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Regular paper

Glutathione reductase activity correlates with concentration of extracellular matrix degradation products in synovial fluid from patients with joint diseases

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The mechanisms underlying cartilage matrix degradation in joint diseases is not fully understood but reactive oxygen species are implicated as main causative factors. Comparative studies of glutathione reductase (GR) activity in synovial fluid from patients with rheumatoid arthritis (RA), reactive arthritis (ReA) and osteoarthritis (OA) as well as correlations between GR activity and concentration of the major cartilage components in synovial fluid are presented in this study. We found significantly higher activity of GR in RA (about three-fold) and ReA (about two-fold) than in OA. In RA and ReA patients, GR activity in synovial fluid correlates negatively with the concentrations of collagen and degradation products of sulfated glycosaminoglycans. In OA patients the activity of GR was significantly lower than in RA and ReA, which positively correlated with the concentration of collagen and showed a tendency for positive correlation with the degradation products of sulfated glycosaminoglycans. Our results suggest that in RA and ReA patients increased activity of GR does not prevent the increased degradation of collagen and proteoglycans by ROS.

Keywords: glutathione reductase, collagen, glycosaminoglycans, synovial fluid

INTRODUCTION

Rheumatoid arthritis (RA), reactive arthritis (ReA), and osteoarthritis (OA), are a group of joint diseases which differ in pathogenesis intensity and rapidity (Biernat-Kałuża, 2001; Eguchi, 2007; Rousseau & Delmas, 2007). RA is an autoimmunological inflammatory joint disease. Reactive arthritis is an aseptic arthritis following infection of the alimentary, genitourinary or respiratory tracts (Sieper & Braun, 1999; Biernat-Kałuża, 2001). ReA is clinically characterized by acute-onset polyarthralgia mainly in the lower extremities after infection by various microorganisms, although its pathogenetic mechanism remains unclear (Sieper & Braun, 1999). Osteoarthritis is a chronic degradation of articular cartilage, with a possible secondary inflammatory process (Rousseau & Delmas, 2007).

In RA and ReA, macrophages and neutrophils activated during an inflammatory process attack cellular pathogens with an involvement of reactive oxygen species (ROS) (Filippin *et al.,* 2008). ROS, and some related highly reactive agents, besides injuring the invading pathogens, also cause lesions in the knee joint (Carlo & Loeser, 2003; Henrotin *et al.,* 2003; Olszowski *et al.,* 2003). ROS oxidize and subsequently impair numerous components of the joint (Ostalowska *et al.,* 2006). ROS can damage collagen

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Abbreviations: CII, collagen type II; ECM, extracellular matrix; GAGs, glycosaminoglycans; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; OA, osteoarthritis; RA, rheumatoid arthritis; ReA, reactive arthritis; ROS, reactive oxygen species; SF, synovial fluid.

by a direct or indirect action, *via* the activation of latent collagenase and neutralization of protease inhibitors (Rajagopalan *et al.,* 1996).

Oxygen radicals are inactivated by, among others, the glutathione system (Ostrowska *et al.,* 2004) with an involvement of glutathione reductase which regenerates reduced glutathione (GSH) from oxidized glutathione (GSSG) at the expense of NADPH (Bazzichi *et al.,* 2002):

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

A decreased antioxidant capacity of the glutathione system has deleterious effects on articular cartilage. An increased level of endogenous ROS resulting from decreased levels of GSH can reduce the synthesis of proteoglycan and hyaluronic acid, which are components of the articular cartilage extracellular matrix (ECM) (Carlo & Loeser, 2003). Osteoarthritis and rheumatoid arthritis are characterized by irreversible damage to the cartilage matrix caused by enzymatic degradation of the proteins, e.g., collagen type II (CII), and proteoglycans of cartilage (e.g., aggrecan) (Billinghurst *et al.,* 1997; Lark *et al.,* 1997). CII is the primary collagen in the cartilage matrix. As a result of the breakdown of the proteins and proteoglycans, CII degradation products and sulfated glycosaminoglycans (GAGs) appear in synovial fluids (SF) of the affected joints. The level of GAGs in SF indicates the extent of proteoglycan degradation (Lark *et al.,* 1997).

The aim of our work was a comparative study of GR activity, concentration of degradation products of collagen and sulfated GAGs in SF of patients with RA, ReA and OA, as well as determination of correlations between the GR activity and the concentration of the degradation products.

