Distribution and form of gadolinium in tissue of normal rats, and the relevance to nephrogenic systemic fibrosis

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Organs from normal rats treated with Gadolinium (Gd) agents were studied using Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM), as well as bulk compositional analysis using ICP-AES, to ascertain the presence, concentration, distribution, and form of any Gd compounds present. Nephrogenic systemic fibrosis (NSF) is a rare condition characterized by the increased formation of connective tissue in the skin which becomes thickened, coarse, and indurated, sometimes leading to contractures and joint immobility [1, 2]. Patients with NSF may have systemic involvement of other organs including the lungs, liver, diaphragm, muscles, and heart. To date, NSF has been reported almost exclusively in patients with severe (stage 4 and 5) renal insufficiency, and frequently in patients who have also been exposed to a gadolinium (Gd)-based contrast agent (GBCA). In early 2006, a possible link between GBCA administration and the development of NSF was proposed. It was reported that 5 of 9 patients with end-stage renal disease (ESRD) who had received GdDTPA-BMA (Omniscan; GE Healthcare) developed NSF 2 to 4 weeks later [3]. Shortly after this publication, a separate study reported that an additional 13 ESRD patients with NSF had all received Omniscan [4]. The reason why patients with ESRD are at risk is speculative, but one theory is related to the prolonged retention of GBCA in the circulation due to the reduced rate of urinary excretion. The blood half-life of gadodiamide increases from 1.3 hours in healthy volunteers to over 30 hours in patients with ESRD [5,6]. However, the influence of prolonged retention per se of the administered GBCA in the circulation is uncertain (7). As only 3-5% of patients with ESRD exposed to a GBCA develop NSF [8], and not all patients with NSF have documented exposure to a GBCA, investigators have proposed that additional cofactors beyond GBCA exposure must play a role in the development of NSF in the at-risk patient group [3,4,9 -11]. Recently, results have been published which used Scanning Electron Microscopy (SEM) to investigate the location, distribution, quantity, and form of Gd-containing complexes in rat [12] and human [13-15] tissues after the administration of GBCA. The SEM images and associated x-ray microanalysis showed micron-size features containing Gd. In some studies there was speculation on the nature of the Gd complexes, suggesting that they might be inorganic apatite-like precipitates, although more recently they are described as a gadolinium-sodium-calcium-phosphate material. In a prior publication Grant et al [16] have described a study in which GBCA were administered to normal and also to 5/6 nephrectomized rats in an attempt to induce conditions similar to those in patients at risk of developing NSF. In the current study, selected tissues from the normal rats in the Grant study were examined using SEM and TEM.
Methods and Materials

