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CONTRAST MEDIA

Experimental studies investigating the pathophysiology of nephrogenic systemic fibrosis; what did we learn so far?

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The association between nephrogenic systemic fibrosis (NSF) and gadolinium based contrast agents (Gd-CAs) was first suggested in 2006 [1, 2]. This was followed by several reports confirming this association with the vast majority of published cases were associated with the non-ionic linear agent gadodiamide "Gd-DTPA-BMA" (Omniscan, GE Healthcare, USA) [3]. Smaller percentages of cases have been directly linked to the ionic linear chelate gadopentate dimeglumine "Gd-DTPA" (Magnevist, Bayer-Schering, Germany), and the non-ionic linear chelate gadoversetamide "Gd-DTPA-BMEA" (OptiMARK, Covidien, USA) [3]. The high temporal association with the administration of Omniscan which has low kinetic and thermodynamic stability raised the possibility that NSF is caused by the release of toxic Gd ions from unstable Gd-CA molecules [4]. This probable explanation promoted extensive investigation of the importance of stability of Gd-CAs in the pathogenesis of NSF with several experimental studies had been published in the literature over the last few years. The extent to which these studies increased the understanding of the pathophysiology of NSF is the main focus of this editorial. Concise summary of key studies in this field will be presented including those evaluating the stability of Gd-CAs and their biological effects, in vivo and in vitro.

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Studies evaluating the stability of Gd-CAs

The *kinetic stability* of Gd-CAs, has been suggested as a suitable marker for predicting the stability of these agents in vivo [5]. It can be assessed by measuring the dissociation half life ($T_{1/2}$) under very acidic conditions (pH 1). However, dissociation half life data of different Gd-CAs has been obtained from different laboratories making direct comparison between these agents rather difficult. A recent study evaluated the dissociation half life ($T_{1/2}$) of Gd-CAs under the same laboratory conditions, confirmed the low kinetic stability of linear chelates and high stability of the macrocyclic agents [6].

The dissociation of Gd-CAs incubated in human serum at 37°C is another suitable method for predicting the stability of these agents in vivo. The highest release of gadolinium (Gd^{3+}) was observed with the non-ionic linear chelates Omniscan and OptiMark. The addition of phosphate to serum markedly increased the release of Gd^{3+} and the total amount of released Gd^{3+} at day 15 increased from 20% to around 35% of the total dose of these agents. For the ionic linear chelate Magnevist, phosphate did not increase the total amount of released Gd^{3+} which remained at 2%, only the speed of the release was increased in day one to 2% and remained at this level up to day 15. The small release of free Gd^{3+} from the ionic linear chelates in comparison to the non-ionic ones is due to a higher thermodynamic stability of the former. No release of Gd^{3+} was observed with the macrocyclic Gd-CAs even after the

addition of phosphate to the serum [6]. This study again confirms the high stability of the macrocyclic Gd-CAs and shows that the non-ionic linear chelates have the lowest stability. [7].

In vivo animal studies

Experimental studies using normal rats and rats with reduced renal function secondary to 5/6 subtotal nephrectomy have been carried out to investigate the association between Gd-CAs and NSF.

Studies in the normal rat

The biological effects of multiple injection of Gd-CAs of different stability at the dose of 2.5 mmol/kg/day administered intravenously 5 days a week over 4 weeks were investigated in the normal rat. Omniscan and OptiMark but to a lesser extent induced skin lesions (fibrosis, CD34+ cell infiltration and increased cellularity) comparable to those observed in patients with NSF. No skin lesions were observed with either the Omniscan ligand, caldiamide (Ca-DTPA-BMA) or with ionic linear chelates and macrocyclic agents [8, 9]. Correlation between the Gd concentration in skin tissue and occurrence of NSF-like lesions was observed. The highest Gd concentration was observed after treatment with non-ionic linear Gd-CAs and the lowest after treatment with macrocyclic agents [9].

Depletion of endogenous zinc (Zn) ions caused by the administration of low stability Gd-CAs was suggested as a possible cause for the skin lesions induced by these agents. However, in a study employing a similar experimental protocol to the above mentioned in vivo studies with the exception of offering a group of animals Zn enriched diet and another group a Zn deficient diet the possible role of Zn deficiency was excluded. Omniscan induced NSF like lesions associated with high Gd retention in the skin in all the groups treated with this agent whether the rat was on normal, Zn enriched or Zn deficient diet [10].

The contribution of iron and erythropoietin to the pathophysiology of NSF was also evaluated in the normal rat [11]. This study revealed that the administration of erythropoietin and free iron enhances the histological and macroscopic changes in the skin in response to Omniscan [11].