MATERIALS AND METHODS

Synovial fluid. The study involved patients with ReA, OA or RA (n=14 in each group). The group with ReA comprised 5 women and 9 men with a mean age of 17.4 years (range 15–21 years) and disease duration of 4–20 weeks. Patients were classified according to Sieper and Braun (1999), eight patients had a preceding infection with *Chlamydia trachomatis* (genitourinary infection) and six with *Yersinia enterocolitica* (alimentary tract). The OA group consisted of 9 women and 5 men with a mean age of 63.2 (range 55–76 years) with primary medium gravidity knee OA, fulfilling the classical and radiological criteria of the American College of Rheumatology (Brandt *et al.,* 1991). Patients with knee OA all had radiological evidence of narrowing of the joint space and osteophyte in one or more knee compartments. The patients with RA (according to the 1987 criteria of the American College of Rheumatology — formerly the American Rheumatism Association) (Arnett *et al.,* 1987) (9 women and 5 men with a mean age of 45.3, range 25–72 years) had a clinically inflamed knee joint with effusion, joint swelling and pain.

SF samples were aspirated from the knee joints of patients during routine outpatient therapeutic procedures. Immediately after aspiration, fluids were centrifuged at $1700 \times g$ for 15 min at 4°C. Supernatants were collected and stored at –70°C until use. All the patients had given their written consent to the participation in the study.

Glutathione reductase (GR) activity determination. Activity of GR was determined in SF by measuring the decrease in absorbance of the reaction mixture at 340 nm, which is a function of the oxidation of NADPH (Bazzichi *et al.,* 2002). The reaction mixture (2 mL) contained 50 mM Tris/HCl (pH 7.6), 0.1 mM EDTA, 0.14 mM NADPH, 1 mM GSSG, and 50 μl of SF. The reaction mixture was incubated for 30 min at 37°C. The concentration of the enzyme activity was expressed as mU.E./mL (one unit reduces 1 μmole of GSSG/min at 37°C). The molar absorption coefficient of NADPH was taken as: $\varepsilon = 6.2 \times 10^3$ $mol^{-1} \times cm^{-1}$.

Determination of collagen degradation products. The concentration of collagen degradation products in SF was determined according to Komsa-Penkova *et al.* (1996).

Sulfated GAG assay. Sulfated GAGs were assayed in SF samples by the 1,9-dimethylmethylene blue binding method of Farndale *et al.* (1982).

Statistical analysis. The results were subjected to statistical analysis by Statistica 6.0 PL program. Data are expressed as mean±standard deviation (S.D.). Normal distribution of data was assessed by the Kolmogorov-Smirnov test. Since the data were not normally distributed, U-Mann-Whitney nonparametric test for unrelated results was used to compare differences between the groups, accepting $P<0.05$ as statistically significant. Correlations between glutathione reductase activity and concentration of collagen degradation products and sulfated GAGs were performed by Spearman's rank correlation test.

RESULTS

GR activity was found in the SF of all studied groups (Fig. 1). The lowest values of activity was found in OA SF (15.07±5.81 mU.E./mL). Significantly higher GR activities were found in RA SF (47.81±20.93 mU.E./mL) ($P<0.001$) and ReA SF (34.01±14.24 mU.E./mL) ($P<0.01$). The GR activities in RA and ReA patients were not significantly different ($P=0.12$).

The concentration of GAGs in SF from patients with RA (0.41±0.15 mg/mL; $P=0.42$) was not significantly different and in ReA showed a weak tendency to increase (0.52±0.17 mg/mL; $P=0.19$) in comparison with 0.37±0.11 mg/mL in OA (Fig. 2a). The collagen degradation products had the lowest concentration in SF from patients with OA (4.54±1.38 mg/mL), and their concentration were significantly higher in RA (6.98±2.44 mg/mL) ($P<0.05$) and ReA (6.22±2.18 mg/mL) ($P<0.05$) (Fig. 2b).

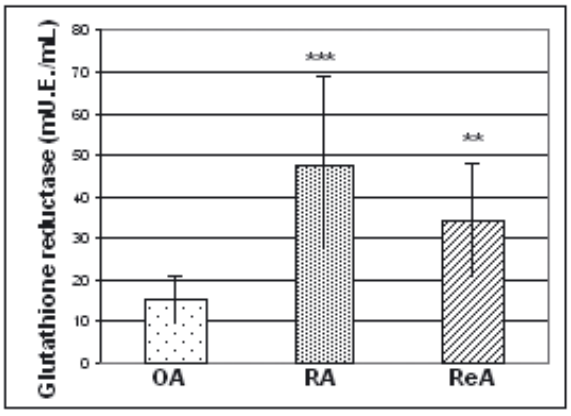

Figure 1. GR activity in SF of OA, RA and ReA patients.
$^{**}P<0.01$, $^{***}P<0.001$ compared with OA.

