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Nanomolar aluminum induces expression of the inflammatory systemic biomarker C-reactive protein (CRP) in human brain microvessel endothelial cells (hBMECs)

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ABSTRACT

C-reactive protein (CRP; also known as pentraxin 1, PTX1), a 224 amino acid soluble serum protein organized into a novel pentameric ring-shaped structure, is a highly sensitive pathogenic biomarker for systemic inflammation. High CRP levels are found in practically every known inflammatory state, and elevated CRP levels indicate an increased risk for several common age-related human degenerative disorders, including cardiovascular disease, cancer, diabetes, and Alzheimer's disease (AD). While the majority of CRP is synthesized in the liver for secretion into the systemic circulation, it has recently been discovered that an appreciable amount of CRP is synthesized in highly specialized endothelial cells that line the vasculature of the brain and central nervous system (CNS). These highly specialized cells, the major cell type lining the human CNS vasculature, are known as human brain microvessel endothelial cells (hBMECs). In the current pilot study we examined (i) CRP levels in human serum obtained from AD and age-matched control patients; and (ii) analyzed the effects of nanomolar aluminum sulfate on CRP expression in primary hBMECs. The three major findings in this short communication are: (i) that CRP is up-regulated in AD serum; (ii) that CRP serum levels increased in parallel with AD progression; and (iii) for the first time show that nanomolar aluminum potently up-regulates CRP expression in hBMECs to many times its '*basal abundance*'. The results suggest that aluminum-induced CRP may in part contribute to a pathophysiological state associated with a chronic systemic inflammation of the human vasculature.

1. Introduction

The ~100,000 km of vasculature — arteries, veins and capillaries — in a normal human adult is lined by a single layer of highly specialized mesoderm-derived endothelial cells [1–3]. While large CNS cerebrovascular vessels contain a single endothelial cell layer and an additional layer of basal lamina interspersed with contractile pericytes, the smallest of these vessels, typically ~5 μm in diameter, consists only of a single layer of endothelial cells that normally develop into tube segments and form the basis of the cerebral vasculature [1]. These smallest diameter cerebrovascular vessels allow the passage of only single ~5 μm diameter red blood cells essential for O_2–CO_2 exchange and nutrient transfer [2–6]. A 1400 g healthy adult human brain contains about 4000 km of vasculature, and most of this vasculature consists of ~5 μm diameter vessels comprised exclusively of a single layer of human brain microvessel endothelial cells (hBMECs) [1,7,8]. hBMECs (i) form the basis for the blood–brain barrier (BBB) [1,4]; and (ii) are responsible for the regulation of the transit of neurochemical signals, O_2–CO_2 and nutrients between the systemic circulation and the brain [1–4]. Endothelial cell function is impaired in patients with AD and vascular factors have long been known to make a significant contribution to AD pathogenesis [3,6,8–10]. For example cerebral blood perfusion appears to be reduced in AD, perhaps as a result of endothelial cell generated endothelin, a potent vasoconstrictor elevated in the cerebral cortex of AD brain [10,11]. Endothelial cells have pleiotropic roles; not only are they potent regulators of pathological vascular changes, with implications for hypoperfusion, but also impact 42 amino acid amyloid beta (Aβ42) peptide production [10–13]. One understudied secretory element of endothelial cells is CRP, a soluble serum protein recently found to be significantly up-regulated in AD patients [14–16, this report] (Table 1, Fig. 1A). Interestingly increased CRP has recently been shown to exacerbate Aβ42 peptide production and induce tau hyperphosphorylation and may be an important trigger in the early development of AD pathogenesis by multiple pathogenic mechanisms [16–18].

