Recombinant alpha-1-microglobulin: a potential treatment for preeclampsia

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Preeclampsia is a serious pregnancy-specific condition, affecting 10 million women annually worldwide. No specific treatment is currently available. Recent studies have demonstrated abnormal production and accumulation of free fetal hemoglobin in the preeclamptic placenta, and identified subsequent leakage into the maternal circulation as an important factor in the development of preeclampsia. A recombinant version of alpha-1-microglobulin, an endogenous well-characterized heme and radical scavenger, has been developed. Intravenous administration of recombinant alpha-1-microglobulin in animal models has been proved to eliminate or significantly reduce the manifestations of preeclampsia. Recombinant alpha-1-microglobulin has the potential to become the first specific therapy for preeclampsia.

Introduction

Preeclampsia is a serious pregnancy-specific complication characterized by hypertension and proteinuria or one of the following signs or symptoms: renal insufficiency, thrombocytopenia, hepatic dysfunction, pulmonary edema or cerebral and/or visual impairment according to the International Society for the study of Hypertension in Pregnancy (ISSHP) classification [1]. The condition is associated with increased maternal and fetal morbidity and mortality [2]. There is currently no specific therapy available to treat this condition; the only curative intervention is to induce delivery. Consequently, there is a clear need for a safe and effective pharmacological therapy. In this short review, we discuss recent research on the involvement of fetal hemoglobin (HbF) within the maternal circulation in the pathophysiology of preeclampsia, and describe results strongly supporting a pharmacological therapy based on recombinant alpha-1-microglobulin (A1M), a naturally occurring human heme and radical scavenger.

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Development of preeclampsia

Preeclampsia is generally considered to evolve in two stages [3]. The first is characterized by a defect in the formation of the placenta [4]. The invasion of the placental cells, the extravillous trophoblasts, into the muscle layers of the spiral arteries has been shown to be impaired in preeclampsia [5]. This contributes to reduced utero–placental blood flow that results in fetal intrauterine growth restriction, a condition seen in approximately one in four preeclampsia cases [6]. Clinical manifestations of preeclampsia typically become apparent from 20 weeks of gestation onwards, with the development of hypertension and proteinuria representing stage two of the disease The vascular hallmarks of preeclampsia, general endothelial dysfunction and diminished ability to vasodilate [7] give rise to organ dysfunction and diffuse symptoms such as edema and weight gain. It has been proposed that endothelial damage is central for the progression of general organ damage [8].

Abnormal placental formation of free HbF: role in preeclampsia

Free HbF has been shown to play an important part in the pathophysiology of preeclampsia (reviewed in [9,10]). Independent

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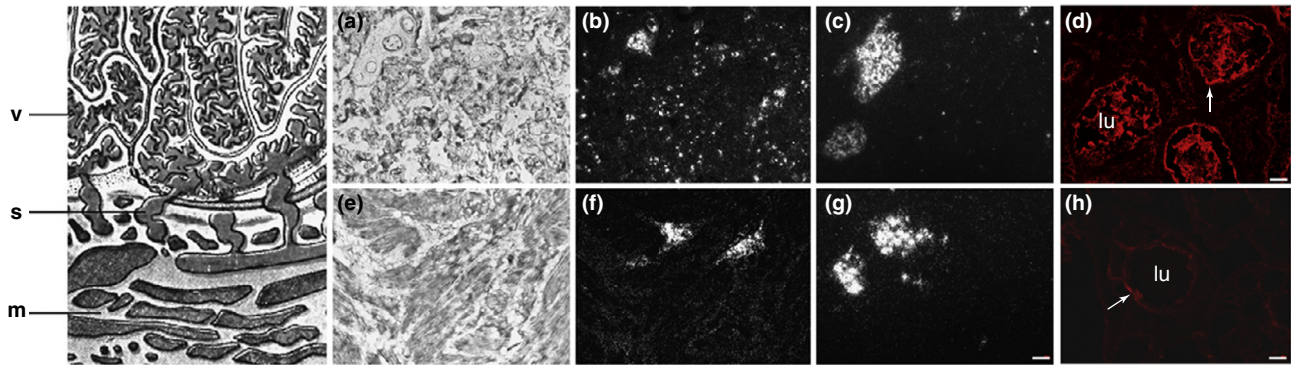