**Tissue Samples:** The protocols associated with the administration of the GBCA to normal rats and the preparation of the tissue samples are described in detail in Grant [16]). Test animals were dosed with 5 or 10 mmol/kg Omniscan; 5 mmol/kg Magnevist; 1 mmol/kg caldiamide; 1, 2.5, or 5 mmol/kg gadodiamide (GdDTPA-BMA without excess caldiamide ligand); 25 µmol/kg GdCl3; or 25 µmol/kg Gd citrate (Tables 1 and 2). Dosing was on consecutive week days, ie, days 1-5, 8-12, and 15-17, a total of 13 doses, with termination on day 18, one day after the last dose. Protocol tissues were removed into 10% neutral buffered formalin (NBF) for histology. After formalin fixation, samples were processed into paraffin wax. Samples of selected tissues were frozen and stored at -20C before analysis by inductively coupled plasma atomic emission spectrometry (ICP-AES) for total Gd and Zn (tissues). Only animals that survived to scheduled termination were included in these analyses. Tissues from rats that had been given Omniscan, Magnevist, and GdCl3 were selected for microscopy analysis. Liver and skin samples from rats given all three substances were examined. Selected kidney and spleen tissues, and tissues from rats that had received saline as a control were also imaged. Table 2 gives the matrix of conditions used in the microscopy study. Spleen and kidney results are not discussed in this paper but were similar to the liver and skin studies. **Microscopy Methods:** Samples for histology were sectioned at a nominal thickness of 5 microns, stained with hematoxylin and eosin (H&E), and examined by light microscopy. Samples were prepared for SEM by taking a standard 5 micron histology slice from tissue blocks. These slices placed on glass histology slides back coated with Pt (for bottom conductivity). Remaining paraffin wax was removed using xylene. The sample slide was coated with thin layer of carbon (for top conductivity). The SEM was operated at high vacuum, 15kV. Images were collected using the secondary electron (SE) and backscatter electron (BSE) detectors that give image contrast based on topography and composition, respectively. X-ray spectra showing elements present were taken using a thin-window energy-dispersive x-ray detector. Compositional maps were also produced using the x-ray signal. Samples were prepared for TEM by cutting a small chunk of tissue from the paraffin block. The tissue was cut into shape for microtomy and glued to the microtome pin stub. A cryoultramictotome was then used to perform cryomicrotomy dry at -90C, producing 100-120nm thick sections that were collected onto 400 mesh copper and 300 mesh lacey formvar/carbon copper grids. TEM imaging, diffraction, and microanalysis were conducted at 200kV. X-ray spectra showing elements present were taken using a thin-window energy-dispersive x-ray detector. In some cases peak intensities were converted into elemental concentrations using a thin-film (Cliff-Lorimer) approximation with calculated k-factors.
Fig. 0: (Table) Nomenclature and composition of the different Gd substances used.

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<table>
<thead>
<tr>
<th>Test Item</th>
<th>Active Ingredient</th>
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<tbody>
<tr>
<td>Omniscan (Gadodiamide injection)</td>
<td>GdDTPA-BMA + 5 mol% excess of the Ca-chelate (caldiamide, CaDTPA-BMA)</td>
</tr>
<tr>
<td>GdCl3</td>
<td></td>
</tr>
<tr>
<td>Magnevist</td>
<td>(Gadopentetate dimeglumine) GdDTPA + 0.1% excess Ca chelate (CaDTPA, meglumine salt)</td>
</tr>
</tbody>
</table>

Fig. 0: (Table) Tissue samples chosen for microscopy study.

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Results

Figure 1a shows the overall Gd concentration in liver and skin tissue samples measured by ICP-AES as a function of treatment and dose (from Grant et al. 2009 [16]). The highest levels of Gd are seen for GdCl3 in both organs. However, Figure 1b shows that understanding the form of Gd is critical to interpreting the data. Although on a %ID basis there is more Gd in skin after GdCl and Gd citrate, its presence did not cause lesions. **Liver:** Figure 2 shows typical SEM images of liver tissue samples after administration of Omniscan, Magnevist, and GdCl3. The left are the backscattered electron (BSE) images which show composition contrast, and on the right are secondary electron (SE) images which show topography. In the BSE images, larger bright regions can be seen which are tears or thin regions in the section. In addition, there is a distribution of smaller bright features, most visible in 2e, which appear to be particles of higher average atomic number. Figure 3 shows a comparison of the SEM images (figs. 2a,b) with the H&E-stained histology section at the same magnification; while some features are obscured in the SEM view, good correspondence is seen. Figure 4 shows higher-magnification BSE-SEM images of the sections in Fig 2. Here the smaller bright features are seen to be particles or particle clusters ranging in size from 0.3 microns to several microns in size. Figure 5 shows x-ray microanalysis (EDX) from specific regions in Fig. 4. Fig 5 a,b,c show the spectra from the bright particles from the Omniscan, Magnevist, and GdCl3 sections. In each case Gd is seen, along with Ca, P, Si, Na, O, and small amounts of Fe and Al. The spectra are qualitatively similar for the three samples. Fig. 5d shows a spectrum from adjacent tissue area where no particles are present, and Ca, S, P, K, Si, Al, Mg, Na, and O are seen. Finally, Fig. 5e shows the spectrum from the underlying glass slide, showing Ca, Si, Al, Mg, Na, and O. Comparing Figs. 5a,b,c to 5d shows that the clearest result is that enhanced P is associated with the Gd in the particles. This conclusion is reinforced by Figure 6, where x-ray maps of the region in fig. 4f are shown. In the maps, the bright pixels correspond to high levels of the element indicated. Again, the clearest result is that Gd and P are associated in the particle clusters. Although only the maps for the GdCl3 sample are shown here as an illustration, similar results were obtained for Omniscan and Magnevist. TEM was performed on thin sections of the three samples in an attempt to further clarify the nature of the particles. Figure 7 shows TEM images from the Omniscan (a,b), Magnevist (c,d) and GdCl3 (e,f) samples. These sections are unstained and so biological structures are not visible. Particles are clearly seen in all images, with the largest clusters again occurring for the GdCl3 section, consistent with the ICP results. The images show that the particles or clusters seen in fig. 4 are comprised of smaller particles of a few nm in size, as seen in the higher magnification view in fig. 8. X-ray microanalysis was performed on the particles in fig. 7. EDX in the TEM has the advantage that the contribution from the surrounding tissue can be minimized, and there is no substrate. Figure 9 shows typical spectra from particles in two of the samples, plus the adjacent tissue. From these spectra it is possible to get semi-qualitative compositions of the inorganic elements, and those results are also shown (C and O omitted). The particle compositions are similar in the
two samples and confirm that P is primarily associated with Gd, with lesser amounts of Ca, Fe, and in some cases S and K. Electron diffraction in the TEM was also used to determine the crystallographic nature of the particles. Those results are shown in Figure 10 for the Omniscan and GdCl3 samples. The diffuse rings in the electron diffraction patterns show that the particles are amorphous and not crystalline.

