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Dermal inorganic gadolinium concentrations: evidence for *in vivo* transmetallation and long-term persistence in nephrogenic systemic fibrosis

J.L. Abraham, C. Thakral, L. Skov,* K. Rossen† and P. Marckmann‡

Department of Pathology, SUNY Upstate Medical University, Syracuse, NY 13210, U.S.A.
*Departments of *Dermatology and †Pathology, Gentofte Hospital, DK-2900 Hellerup, Denmark*
‡Department of Nephrology, Herlev Hospital, DK-2730 Herlev, Denmark

Correspondence
Jerrold L. Abraham.
E-mail: abrahamj@upstate.edu

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Conflicts of interest
None declared.

Summary

Background Gadolinium (Gd)-based magnetic resonance contrast agents (GBMCA), including gadodiamide, have been identified as the probable causative agents of the serious disease, nephrogenic systemic fibrosis (NSF).

Objectives To investigate retained Gd-containing deposits in skin biopsies from patients with NSF and to determine their relative concentrations over time from administration of GBMCA.

Methods An investigator-blinded retrospective study, analysing 43 skin biopsies from 20 patients with gadodiamide-related NSF and one NSF-negative gadodiamide-exposed dialysis patient, ranging from 16 days to 1991 days after Gd contrast dose. Utilizing automated quantitative scanning electron microscopy/energy-dispersive X-ray spectroscopy we determined the concentration of Gd and associated elements present as insoluble deposits *in situ* in the tissues.

Results We detected Gd in skin lesions of all 20 patients with NSF, whereas Gd was undetectable in the NSF-negative patient. Gd concentration increased over time in 60% of patients with multiple sequential biopsies ($n = 10$), decreasing only when the initial sampling time was > 23 months after first gadodiamide dose. All Gd-containing deposits contained phosphorus, calcium and sodium. The ratio of Gd to calcium in tissue deposits correlated positively with the gadodiamide dose and with serum ionized calcium at the time of Gd exposure.

Conclusions These findings demonstrate the *in vivo* release (through transmetallation) of the toxic free Gd^{3+} from gadodiamide, and its retention in apatite-like deposits. We suggest that Gd may be mobilized over time from bone stores, explaining variably delayed onset of NSF and increasing skin concentration over time in patients with NSF.

Gadolinium (Gd) is a rare earth metal that occurs in only trace amounts biologically and has no known metabolic role. This lanthanide element (lanthanides are any members of the rare earth element series, atomic numbers 57–71) is used in magnetic resonance (MR) imaging because of its high paramagnetic properties (a paramagnetic material is not naturally magnetic but can be made magnetic, e.g. by application of an external magnetic field in MR imaging) and long relaxation time (relaxation time is a measure of the time taken for a substance to return to the resting or ground state following excitation). The first experience in humans with Gd-based MR contrast agents (GBMCA) was in 1988.[1] GBMCA are manufactured by a chelating process, in which organic ligand molecules form a stable complex around Gd. As free Gd^{3+} is extremely toxic, the criteria for the ligand were to provide a highly soluble, stable and therefore nontoxic complex. In 2000, 15 haemodialysis patients were reported with a new scleromyoxedema-like skin condition, distinct from morphoea and scleroderma.[2] First recognized in 1997, the clinical spectrum and nomenclature of this disease have evolved during the last decade. This unique cutaneous disease was named nephrogenic fibrosing dermopathy in 2001.[3] Subsequent recognition of this as a systemic disorder led to the renaming as nephrogenic systemic fibrosis (NSF).[4–6] In January 2006 the first report linking GBMCA with NSF was published.[7]

Patients with NSF present with painful or pruritic skin lesions, primarily of the extremities. Skin biopsies show prominent dermal collagen bundles, often with a haphazard arrangement, increased number of dermal fibroblast-like cells and increased number of dermal macrophages and dendritic cells. The changes extend into the subcutaneous tissue along fibrous septae. Infiltrates of circulating fibrocytes (CF), which are dual-positive for both CD34 and procollagen-1, are characteristic.[8] The clinical course of NSF may be progressive, with development of contractures, loss of mobility and in some cases a fulminant course that may be fatal. No specific treatment modality has been consistently effective.