FIGURE 1

In situ hybridizations and immunohistochemistry from placenta and placenta bed samples modified, with permission, from Centlow *et al.* [12]. Schematic image of the human placenta showing the villous section (v) of the placenta and below a placenta bed section (m) with spiral arteries (s) in between. **(a)** A light field image of Hbα (common chain i all Hb) mRNA expression in a representative preeclampsia placenta sample. Hbα expression was especially seen in and around blood vessels. However, several scattered cells in the villous section are also seen. **(b)** Dark field image of the same section. **(c)** A dark field image of Hbα mRNA expression from a representative control placenta. Compared with preeclampsia (PE) placentas, the control placentas show fewer Hbα-expressing cells in the villous section. **(e)** Light field image from a representative myometrial sample from a preeclamptic patient. Hbα expression is only seen in the spiral arteries, no expression is seen in the myometrial tissue. **(f)** The same myometrial section in dark field. **(g)** A myometrial sample from a control placenta. Hbα mRNA expression is similar to the expression seen in preeclamptic myometrium. mRNA expression for Hbγ (specific chain in HbF) and Hbβ (specific chain in adult Hb) were similar to that for Hbα (data not shown). Scale bar 100 μm. Protein expression is shown with a red fluorescent marker. In the preeclamtic placenta there is a strong expression of Hbγ in the vascular lumen (lu), but Hbγ is also expressed in the vascular endothelium (arrow) as well as in the extravascular section of the villous stroma **(d)**. The placenta from normotensive controls, however, showed no expression of Hbγ in the vascular lumen (**h**), but Hbγ is expressed in the vascular endothelium (arrow). Scale bar: 25 μm.

evidence from gene profiling and proteomic studies has shown the expression, production and accumulation of HbF to be abnormal in the preeclamptic placenta (Fig. 1) [11,12]. As a result of increased HbF gene expression, excessive amounts of free HbF accumulate in the vascular lumen, causing damage to the placental barrier. Furthermore, it has been demonstrated *ex vivo* that the damage caused to the placental barrier leads to leakage of free HbF into the maternal circulation [13]. This leakage has been confirmed *in vivo*, where elevation of free HbF has been reported to be evident in the maternal circulation up to 2 months before the onset of clinical symptoms (as early as 10 weeks of gestation) in women who went on to develop preeclampsia [14]. The fraction of HbF in plasma was 3–5-fold increased in women who developed preeclampsia and in full-term pregnancies the levels correlated with blood pressure (*i.e.*, the severity of the disease) [15]. Furthermore, Anderson *et al.* [16] suggested that an increased HbF:Hb ratio could be predictive of preeclampsia. Free HbF has also been demonstrated in cerebrospinal fluid (CSF) in women with preeclampsia [17].

The placenta is an extra-medullary hematopoietic organ providing the fetus with hematopoietic stem cells throughout gestation [18–20]. What stimulates the formation of free HbF in the placenta has not been clarified but recent studies have shown that the inflammation seen in preeclampsia can induce hematopoiesis [21]. The abnormal presence of free HbF within the maternal circulation contributes to the endothelial damage, inflammation and vasoconstriction caused by heme binding of nitric oxide (NO) [9,10]. Hemoglobin (Hb) and its toxic metabolites, methemoglobin, heme and iron, are well known from the field of transfusion medicine to have pro-inflammatory, pro-oxidative, tissue damaging and vasoconstrictive properties [22]. In preeclampsia, free HbF and its metabolites drive the formation of free radicals and reactive oxygen species which cause membrane damage, in turn leading to the formation of microparticles or syncytiotrophoblast-derived microparticles that contain HbF [23]. Based on this and other studies, it has been suggested that HbF is an important factor linking stages one and two in preeclampsia [10].

Alpha-1-microglobulin

A1M, first described in 1975 [24,25], belongs to the lipocalin protein family, a group of structural proteins with a similar one-domain fold that are found in bacteria, plants and animals [26,27]. The brown color and heterogeneous charge of A1M are caused by size-heterogeneous covalent modifications of a cysteinyl and three lysyl residues [28,29]. A1M has been shown to be a reductase and a binder of heme groups and small organic radicals [30–32] as described in more detail below. Today, A1M is recognized as a physiological antioxidant with powerful cell- and tissue-protective properties [33].

A1M structure

A1M is a one-domain, 183-amino-acid, glycosylated protein; the crystal structure of a large fragment was published recently [34]. A1M shares the common 3D structure of the lipocalin family, consisting of eight antiparallel β-strands forming a barrel with one closed end (bottom) and one open end (top) (Fig. 2a). The barrel functions as a pocket for hydrophobic ligands in most lipocalins. In A1M, a handful of amino acid side-groups located on four loops, which make up the rim of the open end of the barrel, or on the inside of the pocket, have been shown to be important for the functions of the protein.

