealed by an increased GR-Cr (> 0.6 IU/g Hb). To exclude a mutation in the G6PD gene, sequencing was performed in cases with an abnormal GR-Cr. Sarcoidosis pulmonary disease severity was evaluated by means of laboratory data, radiographic staging, HRCT scoring, pulmonary function- and exercise capacity testing.

Results

Fourteen (29.2%) females and one male (2.5%) demonstrated an increased GR-Cr test, indicative of a decreased NADPH level. Patients with an abnormal test result demonstrated also significantly increased ACE and GR values ($p < 0.05$). Only one female case (of 6 tested) appeared to have a mutation in the G6PD gene.

Conclusion

In a considerable percentage of female patients with sarcoidosis, a decreased level of NADPH in the erythrocytes was found.

Introduction

Sarcoidosis, a disease predominantly affecting young adults, is the commonest diffuse lung disease, with a population prevalence ranging from 1.4 to 64 per 100.000 population[1]. The disease etiology remains undetermined, although genetic predisposition is likely to play an important role[1,2]. Environmental factors, which are mostly unknown, may determine the disease course. A consequence of environmental factors is often oxidative stress, inducing the production of free radicals[3].
During oxidative stress, either intracellular in origin or initiated by drugs or environmental chemicals, reduced glutathione (GSH) is critical for protection of the cells[4,5]. GSH is involved in the detoxification of reactive oxygen species through its donation of reducing equivalents. Furthermore, GSH reduces oxidized protein thiol groups back to reduced thiol groups (-SH)[5,6]. The enzyme glutathione reductase (GR) maintains high intracellular concentrations of GSH by reducing the oxidized form of glutathione (GSSG) back to GSH. For this reaction, the reducing equivalents are derived from reduced nicotinamide adenine dinucleotide phosphate (NADPH) ($GSSG + NADPH + H^+ \leftrightarrow 2GSH + NADP^+$)[6-9]. The only pathway to generate NADPH in erythrocytes is the hexose monophosphate shunt (HMS). In this pathway, glucose-6-phosphate dehydrogenase (G6PD) is the first and rate-limiting enzyme[5,10].
There is clearly lack of international data about the relation of cellular redox state and sarcoidosis. We hypothesized that the malfunctioning of this protective system might be involved in sarcoidosis, and recognition of this phenomenon might be of clinical importance. Avoiding certain drugs and other causes of oxidative stress might then limit the extent of the inflammatory response in sarcoidosis.
Therefore, the erythrocyte capacity to maintain NADPH concentrations was evaluated in a population of sarcoidosis patients by means of the chromate inhibition test[11,12].

Materials and Methods

Study population

Eighty-eight mainly Caucasian sarcoidosis patients were included in this study. These patients visited the Sarcoidosis Management Center of the University Hospital Maastricht, a Dutch referral center for sarcoidosis, between January 2000 and January 2001. The patients' diagnosis was based on consistent clinical features and bronchoalveolar lavage fluid analysis[13], according to the ATS/ERS/WASOG guidelines[1]. The diagnosis was histologically confirmed in 91% of the cases. Informed consent was obtained from all participating subjects. Patient characteristics are summarized in Table 7.1.

Erythrocyte redox-state evaluation

The activities of G6PD and GR, as well as the chromate inhibition test (GR-Cr) were measured spectrophotometrically in hemolysates of washed erythrocytes according to

the method of Zürcher *et al*.[11], with minor modifications as described by Jonges *et a.l*[12]. G6PD activity was measured to screen for possible G6PD deficiency and GR for its capacity to regenerate GSH, both by means of a direct spectrophotometric method[11,12]. To screen for levels of NADPH in the erythrocytes, the chromate inhibition test was used. This test is based on the inhibition by chromate (Cr^{6+}) of GR activity in erythrocytes with a normal level of NADPH, but not in erythrocytes with decreased levels of NADPH. Chromate inactivates GR with reduced, catalytically active (-SH) thiol groups (this reduction is dependent on NADPH concentration) in the active center of the enzyme, but not GR with oxidized (-S-S-) thiol groups. The remaining glutathione reductase activity, will be thus higher in NADPH-deficient cells than in normal cells, and is thus a reflection of the intracellular redox state.

