A novel method for assessing the role of iron and its functional chelation in fibrin fibril formation: the use of scanning electron microscopy

Etheresia Pretorius¹, Natasha Vermeulen¹, Janette Bester¹, Boguslaw Lipinski², and Douglas B. Kell³

¹Department of Physiology, Faculty of Health Sciences, University of Pretoria, Arcadia, South Africa, ²Joslin Diabetes Center, Harvard Medical School, Boston, MA, USA, and ³School of Chemistry and Manchester Institute of Biotechnology, University of Manchester, Manchester, UK

Abstract

Aims: Inflammatory diseases associated with iron overload are characterized by a changed coagulation profile, where there is a persistent presence of fibrin-like material of dense-matted deposits (DMDs). It is believed that one source of such material is a result of the activation of blood coagulation without the generation of thrombin, causing clots to become resistant to fibrinolytic dissolution. The aim of the current manuscript therefore is to apply a novel scanning electron microscopy method for assessing the role of functional chelation in the prevention or reversal of iron-induced fibrin formation.

Methods and results: Purified fibrinogen and platelet-rich plasma were exposed to chelating agents followed by iron, to determine the chelating effects. We show that there is another, pathological pathway of fibrin formation initiated by free iron (initially as Fe (III)), leading to the formation of highly reactive oxygen species such as the hydroxyl radical that can oxidize and insolubilize proteins, a process that might be inhibited by iron-chelating compounds. The final product of such a pathway is a fibrin-like material, termed DMDs that are remarkably resistant to proteolytic degradation.

Conclusions: Scanning electron microscopy shows that iron-chelating agents are effective inhibitors of DMD formation. The most active inhibitors of DMD formation proved to be Desferal, Clioquinol and Curcumin, whereas Epigallocatechin gallate and Deferriprone were less effective. The functional model we describe may point the clinical utility of various substances in iron-mediated degenerative diseases.

Introduction

It is known that human body contains 3–5 g of total iron with the pool of “labile” iron being <70–90 mg (Shah 2009; Shah & Houser, 2008). High contents of tissue iron (iron overload) have been associated with many pathological conditions, including liver and heart disease, neurodegenerative disorders, diabetes, hormonal abnormalities and immune system abnormalities (Aderibigbe et al., 2011; Altamura & Muckenthaler, 2009; Bae et al., 2009; Zacharski et al., 2008, 2011). Chronic iron toxicity is a condition associated with primary hemochromatosis, high dietary iron intake and frequent blood transfusions. The pathological role of poorly liganded iron was reviewed extensively (Kell, 2009, 2010).

It is important to note that of the pathologic conditions, fibrin-like deposits in the affected organs are well documented, which might suggest the involvement of thrombotic episodes. The presence of fibrin-like material in atherosclerotic plaques was reported first by Duguid (1946), and later on by others (Haust et al., 1964; More et al., 1957; Shainoff & Page, 1972; Woolf, 1961). Elspeth Smith in a series of elegant papers has documented the existence of soluble fibrin(ogen)-related antigens in both soluble and insoluble fractions of atherosclerotic intima (Smith, 1994; Smith et al., 1996). Atherosclerotic plaques (in contrast to nearby control tissue) may also be heavily laden with iron (Stadler et al., 2004). We have previously shown that iron, in the form of ferric ions, converts soluble human fibrinogen into an insoluble fibrin-like polymer that, in contrast to thrombin-induced fibrin, is remarkably resistant to fibrinolytic degradation (Lipinski & Pretorius, 2012). In this paper, we present evidence for the inhibitory effects of various iron-chelating agents on the non-enzymatic conversion of fibrinogen into fibrin-like deposits in human blood, a method that may find clinical applications in terms of assessing agents suitable for combating degenerative disease.

Ethics statement

Ethical clearance was obtained from the University of Pretoria Human Ethics committee and this conforms to the
Declaration of Helsinki. All experiments were performed at this institution.

**Materials and methods**

**Preparation of purified human fibrinogen solution**

Human fibrinogen was purchased from Sigma-Aldrich (St. Louis, MO; Catalogue no. F3879-250MG). This was dissolved by pouring 10 ml of warm phosphate buffered saline (PBS; pH of 7.4) over the content and gently mixed with a glass rod until completely dissolved. A working solution of 0.166 mg/ml was prepared. This concentration was found to be the optimal concentration to form fibrin fibers similar to that of platelet-rich plasma (PRP) fibers from healthy individuals. The fibrin fiber experiments were repeated six times and found to be consistent each time.