The involvement of pro-inflammatory cytokines in NSF was also investigated [12]. Intravenous injections of gadodiamide (without caldiamide) at a dose of 2.5 mmol/kg b. w per injection in the normal rat were employed [one group of animal received only a single injection and sacrificed 6 h later, another group received daily single injections for 3 consecutive days and sacrificed 24 h after last injection and another group received single daily injections for 8 consecutive days and sacrificed shortly after last injection]. Macroscopic skin lesions were observed in one animal after the third daily injection and in all animals after 8 daily administrations. Significant elevation of several cytokines shortly after first injection was observed but only 8 stayed elevated over all time points which include; monocyte chemotactic proteins, macrophage inflammatory proteins, tumour necrosis factor (TNF-α), tissue inhibitor metalloproteinase type 1 (TIMP-1), vascular epithelial growth factor (VEGF) and osteopontin. The report postulated that up-regulation of the production of these cytokines, growth factors and enzymes is elicited by Gd released from low stability Gd-CA molecules and these substances would increase vascular permeability, recruit numerous immune cell types and promote fibrosis. [12].

The long term retention of gadolinium in the skin after intravenous administration of commercially available Gd-CAs was also evaluated in the normal rat [12]. The Gd-CA was injected daily at the dose of 2.5 mmol/kg bodyweight for five consecutive days. Skin biopsies were taken at various time points up to a year after the last injection for measuring gadolinium concentration using Inductively Coupled Plasma Spectrometry (ICP-MS). Linear Gd-CAs produced retention of gadolinium in the skin throughout the observation period with correlation between the stability of the Gd-CA and the amount of residual gadolinium in the skin. The amount of gadolinium retention in the skin followed the order Omniscan>OptiMARK>Magnevist. Only minimal amount of gadolinium retention was observed with the macrocyclic agents [13]. The persistence of Gd in skin tissue for such a long period suggests that it is likely to be in an insoluble form that would make it less subject to elimination [12].

Rats with 5/6 subtotal nephrectomy

Grant et al. [14] investigated the effects of Gd-CAs in partially nephrectomised rats. Rats received a dose of 5 mmol/kg body weight of Omniscan, Magnevist, 1 mmol/kg body weight of gadodiamide, Gd chloride 25 micromol/kg body weight or gadolinium citrate 25 micromol/kg body weight daily except weekends (total of 10 doses). Rats were sacrificed on day 15, 3 days after the last injection. Both gadodiamide (1 mmol/kg/day) and Omniscan (5 mmol/kg/day) induced macroscopic and histological skin lesions. Skin lesions were greater and developed more rapidly in animals which received gadodiamide in comparison to those which received Omniscan (gadodiamide + excess ligand) confirming initial beneficial effects of the excess ligand,

caldiamide in the Omniscan preparation. No macroscopic or histological skin lesions were observed with Magnevist or with either gadolinium salts chloride or citrate. Grant et al. concluded that the skin lesions detected did not represent NSF, due to absence of fibrosis and CD34 positive cells. The authors indicated that the ulcerative skin lesions were produced secondary to scratching precipitated by an allergic response since there was some elevation of plasma histamine and an increase in dermal mast cells [14]. The possibility that the dermatological changes could be a reaction to Gd deposition in the skin was not considered although itching and macroscopic skin lesions were observed only in animals with high skin Gd content and no acute inflammatory response was observed when the skin Gd content was low. Furthermore, the development of NSF like lesions requires time and allowing only 3 days following cessation of Gd-CA administration is too short for detection of typical histological changes of NSF.

Grant et al. also commented that Gd salts are not suitable to investigate the role of free Gd^{3+} in vivo. Free Gd^{3+} ions within the intravascular compartment following intravenous administration of Gd chloride/citrate will be attached to plasma proteins and may also form insoluble Gd hydroxide colloid and eventually will be phagocytosed by the reticuloendothelial system in the liver and spleen [15]. For Gd^{3+} to be delivered to the skin or other organs it has to be in a soluble form i.e. while it is still in the chelated form. Retention of Gd in the skin is most likely due to dissociation Gd-chelate molecules in the extravascular extacellular compartment [4].

The long term retention of Gd in the skin of rats with subtotal nephrectomy over 168 days was also investigated employing a similar protocol to the study performed in the normal rat. Again the highest Gd retention in the skin was observed with the non-ionic linear Gd-CAs and minimal Gd retention was found with macrocyclic agents. The measured Gd values in the skin were also significantly higher at all time points than the Gd values observed in the normal rat which illustrates the impact of reduced renal function on increasing the extent of Gd retention in tissues [16]. A more recent study also demonstrated the impact of reduction in renal function on the extent of Gd retention in tissues and dermatological response after administration of Omniscan in rats with 5/6 subtotal nephrectomy (SNx) [17]. Rats with GFR 20% of normal had an increased tissue Gd concentration in bone (2.5-fold), skin (3-fold) and liver (10-fold) compared to sham-operated controls. No macroscopic skin lesions were observed in this study but the dermal cellularity of rats with the GFR reduced down to 20% of normal was increased following Omniscan together with positive immunostain for CD34 and prolyl-4-hydroxylase [17].

