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Speciation of non-transferrin-bound iron ions in synovial fluid from patients with rheumatoid arthritis by proton nuclear magnetic resonance spectroscopy

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Introduction

Knee-joint synovial fluid of patients with rheumatoid arthritis and other inflammatory arthritides has previously been reported to contain non-transferrin-bound iron, present as low-molecular-mass, redox-active iron complexes [1]. This non-transferrin-bound iron can readily promote the generation of the hydroxyl radical (·OH) from superoxide (O_2^-) and hydrogen peroxide (H_2O_2) released by activated polymorphonuclear leucocytes and macrophages which are present at sites of inflammation [2]. The ·OH radical can attack a wide range of endogenous molecules to produce modified chemical species that are detectable in synovial fluid samples. These degradation products can themselves stimulate synovitis and progressive joint destruction [3, 4].

Non-transferrin-bound iron ions can be measured in rheumatoid synovial fluid by the bleomycin assay [15], an analytical system that appears to measure iron ions in a form capable of accelerating free radical reactions involving oxygen. However, to date there have been no attempts to investigate its precise chemical nature. Since the chromatographic fractionation of human biological fluids appears to give rise to the degradation of proteins and release of metal ions [6], studies of the chemical nature of non-protein-bound iron require methodologies that involve little or no pre-treatment of samples. Nuclear magnetic resonance spectroscopy has been used extensively for the study of body fluids [7], particularly for monitoring low-molecular-weight metabolites and endogenous compounds. Hence, in the present study we have employed proton Hahn spin-echo nuclear magnetic resonance (NMR) spectroscopy combined with the use of the powerful hexadentate iron(III) chelator desferrioxamine to "speciate" catalytic, low-molecular-mass complexes of iron in synovial fluid obtained from patients with inflammatory joint diseases.

Materials and Methods

Reagents

Analytical grade iron(III) chloride was obtained from BDH Pharmaceuticals Ltd. Desferrioxamine (as Desferal®, desferrioxamine B methanesulphonate) was generously provided by Ciba-Geigy.

NMR measurements

Proton NMR measurements were carried out on a JEOL GSX-500 NMR spectrometer operating at 500 MHz. All spectra were recorded at ambient probe temperature. Typically, 0.60 ml of a freshly obtained synovial fluid sample was placed in a 5-mm diameter NMR tube, and 0.10 ml of deuterium oxide was added to provide a field-frequency lock. The broad protein resonances and the intense

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water signal were suppressed by a combination of the Hahn spin-echo sequence D[90°x-τ-180°y-τ-collect], where τ = 60 ms [8], and the application of a gated secondary irradiation at the water frequency. The Hahn spin-echo sequence was repeated 342–354 times. Synovial fluid spectra were referenced to external sodium-3-(trimethylsilyl)-1-propane-sulphonate (TSP, δ = 0 ppm).

Synovial fluid and serum samples

Knee-joint synovial fluid was drawn into heparinized tubes from rheumatoid patients (n = 9). The samples were centrifuged immediately (400 ***g***, 15 min, ambient temperature) and then either analysed within a few hours after collection or stored at −70°C. Serum samples were prepared by allowing freshly drawn non-heparinized blood to clot, and analysed and stored as above. Relevant control experiments established that none of the criteria studied changed significantly during periods of storage.

Results

The Hahn spin-echo technique suppresses broad resonances arising from macromolecules leaving an NMR spectrum that has well resolved resonances arising from highly mobile components. Figure 1 shows the low frequency region of the 500-MHz proton Hahn spin-echo NMR spectrum of a typical synovial fluid sample obtained from a subject with moderately severe rheumatoid arthritis. Incubation of serum from healthy or rheumatoid human subjects with the powerful iron chelator desferrioxamine (3.00×10^{-4} mol. dm^{-3}) for periods of 30 h gave no significant modifications in serum NMR spectra. However, treatment of rheumatoid synovial fluid with desferrioxamine (2.44×10^{-4} mol. dm^{-3}) produced small increases in the intensities of the citrate resonances (Fig. 2) following prolonged incubation (>6 h). Similar modifications in the spectra were observed in four different samples studied. These increases in the intensity of the citrate resonances following the addition of desferrioxamine suggest that at least some of the non-transferrin-bound iron present in synovial fluid samples is bound to the endogenous chelator citrate.