A1M gene and expression

A1M is encoded by the alpha-1-microglobulin–bikunin precursor gene (*AMBP*) located on chromosome 9. The gene consists of ten

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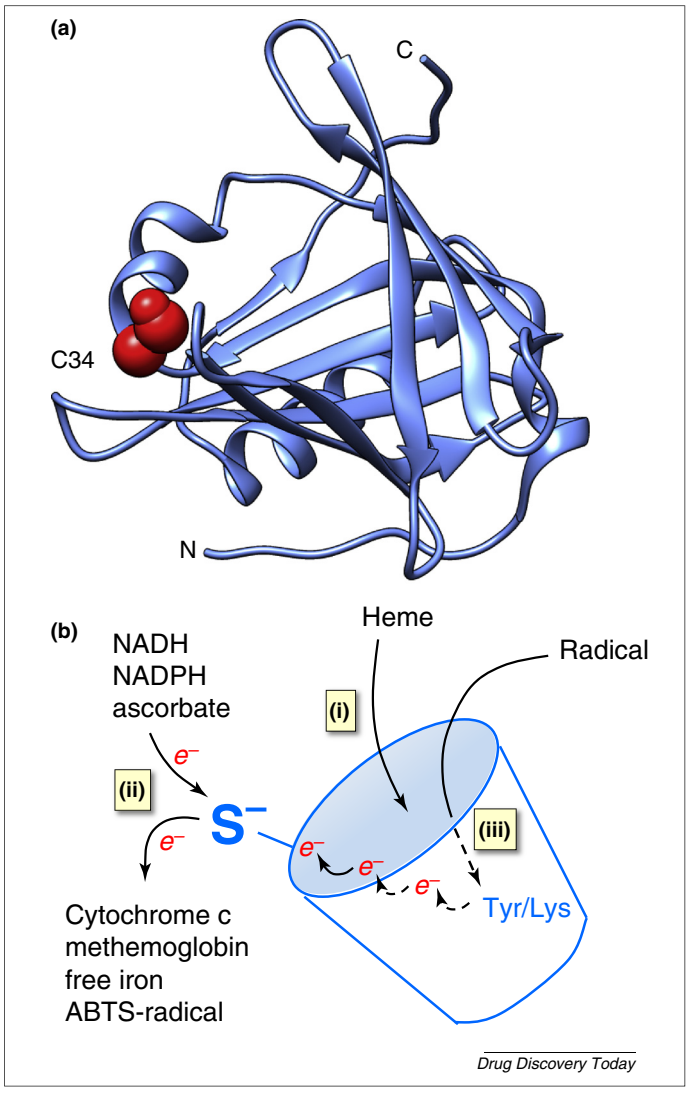

FIGURE 2

Structure and molecular mechanisms associated with human alpha-1-microglobulin (A1M). **(a)** Three-dimensional structure of a human A1M-fragment. The unpaired cysteine in position 34 (mutated to serine in the crystal structure) is highlighted in red. Reproduced, with permission, from [34]. **(b)** Molecular mechanisms implicated in the actions of A1M including (i) heme binding, (ii) reductase and dehydrogenase activity and (iii) radical binding. *Abbreviation*: ABTS, 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid).

exons, the first six of these encoding A1M, and the last four encoding another protein, namely bikunin [35]. *AMBP* is transcribed and translated into a precursor protein: alpha-1-microglobulin–bikunin [36]. The two proteins are joined together by a short linker but are separated by proteolytic cleavage, processed and secreted from the cell as two different proteins with distinct functions [37–39]. This co-synthesis is found in all cells and species where A1M and bikunin have been found, but the reason for the peculiar co-expression is not known. Whereas A1M is a heme and radical scavenger, and a reductase, bikunin is a structural component of extracellular matrix and has protease inhibitor and anti-inflammatory properties. No reports have suggested the involvement of bikunin in preeclampsia. A1M is evolutionarily conserved, and expression of *AMBP* has been shown in fish, birds, mammals and other vertebrates. In humans, the majority of A1M is synthesized in the liver, but smaller quantities are also expressed in most other cells in the body. In the blood, equal quantities of two forms of A1M can be found: a free monomeric form and a covalent high-molecular weight complex bound to immunoglobulin A, albumin and prothrombin [40,41]. Endogenous A1M has also been described to be localized ubiquitously in the dermal and epidermal layers of skin [42], and in syncytiothrophoblasts, monocytes/macrophages, vascular endothelium and extracellular matrix of placental tissue and extracellular matrix [13,43].