Table 7.1. General characteristics of the studied sarcoidosis patient population.

	Female	Male	Total
Number of cases	48	40	88
Age at diagnosis (yr)[a]	36.1 ± 13.0 (12 – 68)	36.8 ± 8.3 (25 – 55)	36.4 ± 11.1 (12 – 68)
Smoking (no / yes)	42 / 6	38 / 2	80 / 8
Use of prednison (no / yes)	41 / 7	22 / 18	63 / 25
Race (caucasian / negroid)	41 / 7	37 / 3	78 / 10

[a] Data are mean ± SD with range in parenthesis

Molecular genetic studies

Nucleotide sequence analysis of the G6PD gene was performed in 6 cases with an increased GR-Cr (> 0.6 IU/g Hb). Genomic DNA was isolated from 5 ml of K_3-EDTA anti-coagulated whole blood (buffy coat) with the High Pure PCR Template Preparation Kit, cat no. 1796828 (Roche, Mijdrecht, The Netherlands). The whole G6PD coding sequence was amplified from genomic DNA by polymerase chain reaction (PCR) with primers complementary to the intron regions, to ensure analysis of all exon and intron splicing regions of the G6PD gene (Table 7.2). Aliquots of 50 – 200 ng genomic DNA were amplified in the Air Thermo-cycler 1605 (Idaho Technology, Inc., Idaho Falls, ID, USA). The protocol used was as follows: denaturation at 95^{o}C for 5 minutes, followed by 40 – 50 cycles of annealing at 60^{o}C for 30 seconds and extension at 72^{o}C for 15 seconds. For nucleotide sequencing, the dideoxynucleotide chain termination method was used, with an ABI PRISM Big Dye Terminator Cycle sequencing kit on the ABI PRISM 377 DNA Sequencer (Perkin-Elner, Norwalk, CT, USA).

Evaluating Sarcoidosis Pulmonary Disease Severity

Serum ACE was measured with a colorimetric method (Fujirebio Inc., Tokyo, Japan, cat. nr. FU 116). ACE hydrolyzes p-hydroxybenzoyl-glycyl-L-hystidyl-L-leucine and generates p-hydroxybenzoyl-glycine, which is converted in two subsequent reactions in quinoneimine dye. The absorbance of the quinoneimine dye is measured at 505 nm to evaluate the ACE activity.

Serum calcium concentration was measured by indirect potentiometry on a Synchron LX®20 with a detection limit of 0.5 mmol/L and a measuring range of 0.5 to 5.0 mmol/L (Beckman Coulter, Inc. Fullerton, CA, US; electrolyte buffer reagent (P/N 467915) and electrolyte reference reagent (P/N 467935)).

Table 7.2. Primers used for the sequencing of the G6PD gene.

