Haemoglobin scavenging after subarachnoid haemorrhage

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SUMMARY

Rapid and effective clearance of cell-free haemoglobin after subarachnoid haemorrhage is important to prevent vasospasm and neurotoxicity, and improve long-term outcome. Haemoglobin is avidly bound by haptoglobin, and the complex is cleared by CD163 expressed on the membrane surface of macrophages. We studied the kinetics of haemoglobin and haptoglobin in cerebrospinal fluid after SAH. We show that haemoglobin rises gradually after SAH. Haptoglobin levels rise acutely with aneurysmal rupture as a result of injection of blood into the subarachnoid space. Although levels decline as haemoglobin scavenging occurs, complete depletion of haptoglobin does not occur and levels start rising again, indicating saturation of CD163 sites available for haptoglobin-haemoglobin clearance. In a preliminary neuropathological study we demonstrate that meningeal CD163 expression is upregulated after SAH in keeping with a pro-inflammatory state. However loss of CD163 occurs in meningeal areas with overlying blood, compared to areas without overlying blood. Since ADAM17 is the enzyme responsible for shedding of membrane-bound CD163, its inhibition may be a potential therapeutic strategy after SAH.

KEYWORDS

Subarachnoid haemorrhage

Vasospasm

Haemoglobin

Haptoglobin

Delayed neurological deficit

INTRODUCTION

Spontaneous subarachnoid haemorrhage (SAH) is an acute cerebrovascular event which causes significant morbidity and mortality. The majority of cases result from ruptured intracranial aneurysms. Following SAH, evidence suggests that extracellular haemoglobin (Hb) released from erythrocytes within the subarachnoid space is the agent responsible for poor outcome [9], mediated by effects on cerebral vessels and neurons, leading to ischaemia due to vasospasm and direct neurotoxicity.

Outside the brain, the CD163-haptoglobin axis is responsible for haemoglobin scavenging. Haptoglobin has a very high affinity for Hb. The resulting haptoglobin-Hb complex formation uncovers a neo-epitope on haptoglobin which enables recognition of the complex by CD163 expressed on the membrane surface of myeloid cells [8]. This results in receptor-mediated endocytosis of the complex and further intracellular degradation of Hb into bilirubin. ADAM17 enzyme is co-expressed by CD163-expressing myeloid cells and proteolytically cleaves CD163 from the cell membrane surface, resulting in constitutive circulating levels of soluble CD163 [3]. ADAM17 may be upregulated by ischaemia [2] and inflammation [7], which results in increased soluble CD163 shedding.

In the brain, CD163 is expressed by perivascular and meningeal macrophages in both rodents and humans [4, 5]; these macrophages are strategically located to scavenge blood products after SAH. We have recently shown saturation of the CD163-haptoglobin system after SAH [6], by examining cerebrospinal fluid (CSF) from patients with SAH and normal individuals. We demonstrated that CSF haptoglobin co-existed with free uncomplexed Hb, suggesting that CD163-mediated clearance of haptoglobin-Hb complexes was saturated after SAH. This could be secondary to pure overwhelming of CD163 sites available for binding in the CNS, or proteolytic loss of membrane-bound CD163 mediated by ADAM17, or both. Preliminary evidence suggested that CD163 was indeed being shed since CSF soluble CD163 levels were higher after SAH compared to controls, and most of it was synthesized intrathecally. We could not examine the levels of membrane-bound CD163 available for binding in these patients since this requires availability of tissue for immunohistochemistry or Western blot, and only CSF was available from these patients.

Here we sought additional evidence for saturation of the CD163-haptoglobin system by examining the kinetics of CSF Hb and haptoglobin levels with time. We also examined post-mortem tissue from brains of SAH and control patients to determine the level of membrane-bound CD163.

MATERIALS AND METHODS

Cerebrospinal fluid (CSF) from patients with SAH (n=30) and control individuals (n=20) was analysed by derivative spectrophotometry for Hb and enzyme-linked immunosorbent assay for haptoglobin (AssayPro, MO, USA). In patients with SAH, CSF was obtained from external ventricular drains on insertion. However, unlike serial samples taken after drainage, these CSF samples therefore truly reflect the composition of CSF at different time points after SAH. Control individuals were patients with non-inflammatory/non-hemorrhagic conditions undergoing lumbar puncture; their CSF was subsequently found to be normal with respect to protein, glucose, cell count, cytology, albumin CSF/serum quotient and isoelectric focusing for oligoclonal bands. Samples were collected with Research Ethical Committee approval (04/Q2707/236 and 07/H0304/71).