In vitro cell culture studies

Effects of Gd-CAs of different stability on human fibroblasts and production of collagen and other matrix substances

In a study by Edward et al. [18] Omniscan(10–500 μmol/L) or serum from NSF patients stimulated proliferation of normal human skin fibroblasts in culture [18]. A subsequent study by Varani et al. [19], confirmed the stimulant effect of Omniscan on the proliferation of human fibroblasts over a lower concentration range (0.5–25 μmol/L). A proliferative response of Gd chloride was also detected, an effect inhibited in the presence of the free ligand DTPA. More stable Gd-CAs also induced proliferation but at a higher minimum concentration, 50 μmol/L for Magnevist and Multihance (ionic linear Gd-CA, Bracco, Italy) and 25 mmol/L for the macrocylic Prohance (non-ionic macrocyclic Gd-CA, Bracco, Italy) a 50,000 higher concentration than Omniscan.

The effect of Gd-CAs on collagen synthesis in vitro is somewhat controversial. Some studies suggested that low stability Gd-CAs modulate the synthesis of the enzymes that control the breakdown of collagen in tissues [matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinases-1 (TIMP-1)] causing reduction in collagen breakdown resulting in an increase in the amount of collagen [19, 20]. However, a more recent study has shown that Omniscan and also Magnevist can stimulate the production of collagen 1 by fibroblasts after 48 h incubation. The increase in collagen production with Omniscan was higher than Magnevist at least by a factor of two [21].

Effects of gd-CAs on human peripheral blood mononuclear cells (PMBC)

The effects of various high concentrations of Omniscan, Magnevist [5, 10, 25, and 50 mM] and Gd chloride (GdCl3) at the concentrations of 2.7, 27 and 270 μmol on adherent human peripheral blood mononuclear cells (PMBC) after incubation for 12 or 24 h were investigated. The effects of different concentrations of the ligand caldiamide without Gd were also investigated. In addition, conditioned media isolated from Gd-exposed PBMC were added to cultured normal human dermal fibroblasts. Gd compounds caused significant up-regulation of the expression of multiple profibrotic cytokines and growth factors, such as interlukin (IL) IL-6, IL-13, transforming growth factor β (TGF-β) and VEGF. Magnevist induced higher stimulation than Omniscan however, no osmotic control groups was included in this study to assess the contribution of high osmolality to the observed effects. low concentrations of

GdCl also induced a stimulatory effect but caldiamide induced a mixed response. Culture supernatants from Gd-exposed PBMC added to cultured normal dermal human fibroblasts induced an increase in the production of extracellular matrix proteins such as type I and type III collagen, fibronectin and α-smooth muscle actin. The study concluded that Gd-CAs are potent activators of cytokines and growth factors expression and production in human PBMC [22].

What did we learn so far?

Considering the above mentioned studies there is a consistent pattern of knowledge is emerging supporting the concept that dissociation of low stability Gd-chelates and release of free gadolinium is an important factor in the pathogenesis of NSF. The outcome of these studies can be summarised as follow:

1. Non-ionic linear chelates are the least stable Gd-CAs available for clinical use with propensity to release free Gd^{3+} in vivo.
2. Low stability non-ionic linear Gd-CAs can induce skin lesions in the rat and high retention of Gd in tissues. The highly stable macrocyclic agents induce minimal retention of Gd in tissues and no dermatological changes.
3. Low stability Gd-CAs can stimulate the proliferation of human fibroblasts and increase the accumulation of collagen in the extracellular matrix. They also up-regulate the production of proinflammatory and profibrotic cytokines and growth factors from monocytes. These effects seem to be Gd dependant since Gd CL can also induce similar stimulant effects which are absent with the ligand of the Gd-CA.

In conclusion, released Gd^{3+} from low stability Gd-CA seems to be the culprit of the fibrogenic effects of these agents in patients with advanced renal impairment. The biological effects of Gd^{3+} which belongs to the lanthanide series of elements could be partly explained by the fact that Gd^{3+} has an ionic radius (107.8 pm) similar to that of calcium ion (114 pm). Gadolinium is often used as isomorphous replacement for calcium ions in biochemical studies and has the ability to mimic the effects of calcium ions including activation of calcium-dependant enzymes such as transglutaminase an important enzyme in the progression of fibrosis [23]. The fibrogenic effects of lanthanides were recognised as early as 1983 [24]. In vitro studies have shown that lanthanides enhance the polymerisation of skin collagen to a higher extent than calcium and could be involved in the promotion of fibril formation [24, 25].

The details of how Gd^{3+} alters the biological behaviour of important target cells such as fibroblasts and monocytes/macrophages remain to be established.

Conflict of interest declaration

1. The author received lecture fees from Bracco, Italy and Guerbet, France over the last 12 months.
2. A research grant was received by the University of Sheffield from Guerbet, France for experimental studies investigating the pathophysiology of nephrogenic systemic fibrosis (NSF).
3. The author is currently acting as an expert witness in NSF litigation in the USA.

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