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Synchrotron X-ray analyses demonstrate phosphate-bound gadolinium in skin in nephrogenic systemic fibrosis

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Conflicts of interest
Two of the authors (S.P.C. and J.L.A.) have served as expert witnesses for plaintiffs in gadolinium-related litigation. The investigations reported herein and previous studies by J.L.A. were not performed for litigation.

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Summary

Background Nephrogenic systemic fibrosis (NSF) is an incurable, debilitating disease found exclusively in patients with decreased kidney function and comprises a fibrosing disorder of the skin and systemic tissues. The disease is associated with exposure to gadolinium (Gd)-based contrast agents (GBCA) used in magnetic resonance imaging (MRI). Tissue samples from many patients with NSF contain micron-sized insoluble Gd-containing deposits. However, the precise composition and chemical nature of these particles is unclear.

Objectives To clarify the precise chemical structure of the Gd-containing deposits in NSF tissues.

Methods Autopsy skin tissues from a patient with NSF were examined *in situ* using synchrotron X-ray fluorescence (SXRF) microscopy and extended X-ray absorption fine structure (EXAFS) spectroscopy and in correlation with light microscopy and the results of scanning electron microscopy/energy dispersive spectroscopy analyses.

Results The insoluble Gd deposits were shown to contain Gd no longer coordinated by GBCA chelator molecules but rather in a sodium calcium phosphate material. SXRF microscopy shows a clear correlation between Gd, Ca and P. EXAFS spectroscopy shows a very different spectrum from the GBCAs, with Gd–P distances at 3·11 Å and 3·72 Å as well as Gd–Gd distances at an average of 4·05 Å, consistent with a $GdPO_4$ structure.

Conclusions This is the first direct evidence for the chemical release of Gd from GBCA in human tissue. This supports the physical–chemical, clinical and epidemiological data indicating a link between stability and dose of GBCA to the development of NSF.

The potentially fatal disease, nephrogenic systemic fibrosis (NSF), is associated with decreased kidney function and exposure to gadolinium (Gd)-based contrast agents (GBCA) used in magnetic resonance imaging (MRI).[1,2] Scanning electron microscopy/energy dispersive spectroscopy (SEM/EDS) of patient tissues has consistently demonstrated micron-sized insoluble deposits containing Gd together with Ca, P and Na.[3,4] GBCAs are the only significant source of human Gd exposure. As Gd is toxic, they comprise Gd^{3+} bound to a chelator designed to both solubilize the metal ion and render it biologically inaccessible. Whether the deposits observed in tissues contain precipitated intact GBCA or mineralized Gd dissociated from the chelating ligand remains an intractable biomedical problem key to understanding the pathogenesis of NSF.

In this study we used synchrotron X-ray fluorescence (SXRF) microscopy and extended X-ray absorption fine structure (EXAFS) spectroscopy to characterize chemically the Gd deposits in skin from a patient with NSF. SXRF applies raster scanning with a focused monochromatic X-ray beam across a sample, collecting an X-ray fluorescence spectrum at each point and yielding images showing distributions of specific elements.[5] EXAFS is a structural technique that yields numbers, types and distances of neigh-

bouring atoms around a specific element within a radius of about 6 Å. Fourier transforms of EXAFS spectra are used to visualize atomic structure, as they comprise plots of intensity vs. distance with peaks corresponding to neighbouring atoms.[6]

Materials and methods

The 64-year-old patient had been diagnosed with end-stage renal disease at age 43 years. While on peritoneal dialysis he had three MRI studies on one day, receiving 180 mL of

