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Published in:
Journal of Alzheimer's Disease

DOI:
10.3233/JAD-141200

2014

Increased Levels of Hyaluronic Acid in Cerebrospinal Fluid in Patients with Vascular Dementia

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Running title: Hyaluronic Acid in CSF and Vascular Dementia

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Keywords: hyaluronic acid, vascular dementia, cerebrospinal fluid, biomarker, glycocalyx

Abstract

Hyaluronic acid (HA) has been shown to affect angiogenesis and the function of the blood–brain barrier (BBB) and a crucial role for HA in atherosclerosis has been described. We have recently demonstrated changes in the levels of HA in cerebrospinal fluid (CSF) in patients with Alzheimer's disease (AD) with documented vascular alterations. To further investigate if the level of HA in CSF can be used as a clinical diagnostic biomarker to identify vascular pathology in dementia, we analyzed the levels of HA in the CSF of patients with vascular dementia (VaD) (*n*=46), AD (*n*=45), and controls without dementia (*n*=26). In line with our previous data, we found significantly increased levels of HA in CSF from patients with VaD compared with controls, whereas the levels of HA in patients with AD were found to be unaltered compared with controls and patients with VaD. We also detected increased levels of HA in individuals with vascular changes determined as significant white matter changes or previous infarction on cranial computed tomography or magnetic resonance imaging, compared with individuals without these findings. Furthermore, we found a significant positive correlation between the levels of HA and the CSF/serum albumin ratio, an indicator of BBB integrity, in patients with VaD and AD, supporting the role of HA in vascular changes in the brain. Our results indicate a potential diagnostic value for the detection of vascular brain changes in dementia using CSF levels of HA, but emphasize the importance of further development of more sensitive HA assays.

INTRODUCTION

Hyaluronic acid (HA), chains of nonsulfated glycosaminoglycan, is often referred to as the backbone of the extracellular matrix (ECM), which provides biochemical and physical support to multicellular structures [1]. In vessels, HA together with other ECM molecules form a luminal mesh called glycocalyx [1]. The function of glycocalyx is to modulate vascular integrity, for example, by sieving molecules along the capillary wall [2, 3] and regulating adherence and migration of blood-derived immune cells [4]. The role of HA and glycocalyx in vascular integrity became apparent when pathologic changes in vessel function were investigated. Neointimal formation is markedly enhanced when HA is overexpressed [5] and degradation/shedding of HA increases glycocalyx permeability [6]. Moreover, inhibition of HA production reduces the thickness of glycocalyx, increases leukocyte rolling, and accelerates the burden of aortic plaque [7]. A crucial role for HA in atherosclerosis has been demonstrated in a number of studies [8].

Levels of shedded soluble HA can be measured in blood and cerebrospinal fluid (CSF) [9-11] and could thus theoretically function as a diagnostic marker reflecting glycocalyx degradation and vascular dysfunction. Increased levels of HA in CSF from stroke patients have been reported [9]. In addition, we have recently demonstrated increased levels of HA in CSF from patients with Alzheimer's disease (AD) with vascular changes and a strong correlation between the levels of HA in CSF and Q-albumin (CSF/serum albumin ratio), an indicator of the function of the blood–brain barrier (BBB) [10]. To further investigate the potential diagnostic value of HA for vascular dementia (VaD), in this study we analyze HA in a new patient cohort consisting of controls without dementia and patients with AD and VaD.

MATERIALS And METHODS

Patients

Samples of CSF from patients diagnosed with AD (*n*=45) or VaD (*n*=46), and controls without dementia (*n*=27) were obtained at the Memory Clinic at Skåne University Hospital (Sweden). The controls were individuals referred for evaluation of subtle cognitive symptoms. After the diagnostic dementia work-up, they were judged to be cognitively healthy. The dementia diagnoses were made according to the DSM-IIIR criteria of dementia [12] combined with the NINCDS-ADRDA criteria [13] for AD and NINDS-AIREN criteria for VaD [14]. All individuals underwent computed tomography (CT) or magnetic resonance imaging (MRI) of the brain before lumbar puncture. CT (*n*=91) or MRI scans (*n*=15) were retrieved for the controls (*n*=26), patients with AD (*n*=35) and patients with VaD (*n*=45), and rated for signs of vascular changes defined as the presence of (1) white matter changes (WMC) grade 2 according to Wahlund (beginning confluence of lesions) in any region, left and right frontal and parietal lobes as well as the basal ganglia and insula [15], or (2) tissue defects with a characteristic appearance of previous infarction or hemorrhage. Individuals were classified as having cerebrovascular changes or not (Table 1). The number of individuals exposed to head trauma, inflammatory diseases, increased levels of C-reactive protein (CRP) or peripheral tumors earlier in life was identified through review of the medical records (Table 1). The Mini Mental State Examination (MMSE) [16] was used to evaluate the cognitive status of the patients and controls. The ethics committee of Lund University approved the study and the study procedures were conducted in accordance with the Helsinki declaration of 1975 (revised in 2000). All participants gave informed consent to the research.