Serum levels of A1M

A1M is found within the circulation throughout life. Serum levels of A1M are reported to be in the range 15–50 mg/ml [44] with males having slightly higher levels than females [45]. A1M serum concentrations also tend to increase with age. No clinical condition linked to a deficiency of A1M has so far been described. Levels of A1M are, however, known to be influenced by renal and hepatic function; levels increase with impaired renal function and decrease with reduced liver function. The association with renal function makes determinations of A1M levels useful in the assessment of renal disease [46]. Furthermore, the inverse relationship between A1M levels and renal function could explain the tendency for A1M levels to increase with age [46], owing to the gradual decline in renal function with age that is normally observed. A1M concentrations are slightly elevated in preeclampsia – in plasma as well as in the CSF [15,47].

Molecular mechanisms associated with A1M function in humans

Four different molecular mechanisms contribute to the role of A1M as an antioxidant tissue protection protein (Fig. 2b). These are heme binding, reductase activity, radical scavenging and binding to mitochondria, and are described briefly below with references to studies using human A1M extracted from urine or plasma or produced as a recombinant protein in an *E. coli* system. A1M has been shown to bind to heme (Fig. 2b, *i*) at the molar ratio 2:1, such that two heme groups are bound to each A1M molecule [30,48]. The dissociation constant is around 10^{-6} M for both heme groups [49]. Molecular simulation suggests there to be one binding site in the lipocalin pocket, and a second more-superficial site between loops 1 and 4, where the iron atom is coordinated by Cys34 and His123 [50]. The heme-binding property of A1M is evolutionarily conserved, and has been shown for human, mouse, rat, guinea pig, cow, chicken and plaice A1M [51]. Although initial binding is noncovalent, covalent binding of heme degradation products has been reported [30,49]. The heme degradation activity of A1M is enhanced in a truncated form of A1M (t-A1M) which is formed in the presence of free Hb [30]. The t-A1M form, which lacks the C-terminal tetrapeptide of full-length A1M, has been found *in vivo* in the urine, skin and placenta [15,30,43,52].

A1M has the capacity to reduce several biological substrates owing to the possession of a free cysteine, Cys34, with a negative reduction potential [31]; these include methemoglobin, cytochrome c, oxidized collagen I, oxidized low density lipoprotein, free iron and nitroblue tetrazolium (Fig. 2b, *ii*). The reduction potential of A1M is catalytic in the presence of strong electron donors such as NADH, NADPH and ascorbate. This catalytic reductase and dehydrogenase activity of A1M is dependent on

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the Cys34 free thiol group, and becomes much weaker in the absence of the three lysyl residues Lys92, Lys118 and Lys130. Based on the crystal structure of A1M, these four residues are located in the vicinity of each other at the open end of the lipocalin barrel.

A radical-trapping mechanism has been described for A1M that involves the capture of the synthetic, long-lived radical 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) [32] (Fig. 2b, *iii*). This reaction again involves the reduction potential of Cys34, and leads to a reduction of five to six ABTS radicals, and covalent trapping of a further three to four ABTS radicals on tyrosyl residues by an unknown intramolecular electron-transfer mechanism. Supporting radical-binding *in vivo*, the lysyl residues Lys69, Lys92, Lys118 and Lys130 were shown to carry brown-colored modifications in A1M isolated from human urine and amniotic fluid [29,53].

Specific binding of A1M to mitochondria has been shown in several different cell types [54]; the uptake mechanism has not been elucidated. In this context, Complex I of the respiratory chain was identified as the main target of A1M binding. Increased mitochondrial uptake of A1M has been demonstrated during early phases of the apoptotic process, as well as in necrotic cells after exposure to free heme and oxidative stress. The binding of A1M resulted in inhibition of heme- and reactive-oxygen-species-induced mitochondrial swelling and abrogated ATP production.

Production of recombinant human A1M

A recombinant human version of A1M, denoted RMC-007, has been developed for pharmaceutical evaluation using a process suitable for large-scale production [55]; the protein, which is expressed in an *E. coli* system, has a molecular weight of 22 kDa and a peptide chain identical to the human sequence although it is not glycosylated. Recombinant human A1M (rA1M) has been shown to be fully functional in comparison with endogenous A1M derived from human plasma or urine [31,32]. Several variants of rA1M that have modified amino acid sequences but retain the same biological effects have been developed and evaluated. One of these variants, RMC-035, has several physicochemical advantages over RMC-007 that make it more suitable for drug development. rA1M lacks the glycosylation of endogenous human plasma A1M and therefore is less hydrophilic and more prone to aggregate. One improvement of RMC-035 over RMC-007 is an increased hydrophilicity and hence higher solubility and stability.