The aetiology and pathogenesis of this entity are incompletely understood. There is reduced clearance of GBMCA in patients with renal insufficiency. Depending on the stability constants and transmetallation kinetics, these agents can dissociate resulting in release of toxic Gd^{3+} ions. These Gd ions may act as a target or trigger for recruitment of CF and result in release of certain growth factors and/or cytokines leading to fibrosis.

In late 2006, two groups identified Gd in skin lesions of NSF using scanning electron microscopy/energy-dispersive X-ray spectroscopy (SEM/EDS).[9,10] We extended our investigation to quantify the amount of detectable Gd in tissue from the Danish series of patients with NSF reported by Marckmann *et al.*[11] using our quantitative SEM/EDS method.[12] This *in situ* analysis also reveals the spatial distribution and the association of other elements in Gd-containing deposits. Our goal was to examine the association of the skin concentration of Gd with clinical data. We hypothesized that Gd chelates dissociate under certain metabolic conditions favouring transmetallation, with release of Gd ions – the suspected underlying cause of NSF.

Materials and methods

An investigator-blinded study was conducted for detection and quantification of Gd in tissue of patients with a clinical and histopathological diagnosis of NSF. The analytical pathologists (J.L.A. and C.T.) received 43 coded paraffin-embedded tissues from 20 patients with gadodiamide-related NSF and one NSF-negative, gadodiamide-exposed patient with renal failure (biopsied for other skin reaction) followed at the nephrology department of Herlev Hospital, Denmark. Skin biopsies were taken as part of diagnostic efforts. Punch biopsies (performed by a dermatologist) were 4 mm diameter and contained epidermis, dermis and in most cases at least a sampling of subcutaneous tissue. Surface cut sections for light microscopy were prepared, leaving a freshly cut surface of the paraffin block for examination in SEM. Analysis was done using the variable pressure ('Environmental') mode of the Aspex® (Delmont, PA, U.S.A.) scanning electron microscope, allowing direct examination of nonconductive specimens without any further preparation. Operating conditions were 20 keV accelerating voltage, 15–16 mm working distance, 0·15 torr pressure in the specimen chamber, and beam current approximately 500 pA. Backscattered electron imaging (BEI) reveals the relatively high atomic number inorganic materials in a low atomic number organic matrix (tissue). The X-ray spectra of detected features were compared with standard reference spectra of chemical elements.

Based on established methods for automated computer-controlled SEM of individual particles or 'features' in environmental samples,[13] we developed a method for quantitative measurement of Gd deposits in tissues.[12] A polygon containing the tissue area (excluding the epidermis) of each specimen was defined, and searched in random fields of view at ×500 magnification, with thresholding on the brightness of the BEI signal to detect features of atomic number greater than that of the organic tissue matrix. Each detected feature was measured and an EDS spectrum collected. Features were classified based on their elemental composition. Elements included in the rules for these analyses were Na, Mg, Al, Si, P, K, Ca, Ti, Fe and Gd. The percentage of collected X-rays for each of these elements is tabulated. Digital images of each searched field and of a subset of individual features and their X-ray spectra are saved. Each analysis takes from 1 to 3 or more hours, depending on the area of the tissue sample and the number of features detected and analysed. The Gd concentration of each sample is reported as the sum of the Gd counts per second (cps) for all the Gd-containing features, divided by the total tissue area scanned, resulting in Gd cps mm^{-2}. Additional data are available for each of the elements noted, and this individual feature analysis documents the association of and relative amounts of each element in each deposit. The spatial distribution of the various classes of deposits within the tissue is also recorded. This method is highly reproducible, with median coefficient of variation for repeat analyses $< 10\%$. The tissues examined have been processed through standard aqueous formalin fixation, alcohols, xylenes and paraffin; thus, only insoluble elements remain for analysis. We cannot exclude the presence of water-soluble forms of Gd which are no longer retained in the tissues, nor can we detect concentrations below the detection limit of our SEM/EDS system (minimum detectable Gd in a scanned tissue area would be a single 0·5 μm deposit with 1·0% Gd by weight, which would be equivalent to approximately $1{\cdot}0 \times 10^{-15}$ g. This would be in the order of 1 cps mm^{-2}, depending on the area examined). This measurement is volumetric and cannot be precisely converted to gravimetric as the density of the tissues is not uniform.

