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

Peter N. Alexandrov [a], Theodore P.A. Kruck [b], Walter J. Lukiw [c,d,e,*]

[a] Russian Academy of Medical Sciences, Moscow 113152, Russia
[b] Department of Physiology, University of Toronto, Toronto, ON M5S 1A8, Canada
[c] LSU Neuroscience Center, Louisiana State University Health Science Center, New Orleans, LA 70112, USA
[d] Department of Ophthalmology, Louisiana State University Health Science Center, New Orleans, LA 70112, USA
[e] Department of Neurology, Louisiana State University Health Science Center, New Orleans, LA 70112, USA

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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

* Corresponding author at: LSU Neuroscience Center of Excellence, Louisiana State University Health Sciences Center, 2020 Gravier Street, Suite 904, New Orleans, LA 70112-2272, USA.
E-mail address: wlukiw@lsuhsc.edu (W.J. Lukiw).

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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,

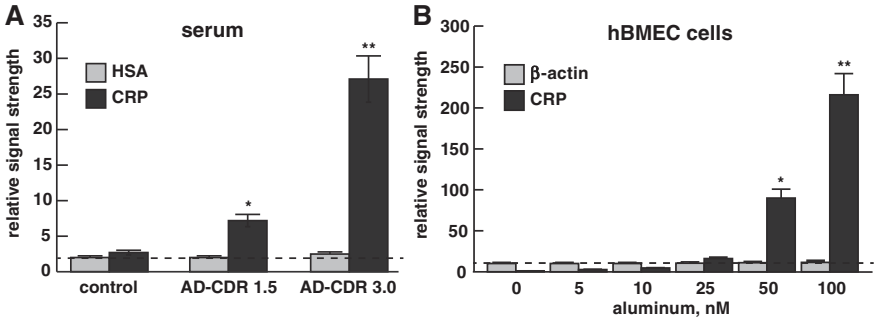

Fig. 1. (A) Abundance of CRP in human control and AD serum compared to human serum albumin (HSA) in the same sample; we used human serum albumin (HSA; ~66.5 kDa) as an internal serum control as it is the most abundant protein in human blood serum [35,000–50,000 mg/L (3.5–5.0 g/dL)]; in our studies control 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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moderately-demented AD subjects with a CDR ~1.5 had a CRP levels elevated to a mean of 3.6-fold over age-matched controls; similarly advanced AD subjects with a CDR ~3.0 had mean CRP levels elevated to nearly 16-fold over age-matched controls (see Legend to Fig. 1). These results are comparable to other studies of significantly increased CRP levels in AD patient's blood serum [14–17], although there is a report to the contrary [38]. Because endothelial cells that line the vasculature are an inducible source of CRP we next measured released CRP levels in hBMEC cells, a specialized endothelial cell type that represents the major endothelial cell type that lines the vasculature of the human CNS [8,21–23]. The results for CRP content of the medium surrounding aluminum-stressed hBMEC cells are shown in Fig. 1B. Aluminum sulfate added to cultured hBMEC cells displayed a classical dose–response relationship. For example, just 10 nM added aluminum (sulfate) to the hBMEC cells increased CRP in the external medium 4-fold, similarly 25 nM added aluminum added to the hBMEC cells increased CRP 9-fold, and 50 nM and 100 nM aluminum (sulfate) increased CRP 75-fold and 222-fold respectively, compared to samples treated in parallel with magnesium sulfate (Fig. 1B). For the first time this indicates a highly significant aluminum-mediated effect on the induction of CRP in hBMEC cells that line the human cerebrovasculature at physiologically relevant concentrations.

4. Discussion and conclusions

Aluminum's neurotoxic, pathogenic effects and potential contributions to neurodegenerative diseases such as AD are abundantly represented in the literature and have recently been reviewed and updated in a Special Research focus of *Frontiers in Neurology* [39]. In this compilation of recent aluminum neurotoxicity papers, Bhattacharjee et al. [29] provided evidence of the selective accumulation of aluminum in the cerebral arteries in AD brain and CNS, suggesting that hBMECs: (i) possess an extremely high affinity for aluminum when compared to other types of brain cells; and (ii) that endothelial cells that line the cerebral vasculature may have aluminum receptors, acceptor molecules or related biochemical attributes conducive to binding and targeting aluminum to selective anatomical regions of the brain, such as the hippocampus, with potential downstream pro-inflammatory and pathogenic consequences [29–32]. One reason for this selective targeting may be the slightly different phospholipid composition of various endothelial cell types, including hBMECs that line the cardio- and cerebral-vasculature [30,31]. As suggested by the current experiments it is reasonable to speculate that the targeting of systemic aluminum to highly specialized hBMECs induces the release of soluble secretory factors such as CRP from these endothelial cells that may in turn further propagate systemically to up-regulate inflammatory signaling in the brain and CNS [29–32]. It may be of relevance that CRP protein is encoded in a region of the long arm of human chromosome 1 (chr1q21-1q25) that also encodes other inflammation related genes including cyclooxygenase-2 (COX-2; chr1q25) and cytosolic phospholipase A_2 (cPLA2; chr1q25-1q31), key elements of the arachidonic acid cycle that generates eicosanoids and other metabolites which further promote systemic inflammation [40,41]. Indeed the arachidonic acid cycle appears to be chronically over-stimulated in AD brain [41]. It is further interesting to note that while liver-derived CRP passes directly into the systemic blood circulation that must cross the endothelial-cell mediated blood brain barrier (BBB) to access brain compartments, endothelial cell-derived CRP has no BBB to cross and thus may be immediately available to the brain and CNS [1,8]. Of further interest is that although we found that in cell culture as little as 10 nM aluminum sulfate induced CRP four-or-more-fold over untreated control hBMECs, aluminum is a particularly 'sticky' entity when used in vitro (i.e. has multiple biological targets both inside and outside of the cell) so the effective physiological concentration of aluminum's effects on hBMECs may even be much lower than 10 nM (unpublished observations). This has a bearing on aluminum's tremendous potential for interaction with, and the up-regulation of, inflammatory signaling, including gene signaling, within the systemic vasculature [29–32,36]. Interestingly, the homeostatic or pathogenic interaction between liver- and vasculature-endothelial cell generated CRP is not well understood but may be involved in a number of physiological situations in both health and disease. These include angiogenesis, alterations in vasoconstriction both inside and outside of the BBB, endothelial cell inflammation, thrombus formation in cardiovascular or cerebrovascular disease, and in the production of neurotoxic Aβ42 peptides chronically released into and transported by the systemic circulation [18,19,42]. It is important to further note that increases in systemic CRP have been shown recently to both: (i) enhance the production of Aβ42 peptides in the brain vasculature, and (ii) induce tau hyperphosphorylation within the CNS, and these two features are intimately linked to the development of AD neuropathology [16–18,43].

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