**Human control PRP**

Blood was obtained from healthy individuals (ranging between 20 and 25 years; Ethical clearance from the University of Pretoria Ethics committee was granted). These individuals did not have any chronic conditions, did not smoke and did not use any medication. The micrographs of fibrin fibers formed in the plasma of healthy individuals were compared to our database of thousands of micrographs, and found to be comparable. Each experiment was repeated with PRP from 10 healthy individuals.

**Preparation of iron solution**

Previously, we showed that 5 ml of a 15 mM FeCl₃ solution caused the formation of dense-matted fibrin deposits (DMDs) in PRP. In the current work, the first set of experiments was performed with 5 ml of a 15 mM FeCl₃ solution.

Table 1 shows a comparison of the different types of iron stores found in plasma and serum and their levels in healthy individuals and iron overload (Arutona et al., 1988; Balan et al., 1994; Gackowski et al., 2002; Grootveld et al., 1989; Piga et al., 2009; Pootrakul et al., 2004, 2005; Srichairatanakool et al., 2006; Taher et al., 2009). Therefore, in addition to the first set of experiments, a 100× and 500× dilution of the 15 mM FeCl₃ solution was also prepared (0.15 mM and 0.03 mM). We mix PRP/fibrinogen, chelators and thrombin together with iron. We therefore took the normal range of serum iron in blood to be 30–170 μg/dl; that is equal to 0.000011–0.0063 mM.

As mentioned in the previous paragraph, for the lower iron experiments we used a starting concentration of 0.15 and 0.03 mM iron. This is equal to 0.006 and 0.03 mM iron in the final experiment (chelator + PRP + iron + thrombin). Therefore, these two final iron concentrations (0.006 and 0.03 mM) are in the upper range of normal, as well as within the range of individuals with iron overload (>180 μg/dl or 0.00666 mM; Table 1).

**Chelating compounds used**

Desferal, Clioquinol, Epigallocatechin gallate, 3-hydroxy-1,2-dimethyl-4(1H)-pyridone (Deferiprone) and Curcumin (crystalline from *Curcuma longa*) were prepared at 10 mM concentrations. The final chelator concentration in our experiments, where 10 mM of chelators were added (chelator + PRP + iron + thrombin), is therefore is 2 mM.

However, here we also tested physiological levels of Desferal and Deferiprone. We used a final chelator concentration for Desferal and Deferiprone that is similar to a single daily dosage, the concentrations used are as follows: for Desferal, a single daily dosage is equal to 50 mg/kg/day (average male weight was used at 80 kg). This therefore is a 0.0973 mM (97.3 μM) concentration. In order to have a final chelator/iron/PRP/thrombin concentration of 0.973 mM, we added 0.487 mM Desferal to our experiment (chelator + PRP + iron + thrombin). Similarly, the daily dosage of Deferiprone is 100 mg/kg/day. The final concentration that was used was therefore 0.206 mM for Deferiprone.

**Preparation for SEM samples (purified fibrinogen and PRP)**

All samples for SEM were prepared as follows. 10 μl of either purified fibrinogen or PRP was placed directly on a glass cover slip and immediately mixed with various volumes.
(either 2, 4 or 5 μl) of the above-mentioned chelating compounds. The cover slips were incubated for 3 min, followed by the addition of 5 μl of different FeCl₃ concentrations. To create extensive fibrin fiber networks, 5 μl of thrombin (10 U/ml) was added.

The cover slips with prepared clots were then incubated at room temperature for 5 min followed by immersion in 0.075 M sodium phosphate buffer (pH 7.4) and finally placed on a shaker for 2 min. Smears were fixed in 2.5% glutaraldehyde/formaldehyde in the PBS solution with a pH of 7.4 for 30 min, followed by rinsing 3× in phosphate buffer for 5 min before being fixed for 30 min with 1% osmium tetroxide (OsO₄). The samples were again rinsed 3× with PBS for 5 min and were dehydrated serially in 30%, 50%, 70%, 90% and three times with 100% ethanol. The material was critical point dried, mounted and coated with carbon. A Zeiss UltraPlus FEG-SEM (Oberkochen, Germany) with InLens capabilities was used to study the surface morphology of platelets and micrographs were taken at 1 kV. This instrument is located in the Microscopy and Microanalysis Unit of the University of Pretoria, Pretoria, South Africa.