Following the SEM/EDS analyses, we examined the histopathological features in the haematoxylin and eosin-stained sections. After all the data collection in the SUNY laboratory, the Danish group provided the clinical information for further analyses in each case. Data for each patient included date and dose of Gd-enhanced MR imaging scan, dates and sites of skin biopsies, renal status at the time of scan and clinical evaluation of the severity of NSF. Plasma ionized Ca and P were noted, as these were found to be significantly higher in NSF cases compared with controls in a current analysis.[14]

Table 1 Patient demographics and scanning electron microscopy/energy-dispersive X-ray spectroscopy results for all biopsies studied

Patient	Sex, birth year, renal diagnosis	Date of biopsy (day/month/year)	NSF lesion (Y/N)	Gadodiamide exposure date (day/month/year)	First (cumulative[a]) gadodiamide dose (mmol kg^{-1})	Serum Ca (mmol L^{-1})[b]	Serum P (mmol L^{-1})[b]	Gd (cps mm^{-2})	Sum (Gd cps)/ sum (Ca cps) in Gd-containing deposits
1	F, 1967, GN	4/11/05	Y	14/9/05	0·19	1·16	2·25	22·4	0·49
		4/11/05	Y	14/9/05	0·19	1·16	2·25	3·4	0·41
		21/11/05	Y, border of lesion	14/9/05	0·19	1·16	2·25	45·5	1·20
		21/11/05	N	14/9/05	0·19	1·16	2·25	0·0	0·00
2	F, 1955, GN	9/2/06	Y, acute eczema-like	24/1/06	0·32	1·2	2·07	318·7	0·89
		13/3/06	Y	24/1/06	0·32	1·2	2·07	76·2	0·77
		13/9/06	Y	24/1/06	0·32	1·2	2·07	1127·3	1·28
3	M, 1939, Hyp	11/8/03	Y	8/5/03	0·36	1·27	2·76	1·9	1·88
		13/11/06	Y	8/5/03	0·36	1·27	2·76	217·5	1·16
4	F, 1964, GN	16/12/02	Y	18/9/02	0·32	1·23	2·06	3·3	0·44
		4/3/03	Y	18/9/02	0·32	1·23	2·06	1032·0	2·40
		26/3/03	Y	18/9/02	0·32	1·23	2·06	565·4	1·98
		10/4/06	Y	18/9/02	0·32	1·23	2·06	1221·1	2·72
5	F, 1939, GN	15/4/03	Y, severe	16/12/02	0·27	1·25	1·83	139·8	1·17
		15/4/03	N, lichen chronicus	16/12/02	0·27	1·25	1·83	239·9	1·83
6	M, 1938, GN	18/4/05	Y	17/1/05	0·22	1·11	2·19	0·0	0·00
		18/4/05	N, actinic keratosis	17/1/05	0·22	1·11	2·19	9·3	0·71
		2/5/05	Y	17/1/05	0·22	1·11	2·19	37·0	0·42
7	F, 1957, Hyp	21/11/05	Y	17/8/05	0·23	1·23	1·48	21·2	0·63
8	M, 1941, Hyp	24/5/06	No NSF	18/1/06	0·28	0·98	1·51	0·0	0·00
9	M, 1953, PCKD	29/5/06	Y	3/1/02	0·37	1·29	2·22	168·7	0·74
10	M, 1953, GN	7/6/06	Y	2/1/06	0·24	1·14	2·21	4·8	0·22
		7/6/06	Y	2/1/06	0·24	1·14	2·21	49·5	0·31
11	M, 1972, HUS	9/6/06	Y, mild changes	2/10/03	0·39	1·36	1·82	1184·6	0·06
		9/6/06	Y	2/10/03	0·39	1·36	1·82	810·7	3·70
		6/12/06	Y	2/10/03	0·39	1·36	1·82	249·6	0·68
12	M, 1958, GN	11/9/06	Y	16/6/04	0·34	NA	NA	47·5	1·72
		13/12/06	Y	16/6/04	0·34	NA	NA	1·0	0·18
13	M, 1960, Hyp	9/10/06	Y, atypical	15/4/03	0·09	NA	NA	154·3	2·01
		3/11/06	Y, atypical	15/4/03	0·09	NA	NA	10·1	1·90
14	M, 1953, DM	17/5/05	Y, acute eczema-like	4/9/03, 11/4/05	0·21 (0·4)	1·18	3·07	805·3	0·04
		14/9/05	N	4/9/03, 11/4/05	0·21 (0·4)	1·18	3·07	40·3	2·51
		14/9/05	Y, border of lesion	4/9/03, 11/4/05	0·21 (0·4)	1·18	3·07	1666·8	1·18
		14/9/05	Y	4/9/03, 11/4/05	0·21 (0·4)	1·18	3·07	39·7	0·20
15	M, 1947, Li	5/12/05	N, dermatophytosis	5/2/05, 24/6/06	0·32 (0·6)	1·25	1·61	135·5	1·10
		5/12/05	Y	5/2/05, 24/6/06	0·32 (0·6)	1·25	1·61	192·3	0·88
16	F, 1954, GN	30/1/06	Y, severe	13/6/05, 15/7/05	0·36 (0·9)	1·11	2·82	121·3	1·61