Primer sequences[a] (5' to 3')		Amplified fragment bp from … – to…(size)	G6PD protein part
PRS	CTCTGCAGGCCCGCGGAAGCTCGGT	2015 – 2443 (428)	Promotor region
PRAS	CCGCTGCCGCTGCTCTGCATCCCC A		
1S	CGGCGATGGGGATGCGGGAGCACTA	2364 – 2773 (409)	Exon I: no coding sequence
1AS	GCGCAGCGCGGGACAGTACGCTCCT		
2S	AGGAACTCTCAAGAAAGGGGCTAAC	3264 – 3533 (269)	Exon II: Met1 – Ser 39
2AS	AAAAGCTGAGGCATGGAGCAGGCAC		
3S	AAGGGTGGAGGATGATGTATGTAGG	13253 – 13623 (370)	Exon III: Gly41 – Trp53 Exon IV: Leu54 – Lys88
4AS	TGGGGGCTGGTAGAGAGGGCAGAAC		
5S	CTGGGGCAGAACACACACGGACTCA	14042 – 14403 (361)	Exon V: Ala89 – Ile162
5AS	ATAGAGTGGTGGGAGCACTGCCTGG		
6S	TGGGAGGGCGTCTGAATGATGCAGC	14920 – 15227 (307)	Exon VI: Gly163 – Arg215
6AS	GGCCAGGTGAGGCTCCTGAGTACCA		
7S	GGGTGACCCCTCACATGTGGCCCCT	15268 – 15517 (249)	Exon VII: Phe216 – Arg257
7AS	GGCTCTGCCACCCTGTGCCAGCCT		
8S	GTTTGGGGTCCCCATGCCCTTGAAC	15756 – 15979 (223)	Exon VIII: Asp258 – Lys288
8AS	CAGATGGGCCTGCGACAGGGCATGC		
9S	TGCACATCTGTGGCCACAGTCATCC	16294 – 16619 (325)	Exon IX: Val289 – Asp350
9AS	TGCCCGCACACAGGGCATGCCCAGT		
10S	GCTCCCACTGAGACACTCACGCACT	16624 – 16982 (358)	Exon X: Gly351 – Lys429
10AS	GGCCCAGGCCGCCCACCCTCCACA		
11S	CTGGGGCCCGGGGGACTCCACATGGT	16975 – 17162 (187)	Exon XI: Asn430 – Ser455
11AS	ACCCCATAGCCCACAGGTATGCAG		
12S	GGGGTGGCCTTTGCCCTCCCTCC	17157 – 17388 (231)	Exon XII: Asp456 – Ser486
12AS	GGCATGAGGTAGCTCCACCCTCAC		
13S	AGGAAAGGGTGGGGGCTGGGGACAGA	17318 – 17590 (272)	Exon XIII: Arg487 – Leu515
13AS	GTCAATGGTCCCGGAGTCCTCCCGA		

[a] Primers were designed based on the Genbank sequence with accession number X55448. (Homo sapiens gene for G6PD, glucose-6-phosphate dehydrogenase) and the numbering of amino acids was deduced from the cDNA accession number X03674.

Soluble IL2 receptor (sIL-2R) was measured on the IMMULITE automated analyzer, which is a two-site chemiluminescent enzyme immunometric assay with a detection limit of 50 kU/L and a measuring range of 50 – 7500 kU/L (Diagnostic Product Corporation, Los Angeles, CA, cat no LKIP1).

Chest radiographs were graded according to the radiographic staging of DeRemee (0 to III), adding stage IV, *i.e.* the end stage of lung fibrosis[1,14].

By means of high-resolution computer tomography (HRCT), thin-section scans with 1-mm collimation were obtained at 10-mm intervals through the chest. The scanning parameters included 137 kVp, 255 mA, and 1-second scanning time. Both mediastinal

(width, 400 HU; level, 40 HU) and lung (width, 1,600 HU; level, -800 HU) window images were obtained. The semiquantitive HRCT scoring system was used as described previously[15]. This HRCT score measures parenchymal, as well as lymphatic, and pleural involvement and grades each of these pathological features (six in total) into four categories. The total HRCT score was obtained by counting the six individual scores together, obtaining a maximal score of 18.
Lung function parameters, including the forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC), were measured with a pneumotachograph. The diffusing capacity for carbon monoxide (DLCO) was measured by the single-breath method (both Masterlab, Jaeger, Würzburg, Germany). Values were expressed as a percentage of those predicted[16].
Patients performed a symptom-limited incremental exercise test as described previously[3]. Breath-by-breath gas exchange was measured by a ventilated hood system (Oxyconbeta; Jaeger, Bunnik, The Netherlands). In rest and during maximal exercise, arterial blood samples were taken for blood gas analyses. The alveolar-arterial oxygen differences at rest (PO_2rest) and at maximal exercise ($AaPO_2$max) were calculated.

Statistics

All analyses were performed with the Statistical Package for Social Science (SPSS) for Windows (SPSS version 10.0, Chicago, IL, USA). Group comparisons were performed by means of parametric tests (Independent Samples t-test) for continuous data and by means of non-parametric tests (Mann-Whitney U test) for categorical data. A two-sided p-value of less than 0.05 was considered to be statistically significant.