**Results**

**Experiments with 15 mM FeCl₃ and 10 mM chelators**

Human purified fibrinogen and PRP form an expansive fibrin fiber net following the addition of human thrombin (Figure 1a and c). In both cases, individual fibrin fibers can be identified. If thrombin is not added, no extensive net is formed and in the case of PRP smears, individual platelets are visible and with fibrinogen, cover slips are mostly empty, with few scattered plasma protein molecules. When ferric chloride ions are added, this typical fibrin fiber net changes to form DMDs; these changes are noted in Figure 1(b) and (d).

The aim of this study was to determine the functionally protective capability of chelating agents, when added to either fibrinogen or PRP, before the addition of FeCl₃. Also, the ultrastructural technique used here is a novel technique that is very sensitive and might be developed further to be used in the clinical setting. Here, five chelating agents were chosen.

Some of the iron-chelating compounds may have the ability to scavenge hydroxyl radicals directly, thus increasing their potency in comparison with other chelators. Desferal was chosen, as it is a medication known to remove excess iron from the body or to treat acute iron poisoning. Clioquinol acts as a mild metal chelator, binding metals like iron, copper and zinc in the body (Bush, 2002; Cooper, 2011). Epigallocatechin gallate, 3-hydroxy-1,2-dimethyl-4(1H)-pyridone (Deferiprone) and Curcumin (obtained as crystals from C. longa) are all phenols or polyphenols with chelating effects (Perron & Brumaghim, 2009). Desferal was prepared to a 10 mM concentration and the effects of three volumes (2, 4 and 5 μl) were studied using purified fibrinogen. DMD formations were clearly visible at the lowest volume (2 μl; Figure 2a); however, 4 μl showed a marked change in DMD formation with fibers starting to form (Figure 2b). The highest volume (5 μl) showed a fibrin net similar to controls (Figure 2c). The highest volume was then used with PRP from healthy individuals, and here also no DMD formation was visible (Figure 2d).

Clioquinol (10 mM) was also tested using the three different volumes before ferric iron exposure. Here, the lowest volume added to fibrinogen showed a beaded fibrin fiber formation with fine fibers scattered in between (Figure 3a), while both 4 and 5 μl compared well to control experiments (Figure 3b and c). The addition of 5 μl Clioquinol to PRP led to images that compared well to control experiments (Figure 3d).

As 5 μl of a 10 mM concentration seems to show protective effects against the harmful effects of ferric ions, this volume and concentration were used for the three polyphenols Epigallocatechin gallate, Deferiprone and Curcumin. Here varying results were seen (PRP micrographs shown in Figure 4a–c). A beaded fibrin ultrastructure was noted with Epigallocatechin gallate (Figure 4a); however, no fine fibers were noted as in the case with 2 μl of a 10 mM solution of Clioquinol. A total of 5 μl of a 10 mM solution of Deferiprone showed fiber formation, but with beaded fibers still present (Figure 4b). Curcumin, however, proved to be the most protective of the three polyphenols, with no DMD formation and fibers similar to that of control smears (Figure 4c).
Figure 2. (a) Purified human fibrinogen (10 μl) + 2 μl of 10 mM Desferal wait 3 min + 15 mM FeCl₃ (5 μl) + thrombin (5 μl), (b) purified human fibrinogen (10 μl) + 4 μl of 10 mM Desferal wait 3 min + 15 mM FeCl₃ (5 μl) + thrombin (5 μl), (c) purified human fibrinogen (10 μl) + 5 μl of 10 mM Desferal wait 3 min + 15 mM FeCl₃ (5 μl) + thrombin (5 μl) and (d) PRP (10 μl) + 5 μl of 10 mM Desferal wait 3 min + 15 mM FeCl₃ (5 μl) + thrombin (5 μl). Scale = 1 μm.

Figure 3. (a) Purified human fibrinogen (10 μl) + 2 μl of 10 mM Clioquinol wait 3 min + 15 mM FeCl₃ (5 μl) + thrombin (5 μl), (b) purified human fibrinogen (10 μl) + 4 μl of 10 mM Clioquinol wait 3 min + 15 mM FeCl₃ (5 μl) + thrombin (5 μl), (c) purified human fibrinogen (10 μl) + 5 μl of 10 mM Clioquinol wait 3 min + 15 mM FeCl₃ (5 μl) + thrombin (5 μl) and (d) PRP (10 μl) + 5 μl of 10 mM Clioquinol wait 3 min + 15 mM FeCl₃ (5 μl) + thrombin (5 μl). Scale = 1 μm.

