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Iron nanomedicines induce Toll-like receptor activation, cytokine production and complement activation

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ABSTRACT

Approximately a dozen of intravenous iron nanomedicines gained marketing authorization in the last two decades. These products are generally considered as safe, but have been associated with an increased risk for hypersensitivity-like reactions of which the underlying mechanisms are unknown. We hypothesized that iron nanomedicines can trigger the innate immune system. We hereto investigated the physico-chemical properties of ferric gluconate, iron sucrose, ferric carboxymaltose and iron isomaltoside 1000 and comparatively studied their interaction with Toll-like receptors, the complement system and peripheral blood mononuclear cells. Two out of four formulations appeared as aggregates by Scanning Transmission Electron Microscopy analysis and were actively taken up by HEK293T- and peripheral blood mononuclear cells in a cholesterol-dependent manner. These formulations triggered *in vitro* activation of intracellular Toll-like receptors 3, -7 and -9 in a dose- and serum-dependent manner. In parallel experiments, we determined that these compounds activated the complement system. Finally, we found that uptake of aggregation-prone iron nanomedicines by peripheral blood mononuclear cells in whole blood induced production of the proinflammatory cytokine IL-1β, but not IL-6.

1. Introduction

Iron deficiency anemia is a nutritional disorder affecting almost 30% of the world's population [1]. Besides relative low uptake from diet, iron deficiency anemia is seen during pregnancy, hemorrhage and diseases, such as inflammatory bowel disease and chronic kidney failure [2]. Although iron deficiency can be treated with oral iron, patients may require such amounts that are unable to be restored by this route [3]. All commercial intravenous iron formulations consist of a polynuclear Fe(III)-oxyhydroxide/oxide core surrounded by carbohydrates [4]. This carbohydrate shell functions as a stabilizer preventing direct release of bioactive iron and particle aggregation [5]. As such, these products have a diameter between 10 and 30 nm and are considered to be nanomedicines [6]. Examples of carbohydrates used in commercial iron nanomedicines include: sucrose, gluconate, dextran, isomaltoside 1000 and carboxymaltose.

The number of different intravenous iron nanomedicines on the market was limited until the 1990's, but an ever increasing number of products have since entered the clinic [5]. Although iron nanomedicines are considered safe, they are associated with a product dependent risk for (acute) hypersensitivity-like reactions, such as wheezing, bronchospasm, periorbital edema and circulatory collapse; both of which can result in death [7,8]. Although iron dextran is associated with the greatest risk of hypersensitivity-like events, newer developed non-dextran iron nanomedicines are also linked to these reactions, however less frequent [7,9–11]. As such, the European Medicine Agency send out a warning for all commercially available intravenous iron products in 2013 stating that physicians should take precautions when administering these products [12].

Whereas high molecular weight iron dextran formulations have been associated with anti-dextran antibodies, the mechanisms how non-dextran formulations can induce these hypersensitivity-like reactions remain inconclusive [10,13,14]. As such, high molecular weight iron formulations are no longer available [13]. Possible factors triggering these reactions are the release of high

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concentrations of unbound iron into the circulation, recognition of carbohydrates by the immune system and or destabilization of the formulation by dissociation of these carbohydrates [4,9,11,15].

In this study, we investigated the physico-chemical characteristics of four different intravenous iron nanomedicines and their interaction with the innate immune system. We found differences in aggregation behavior among the various formulations investigated which associated with higher cellular uptake and activation of Toll-like receptors, the complement system and specific cytokine release.

2. Materials and methods

2.1. Materials

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), adenosine triphosphate (ATP), ammonium iron (III) citrate, propidium iodide, chlorpromazine, methyl-beta-cyclodextrin, transferrin, Dulbecco's Phosphate Buffered Saline (PBS) without calcium chloride and magnesium chloride, PBS with calcium chloride and magnesium chloride, fetal bovine serum (FBS), trypsin, ethylenediaminetetraacetic acid (EDTA), RPMI-1640 containing both 20 mM HEPES, L-glutamine and sodium bicarbonate, Dulbecco's Modified Essential Medium containing 4.5 g/L glucose and L-glutamine, and antibiotic–antimycotic solution containing penicillin–streptomycin and antimycotics were all obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Ficoll-Paque Plus was obtained from Fisher Scientific (Landsmeer, the Netherlands). Normocin, blasticidin, zeocin, HEk-Blue Selection, PAM3SCK, Poly(I:C) LPS-EK, CL264, ODN2006, and QUANTI-Blue were obtained from Invivogen (Toulouse, France). Ferric gluconate, iron sucrose, ferric carboxymaltose and iron isomaltoside 1000 were kindly provided by Vifor Pharma (Glattbrug, Switzerland) and freshly used before the expiration date. All iron nanomedicines were endotoxin free, determined by the Pyrogene Recombinant Factor C assay (Lonza Benelux bv, Breda, the Netherlands).

