Osteoarthritis and Cartilage (2008) **16,** 515–521

doi:10.1016/j.joca.2007.09.001

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Joint fluid antioxidants are decreased in osteoarthritic joints compared to joints with macroscopically intact cartilage and subacute injury

E. A. Regan M.D., Ph.D.*, R. P. Bowler M.D., Ph.D. and J. D. Crapo M.D.

Department of Medicine, National Jewish Medical and Research Center, Denver, CO, United States

Summary

Objective: Excess reactive oxygen species and oxidative damage have been associated with the pathogenesis of osteoarthritis (OA). Extracellular superoxide dismutase (EC-SOD or SOD3) scavenges superoxide is the major catalytic antioxidant in joint fluid and is decreased in OA cartilage. We studied human joint fluid samples to test whether there is an association between OA and EC-SOD or other low molecular antioxidants in the joint fluid.

Methods: Joint fluid samples were obtained from 28 subjects with severe OA undergoing arthrocentesis or knee joint replacement and compared to joint fluid from 12 subjects undergoing knee arthroscopy for chronic knee pain, meniscal tears or anterior cruciate ligament reconstruction. EC-SOD protein was assayed by enzyme-linked immunosorbent assay (ELISA). Ascorbate and urate were measured with high performance liquid chromatography (HPLC) and total nitrates by the Greiss reaction. Glutathione (GSH) and oxidized glutathione were measured using a colorimetric method. Interleukin-6 (IL-6) and transforming growth factor-beta (TGF-beta) were both measured with ELISA.

Results: Human joint fluid contains significant amounts of the extracellular, catalytic antioxidant EC-SOD. Joint fluid from OA subjects is characterized by significantly decreased EC-SOD levels and significant decreases in GSH, and ascorbate compared to the reference group of knee joints with pain or subacute injury but macroscopically intact cartilage. GSH and ascorbate show only an age effect with no effect from disease state on regression modeling. Urate is present in joint fluid but does not show a significant difference between groups. IL-6 and TGF-beta both show non-significant trends to increases in the arthritic subjects. There was no correlation of EC-SOD levels with IL-6 as a marker of inflammation in either the comparison group or the OA group.

Conclusions: EC-SOD, the major scavenger of reactive oxygen species (ROS) in extracellular spaces and fluids, is decreased in late stage OA joint fluid compared to fluid from injured/painful joints with intact cartilage. Injured joints may be able to increase or maintain secretion of EC-SOD but it appears that late stage OA joints fail to do so in spite of increased oxidative stress seen in the disease. Associated age related declines in GSH and ascorbate might also contribute to the development of severe OA. The net effect of these changes in joint fluid antioxidants is likely to accelerate the damaging oxidant effects on extracellular matrix stability in cartilage tissue.

Key words: Cartilage, Joint fluid, Synovial fluid, Oxidative stress, Antioxidant, Osteoarthritis, Extracellular superoxide dismutase, SOD3, Glutathione, Urate, Ascorbate, Redox, TGF-beta, IL-6.

Osteoarthritis (OA) is a disabling disease of the joints that affects up to half of the aging population, but has neither a clear etiology nor an effective treatment[1]. It causes stiffness, pain, and deformity, most often in the major weight-bearing joints and results in a focal, progressive loss of articular cartilage with formation of bony osteophytes around the joint margins. Advancing age, genetic factors and mechanical damage all play a role in OA[2]. There are no diagnostic tests to confirm the disease, and radiographic evidence often occurs late, after significant loss of cartilage tissue. Although OA is thought to be a disease of articular cartilage, it has effects throughout the joint, including the formation of marginal osteophytes, increased subchondral bone stiffness, variable degrees of synovial hypertrophy and decreased joint fluid viscosity.

*Address correspondence and reprint requests to: Dr Elizabeth A. Regan, M.D., Ph.D., National Jewish Medical and Research Center, 1400 Jackson Street, Denver, CO 80206, United States. Tel: 1-303-398-1531; Fax: 1-303-270-2249; E-mail: regane@njc.org

Received 12 December 2006; revision accepted 1 September 2007.