Figure 4. (a) PRP (10 μl) + 5 μl of 10 mM Epigallocatechin gallate wait 3 min + 15 mM FeCl₃ (5 μl) + thrombin (5 μl), (b) PRP (10 μl) + 5 μl of 10 mM 3-hydroxy-1,2-dimethyl-4(1H)-pyridone (Deferiprone) wait 3 min + 15 mM FeCl₃ (5 μl) + thrombin (5 μl) and (c) PRP (10 μl) + 5 μl of 10 mM Curcumin wait 3 min + 15 mM FeCl₃ (5 μl) + thrombin (5 μl). Scale = 1 μm.
Experiments with 0.03 and 0.15 mM FeCl₃ (0.006 and 0.03 mM final iron concentrations in the experiments)

Due to the fact that 15 mM is a high ferric iron concentration, a 100× and 500× dilution was also made. The final iron concentrations in the experiments are comparable with individuals with high iron levels (Table 1). PRP experiments were repeated with Desferal and 3-hydroxy-1,2-dimethyl-4(1H)-pyridone (Deferiprone) wait 3 min + 0.03 mM FeCl₃ (5 μl) + thrombin (5 μl) and (d) PRP (10 μl) + 5 μl of 0.15 mM 3-hydroxy-1,2-dimethyl-4(1H)-pyridone (Deferiprone) wait 3 min + 0.15 mM FeCl₃ (5 μl) + thrombin (5 μl) and (f) PRP (10 μl) + 5 μl of 0.15 mM Deferal wait 3 min + 0.15 mM FeCl₃ (5 μl) + thrombin (5 μl). Scale = 1 μm.

Experiments with 0.03 mM final iron concentrations in the experiments and daily dosage of Desferal and Deferiprone

The daily Desferal and Deferiprone dosages are equivalent to 0.0973 and 0.04122 mM. We used these calculated final concentrations of these products in our experiments. Even with these low levels of product additions, we did see a protective effective effect (Figure 6a and b).

Discussion

Fibrinogen is a plasma protein of a molecular weight 340 kDa, composed of three pairs of polypeptide chains termed A alpha (68 kDa), B beta (56 kDa) and gamma (48 kDa) held together by means of disulfide bridges. A characteristic feature of fibrinogen is its loss of solubility on a brief heating (3 min) at 55–56°C that causes a conversion of intra-molecular to intermolecular disulfide bonds with the formation of insoluble aggregates. The most important function of fibrinogen is its enzymatic conversion into an insoluble fibrin clot. In normal hemostasis, fibrin has to be removed eventually to make room for the growth of connective tissue and proper wound healing. If it does not, the wound never heals and becomes a source of a chronic inflammation. Fibrinolysis is brought about by
enzymatic activation of plasminogen by various plasminatic and/or tissue activators to form a proteolytic enzyme plasmin that catalyzes hydrolysis of peptide bonds. Although inhibition of fibrinolysis has been observed in numerous disease states, no causative factors have been identified.

In addition to the enzyme-induced modification of fibrinogen molecules, certain chemical agents and free radicals can also alter its properties. For example, exposure of purified fibrinogen to the ascorbic acid/cupric ion system results in the formation of an insoluble fibrin-like precipitate (Marx & Chevion, 1985). Such conditions can contribute to what is known as the Fenton-type reaction, leading to the generation of the most biologically reactive hydroxyl radical (HO; Galaris & Pantopoulos, 2008; Halliwell & Gutteridge, 1990; Marx & Chevion, 1985; Wardman & Candéias, 1996). This type of free radical reacts with double bonds of aliphatic chains and aromatic rings to form corresponding hydroxyl or oxo-derivatives. Formation of insoluble aggregates under the influence of hydroxyl radicals is believed to be due to a limited disruption of intra-molecular disulfide bridges followed by the exposure of buried hydrophobic residues that form non-covalent and extremely strong intermolecular bonds.

Accumulating evidence within last several decades points up the important role of hematic abnormalities in the pathogenesis of atherosclerosis and cardiovascular disease. It should be noted, however, that the mere activation of blood coagulation does not offer a satisfactory explanation in view of the powerful fibrinolytic potential of human blood that efficiently removes fibrin clots from the circulation. In view of a dramatic modification of fibrin properties by ferric ions, as well as voluminous evidence set down elsewhere (Balan et al., 1994; Kell, 2009, 2010). We argue that such a process may be involved in the pathogenesis of numerous degenerative conditions, including cardiovascular disease.