Table 1 (*Continued*)

Patient	Sex, birth year, renal diagnosis	Date of biopsy (day/month/year)	NSF lesion (Y/N)	Gadodiamide exposure date (day/month/year)	First (cumulative[a]) gadodiamide dose (mmol kg^{-1})	Serum Ca (mmol L^{-1})[b]	Serum P (mmol L^{-1})[b]	Gd (cps mm^{-2})	Sum (Gd cps)/sum (Ca cps) in Gd-containing deposits
17	F, 1948, PN	3/4/06	Y, severe	14/1/02, 29/6/04	0·5 (1·0)	1·42	1·59	375·3	2·59
18	F, 1937, Hyp	12/6/06	Y	7/5/01, 1/12/05	0·26 (0·5)	1·15	1·97	50·3	0·83
19	M, 1947, Hyp	11/10/05	Y, severe	19/10/00, 10/6/02, 5/12/03	0·29 (0·5)	1·22	2·24	0·0	0·00
		2/4/06	Y, severe	19/10/00, 10/6/02, 5/12/03	0·29 (0·5)	1·22	2·24	6·5	1·35
20	M, 1940, Hyp	20/3/06	Y	25/5/01, 21/10/02, 17/5/05	0·1[c]	1·3	1·85	4·5	0·59
21	F, 1971, PN	19/9/05	Y, severe	15/9/04, 1/12/04, 18/1/05	0·31 (0·5)	1·14	3·48	38·1	0·44

NSF, nephrogenic systemic fibrosis; GN, glomerulonephritis; Hyp, hypertensive nephropathy; PCKD, polycystic kidney disease; HUS, haemolytic uraemic syndrome; DM, diabetes mellitus; Li, lithium; PN, pyelonephritis; NA, not available. [a]Total dose in patients with more than a single exposure. [b]Ca and P near time of single Gd dose or time nearest preceding onset of NSF. [c]Patient had Omniscan 0·1 and 0·31+ Multihance (15 mL).

JMP statistical software (SAS Institute, Cary, NC, U.S.A.) was used for exploratory data analysis and for linear regression analyses of associations between variables of interest.

Results

We studied 21 patients who had been exposed to gadodiamide while in chronic kidney disease stage 5. Of these, 20 patients had clinical signs and skin histology consistent with NSF. The remaining one patient developed no signs of NSF.

Thirteen patients had only one dose of MR contrast, while five patients had two and three patients had three exposures to GBMCA. All 21 patients were exposed to gadodiamide, with one patient having additional exposure to gadobenate dimeglumine. The cumulative gadodiamide dose ranged from 0·09 to 1·0 mmol kg^{-1}. Ten patients had sequential skin biopsies after gadodiamide exposure, owing either to an inconclusive result in an earlier biopsy or to look for progression of disease. Biopsies were usually from the most severe lesion at the time of examination. Five patients with NSF had additional biopsies from sites that were either clinically normal or involved with another dermatological condition. Demographics of the patients in this study are presented in Table 1.