We have recently shown that the primary, catalytic antioxidant of the extracellular spaces, extracellular superoxide dismutase (EC-SOD or SOD3) is present in articular cartilage and profoundly decreased in human OA cartilage[3]. Furthermore, we found transient decreases in EC-SOD in pre-lesional cartilage associated with progressive increases in nitrotyrosine residues in a spontaneous animal model of OA, the STR/ort mouse. Evidence has continued to accumulate that OA is mediated in part by reactive oxygen and nitrogen species[4–6].

There are three distinct SODs found in the human body: SOD1 or CuZn SOD which localizes primarily to the cytosol, SOD2 or Mn SOD found in the mitochondria, and SOD3 or EC-SOD. EC-SOD is a secreted, tetrameric, glycoprotein with a positively charged heparin-binding site[7]. The active center contains copper and zinc and is homologous with SOD1, but the EC-SOD molecule is immunologically distinct from SOD1. It localizes to the surface of cells and the extracellular matrix of tissues by binding to the negatively charged molecules including proteoglycans and collagen[8,9]. In this extracellular location it can protect the vulnerable proteins and macromolecules of the extracellular matrix from oxidant injury. McCord[10] found that SOD could protect joint fluid hyaluronate from being oxidatively cleaved

by superoxide. Marklund *et al.*[11] measured EC-SOD activity in joint fluids and plasma finding that EC-SOD is three times higher in joint fluid compared with plasma and decreased in rheumatoid arthritis (RA) joint fluid. He did not study EC-SOD in OA joint fluid.

Joint fluid is produced as a transudate of plasma from synovial cells and provides nutrition to the articular cartilage by diffusion of oxygen and other molecules. Changes in the constituents of joint fluid may reflect physiologic events in the articular cartilage. We expected that OA joint fluid would show altered levels of EC-SOD and other antioxidants in response to a state of oxidant stress within the OA cartilage and joint. We chose to study specific non-catalytic antioxidants [ascorbate, urate, oxidized glutathione (GSSG) and reduced glutathione (GSH)] in addition to EC-SOD. The GSH couple is known to be an intracellular antioxidant, and chondrocytes stressed oxidatively had greater incidence of cell death when they had been depleted of GSH or had higher levels of the oxidized form of GSH[12]. Ascorbate was chosen for the study because it is important in collagen biosynthesis and has antioxidant effects. Urate was also studied because it is a known constituent of joint fluid, and has the ability to act as a scavenger of reactive oxygen species[13,14].

We studied joint fluid from patients with severe knee OA at joint replacement or when patients were having arthrocentesis performed for diagnostic or therapeutic purposes. We compared these to joint fluid from patients undergoing knee arthroscopy for meniscal tears or knee pain in which the articular cartilage was evaluated arthroscopically and there was no evidence of OA. We looked to confirm that EC-SOD was present in joint fluid and compared the levels in OA joint fluid to fluid from joints with macroscopically intact cartilage. We also looked at the pattern of selected non-catalytic antioxidants in joint fluid to see if there were other compensatory changes in the antioxidants in OA joint fluid.

Materials and methods

HUMAN SYNOVIAL FLUID

Joint fluid was collected from patients undergoing total knee replacement or who were having arthrocentesis in association with either steroid injection or hyaluronate injection for the group with advanced degenerative arthritis. This group is described as OA patients ($n = 27$). The comparison group joint fluid was obtained from patients with normal knee radiographs who were having their knee arthroscoped for a meniscal lesion, knee pain or anterior cruciate ligament (ACL) reconstruction (controls, $n = 12$). Exclusion criteria for both groups included a history of RA, local joint infection or gout. Joint fluid was aspirated from the joint with a 20 gage or larger needle before the joint was infiltrated with local anesthesia during arthroscopy. Subjects were excluded from the comparison group if at the time of arthroscopy, visual inspection of the articular surfaces demonstrated lesions consistent with OA – specifically areas of exposed bone and or extensive areas of chondromalacia (more than one compartment involved or more than 1 cm diameter of chondromalacia). Subjects were included in the comparison group if they demonstrated small (less than 1 cm diameter), partial thickness lesions (grade 2 chondromalacia) in only one compartment of the knee during the arthroscopic inspection of the joint. Written informed consent was obtained from all human subjects and the protocol was reviewed and approved by the Exempla Hospital IRB and National Jewish IRB. Height, weight, age and gender information was recorded for each subject, and body mass index (BMI) [BMI = (weight in kg)/height in m^2] was calculated. Each joint fluid sample was centrifuged at 3000 rpm to remove any cells and the supernatant stored at −80°C.