This current pilot study consisted of two parts: (i) we analyzed CRP levels in blood serum derived from moderate and advanced AD patients compared to healthy age-matched controls; and (ii) we examined the

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Table 1
Whole blood serum samples from neurologically normal controls ('C1–C8'; N = 8; CDR ~0), a moderate Alzheimer's disease group ('AD1–AD6'; N = 6; CDR ~1.5) and an advanced AD group ('AD7–AD12'; N = 6; CDR ~3.0) analyzed in this study; CDR is the clinical dementia rating given to the patient after neurological assessment (see text) [26]; all blood samples were from female Caucasians; there were no significant age or gender differences between the control or AD groups; c-reactive protein (CRP) as determined by ELISA is expressed as mg/mL; SD = standard deviation; see text for further details.

Blood samples	CDR	Age (yr)	CRP mg/mL
C1	0	58.4	5.9
C2	0	65.2	6.3
C3	0	77.0	8.1
C4	0	62.3	4.5
C5	0	71.2	2.7
C6	0	66.0	5.4
C7	0	62.2	4.8
C8	0	67.0	5.5
Control mean ± 1 SD	0	66.2 ± 5.8	5.4 ± 1.6
AD1	1.5	60.8	22.7
AD2	1.0	57.8	16.1
AD3	2.0	66.8	24.3
AD4	1.5	66.9	14.5
AD5	1.5	62.8	25.2
AD6	1.5	73.4	13.6
AD Group 1 mean ± 1 SD	1.5	64.8 ± 5.5	19.4 ± 5.2
AD7	3.0	54.7	75.3
AD8	3.0	69.4	83.5
AD9	3.0	67.1	85.4
AD10	3.0	66.3	71.7
AD11	3.0	77.9	92.9
AD12	3.0	65.2	75.5
AD Group 2 mean ± 1 SD	3.0	66.8 ± 7.5	80.7 ± 7.9

role of the potent neurotoxin aluminum (as aluminum sulfate) on its potential effects on CRP generation in hBMEC cells, the same cell type that is highly enriched in the cerebral vasculature of the human CNS [19,20].

2. Experimental procedures

In these experiments whole blood serum was obtained from AD (N = 12; mean age 65.7 ± 6.4 yr) and age-matched control (N = 8; mean age 66.2 ± 5.8 yr) patients, and CRP levels were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits and/or modified CRP immunoassay as previously described [21–24; C-reactive protein colorimetric ELISA kit, STA392; detection limit 1 ng/mL; Cell Biolabs Inc, San Diego CA USA and/or CRP colorimetric ELISA kit, detection limit 1 ng/mL; Catalog # AC9916, Neoscientific, Neobiolabs Woburn MA, USA]; complete assay details are given at two independent websites [20,21]. The normal concentration of CRP in serum from healthy aged humans is usually lower than 10 mg/L but active inflammation, such as encountered during bacterial infections, can raise this to over 200 mg/L [22–28]. The AD samples consisted of 2 groups; a moderate AD group (AD Group 1; N = 6; mean age 64.8 ± 5.5 yr) with a clinical dementia rating (CDR) [26] of ~1.5, and an advanced AD group (AD Group 2; N = 6; mean age 66.8 ± 7.5 yr) with CDRs of ~3.0 (see Table 1 and Fig. 1A). All control and AD cases were from female Caucasians; there were no significant age or gender differences between the control or AD groups; the blood sample donors or their caregiver(s) reported no serious viral or bacterial infection within the last 9 months in any of the blood donors.