With SEM/EDS, we detected Gd in skin lesions in all 20 patients with NSF, including four of five biopsies from non-NSF lesions. The non-NSF patient did not have detectable Gd deposits. The amount of Gd detected ranged from 1·00 to 1666·77 cps mm^{-2}. The median diameter of Gd-containing deposits ($n = 1651$) was 1·02 μm (range 0·28–7·9). Dermal Gd was found associated with P, Ca and usually also Na, and a small number of features also had detectable Fe or Al or Zn. Gd was present in cellular areas of dermis along with areas of specific calcification in basement membranes or vessels, as had been seen in the four cases initially reported.[10] There was no correlation between the amount of detectable Gd and the histological features of NSF. Figure 1 shows examples of the histology of typical NSF lesions, types of Gd-containing deposits and X-ray spectra. Noteworthy is the nonuniform distribution of Gd-containing features within the biopsies. In some deep biopsies, Gd was detected more in the subcutaneous fibrous septa as compared with the dermis (Fig. 2).

Skin Gd concentrations varied over time in patients with repeated biopsies ($n = 10$). We observed a substantial increase in skin Gd concentration over time only in patients in whom initial biopsy was performed within a few months of the gadodiamide dose. Four of five of those sampled for the first time > 600 days from initial dose showed an interval decrease in Gd concentration, some of them with very short time intervals between sequential biopsies. Large increases in Gd cps mm^{-2} were seen up to 3·5 years after a single Gd dose (Fig. 3). There was no interval exposure to GBMCA between sequential biopsies in any case.

The ratio of ΣGd cps to ΣCa cps in the Gd-containing skin deposits varied in different biopsies. Eight of the 13 NSF patients with a single gadodiamide exposure had biopsies within 6 months of exposure. There was a positive correlation