Studies with rA1M: tissue protection

rA1M has been shown to have tissue-protective and -reparative effects *in vitro* and *ex vivo*. The cell-protective effects of rA1M *in vitro* have been studied extensively, and several reports describe protective actions in cell cultures stressed by exposure to free Hb, heme and free radicals. These data have recently been reviewed in detail by Åkerström and Gram [33]. rA1M prevents the cellular oxidation and upregulation of the stress response gene heme oxygenase-1 (*HO-1*) induced by free Hb, heme and free radicals in a number of cell types, including keratinocytes and blood cells [42,56]. rA1M has also been shown to inhibit heme-induced cell death in blood cells by clearance of bound heme from cells [56]. Furthermore, it has been shown that rA1M is imported from the extracellular compartment, and binds, as described above, with high affinity to mitochondrial Complex I, thereby conferring protection to mitochondria by inhibiting swelling and reversing the severely impaired ATP production that is displayed when exposed to heme and free radicals [54]. Interestingly, endogenous expression of A1M has been found to be upregulated in different cell types, including hepatocytes and keratinocytes, following exposure to free Hb, heme and free radicals, indicating a 'natural' attempt to increase the defense system [42,57]. Further confirmation of this protective effect was found in a study that showed that silencing endogenous A1M expression by the addition of siRNA led to increased cytosol oxidation [56].

In an *in vitro* irradiation-induced free radical cell model, it was shown that rA1M inhibits the propagation of cell death induced by low-dose alpha-particle irradiation of liver cells [58,59]. Furthermore, following direct irradiation of a small restricted area, it was shown that addition of rA1M reduced the number of dead neighboring cells by 50–100%, and completely inhibited the induction of apoptosis, as well as the formation of carbonyl groups and upregulation of the stress-response-related genes *HO-1*, *p21* and *p53* normally seen after irradiation. It was suggested that the mechanism of rA1M protection was clearing of oxidants, radicals and reactive oxygen species released from the irradiated cells.

In addition to studies using cell cultures, the protective effects of rA1M have been studied in skin explants and in a perfused placenta model [13,42]. In skin explants, the addition of rA1M was shown to confer significant protection from heme- and free-radical-induced ultrastructural damage. Furthermore, rA1M reversed the oxidative-stress-induced upregulation of *HO-1* and *p21*. The protective effects of rA1M were also investigated in the placenta perfusion model [13]. This model enables study of cell and matrix structure and function, including the feto–maternal barrier [60]. *Ex vivo* perfusion of free Hb into the fetal circulation led to a significant increase in the perfusion pressure and feto–maternal leakage of free Hb. Morphological damage, including disruption of the normal extracellular matrix architecture, was also observed, as was upregulation of genes related to immune response, apoptosis and oxidative stress. Simultaneous addition of rA1M into the maternal circulation prevented the specific leakage of free Hb from the fetal into the maternal circulation. A protective effect following rA1M administration was also supported by ultrastructural observations; the extracellular matrix architecture in free-Hb-exposed placentas perfused with rA1M was indistinguishable from controls. Additionally, the cell organelle structures were intact; there was no mitochondrial swelling or membrane disruption, and there were no apoptotic vesicles [13]. Similarly to the observations in cell culture studies, endogenous A1M was upregulated in skin after exposure to heme and free radicals [42].

In addition to the protective effect of rA1M seen in collagen fibrils and in matrix in the skin and placenta, respectively, a repair mechanism associated with rA1M has been described *in vitro, ex vivo* and *in vivo*. In the work by Olsson *et al.* [42] collagen fibrils were restored to normal by the addition of rA1M after destruction had already begun. It was suggested that this effect was most probably the result of free radical clearing. Furthermore, it was shown in the placenta perfusion model that addition of rA1M induced a significant upregulation of extracellular matrix genes and increased the number of collagen fibrils [13]. The details of how this repair mechanism is exerted are currently under investigation.