Further evidence that endogenous citrate has the ability to chelate non-transferrin-bound iron(III) in synovial fluid was provided by the observation that the addition of iron(III) to

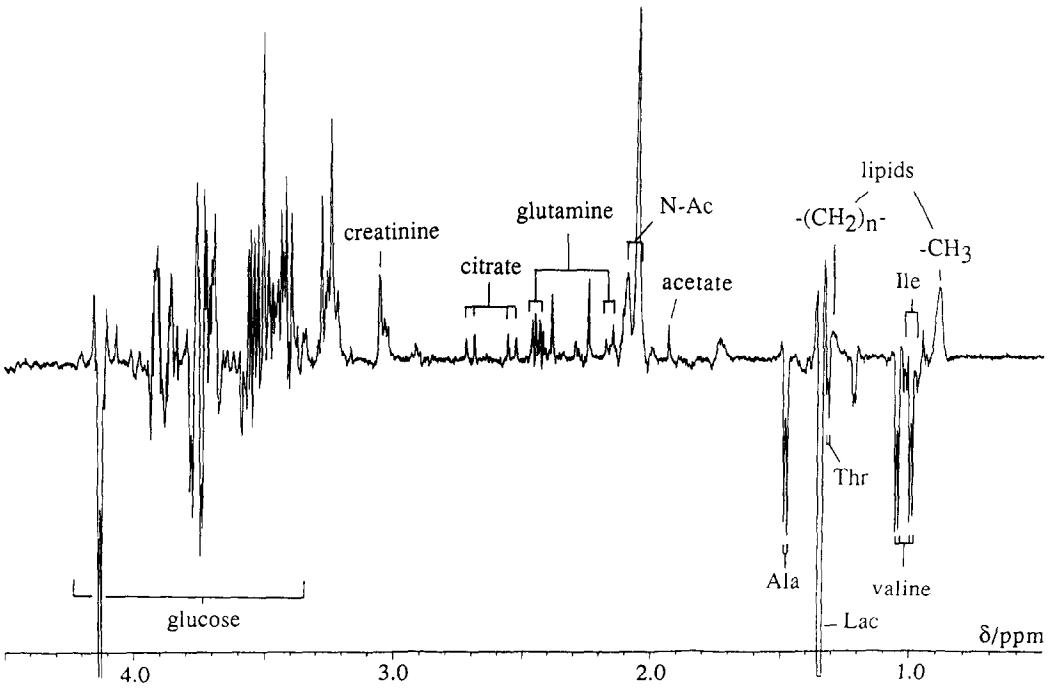

Figure 1
A typical 500 MHz proton spin-echo NMR spectrum of a synovial fluid sample obtained from a patient with rheumatoid arthritis. Abbreviations: Ala, alanine; Ile, Isoleucine; Lac, lactate; N-Ac, mobile portions of *N*-acetylated glycoproteins; Thr, threonine.

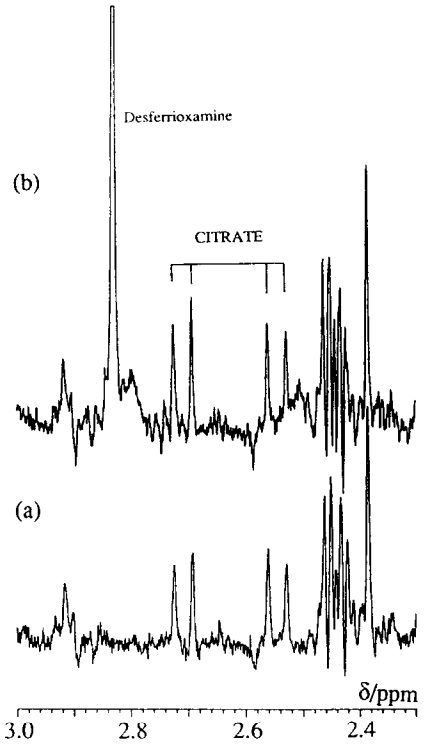

Figure 2
500 MHz proton spin-echo NMR spectra of a typical sample of rheumatoid synovial fluid (a) before and (b) after equilibration with 2.44 × 10^{-4} mol. dm^{-3} desferrioxamine at ambient temperature for a period of 6 h.

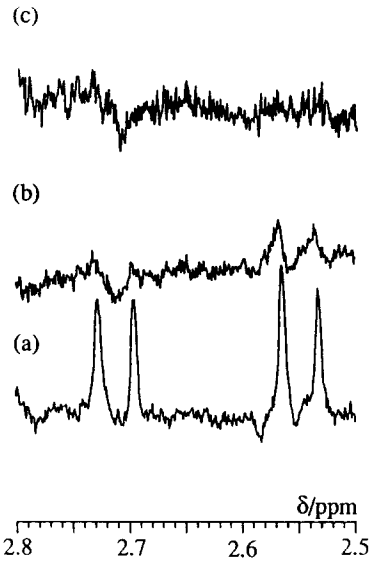

Figure 3
500 MHz proton spin-echo NMR spectra of a typical sample of rheumatoid synovial fluid (a) untreated sample, (b) with the addition of 1.00 × 10^{-4} mol. dm^{-3} iron(III), and (c) with the addition of 2.00 × 10^{-4} mol. dm^{-3} iron(III). Added iron was in the form $FeCl_3$ and equilibration was for a period >2 h in each case.