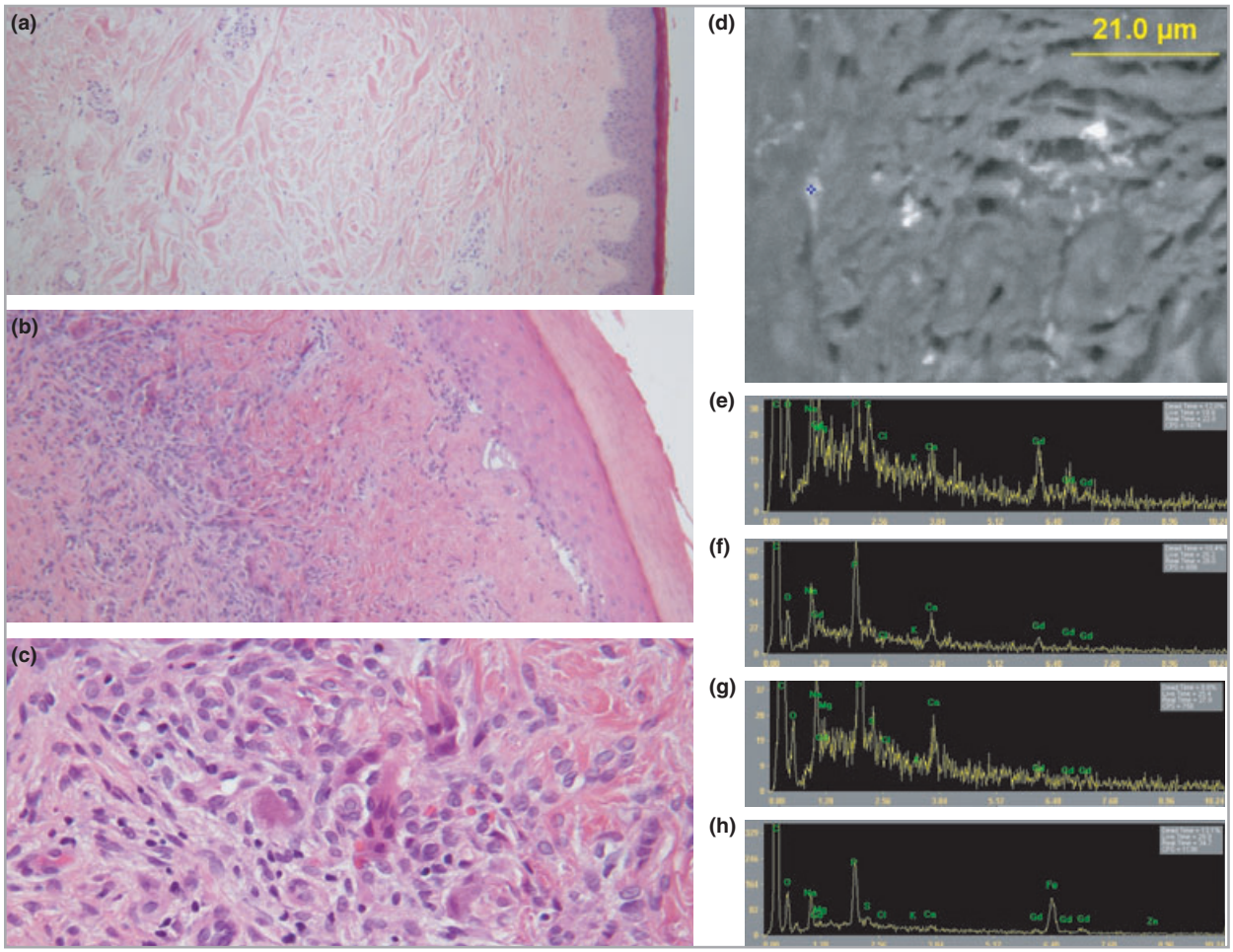

Fig 1. Images of nephrogenic systemic fibrosis biopsies showing bland fibrosis (a), cellular lesion (b) and detail of cellular area showing many small multinucleated histiocytes (c) (haematoxylin and eosin). Scanning electron microscopy, backscattered electron image showing numerous bright features of higher atomic number (d). Representative X-ray spectra showing Gd associated with P, Ca and Na with varying ratio of Gd to Ca (e–g) and with Fe (h).

between gadodiamide dose and Gd/Ca ratio in these eight cases ($r^2 = 0{\cdot}64$, $P < 0{\cdot}02$). However, this correlation was no longer seen for biopsies taken > 6 months after exposure, most probably owing to variation in Gd concentration over time. The serum Ca concentration just prior to gadodiamide exposure was also significantly associated with the Gd/Ca ratio in this group of cases ($r^2 = 0{\cdot}76$, $P < 0{\cdot}005$).

Discussion

At present, exposure to GBMCA and renal insufficiency are the only recognized obligate factors associated with NSF. Our findings provide clear proof of deposition of insoluble, unchelated Gd with phosphates in tissue involved with NSF, underlining the causal role of GBMCA in NSF.

In healthy subjects, soluble Gd chelates are excreted unchanged by passive glomerular filtration within 1·5 h. In renal insufficiency, the clearance is reduced and its elimination half-life may exceed 30 h. During the passage of Gd-chelate complexes through the body, equilibrium exists between Gd, ligand and the complex. This depends on the thermodynamic stability constants of Gd chelates. There is a very large variation in the stability constants of the several Gd chelates in widespread clinical use internationally.[15,16] The reported frequency of NSF cases following differing Gd chelates parallels the relative stability of these chelates. Cacheris *et al.*[17] clearly demonstrated that the amount of Gd released, rather than the total dose of any individual chelated Gd agent, was the determinant of toxic outcome.