Results

Table 7.3. shows clinical and laboratory data of the studied sarcoidosis population. Male patients appeared to have a worse clinical picture than female patients with respect to radiographic staging, total HRCT score, FEV1 and FVC. They also appeared to have significantly lower GR activities in their erythrocytes than female patients.
To assess whether normal levels of NADPH were present in the erythrocytes, the chromate inhibition test was performed. Abnormal GR-Cr values were found in 17.1% of sarcoidosis patients, 14 of 48 females (29%) and 1 of 40 male patients (2.5%), indicating decreased levels of NADPH.
In Table 7.4 the clinical data of the female patients have been divided into two groups, one with GR-Cr test results lower than or equal to 0.6 IU/g Hb (normal) and another with a GR-Cr test result higher than 0.6 IU/g Hb (abnormal). Among clinical parameters, only GR and ACE appeared to be significantly higher ($p < 0.05$) in the group with the abnormal GR-Cr test results, even after correction for the use of prednison.

Table 7.3. Clinical and laboratory findings of the studied sarcoidosis population [a]

Parameter (normal range)	Female (n = 48)	Male (n = 40)	Total
ACE (9-25 U/L)	22.8 ± 9.6 (88 – 60)	20.1 ± 8.4 (38 – 41)	21.6 ± 9.1 (38 – 60)
sIL-2R (241 – 846 kU/L)	861 ± 561 (2168 – 2271)	735 ± 432 (2368 – 2243)	804 ± 507 (2168 – 2271)
TotalCa^{2+} (2.1 – 2.6 mmol/L)	2.4 ± 0.1 (2.38 – 2.6)	2.5 ± 0.1 (2.38 – 2.7)	2.4 ± 0.1 (2.3 – 2.7)
GR-Cr (≤ 0.6) / > 0.6)	34 / 14	39 / 1	73 / 15
G6PD (4.8 – 7.2 IU/g Hb)	5.1 ± 1.0 (1.58 – 8.6)	4.8 ± 0.4 (3.98 – 5.7)	5.0 ± 0.8 (1.58 – 8.6)
GR [b] (2.4 – 4.8 IU/g Hb)	4.5 ± 1.1 (2.78 – 7.1)	3.9 ± 0.7 (2.58 – 5.4)	4.2 ± 1.0 (2.58 – 7.1)
FEV1, % pred [c]	91.2 ± 19.9 (298 – 135)	77.4 ± 20.9 (268 – 108)	85.2 ± 21.3 (268 – 135)
FVC, % pred [c]	99.3 ± 19.2 (498 – 152)	86.7 ± 15.3 (498 – 110)	93.9 ± 18.6 (498 – 152)
DLCO, % pred	81.9 ± 15.7 (378 – 112)	84.7 ± 20.7 (398 – 124)	83.2 ± 18.0 (378 – 124)
D/VA, % pred	76.5 ± 13.3 (468 – 105)	81.8 ± 13.8 (538 – 107)	78.9 ± 13.7 (468 – 107)
PO_2rest,(8.7 – 13.1 kPa)	11.6 ± 1.7 (7.8 – 15.0)	11.3 ± 1.6 (6.48 – 15.6)	11.4 ± 1.6 (6.48 – 15.6)
$AaPO_2$max, (0 – 4.7 kPa)	4.1 ± 1.8 (0.8 – 8.7)	4.4 ± 1.6 (0.98 – 7.5)	4.2 ± 1.7 (0.88 – 8.7)
Radiographic stages [b], 0/I/II/III/IV	10 / 7 / 18 / 6 / 3	3 / 4 / 14 / 9 / 5	13 / 11 / 32 / 15 / 8
HRCTtot [b] (0 – 18)	4.6 ± 3.4 (0 – 12)	6.7 ± 3.6 (8 – 13)	5.5 ± 3.6 (8 – 13)

[a] Data are mean ± SD with range in parenthesis, except for GR-Cr and radiographic stages
[b] $p < 0.05$ (males vs. females)
[c] $p < 0.01$ (males vs. females)

Table 7.4. Clinical parameters in *female* sarcoidosis patients with abnormal chromate-inhibition test as compared to those with normal test result[a]