EC-SOD ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

EC-SOD antibody #97-5 was a rabbit polyclonal antibody raised against alkylated purified human EC-SOD. EC-SOD antibody #4G11G6 was a mouse monoclonal antibody raised against human EC-SOD purified from aortas. Protein concentrations were determined for the joint fluids using the BCA Protein Assay Kit (Pierce; Rockford, IL).

EC-SOD protein was quantified using a sandwich ELISA. Ninety-six well Maxisorb immunoplates (Nunc; Naperville, IL) were coated with capture antibody 4G11G6, Ms α hEC-SOD, at 1 μg/ml in 100 mM sodium phosphate, pH 8.4 at 4°C overnight. The plate was washed with tris buffered saline with Tween (TBS-T) (0.1% Tween-20) after all incubations. Wells were blocked with 1% bovine serum albumin (BSA) (fraction V fatty acid free)/10% normal goat serum/phosphate buffered saline (PBS) at 200 μl/well. Samples, including cartilage lysates and rhEC-SOD standard (31 pg/ml−2 ng/ml), were diluted in 1% BSA (fraction V fatty acid free)/0.05% Tw-20. Detection antibody 97-5, Rb α hEC-SOD, was added at 1:10,000 in blocking solution. Secondary antibody, Gt α Rb-biotin (ABC Elite Vectastain kit for Rb IgG; Vector Labs; Burlingame, CA), was used at 1:200 in blocking solution. The avidin biotin complex (ABC) solution was incubated for 15 min at 37°C. ABTS (product component of ABC kit) substrate solution was added at 100 μl/well. Absorbance was measured at 405 nm. EC-SOD per μg of total protein was calculated for all of the samples.

HPLC DETERMINATIONS OF ASCORBATE AND URATE

Joint fluid samples were treated with 150 μl of 5% metaphosphoric acid added to 350 μl of the fluid supernatant after centrifugation to precipitate proteins, and then incubated on ice for 10 min before repeat centrifugation and separation of the protein depleted supernatant. This supernatant was analyzed for ascorbate and urate by high performance liquid chromatography with electrochemical detection using an ESA Coularray (Chelmsford, MA). Ascorbate and urate standards were obtained from Sigma (St Louis, MO). Detection potentials were set at 100, 250, 600 and 670 mV. Five microliters of sample was injected onto a 4.6 × 250 mm YMC ODS-AQ S5 μM 120 Å column (Waters, Milford, MA). Antioxidants were eluted with mobile phase (potassium phosphate 125 mM, acetonitrile 1%, pH 3.0) at 0.75 mL/min.

GSH and GSSGwere measured using the Bioxytech GSH/GSSG-412 kit (OxisResearch, Portland, OR) that is a colorimetric method for measuring both reduced GSH and GSSG.

TOTAL NITRATES

Nitrites and nitrates were measured using the Greiss reaction[15] and separately analyzed as nitrites (NO_2^-), nitrate (NO_3^-), and total nitrates ($NO_2^- + NO_3^-$). The Griess reaction method includes reduction of nitrate to nitrite with nitrate reductase and colorimetric determination of the diazonium ion. Interleukin-6 (IL-6) and transforming growth factor-beta (TGF-beta) were measured in joint fluid samples using commercially available colorimetric ELISA for human IL-6 and TGF-beta (Quantikine Immunoassay, R&D Systems, Minneapolis, MN).

STATISTICS

Linear regression modeling using EC-SOD protein, the low molecular weight antioxidants or other joint fluid constituents as the outcome variable was used to test for effects of age, gender, and BMI on the relationship between disease status and measured variable. All reported values are mean ± S.E.M.

Results

THE STUDY GROUPS

We compared OA subjects with severe OA of the knee joint to subjects who were undergoing arthroscopy for chronic knee pain, meniscal tears and ACL reconstruction. The OA patients were significantly older and more obese. The baseline characteristics and results of the two groups are shown in Table I.

EC-SOD IN JOINT FLUID

EC-SOD was found in human joint fluid samples. EC-SOD mean concentration in the joint fluid was 7.3 ± 0.9 pg/μg

Table I
Baseline characteristics of study subjects

	Number	Mean age (years)	BMI	Male	Female
Control group	12	37	27	6	6
OA group	28	64	33	11	17