The involvement of labile iron pools in this and other degenerative diseases was suggested by several authors (Galaris & Pantopoulos, 2008; Halliwell & Gutteridge, 1990; Jomova & Valko, 2011a,b, 2012; Jomova et al., 2010; Kruszewski, 2004; Marx & Chevion, 1985; Small et al., 2011; Wardman & Candéias, 1996; Whitnall & Richardson, 2006). Elevation of free iron was also documented in focal brain ischemia suggesting early chelation therapy in stroke patients (Milleret-Serruot et al., 2008). Carotid atherosclerosis was observed to be positively associated with serum ferritin level (Ahluvalia et al., 2010). It is therefore reasonable that the phenomenon of iron-induced fibrinogen polymerization may explain the persistence of fibrin in atherosclerosis and related diseases. Ferric chloride was also shown to potentiate venous and arterial thrombosis induced with lipopolysaccharide (LPS; Wang et al., 2004). This work is relevant to previous findings that LPS in rabbits elicited deposition of a fibrin-like material in vital organs that were resistant to proteolytic degradation (Lipinski et al., 1974).

The rationale for choosing our lower iron concentrations (experiments shown in Figures 5 and 6) was due to the different free iron concentrations in plasma and serum in healthy and iron-overloaded individuals (Table 1). Values for both non-transferrin-bound iron (NTBI) and the LIP cluster around 5μM in many papers.

Our lowest final iron concentration is therefore comparable to the levels of redox-active iron in plasma iron-overloaded individuals. The labile plasma iron (LPI) represents a component of NTBI (Cabanthik et al., 2005) that is both redox active and chelatable (Cabanthik et al., 2005; Gackowski et al., 2001). LPI is furthermore capable of inducing iron overload. Also, NTBI is responsible for catalyzing the formation of reactive radicals in the circulation of iron-overloaded individuals (Esposito et al., 2003). According to Esposito and colleagues (2003), LPI is essentially absent from sera of healthy individuals, but is present in β-thalassemia patients at the levels of 1–16μM. Furthermore, the authors mention that this correlates significantly with those of NTBI measured as mobilizer-dependent chelatable iron or desferrioxamine-chelatable iron.

NTBI is thought to be associated with low molecular weight chelates (such as citrate or acetate) and albumin (Brissot et al., 2012), which are present in both plasma and serum. The only difference between plasma and serum is the clotting factors, which are not expected to bind NTBI (at least in significant amounts).

An unexpected finding described in this paper is that the most biologically reactive hydroxyl radical can potentially be formed in a simple reaction of ferric ions without the simultaneous presence (or at least addition) of oxidants (e.g. hydrogen peroxide) (Fenton, 1894; Lipinski et al., 1974).

The classical iron chelator is Desferal, but due to poor gastrointestinal absorption it has to be administered intravenously or subcutaneously; there is therefore an obvious need to find and develop new effective iron chelators for oral use (Flaten et al., 2012; Ma et al., 2012; Yu et al., 2012). There is convincing epidemiological evidence that regular intake of dietary bioactive polyphenolic compounds promotes human health (Baccan et al., 2012; Fiorani et al., 2002; Kim et al., 2008; Vlachodimitropoulou et al., 2011). The antioxidant activity of a variety of flavonoids may involve their ability to complex body iron in non-redox-active forms. It was found that the catechol flavonoids rutin and quercetin are able to suppress redox-active LPI in both buffered solution and in iron-overloaded sera (Baccan et al., 2012). Both flavonoids are effective in loading the metal into the iron-transport protein transferrin. Iron derivatives of quercetin and rutin are able to permeate cell membranes.

The mechanism of action of novel ligands including curcumin includes alteration in the expression of key regulatory molecules, as well as the generation of iron complexes (Kovacevic et al., 2011), effecting protection via iron chelation mechanisms (Du et al., 2012). Furthermore, curcumin has been shown to exhibit antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal and anticancer activities (Aggarwal et al., 2007). Epigallocatechin gallate, a polyphenolic catechin, is regarded as the most bioactive disease-preventing polyphenol compound in green tea (Aggarwal et al., 2007; Mak, 2012; Tanaka et al., 2011; Tijburg et al., 1997; Weinreb et al., 2009a). The antioxidant activity of this polyphenolic compound is particularly due to its ability to scavenge free radicals and to chelate metals such as iron (Kim et al., 2008). The ability of compounds like Clioquinol to chelate and redistribute iron also plays an important role in diseases characterized by dyshomestasis.
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The role of iron and its functional chelation