Human brain primary microvascular endothelial cells (hBMECs), initiated by elutriation of dispase-dissociated normal human brain cortex and cryopreserved at passage one, were obtained from two commercial sources (Cell Systems, ACBRI 376, Kirkland WA, USA; or ScienCell Research Laboratories, Carlsbad CA, USA). hBMECs, tested negative for HIV-1, HBV, HCV, mycoplasma, bacteria, yeast and fungi at source, have been extensively used for studies on brain cell adherence, transport and permeability of the BBB, angiogenesis and vascular-related disorders, HIV transmission and AIDS-related BBB dynamics, and demonstrate particular markers of differentiation (such as interdigitated cell contact, desmosomes, ZO-1 protein epitopes). hBMECs initially contained about 5×10^5 cells/mL volume and were cultured in fibronectin-coated culture vessels to ~90% confluency in endothelial cell medium (ECM, Cat. #1001) as described in detail [27,28]. In these studies ultrapure reagents for molecular biology, including $MgSO_4$ (63133; used as a control) and $Al_2(SO_4)_3$ (11044; Biochemika MicroSelect©; Fluka Ultraselect©; Fluka Chemical, Milwaukee WI, USA), freshly prepared as 0.1 M stock solutions, were instilled into either serum containing or half serum strength ECM made up in ultrapure water (18 megohm, Milli-Q, Millipore; aluminum content less than 1 ppb) followed by filter sterilization using 0.2-μM spin filters (Millipore Corporation, Billerica MA, USA) [31,33–36]. Freshly prepared aluminum sulfate was added at 5, 10, 25, 50 and 100 nM of aluminum (final concentration; N = 3 to 5 replicates for each aluminum concentration) in pre-warmed ECM medium (37 °C) and cells and conditioned medium were harvested after 12–18 h; CRP was determined in the conditioned medium using ELISA as described above; see also Fig. 1B and references [29–32,36,37].

3. Results

The results for CRP content of control and AD-CDR-1.5 and AD-CDR-3.0 human serum is shown in Table 1 and Fig. 1A. In this small study of 18 age-matched controls and middle-to-advanced AD patients,

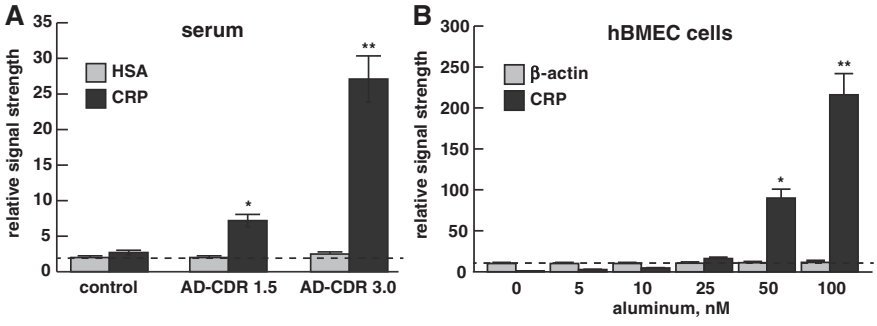

Fig. 1. (A) Abundance of CRP in human control and AD serum compared to human serum albumin (HSA) in the same sample; we used human serum albumin (HSA; ~66.5 kDa) as an internal serum control as it is the most abundant protein in human blood serum [35,000–50,000 mg/L (3.5–5.0 g/dL)]; in our studies control human serum averaged 5.4 mg/mL CRP; moderate AD subjects (CDR ~1.5) had a mean CRP levels averaging 19.4 mg/mL, elevated 3.6-fold over control; advanced AD subjects (CDR ~3.0) had a mean CRP averaging 80.7 mg/mL, elevated to nearly 15-fold over age-matched controls (Table 1); a dashed horizontal line at 2.0 included for ease of comparison; (B) CRP levels in aluminum-treated human brain microvessel endothelial cells (hBMECs) that line the CNS vasculature; we used human β-actin as an unchanging internal control; 0, 5, 10, 25, 50 and 100 nM of aluminum (as aluminum sulfate) increased CRP levels to a mean of 0-, 3-, 5-, 14-, 71- and 260-fold, respectively, over magnesium sulfate treated (control) samples; a dashed horizontal line at 10.0 is included for ease of comparison; for both (A) and (B) bars represent the mean plus 1 standard deviation from that mean; in (B) N = 3 to 5 replicates for each aluminum concentration tested; significance: $^*p < 0.05$; $^{**}p < 0.01$ (ANOVA).

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