**Skin**: Figure 11 shows a comparison of the H&E-stained optical section of the skin with the low-magnification SEM view. The outer skin surface (epidermis) is on the right. The skin is a much less uniform structure compared with the liver. The results from the skin sections were very similar to those discussed above for the liver, and will only be briefly discussed here. Figure 12 shows SEM views of the three samples, showing that Gd-containing particles can be found in each case, and that their size and morphology is similar to those in the liver. For the skin, the highest Gd concentration was seen by ICP for Omniscan, and that is reflected in the SEM results. The x-ray microanalysis on these particles is identical to the results for the liver. Figure 13 shows typical TEM results showing the Gd-containing particles in a skin sample, in this case from the Omniscan samples. At the nanometer scale, the particles in these images seem less well-defined and perhaps more acicular (needle-like) than those in the liver sections. It was also found using EDX in the TEM that these particles appeared to have higher levels of P and Ca compared with Gd than in the case of particles in the liver.
**Fig. 0:** Figure 1. a) ICP-AES results for overall concentration of Gd in liver and skin tissues of rats in this study as a function of treatment and dose (from Grant et al. 2009 [16]).

b) Lesion occurrence in skin of rats in this study [16]. Red boxes show the conditions discussed in this paper.

© Grant D et al. Effects of Gadolinium Contrast Agents in Naïve and Nephrectomized Rats: Relevance to Nephrogenic Systemic Fibrosis. Acta Radiologica 2009 50(2); 156-169
**Fig. 0:** Figure 2. Lower-magnification SEM views of liver tissue after Omniscan(a,b), Magnevist (c,d), and GdCl₃(e,f) administration. Both secondary electron (SE) and backscatter electron (BSE) images are shown of the same area. Very bright areas in BSE views represent "holes" in the tissue slice which exposes the glass slide substrate and "charges" (repels electrons) under the beam. Magnification and micron marker indicated in bar below each image.

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Fig. 0: Figure 3. Comparison of H&E-stained optical histology section with SEM images (a,b in Fig 2) at same magnification. Magnification and micron marker indicated in bar below SEM images.

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Fig. 0: Figure 4. Higher-magnification BSE-SEM views of liver tissue after Omniscan (a,b), Magnevist (c,d), and GdCl₃ (e,f) administration, showing the presence of bright particles. Red boxes on left show enlarged regions on right.