In OA SF we found a tendency to a positive correlation between GR activity and the level of sulfated GAGs (r=0.46; $P=0.098$) (Fig. 3a), as well as significant positive correlation with the concentration of collagen degradation products (r=0.59; $P=0.026$) (Fig. 3b). In SF of RA patients we observed a significant negative correlation between GR activity and concentrations of GAGs (r=–0.573; $P=0.032$) (Fig. 4a) as well as collagen degradation products (r=–0.543; $P=0.044$) (Fig. 4b). In SF of ReA patients we observed a significant negative correlation between GR activity and concentrations of GAGs (r=–0.662; $P=0.01$)

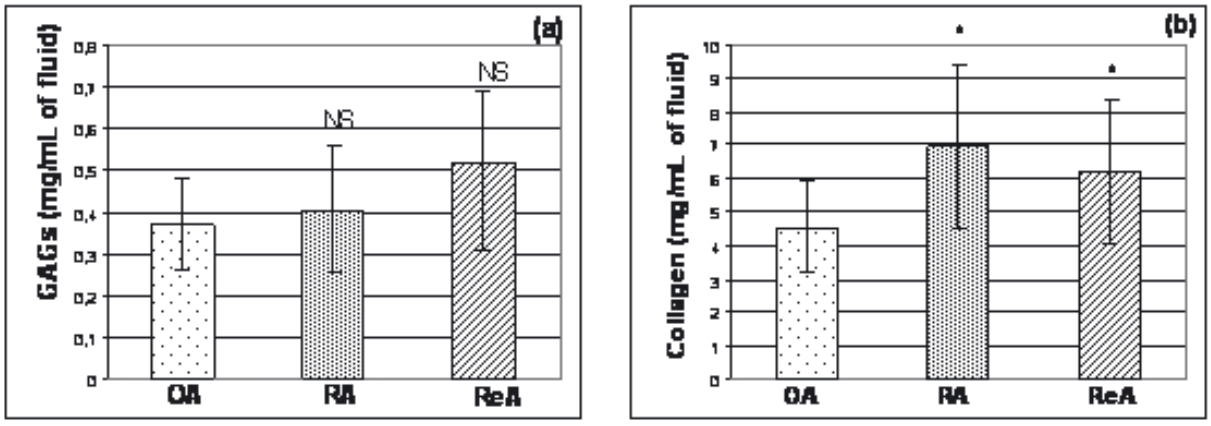

Figure 2. Concentration of sulfated GAGs (a) and collagen degradation products (b) in SF of OA, RA and ReA patients.
$^{*}P<0.05$; NS, no statistical significance as compared with OA.