Different metal ions can compete with Gd ions for the chelator in the complex, resulting in *in vivo* transmetallation. In recent years, there is increasing evidence about transmetallation of GBMCA with body cations such as zinc (evidenced by zincuria), calcium or iron.[18–20] The altered metabolic profile in renal insufficiency can favour transmetallation and shift the equilibrium towards dissociation of Gd ions. The recent case–control study from Denmark supports this concern by showing a significant association between increased serum concentrations of Ca and P at the time of gadodiamide-enhanced scans and the risk of developing NSF. Our finding

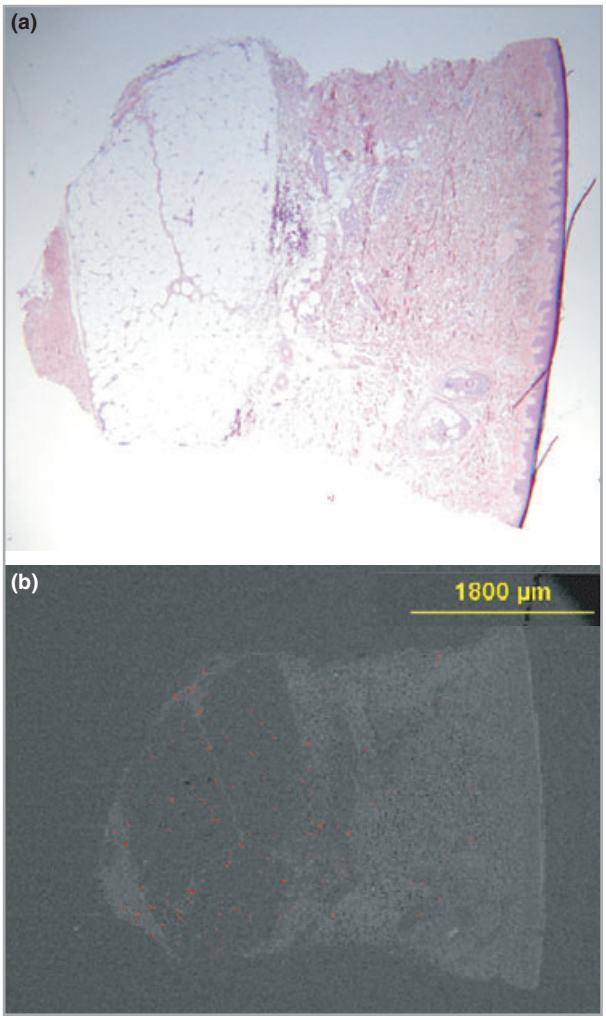

Fig 2. Light micrograph (a, haematoxylin and eosin) and scanning electron microscopy (SEM) image (b) of paraffin block-cut surface of same biopsy, with overlay of distribution of Gd-containing features (red) detected in automated SEM/energy-dispersive X-ray spectroscopy analysis.

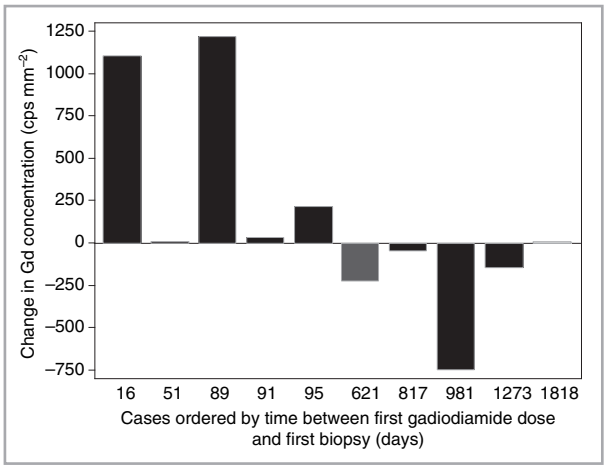

Fig 3. Histogram showing change in Gd concentration (cps mm^{-2}) between sequential (first and last) biopsies of 10 cases displayed according to time from first gadodiamide dose until first biopsy (see text).

of significant correlation of Gd cps/Ca cps in tissue deposits within 6 months of Gd exposure with ionized calcium levels in serum at the time of exposure provides further support for transmetallation. It is known that the complex hydroxyapatite mixture of amorphous calcium carbonate and hydroxide can incorporate Gd, Na, Zn, Fe etc. into apatite in metastatic calcification.[21] Our detection of Fe or Zn in the same Gd-containing deposits is consistent with this process. Although calcification has been described in NSF and one recently reported patient showed improvement of NSF with sodium thiosulphate,[22] the exact role of calcification and Gd-Ca coprecipitation in the development of NSF needs further investigation.