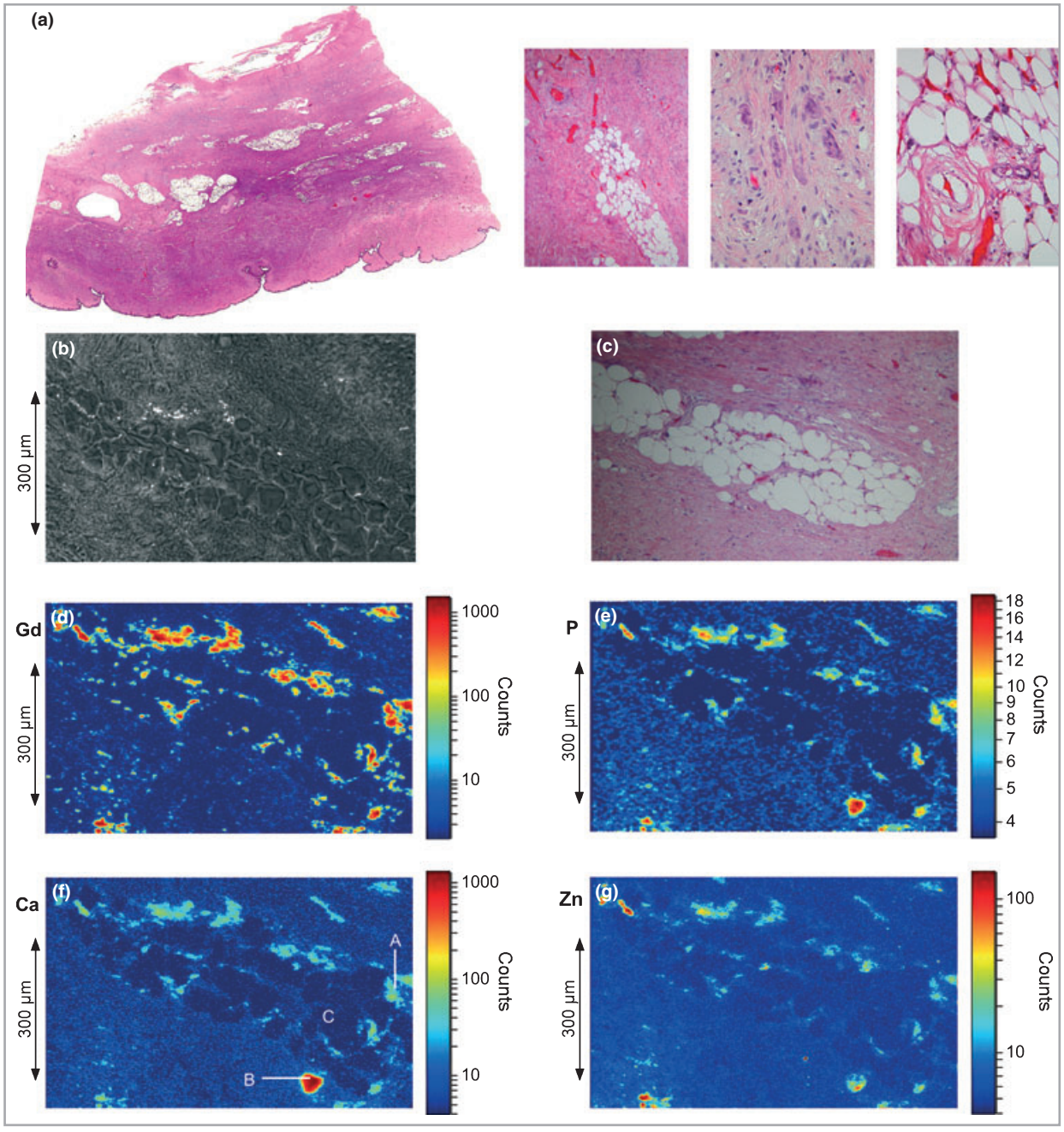

Fig 1. Light and synchrotron X-ray fluorescence (SXRF) microscopy images of the skin tissue showing element distribution. (a) Light microscopy; haematoxylin and eosin (H&E)-stained section of skin with dense fibrosis involving the dermis, extending into the subcutaneous tissue and containing areas of fibrocytes, osteoclast-like giant cells and tissue calcification. (b–g) Images of the tissue area studied by X-ray fluorescence: (b) scanning electron microscopy image; (c) light microscopy image of the same area in an adjacent tissue section with H&E stain; (d) Gd L_α image; (e) P K_α image, for clarity this image has been processed by a 3 × 3 pixel smoothing function; (f) Ca K_α image. A, B and C indicate pixels discussed in the Supporting information; (g) Zn K_α image. For the SXRF images, the field of view is 766 µm horizontal by 482 µm vertical by with a pixel size of 2·8 × 2·0 µm. The sample had approximate thickness of 20 µm. The incident X-ray energy was 13·0 keV.

OptiMARK® (gadoversetamide 0·5 mol L^{-1}; Mallinckrodt Inc., Hazelwood, MO, U.S.A.). NSF symptoms occurred within a few days, and NSF was diagnosed by skin biopsy 4 weeks later. His immediate cause of death 50 months later was multiple organ failure. Autopsy with consent of the family included permission for examination of tissues and access to clinical information for publication.