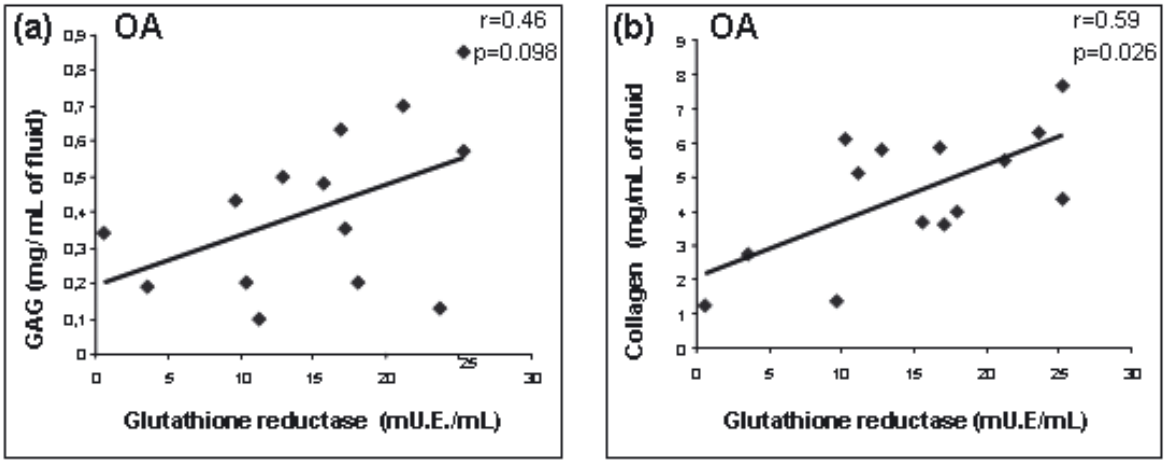

Figure 3. Relationship between GR activity and concentration of degradation products of matrix components in SF from patients with OA.
(a) Correlation between GR activity and GAGs concentration. **(b)** Correlation between GR activity and concentration of collagen degradation products. The Spearman rank correlation coefficient (r) and *P*-value are given.

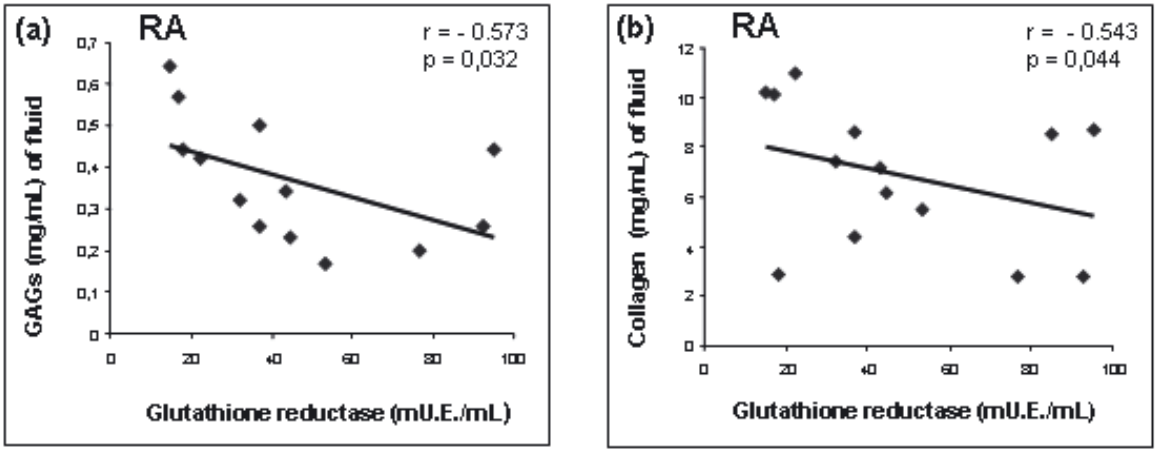

Figure 4. Relationship between GR activity and concentration of degradation products of matrix components in SF from patients with RA.
(a) Correlation between GR activity and GAGs concentration. **(b)** Correlation between GR activity and concentration of collagen degradation products. The Spearman rank correlation coefficient (r) and *P*-value are given.

(Fig. 5a) as well as collagen degradation products (r=–0.717; *P*=0.004) (Fig. 5b). We did not observe a significant correlation between the concentrations of collagen and sulfated GAGs degradation products in SF of patients with OA (r=0.235; *P*=0.417), RA (r=0.429; *P*=0.12) or ReA (r=0.194; *P*=0.5).

DISCUSSION

The major means of destroying pathogens is phagocytosis. The phagosomes are acidified and fuse with lysosomes which contain acid hydrolases and ROS (Alberts *et al.,* 2002). The initially formed reactive oxygen radicals are mostly superoxide radicals ($O_2^{\bullet -}$), which may be converted to more harmful hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) by interaction with intracellular free metal cations (Ostalowska *et al.,* 2006). High levels of free radical reaction products have been reported in the synovial fluids of patients with RA (Taraza *et al.,* 1997). ROS, besides injuring the invading pathogens, also cause knee joint destruction (Carlo & Loeser, 2003; Henrotin *et al.,* 2003). ROS have been shown to degrade aggrecan, a major component of the ECM, and this degradation is one of the initial events in the process of cartilage destruction (Billinghurst *et al.,* 1997). The release of sulfated GAGs is largely reflective of aggrecan degradation in the cartilage. Collagen, which provides tensile strength and forms a network that resists the swelling pressure of aggrecan-hyaluronate aggregates, can be also altered directly by oxygen radicals. Hydroxyl radicals have a direct effects, cleaving collagen in the presence of