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Therapeutic potential of rA1M based on interventional studies in animal models of preeclampsia

A variety of animal models of preeclampsia have been proposed, although all of them have limitations and cannot fully replicate the human disease [61]. The models used can be divided into four mechanistic categories: (i) spontaneous, (ii) pharmacologically and/or substance induced, (iii) surgically induced and (iv) transgenic animals. These were recently reviewed by Erlandsson *et al.* [62]. The effects of rA1M have been investigated in two of these mechanistically different animal models. In these models, as in pregnant women, the initial pathophysiology is assumed to be linked to the damaging effect of free Hb, heme and oxidative stress [9,62]. In the first model, pregnant rabbits were injected with free rabbit HbF, and in the second model pregnant ewes were starved, a procedure that leads to hemolysis and the release of free Hb and heme formation.

rA1M ameliorates effects of free Hb in pregnant rabbits

In a study by Nääv *et al.* [63], preeclampsia-like tissue damage was induced to the kidneys and placenta by infusion of free rabbit HbF. From gestational day 20 onward, the animals received intravenous injections of saline ($n = 5$), free rabbit HbF (20 mg/kg, $n = 8$) or the same dose of rabbit free HbF followed by injections of rA1M (6 mg/kg, $n = 6$). Injections were given every second day until gestational day 28, a total of five occasions. The circulating concentrations of rA1M achieved were ~3–5-fold higher than endogenous levels of A1M (unpublished data). The pregnancy was terminated on gestational day 29 of the normal gestational period of 31 days. Blood pressure and proteinuria were followed throughout the study, and at the end of the study glomerular permeability was assessed by determination of the Ficoll® sieving coefficient [64]. In addition, the ultrastructure of kidney and morphology of the placenta were quantified using transmission electron microscopy, measuring the integrity of extracellular matrix, nuclear membrane and glomerular barrier membrane (kidney) or plasma membrane (placenta). Furthermore, mitochondrial cross sections were determined as heme- and reactive-oxygen-species have been shown to induce mitochondrial swelling [54].

Injections of free HbF caused increased glomerular permeability, measured as an increased Ficoll® sieving coefficient, which was corroborated by the appearance of proteinuria but no hypertension was induced. The findings were further supported by morphological correlates; there was loss of integrity of the extracellular matrix and glomerular basal membrane as well as mitochondria swelling in the podocytes. Endothelial cells showed an aberrant degree of fenestration resulting in areas of obliterated fenestration and areas of increased but aberrant fenestrations. Severe damage was also observed in the placenta displayed as presence of extracellular apoptotic bodies, cell debris, disruption of the electron dens barrier and numerous areas of empty space. Administration of rA1M prevented the morphological effects of free HbF administration in kidney and placenta and resulted in very low levels of proteinuria and an essentially normal Ficoll® sieving coefficient. Thus, this study supports the notion that administration of rA1M, after an insult caused by free HbF, prevents the development of preeclampsia-like placental damage and preserves normal kidney function and morphology.

rA1M counteracts hemolysis-induced preeclampsia-like symptoms in starving pregnant ewes

In the pregnant sheep model of preeclampsia developed by Talosi *et al.* [65], food withdrawal for 96 h led to hemolysis of the