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Fig. 0: Figure 5. Energy-dispersive x-ray (EDX) spectra from particles in Figure 4: a) Omniscan (spectrum 1 in 4b) b) Magnevist (spectrum 1 in 4d) c) GdCl₃ (spectrum 1 in 4f). Also shown are d) spectrum from tissue area (spectrum 4 in 4(d)) and e) underlying glass slide substrate.

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**Fig. 0:** Figure 6. BSE SEM image and associated x-ray maps from GdCl₃ liver section (fig. 4d) showing co-localization of Gd, P, and O. Similar results were seen for particles in Omniscan and Magnevist samples.

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**Fig. 0:** Figure 7. TEM images of liver tissue after Omniscan (a,b), Magnevist (c,d), and GdCl3 (e,f) administration showing the presence of Gd-containing particles (dark).

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**Fig. 0:** Figure 8. Higher magnification TEM image of Gd particle cluster in Fig. 7f (GdCl3) showing Gd-containing clumps in SEM views are made up of aggregated individual particles of a few nm in size.

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Fig. 0: Figure 9. X-ray spectra associated with regions in TEM samples shown in Fig. 7.
a) Spectrum from Gd cluster in 7(a,b) (Omniscan). b) Spectrum from tissue background in above sample. c) Spectrum from Gd cluster in 7(e,f) (GdCl₃).

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**Fig. 0:** Figure 10. TEM images and associated electron diffraction (DP) patterns of Gd-containing particles from a) Omniscan and b) GdCl₃ samples. The circle in the TEM image shows the area from which the DP was obtained. Diffuse rings in DP indicate the amorphous nature of the particles.

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**Fig. 0:** Figure 11. Comparison of H&E-stained optical histology section with low-magnification SEM image of GdCl3 skin section. The epidermis on the right in the SEM image is bright due to charging from non-conductive regions. The red box shows the general type of regions shown in the next figure.

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Fig. 0: Figure 12. SEM views of skin tissue after Omniscan (a,b), Magnevist (c,d), and GdCl₃(e,f) administration. In a) and e) both secondary electron (SE) and backscatter electron (BSE) images are shown of the same area; all other images are BSE. Bright Gd-containing particles are seen. Red boxes on left show enlarged regions on right. In each sample most of the Gd particles were found in the collagen-like region under the epidermis to the left in Fig. 11 (red box).

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Fig. 0: Figure 13. TEM images of Gd-containing particles (dark) in various regions of skin sample after administration of Omniscan.

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Conclusion

The following are the overall conclusions of this study: 1. After administration of the gadolinium-based contrast agents Omniscan and Magnevist, as well as GdCl3, to normal laboratory rats, ICP-AES showed measurable Gd concentration in the liver, skin, kidney, and spleen of the animals, as well as in other organs. Scanning electron microscopy and transmission electron microscopy, coupled with x-ray microanalysis, was able to find Gd-containing particles in all of the tissues where ICP-AES found Gd present. 2. The number of particles found by SEM scaled roughly with the quantitative concentration of Gd in the tissue measured by ICP-AES. 3. In most cases the SEM imaging showed that the Gd was present as individual particle or clumps that were on the order of 0.1 micron to several microns in size. 4. TEM imaging showed that these particles or clumps seen in the SEM consisted of smaller particles on the order of a few nm in size. 5. In particle clumps containing Gd, P was seen to be the primary additional element present. Quantitative analysis using x-ray spectroscopy in the TEM showed that P was present at 40 - 55 at. pct, Gd at 25 - 35 at. pct., Ca and Fe at 8 - 12 at. pct., and in some cases S and K in the 2 - 4 at. pct range. 6. Electron diffraction showed that the Gd-P particles were amorphous, not crystalline, in nature. 7. In general it was difficult to associate the location of particles with any specific cell type or constituent in any organ. 8. In the particular case of the Omniscan imaging agent and the skin tissue, the morphology of the Gd-contending particles seem to be more acicular (needle-like) in shape, with higher levels of P and Ca compared with Gd.
References

Personal Information

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