these samples produced a marked broadening of the characteristic AB coupling pattern of the citrate resonances (a shortening of their spin–spin relaxation time (T_2) with a corresponding increase in line width, so that they disappear from the spectrum), demonstrating that synovial fluid citrate chelates added Fe^{III} ions. Figure 3 displays typical results for a sample of rheumatoid synovial fluid. The citrate resonances are considerably broadened at an added iron(III) concentration of 1.00 × 10^{-4} mol. dm^{-3}. Similar broadenings were seen in a total of five different samples studied. The resonances of alternative potential endogeneous iron(III) chelators (e.g. acetate, glutamine, lactate) remain largely unaffected by the addition of added iron(III) concentrations as high as 2.00 × 10^{-4} mol. dm^{-3}, indicating that synovial fluid citrate is a highly selective ligand for non-transferrin-bound iron(III).

The treatment of synovial fluid with the above concentrations of iron(III) also gave rise to a significant decrease in the intensity of the terminal lipid —CH_3 group resonance (P_2) in all samples studied (data not shown). This resonance is already strikingly reduced in intensity in synovial fluid samples as compared to corresponding plasma from the same patients.

Subsequent prolonged treatment of these iron(III)-loaded samples with an excess of desferrioxamine (2.50 × 10^{-4} mol. dm^{-3} for a period of 6 h at ambient temperature) resulted in the citrate resonances becoming clearly visible again. This demonstrates a transfer of iron(III) from citrate to desferrioxamine under these experimental conditions, consistent with the slow "sharpening" of the citrate resonance observed on treating rheumatoid synovial fluid samples with desferrioxamine (Fig. 2).

Discussion

The simultaneous study of the status of a wide range of molecularly mobile components present in knee-joint synovial fluid samples obtained from patients with inflammatory synovitis by proton Hahn spin-echo NMR spectroscopy, combined with the use of the powerful iron(III) chelator desferrioxamine, provides evidence for the complexation of low-molecular-mass (non-transferrin-bound) iron(III) ions by citrate. There are small, but reproducible modifications in the citrate

proton resonances when the small quantities of this potentially catalytic iron (ca. 1–8 × 10^{-6} mol. dm^{-3}) [5] are transferred from citrate to desferrioxamine after prolonged incubation. Halliwell and Gutteridge [9] postulated that the presence of bleomycin-detectable catalytic iron in rheumatoid synovial fluid could be explained by its release from transferrin in a "sealed off" microenvironment existing between macrophages or other phagocytes and cell or cartilage surfaces, the pH of which can drop to a value of 5 or less, enabling release of iron from this protein. A recent study [10] has suggested that iron can also be released from ferritin by physiological chelators such as citrate or acetate, and hence this process could also give rise to bleomycin-detectable iron. We have recently characterized the chemical nature of non-transferrin-bound iron in blood plasma or serum obtained from patients with idiopathic haemochromatosis by high resolution proton NMR spectroscopy and high-performance liquid chromatography (HPLC) [11]. The results indicated that this low-molecular-mass iron(III) is present as iron–citrate and/or ternary iron–citrate–acetate complexes. However, no evidence for the involvement of acetate in the complexation of non-transferrin-bound iron in samples of rheumatoid synovial fluid was obtained in this study.

Although levels of NMR-detectable (non-protein-bound) citrate in samples of rheumatoid synovial fluid vary widely both between and within patients (ca. 0.5–4.5 × 10^{-4} mol. dm^{-3}), this endogenous chelator is always in large excess over the non-transferrin-bound (bleomycin-detectable) iron. Under these conditions, and at neutral pH the predominant iron(III)–citrate complex is likely to be iron(III)–monocitrate ($[Fe(citrate\ H_{-1})]^-$) with all three carboxylate groups and the hydroxyl group deprotonated. At lower pH values however, the 1:2 $[Fe(citrate)_2]^{5-}$ complex forms, and is 40% mol. fraction of total iron concentration at pH 5 in an isolated aqueous iron(III)–citrate system [12]. Since iron(III)–citrate chelates readily stimulate the adverse production of oxygen-derived free radical species, and can be measured by the bleomycin assay, it is likely that iron–citrate complexes form a major component of bleomycin-detectable catalytic iron in synovial fluid [13].

The suppression of the lipid $-CH_3$ group resonance by the addition of an excess of iron(III) to synovial fluid may arise by the interaction of Fe^{III} ions with pre-formed lipid hydroperoxides further promoting the autocatalytic chain reaction of lipid peroxidation [11]. The reduction of some of the added Fe^{III} to Fe^{II} by synovial fluid reductants (e.g. thiols, ascorbate) could also give rise to the further promotion of this process.

Conclusion

These results demonstrate the value of proton Hahn spin-echo NMR spectroscopy in studies involving the speciation of metal ions in body fluids obtained from patients with inflammatory joint diseases, providing a broad "picture" of abnormalities in metal-ion metabolism with a minimum of sample treatment.

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