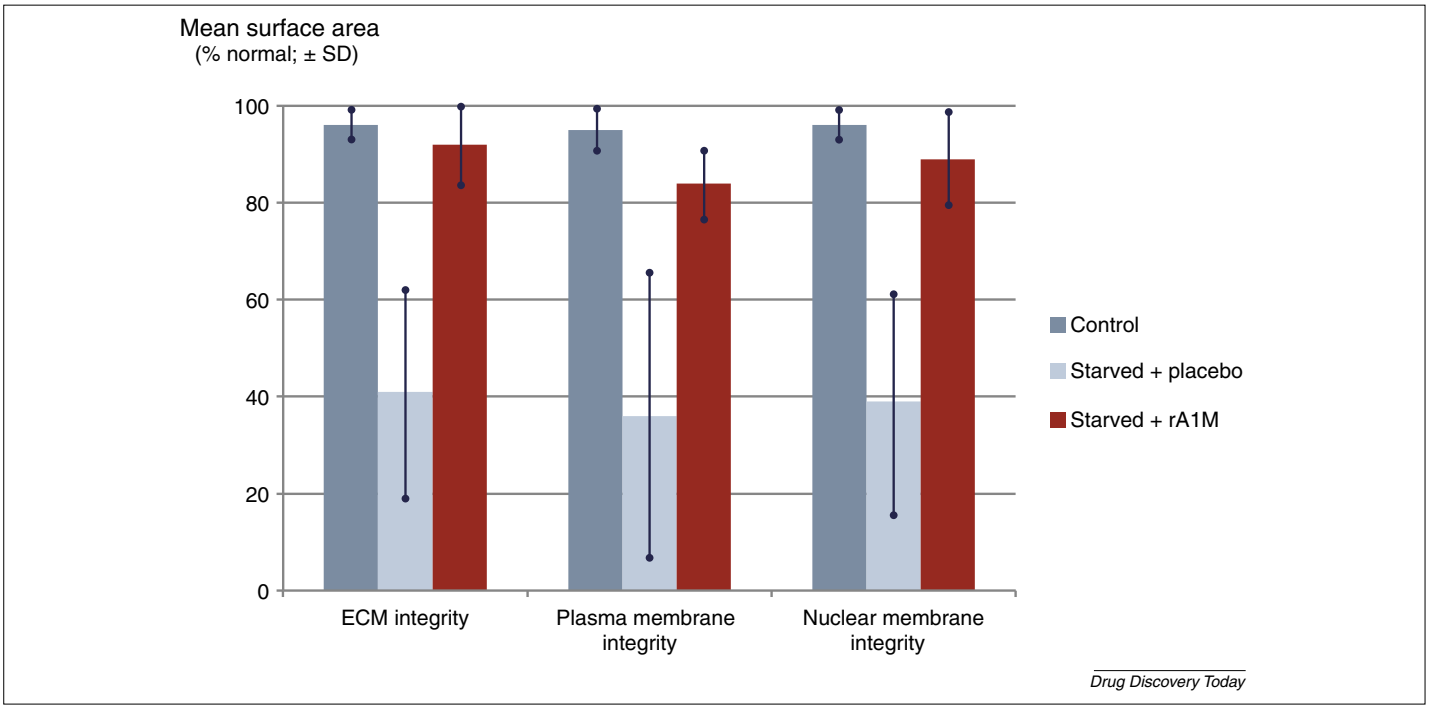

FIGURE 3

Administration of recombinant human alpha-1-microglobulin (rA1M) in starved pregnant ewes, a model for preeclampsia, normalized kidney morphology [measured as extracellular matrix (ECM), plasma membrane and nuclear membrane integrity] as quantified by transmission electron microscopy. Data plotted from Wester-Rosenlöf *et al.* [66].

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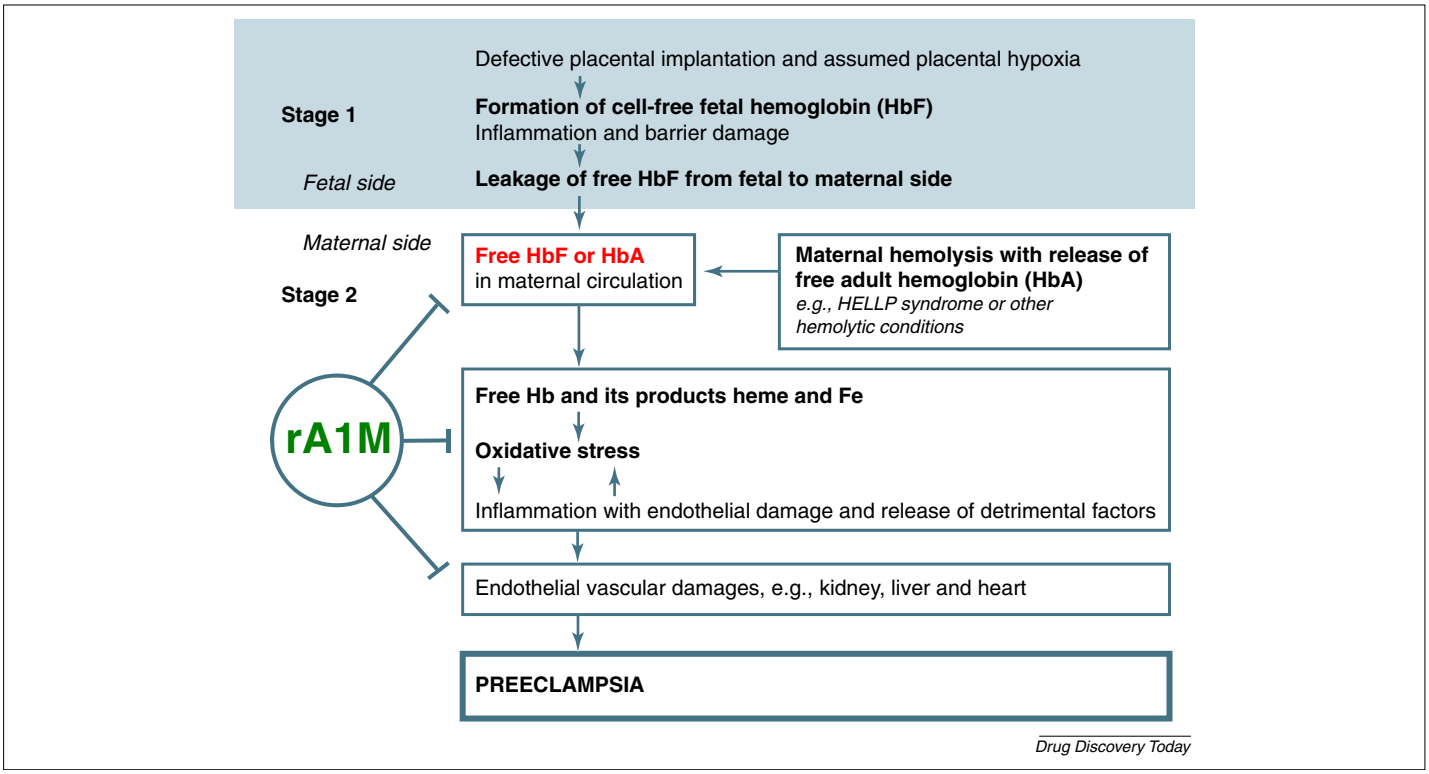