Parameter (normal range)	GR-Cr < 0.6 IU/g Hb (n = 34)	GR-Cr > 0.6 IU/g Hb (n = 14)
ACE[b] (9 – 25 U/L)	20.4 ± 8.2 (8 – 47)	28.4 ± 10.6 (17 – 60)
sIL-2R (241 – 846 kU/L)	843 ± 571 (216 – 2271)	902 ± 553 (373 – 1891)
Total Ca^{2+} (2.1 – 2.6 mmol/L)	2.4 ± 0.1 (2.3 – 2.6)	2.4 ± 0.1 (2.3 – 2.5)
G6PD (4.8 – 7.2 IU/g Hb)	5.2 ± 0.9 (4.0 – 8.6)	5.2 ± 1.3 (1.5 – 6.7)
GR[b] (2.4 – 4.8 IU/g Hb)	4.1 ± 1.0 (2.7 – 6.7)	5.3 ± 1.0 (4.1 – 7.1)
FEV1, % pred	93.9 ± 14.6 (58 – 128)	84.8 ± 28.5 (29 – 135)
FVC, % pred	101.1 ± 13.8 (63 – 128)	95.3 ± 28.2 (49 – 152)
$D_{L_{CO}}$, % pred	83.4 ± 13.3 (58 – 112)	78.6 ± 19.8 (37 – 104)
D/VA, % pred	76.6 ± 12.9 (57 – 105)	76.4 ± 14.6 (46 – 100)
PO_2rest, kPa (8.7 – 13.1 kPa)	11.8 ± 1.7 (7.8 – 15.0)	11.0 ± 1.4 (9.4 – 13.6)
$AaPO_2$max, kPa (0 – 4.7 kPa)	4.2 ± 1.8 (2.3 – 8.7)	3.8 ± 1.9 (0.8 – 6.1)
Radiographic stages[c] 0/I/II/III/IV	6 / 7 / 14 / 3 / 1	4 / 0 / 4 / 3 / 2
HRCTtot (0 – 18)	4.4 ± 3.1 (0 – 12)	5.0 ± 4.1 (0 – 12)

[a] Data are mean ± SD with range in parenthesis.
[b] $p < 0.05$ (normal vs. abnormal GR-Cr test group).
[c] $p < 0.01$ (normal vs. abnormal GR-Cr test group).

Since an abnormal GR-Cr test result is usually caused by G6PD deficiency (leading to decreased levels of NADPH), we also measured the G6PD activity in the patients' erythrocytes. In only one patient a strongly reduced G6PD activity and highly abnormal GR-Cr test result was found, with 1.5 IU/ g Hb for G6PD and 5.1 IU/ g Hb for GR-Cr. To investigate due to which genetic defects in the G6PD gene the abnormal chromate inhibition test results were obtained, DNA was sequenced in 6 of 14 randomly chosen female patients with an abnormal GR-Cr test result (42.8% of cases), as shown in Figure 7.1.
Of the 14 females, two were negroid, whereas all other females with an abnormal GR-Cr test result were Caucasian and from Dutch origin. Only in one negroid female (patient with G6PD activity of 1.5 IU/ g Hb for G6PD and GR-Cr result of 5.1 IU/g Hb) a mutation in the G6PD gene was found. This was a combination of 202G→A (Met68 to Val) and 376A→G (Asp126 to Asn) called G6PD A-, the most common G6PD mutation in the negroid race. The male patient with an abnormal GR-Cr result was Caucasian.

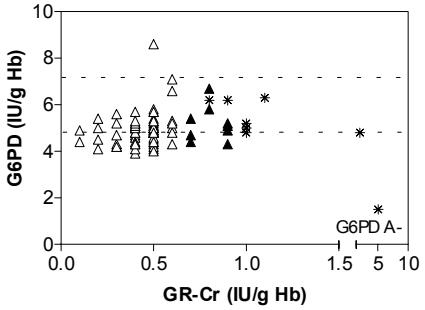

Figure 7.1. G6PD in relation to GR-Cr test and patients selected for sequencing.
Open triangles present patients with normal GR-Cr test values (≤ 0.6 IU/g Hb), whereas closed triangles present patients with abnormal GR-Cr test values (> 0.6 IU/g Hb). Remaining patients were those with abnormal GR-Cr test values, which were sequenced (star symbol). Only one patient of 6 sequenced with GR-Cr = 5.1 IU/g Hb appeared to be a carrier of a G6PD A-).