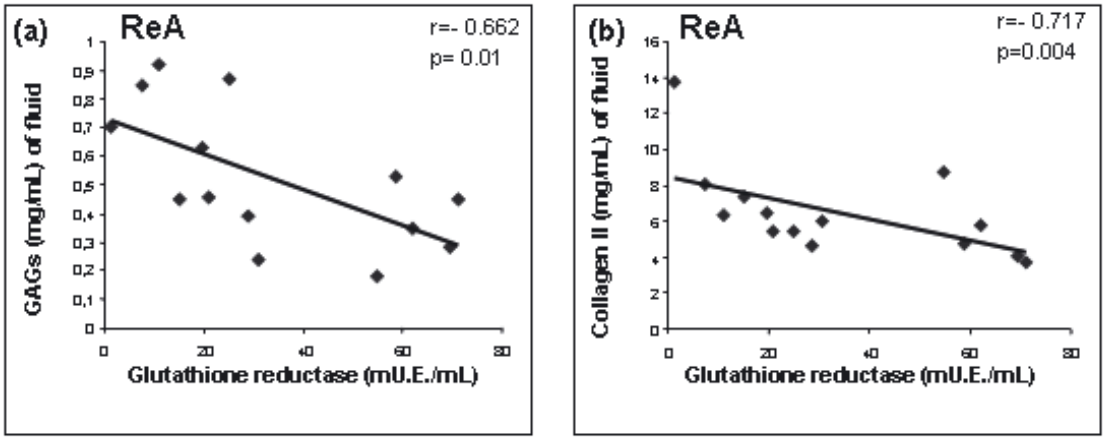

Figure 5. Relationship between GR activity and concentration of degradation products of matrix components in SF from patients with ReA.
(a) Correlation between GR activity and GAGs concentration. **(b)** Correlation between GR activity and concentration of collagen degradation products. The Spearman rank correlation coefficient (r) and *P*-value are given.

oxygen into small peptides; furthermore, free radicals can begin the cleavage of collagen, making it more sensitive to proteolytic enzymes (Monboisse & Borel, 1992). Interestingly, ROS can also directly activate matrix metalloproteinases, enzymes involved in the catabolism of matrix macromolecules (Rajagopalan *et al.*, 1996).

Glutathione reductase which regenerates reduced glutathione from oxidized glutathione plays an important role in the detoxification of oxygen free radicals (Bazzichi *et al.*, 2002; Ostrowska *et al.*, 2004). In our study we found significantly increased, in comparison to OA, GR activity (Fig. 1), concentration of collagen degradation products as well as a tendency to increase in GAGs concentration (Fig. 2) in inflammatory SF, i.e. RA and ReA. The significantly higher GR activity in inflammed SF negatively correlated with the concentration of collagen and GAGs degradation products in RA (Fig. 4) and ReA (Fig. 5), which suggests an involvement of GR activity in the protection of joint tissue collagen and GAGs against degradative action of ROS. Our findings are consistent with the results of Rajagopalan *et al.* (1996), who reported regulation of metalloproteinase activity by ROS, and Monboisse and Borel (1992), who reported oxidative damage to collagen by ROS. In contrast, we found a positive correlation between GR activity and collagen degradation as well as a strong tendency to increased concentration of GAGs degradation products in SF of patients with OA (Fig. 3), which suggests that OA is a non-inflammatory degenerative disease, where ROS are not so heavily involved as in RA and ReA.

It is worthy of note that our data on the increase in GR activity as well as collagen and sulfated GAGs degradation products in synovial fluid of RA and ReA patients are consistent with reports that the inflammatory process in rheumatoid diseases releases to serum and synovial fluid many lysosomal enzymes, including exoglycosidases, which participate in the degradation of articular cartilage and other tissues of the knee joint (Shikman *et al.*, 2000; Ortutay *et al.*, 2003; Popko *et al.*, 2006a; 2006b). In the inflammatory processes of the knee joint increased activity of exoglycosidases has been reported in chondrocytes (Ortutay *et al.*, 2003), synovial membrane (Popko *et al.*, 2006b), and synovial fluid (Ortutay *et al.*, 2003; Popko *et al.*, 2006a).

We can conclude that in the RA and ReA patients studied the increase in SF GR activity is not sufficient to protect collagen and aggrecan against degradation by ROS, since the increased GR activity is accompanied by high SF levels of collagen degradation products and a tendency to increased concentration of sulfated GAGs. In OA, the contribution of ROS to the degradation of collagen and aggrecan seems less significant than in the ReA and RA patients, and the increase in GR activity was much lower in those patients than in RA and ReA.

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