A formalin-fixed paraffin-embedded skin sample showing Gd deposits and fibrosis typical of NSF was selected. SXRF microscopy on 20-μm sections used the Stanford Synchrotron Radiation Lightsource (SSRL; Menlo Park, CA, U.S.A.) beamline 2–3 with a 2·8 × 2·0 μm beam. Fluorescence intensities were calibrated to element concentrations using appropriate standards (Micromatter, Vancouver, BC, Canada). EXAFS measurements on a 3 × 1·5 × 2 mm tissue block used SSRL beamline 7–3. Omniscan™ (gadodiamide; GE Healthcare, Amersham, U.K.) and Magnevist® (gadopentetate dimeglumine; Bayer, Spokane, WA, U.S.A.) were diluted to 20 mmol L^{-1}Gd before measurement. These GBCAs and OptiMARK® are structurally analogous as they are all diethylene triamine pentaacetic acid (DTPA) derivatives. EXAFS analysis employed EXAFSPAK software[7] (see Supporting information for detailed methods).

Results

Microscopy

Figure 1 summarizes the microscopy results. Additional information is presented in Figs S1–S6 (see Supporting information). The tissue shows typical histopathology of NSF.[4] The area studied by SXRF comprises a subcutaneous fat lobule surrounded by fibrotic bands. SEM revealed the presence of insoluble deposits while SEM/EDS confirmed that Gd associates with P, Ca and Na (Fig. S1). The Gd SXRF image shows Gd deposits throughout the tissue especially surrounding the fat lobule. The maximum observed Gd concentration was 0·41 g cm^{-3} with mean and median Gd densities of 0·011 g cm^{-3} and 0·0012 g cm^{-3}, respectively. The median concentration compares well with published gravimetric data.[8] With the exception of a large Ca-rich region containing little Gd, the Gd, Ca and P images show almost identical distributions (see Fig. S2) and analyses of the X-ray fluorescence intensities show a consistent ratio Gd:Ca:P of 1·0 : 0·43 : 1·6 when Gd is present (the P quantitation has ± 0·5 error). Other metals, such as Zn, are present at low concentrations (< 0·035 per Gd). These show no such trend, with the Zn image revealing an inhomogeneous distribution of Zn throughout the Gd and Ca deposits (Fig S3 in Supporting information).

Extended X-ray absorption fine structure spectroscopy

The Gd EXAFS from the skin sample is very different from that of the GBCAs, and analysis indicates that tissue-bound Gd is coordinated by phosphate. Figure 2 compares the Gd EXAFS spectra, Fourier transforms and simulations. Table 1 summarizes the simulation parameters. The data from both GBCAs are very similar and consistent with coordination to the DTPA chelator, with coordinating O atoms at 2·4 Å, a complex pattern of C atoms around 3·5 Å and a split peak at 4·2–4·5 Å from the outer O/N atoms. Analysis of the tissue EXAFS