2.2. Physico-chemical characterization

Iron nanomedicines were characterized by their mean hydrodynamic particle size and polydispersity index measured by dynamic light scattering (DLS), using a Malvern ALV CGS-3 multiangle goniometer equipped with a He–Ne laser source ($\lambda = 632.8$ nm, 22 mW output power) under an angle of 90° (Malvern Instruments, Malvern, UK). Hydrodynamic size and polydispersity were recorded with an optical fiber-based detector and a digital LV/LSE-5003 correlator with a temperature controller set at 25 °C. Samples were diluted in PBS to 1–2.5 mg iron/mL to realize the highest count rate. Zeta-potential of iron nanomedicines was measured with laser Doppler electrophoresis on a Zetasizer Nano-Z (Malvern Instruments, Malvern, UK) following the diffusion barrier method. A sample of 50 μL was dispersed in 10 mM HEPES buffer pH 7.4 at the bottom of the cell and measured at 150.9 V for 100 runs.

Scanning Transmission Electron Microscopy (STEM) was performed on a Philips 200 kV Tecnai 20F transmission electron microscope (FEI Company, Eindhoven, the Netherlands) equipped with a Field Emission Gun and a Twin objective lens. Images were recorded with a Fischione High Angle Annular Dark Field (HAADF) detector. Energy-dispersive X-ray spectroscopy (EDS) was performed to study chemical composition of the sample. The instrument consisted of a VG Escalab 200 R spectrometer with a MgKα X-ray source ($h\nu = 1253.6$ eV). Iron formulations were diluted to 200 μg iron/mL in dH_2O and 3 μL samples were left to dry on carbon coated copper grids.

2.3. Cell culture

HEK-Blue hTLR cells were cultured in DMEM 4.5 g/L glucose to which 10% FBS, 50 U/mL penicillin, 50 mg/mL streptomycin, 100 μg/mL Normocin and 2 mM L-glutamine was added. HEK-Blue hTLR3 culture medium was supplemented with 30 μg/mL blasticidin and 100 μg/mL zeocin, HEK-Blue hTLR7 and -9 culture medium was supplemented with 10 μg/mL blasticidin and 100 μg/mL zeocin and HEK-Blue hTLR4 culture medium with HEk-Blue Selection.

HEK293T cells were cultured in DMEM with 4,5 g/L glucose supplemented with 10% FBS and an antibiotic–antimycotic solution.

2.4. Toll-like receptor activation reporter assay

Activation of hTLR2, -3, -4, -7 and -9 was investigated using the HEK-Blue hTLR reporter cell-lines (Invivogen, Toulouse, France). HEK-Blue hTLR cells express both a specific hTLR gene and a NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. HEK-Blue hTLR cells were seeded at assay-dependent amounts (details in Table 1) and incubated overnight with the various iron nanomedicines or positive controls in culture medium.

The next day, SEAP was quantitatively determined by the addition of 20 μL cell supernatant to 180 μL of QUANTI-Blue and incubated for 1 h at 37 °C with 5% CO_2 and subsequently measured at 650 nm using a Biorad Model 550 Microplate Reader (Biorad, Hercules, United States). hTLR activation was expressed by relative alkaline phosphatase levels, defined as sample level divided by PBS control level. Subsequently, synergy between the iron nanomedicines and hTLR agonists was studied by simultaneous incubation of the cells with their specific hTLR agonists and one of the four iron nanomedicines.

hTLR activation without the presence of serum was investigated with HEK-Blue Detection Medium which enables real-time monitoring of SEAP expression without the need for serum. Here, 20 μL sample was added to 180 μL of HEK-Blue Detection Medium and incubated overnight after which absorbance was recorded at 655 nm. SEAP expression was corrected for the absorbance of iron nanomedicines in HEK-Blue Detection Medium.

2.5. Complement activation

Complement activation was investigated by measuring the amount of SC5b-9, a soluble terminal factor released upon activation of either the classical, lectin or alternative pathway. Activation of the alternative pathway was investigated by SC5b-9 analysis in the presence of EGTA/Mg^{2+} and by the detection of the alternative pathway marker Bb. Ferric gluconate, iron sucrose, ferric carboxymaltose and iron isomaltoside 1000 were incubated in 1:4 diluted serum obtained from healthy donors at 500 μg iron/mL. The incubated serum samples were vigorously shaken for 30 min at 37 °C. Negative and positive controls included respectively the incubation with PBS and 200 μg/mL zymosan. SC5b-9 and Bb release were measured by either the MicroVue SC5b-9 Plus or Bb Enzyme

Table 1
Experimental design of the Toll-like receptor activation study using the HEK-Blue hTLR reporter cell-lines.

Cell line	Cells seeded (cells/cm^2)	Positive control
hTLR2	156,250	10 ng/mL PAM3CSK4
hTLR3	156,250	500 ng/mL Poly I:C
hTLR4	78,125	1 ng/mL LPS-EK
hTLR7	250,000	500 ng/mL CL264
hTLR9	250,000	1 μg/mL ODN2006