Post-mortem formalin-fixed paraffin-embedded tissue from a different set of SAH (n=7) and control cases (n=5) was obtained from the brain bank, Brain UK (Research Ethical Committee approval 09/H0504/68). Control cases were matched for age and sex, did not die from neurological causes, and were selected carefully to exclude inflammatory, haemorrhagic or neurodegenerative pathology; these served as “external” controls. In SAH cases, regions of cerebral cortex with overlying blood and no overlying blood were selected for each case; the cortical areas with no overlying blood were chosen to act as additional “internal” controls (avoiding inter-individual variability). Sections were cut, rehydrated, microwaved in EDTA buffer for 25 minutes to retrieve antigen, incubated with anti-CD163 antibody (EDhu1, AbD Serotec, UK) at 1:1000 dilution for 90 minutes at room temperature, then with secondary antibody, followed by 0.05% 3,3'-diaminobenzidine, and finally counterstained with haematoxylin, dehydrated and mounted in DPX. Sections were stained in the same run. Images were digitally acquired using a camera mounted on a light microscope, at magnification x20, and analysed on ImageJ (version 1.41, NIH US) to obtain a percentage protein load. Mann-Whitney test was used since data was non-parametric (SPSS 19 software, SPSS Inc., US). Plots were generated in GraphPad Prism 6.

RESULTS

Examination of the kinetics of CSF Hb levels showed that they rose progressively between days 1 and 4 after SAH (Figure 1a). There was an additional rise in CSF Hb levels between day 4 and day 6, however the standard deviation on day 6 was very high. CSF haptoglobin levels rose sharply after SAH (Figure 1b), reflecting spillage of blood-derived haptoglobin into the CSF during the acute bleed. This was followed by a steady decline in CSF haptoglobin, reflecting consumption during CD163-mediated scavenging of haptoglobin-

Hb complexes. However depletion did not occur, indicating saturation and/or loss of membrane-bound CD163 available for scavenging. Moreover, CSF haptoglobin levels started rising again on day 4, and there was a statistically significant difference between day 4 and day 6 CSF haptoglobin levels, indicating saturation of the CD163-haptoglobin system.

Our previous study had suggested intrathecal soluble CD163 shedding after SAH [6], which may partially explain the saturation of Hb-haptoglobin clearance via loss of membrane-bound CD163 sites available, if these are not replenished. We therefore proceeded to examine meningeal CD163 expression in postmortem tissue after SAH and in control individuals. CD163 stained cells with the morphological appearance of meningeal (Figure 2) and perivascular macrophages. SAH resulted in upregulation of CD163 expression (Figure 3a, two-fold, $p=0.02$), in keeping with the proinflammatory nature of SAH. Interestingly, in meningeal areas with overlying blood, CD163 levels were lower versus areas without overlying blood (Figure 3a, $p=0.02$), approximating the levels seen in control cases. This was also observed after comparing meningeal areas with and without overlying blood from the same patients, to eliminate intra-individual variability (Figure 3b, $p=0.03$). No such difference was observed in the gray matter (ie perivascular CD163). CD163 meningeal staining is illustrated in Figure 3.

DISCUSSION

In this kinetic study, we show that CSF haptoglobin levels rise acutely after SAH as a consequence of the injection of blood into the subarachnoid space. Under normal circumstances, the CNS is deficient of haptoglobin, with the total Hb-binding capacity in the CSF being x50,000 less than that of blood [6]. The automatic delivery of haptoglobin from serum to CSF during aneurysmal rupture may well protect against and help clear the Hb released during initial haemolysis. Haptoglobin binding to Hb prevents it from participating in toxic redox reactions [1] which damage blood vessels and neurones.

During extracranial haemolysis, haptoglobin never co-exists with free uncomplexed Hb. This is because the affinity of membrane-bound CD163 to haptoglobin-Hb complexes is very high, and the total Hb-binding capacity of the body is not limiting. We show that the situation is different during intracranial haemolysis. Not only does haptoglobin co-exist with Hb, but haptoglobin levels start rising between days 4 and 6. This is clear evidence of saturation of CD163-mediated uptake of haptoglobin-Hb complexes. Such saturation is likely to be of importance since CSF Hb levels rise further between days 4 and 6.

In this preliminary neuropathological study, we demonstrate that a potential mechanism for the persistence of haptoglobin despite high levels of Hb, is the loss of membrane-bound CD163 sites available for scavenging haptoglobin-Hb complexes. Although meningeal CD163 upregulation occurred after SAH, there was loss of CD163 in meningeal areas with overlying blood.

Replication of these findings in a larger number of cases is needed. CD163 is shed by the proteolytic action of ADAM17 expressed by the same macrophages. ADAM17 is a highly regulated enzyme, with at least two known naturally-occurring inhibitors: TIMP3 (tissue inhibitor of metalloproteinase-3) and PDI (protein disulphide isomerase), and can adopt active and non-active conformational states [10, 11]. Further study is needed in order to dissect the mechanism of CD163 shedding after SAH since ADAM17 inhibition may be a potential therapeutic strategy.

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FIGURE LEGENDS

Figure 1. The kinetics of Hb (a) and haptoglobin (b) levels in CSF after SAH

Figure 2. CD163 immunohistochemical staining of meninges following SAH, counterstained with haematoxylin, at magnification x20. Arrow indicates CD163+ve meningeal macrophages (brown).

Figure 3. The CD163 mean percentage protein load in the meninges from control and SAH patients (a). A pairwise comparison of CD163 in SAH meninges with or without overlying blood from the same cases is shown in (b).

CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.