Discussion

The lung, through its large contact surface with the environment, is one of the most oxygen-exposed organs. Therefore, a causal role for oxidative stress in the inflammatory process involved in various pulmonary disorders, including sarcoidosis, has been considered[17,18]. Pulmonary antioxidant defenses are widely distributed and include both enzymatic and non-enzymatic systems. The major enzymatic antioxidants are superoxide-dismutase, catalase and GSH peroxidase. However, the GSH peroxidase activity is dependent on GR for the generation of GSH, a reaction for which NADPH is required. The only pathway to generate NADPH in erythrocytes is the hexose monophosphate shunt (HMS). In this pathway, glucose-6-phosphate dehydrogenase (G6PD) is the first and rate-limiting enzyme[5,10].
Prompted by earlier observations, it was hypothesized that decreased levels of NADPH might be involved in the pathogenesis of sarcoidosis[19,20]. This was indirectly established

by means of the chromate inhibition test (GR-Cr), which gave abnormal results in 29% of female sarcoidosis patients, whereas only one male patient (2.5%) appeared to have an abnormal test result. Although most studies suggest a slightly higher disease rate for women[1], it is hard to find the explanation for this large sex difference.

The chromate inhibition test is considered to be a sensitive biochemical assay that indirectly indicates cellular NADPH concentrations, and is used for the detection of G6PD deficiency in female carriers of this disease[11,12]. In the West-European, Caucasian population, G6PD deficiency is found with a prevalence of less than 0.1%[21], and the GR-Cr test yields an abnormal value in a similar percentage of this population (unpublished data). Thus, our finding of 15 abnormal GR-Cr tests in 73 sarcoidosis patients (17%), *i.e.* in 13 of 68 Caucasians (19%), is very deviant from the values, which are to be expected. One possible reason for the abnormal GR-Cr test could be G6PD deficiency. However, these results could not be explained by genetic mutations in the G6PD genes of the sarcoidosis patients. Only one patient of negroid origin appeared to be a carrier of G6PD A-. G6PD A- represents a mild form of G6PD deficiency, found largely in the African population, but also in other parts of the world[22,23].

Among clinical parameters, only GR and ACE appeared to be significantly higher ($p < 0.05$) in the group with the abnormal GR-Cr test results. Metabolic stress can lead to increased riboflavin uptake by erythrocytes[24]. In modified form (as FAD), riboflavin is incorporated into GR[24]. Slightly decreased NADPH might possibly also cause this phenomenon and lead as compensation to increased GR concentrations, as observed in G6PD deficiency[24,27]. Also ACE appeared to be significantly higher ($p < 0.05$) in the group with the abnormal GR-Cr test results, which might indicate an increased granuloma burden in this patient subgroup[28].

Increased NADPH consumption in erythrocytes of sarcoidosis patients could be a reflection of oxidative stress, which develops during pulmonary damage. NADPH might indirectly be used for instance in protection of hemoglobin from oxidation. Methemoglobin reductase (NADH diaphorase) catalyzes methemoglobin reduction[29]. Through the reduction of methemoglobin the cellular level of NADH decreases. There is an exchange of reducing equivalents between NADH and NADPH (catalyzed by NAD:NADP transhydrogenase), which could subsequently lead to a decrease in NADPH concentration. Decreased NADPH will lead to abnormal chromate-inhibition test results.

Conclusion

A substantial, unexpectedly high number of patients suffering from sarcoidosis appeared to have an abnormal chromate inhibition test, indicating decreased NADPH concentrations in their erythrocytes. Possibly, an increased consumption of reduced NADPH is involved in the inflammatory process triggered by oxidative stress in sarcoidosis.

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