The metabolism of Gd ions in blood is still poorly understood. Experimentally, high doses of lanthanides (including Gd) exceed the complexing ability of plasma and form insoluble precipitates with hydroxides, phosphates and carbonates.[23] Our finding of high Gd cps/Ca cps in tissue deposits of NSF with increasing gadodiamide dose is consistent with the fact that most reported cases of NSF follow high-dose GBMCA. The insoluble, inorganic Gd skin deposits in NSF clearly indicate that Gd was released from its chelate as free Gd ion, but does not resolve whether the Gd is incorporated with phosphates as part of apatite complexes initially or secondarily. Do insoluble precipitates from blood embolize to the skin and other sites where disease appears? Or do these deposits form at the sites after some related or additional injury? The early literature suggests that cells of the reticuloendothelial system clear the precipitates formed in the blood. The presence of macrophages and small multinucleated cells in the skin in the more cellular or active phase seen in some NSF biopsies is perhaps related to this reaction to Gd. Gd is known to cause macrophage apoptosis,[24] which may further explain the temporal transience of the macrophage predominance in the NSF skin lesions. Chelated Gd may be taken up into lysosomes of reticuloendothelial cells where the low pH rapidly promotes transmetallation, resulting in insoluble deposits of Gd with Ca and P.[25]

Skin lesions related to Gd are not novel. In preclinical safety assessment of gadodiamide injection, Harpur *et al.*[26] observed skin lesions occurring at high doses of gadodiamide in rats. This included patchy hair loss, scab formation or thick skin. At that time, this was attributed to disturbance in Zn metabolism. The experimental rat model from Schering also results in skin lesions similar to NSF after gadodiamide, with lesser severity after gadopentetate meglumine (presented at FDA conference, 1 February 2007). The dermal histological changes do not necessarily match the clinical severity of NSF, and in our analyses the amount of detectable Gd did not always correlate with the histology. Also the nonuniform distribution of Gd in tissue may be an important determinant for the performance and interpretation of skin biopsies in NSF.

We believe that our data showing higher dermal concentration of Gd in late than in early biopsies after gadodiamide exposure can best be explained by initial bone storage and subsequent mobilization of Gd. Liver and bone are the main sites of accumulation of lanthanides.[23] Chelators shift the main site of retained lanthanides from liver to bone.[27] Franano *et al.* showed that Gd dissociates from chelate complexes depending on acid dissociation rates and accumulates in bone.[25,28] Two different rare earth metals (Gd and lanthanum, La) have been shown to accumulate in bone in humans after therapeutic use of GBMCA[29] or oral lanthanum carbonate phosphate binder.[30] The amount of Gd deposited in bone of persons with normal renal function 4–8 days after GBMCA administration was found to be greater with gadodiamide than gadoteridol, consistent with the greater relative likelihood of release of free Gd^{3+} from gadodiamide. The mobilization from bone deposits of La over time (years) has been demonstrated, and this has been shown to maintain low but detectable La in plasma.[30] No comparable study of mobilization of Gd from bone stores has yet been reported.

The recognition that rare earth metals are deposited in bone and other sites, with slow long-term clearance,[31] provides strong support for the hypothesis that Gd deposition in bone after Gd-enhanced MR imaging scans may be a long-term internal source of Gd. This may help explain the delayed onset and/or increasing severity of NSF over many months and years. Although NSF has thus far been seen only in patients with severe renal disease, it remains unknown whether persons exposed to GBMCA (regardless of renal function at present) may later develop renal failure or other bone demineralizing conditions, which might lead to Gd toxicity from comobilization of Gd from bone stores.

Our study tries to fill some of the gaps in the understanding of NSF. Although tens of millions of doses of Gd chelates have been administered worldwide, only a few hundred cases of NSF have as yet been recognized. What determines individual susceptibility to develop NSF remains a valid subject for further investigation. To date, this is the largest study of Gd concentration in tissue from patients with NSF, yet it is not sufficient to identify all the variables involved in this complex multifactorial scenario. However, prevention of the disease by eliminating or minimizing the chances for exposure to free Gd would obviate and override the need to understand the precise mechanism(s) immediately.

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