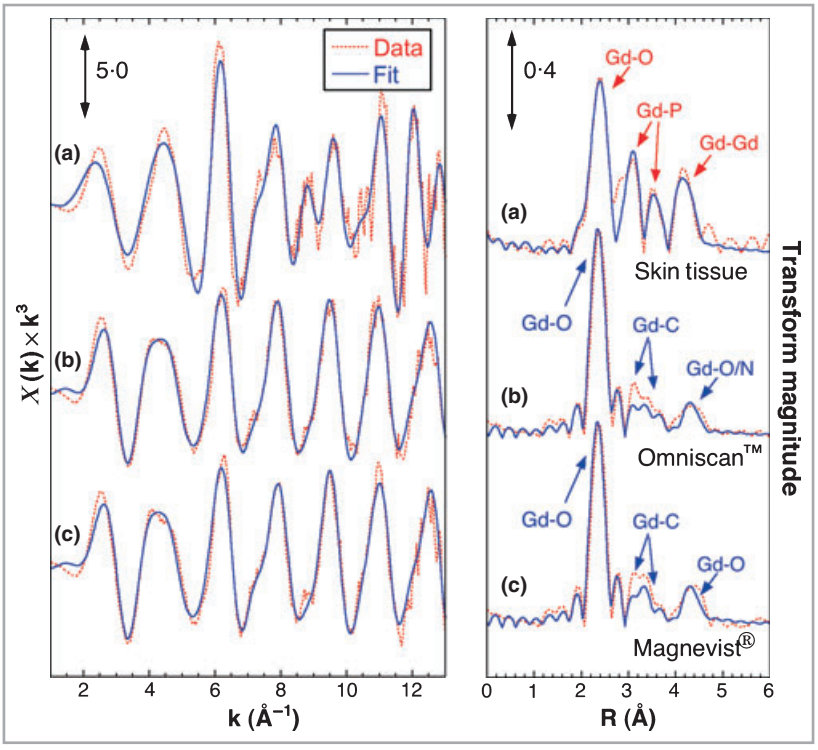

Fig 2. Extended X-ray absorption fine structure (EXAFS) spectra at the Gd L_3-edge and analysis of the tissue sample compared with that from selected gadolinium-based contrast agents. (Left) EXAFS spectra and (right) Fourier transforms (FTs) with simulated fits of (a) skin tissue, (b) Omniscan™, (c) Magnevist®. The Fourier transforms are phase corrected assuming Gd–O interactions. Indicated are the atomic origins of the observed peaks.

Table 1 Key extended X-ray absorption fine structure curve fitting parameters

	Skin tissue		Omniscan™		Magnevist®	
Interaction	N	R(Å)	N	R(Å)	N	R(Å)
Gd–O	(8·0)	2·385	(6·0)	2·391	(6·0)	2·382
Gd–N			(3·0)	2·641	(3·0)	2·634
Gd–P	2·55	3·111				
Gd–C			(9·0)	3·415	(9·0)	3·415
			(5·0)	3·599	(5·0)	3·600
Gd–P (long)	2·43	3·715				
Gd–Gd	2·74	4·053				
Gd–O (long)			(5·0)	4·323	(5·0)	4·323

N, number of backscattering atoms; R, interatomic distance. Parameters fixed in the fit are indicated with parentheses. Full parameters are in Supporting information.

shows around eight O atoms at 2·39 Å, about three P atoms at both 3·11 Å and 3·72 Å, with three Gd atoms at an average of 4·05 Å. These are consistent with the structures of hydrated $GdPO_4$ and mixed metal salts like $KCaGd(PO_4)_2$.[9,10] Although not required for a good fit, Gd–Ca interactions at approximately 4 Å and Gd–Na at shorter distances can also be included (see Supporting information).

Discussion

The SXRF microscopy and EXAFS data provide the first atomic scale constraints on the molecular structure of these Gd deposits in an NSF tissue sample. The SXRF microscopy data show a strong correlation between Gd, P and Ca. These correlations are similar to those seen by SEM/EDS[4,11] and confirm, with substantially greater sensitivity, the co-distribution of Gd and Ca previously reported using secondary ion mass spectrometry[12] and more recently SXRF microscopy,[13] neither of which reported imaging for P. The EXAFS analysis clearly shows Gd present in a $GdPO_4$-like structure. As Ca can substitute Gd sites in hydrated $GdPO_4$ provided Na or K is also coordinated,[9,10] we infer that the observed deposits comprise a predominately $Ca_xNa_xGd_{(1-x)}PO_4$ material.

We conclude that the Gd deposits in this NSF case consist of a Gd phosphate material, with little or no Gd still coordinated to the original organic chelator. That the structure of these deposits in this patient's skin is representative of those seen in other NSF cases is strongly supported by previously published analyses of thousands of similar deposits in tissues from patients with NSF.[11] The enormous thermodynamic stability of Gd phosphate structures (the solubility product for $GdPO_4.H_2O$ has been estimated at 10^{-14})[14] may provide both an explanation for the formation of these deposits and a challenge to chemists and physicians hoping to treat NSF. It appears reasonable to suppose that these deposits in NSF are either causally related to the disease or at least represent a marker of release of Gd from the chelated GBCA.

What's already known about this topic?

- Nephrogenic systemic fibrosis (NSF) is an incurable, debilitating disease found exclusively in patients with decreased kidney function and comprises a fibrosing disorder of the skin and systemic tissues.
- NSF is associated with exposure to gadolinium (Gd)-based contrast agents (GBCA) in magnetic resonance imaging procedures.
- Tissue samples from patients with NSF contain micron-sized insoluble deposits of Gd with P, Ca and Na.
- The chemical nature of these particles is unknown.

What does this study add?

- Synchrotron X-ray fluorescence microscopy and extended X-ray absorption fine structure spectroscopy on skin autopsy samples from a patient with NSF show:
  - nearly invariant ratios of Gd:Ca and Gd:P in the particles;
  - Gd is predominately coordinated by phosphate, and not by the GBCA;
  - Further evidence for a causal link between release of Gd from the GBCA and NSF;
  - State-of-the-art physical–chemical analysis has solved an important and previously intractable biomedical problem.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Data S1. Materials and methods, and Results.

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