FIGURE 4

A conceptual model for the therapeutic actions of recombinant human alpha-1-microglobulin (rA1M) at different stages in the chain of events leading to preeclampsia. rA1M is believed to interfere with preeclampsia in three main ways: it binds radicals formed by free HbF, free adult Hb and free heme; it inhibits the toxic actions of heme and oxidative radicals; it promotes healing and tissue regeneration by repairing damaged molecules and upregulating the expression of specific genes. *Abbreviations*: Fe, iron; HELLP, hemolysis elevated liver enzymes low platelet count.

maternal erythrocytes, rapid elevation of free Hb and heme levels within the circulation and induction of hypertension. A concomitant fall in free thiol groups suggested oxidative stress. In the rA1M treatment study by Wester-Rosenlöf *et al.* [66], five ewes that had been starved for 36 h received two bolus doses of rA1M (1.8 mg/kg/dose, separated by a 2-hour interval) or saline solution (control animals, $n = 6$) at the time that fasting ended. Animals were followed for an additional 72 h with access to free food until sacrificed. Although manifest proteinuria was not observed, significantly increased glomerular permeability (measured as an increased Ficoll® sieving coefficient in the starved *vs* non-starved ewes) was recorded. Light microscopy examinations revealed signs of glomerular endotheliosis, which is typically associated with preeclampsia. Administration of rA1M significantly reduced the Ficoll® sieving coefficient to near-normal levels and there were no signs of glomerular endotheliosis in the renal histology from starved animals that had received rA1M. Morphological examinations of the placenta and kidneys using transmission electron microscopy revealed dramatic effects of rA1M administration; the integrity of the extracellular matrix, plasma membrane and nuclear membrane were all normal after the two injections of rA1M (Fig. 3).

Formulation of a conceptual model

A conceptual model for the therapeutic actions of A1M has been proposed based on the *in vitro, ex vivo* and *in vivo* animal data that support a therapeutic role for rA1M in preeclampsia (Fig. 4). A1M is believed to interfere with preeclampsia in three main ways. First, it binds radicals formed by free HbF, free adult Hb and free heme; second, it inhibits and binds heme and thereby prevents the toxic actions; third, it promotes tissue regeneration by repairing damaged matrix molecules and upregulating the expression of extracellular matrix genes [13].

Concluding remarks

Preeclampsia is the most common cause of death for mothers and children during pregnancy [67], and at present the only curative intervention is to induce delivery. Owing to recent research we now have a greater understanding of the mechanisms responsible for the development of preeclampsia; key events in the pathophysiology are believed to be the formation of free HbF in the placenta, probably induced by regional/local hypoxia, and subsequent leakage across the feto–maternal barrier. Free HbF is very reactive and, *via* degradation to heme and induction of oxidative stress, can cause the endothelial and tissue damage seen in preeclampsia. The endogenous protein A1M has properties that counteract the effects of free HbF and its metabolites (*e.g.*, A1M binds heme and free radicals, thereby reducing oxidative stress) which are crucial for the development of preeclampsia in our model. The potential therapeutic role of rA1M in preeclampsia is supported by *in vitro, ex vivo* and *in vivo* preclinical studies. Based on these findings, rA1M was granted orphan drug designation by the European Commission in 2014 (EU/3/14/1289). A clinical development program for rA1M in preeclampsia is now in

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preparation and the rA1M variant RMC-035 has been identified as a candidate to become the first pharmacological treatment for preeclampsia.

Conflicts of interest

RG is consultant to A1M Pharma, Lund, Sweden. SH, BÅ and MG are co-founders, Directors of the Board and shareholders of A1M Pharma.

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