Analysis of HA, Q-albumin and AD markers

Levels of HA in CSF were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Levels of albumin in serum and CSF were determined by nephelometry using the Behring nephelometer analyzer (Behringwerke AG, Marburg, Germany). The basic CSF AD biomarker (Aβ1-42, T-tau, P-tau181) profile of the patients included in the study was analyzed routinely by commercial ELISA (Innogenetics, Ghent, Belgium) as previously described [17].

Statistical analysis

Statistical analysis was performed using SPSS software (version 20.0 for Windows, SPSS Inc., Chicago, IL). The Kolmogorov–Smirnov test was used to test for normal distribution of the variables. The independent sample *t* test was used for comparisons between two groups. For comparisons between more than two groups, one-way analysis of variance (ANOVA), followed by Bonferroni post hoc correction, was used (comparisons, n=3). Correlations were investigated using the Spearman correlation test. The results are presented as medians or means±standard deviation or range. A P value <0.05 was considered significant.

RESULTS

Demographic data

The demographic data for the individuals included in the study are presented in Table 1. The proportion of men and women was similar in the VaD and the control groups, whereas there were more women than men in the AD group. Patients with AD and VaD were significantly older than controls. Patients with AD and VaD had

significantly lower MMSE scores and CSF Aβ1–42 levels compared with controls. Levels of T-tau and P-tau were significantly higher in patients with AD, but unaltered in patients with VaD compared with controls. The percentage of individuals with increased CRP and peripheral tumors was greater among patients with VaD compared with patients with AD and controls; the percentage of individuals previously exposed to head trauma and inflammatory disease was higher in the AD group and controls compared with the VaD group.

Levels of HA in CSF

The controls were younger than the patients with AD and VaD, therefore we analyzed the association between age and levels of HA in CSF before further statistical analysis. No significant correlation between the two variables was found in any of the groups (data not shown) and hence HA levels were not adjusted for age before comparison analysis. Furthermore, the levels of HA in CSF from individuals with previous head trauma, inflammatory disease, and peripheral tumors did not significantly differ compared with individuals not exposed to similar conditions (data not shown). Similarly, individuals with abnormally high levels of CRP did not show altered levels of HA compared with individuals with normal CRP levels (data not shown).

Multicomparison analysis showed a significant difference in levels of HA in CSF between the groups analyzed (ANOVA, P=0.041); patients with VaD had significantly higher levels of HA compared with controls (P=0.035), but not compared with patients with AD (P=0.948) (Fig. 1A). Levels of HA in patients with AD did not differ significantly compared with controls (P=0.285) (Fig. 1A). We also analyzed HA levels when individuals were categorized based on the presence of

vascular changes. No significant difference was found when individuals with or without vascular changes were compared within the AD and VaD groups (177.62±131.50 pg/ml vs 142.70±68.67 pg/ml, *P*=0.357 and 192.61±89.73 pg/ml vs 156.42±109.62 pg/ml, *P*=0.278, respectively). However, significantly increased HA levels were found when all individuals with vascular changes were compared with individuals without vascular changes (*P*=0.019) (Fig. 1B).

Correlation analysis

In line with our previous study, we found a significant correlation between HA and Q-albumin in patients with both AD and VaD (*r*=0.535, *P*<0.001 and *r*=0.445, *P*=0.002, respectively) (Fig. 2A and B), but not in the control group (*r*=0.039, *P*=0.863). We also found a significant negative correlation between HA and MMSE in the AD group (*r*=−0.322, *P*=0.035) (Fig. 2C), and a weak negative trend in the VaD group (*r*=−0.214, *P*=0.114). No correlations between HA and MMSE were found in the control group (*r*=0.136, *P*=0.509). A weak negative trend to correlation was also found between HA levels and Aβ1–42 levels in the AD group (r=-201, p=0.166), but not in the VaD (r=-0.14, p=0.927) or control group (r=0.156, p=0.457). Neither P-tau nor T-tau were associated with HA levels in any of the groups investigated (data not shown).

DISCUSSION

Studies performed by us and others have demonstrated increased levels of HA in CSF from individuals with pathologic changes in the brain vasculature [9, 10]. In the current study, we aimed to investigate whether the level of HA in CSF could

function as a clinical diagnostic marker for VaD and thus we measured HA levels in a new patient cohort consisting of patients clinically diagnosed with AD and VaD and controls without dementia. Although we found a significant increase in HA in patients with VaD compared with controls, no significant differences between patients with AD and VaD were found. We can therefore conclude that HA is not a suitable biomarker to distinguish VaD from other forms of dementia such as AD. One plausible explanation for the lack of difference between the two groups is the fact that patients with AD often display a varied degree of vascular pathology [18]. The patients with AD included in this study were diagnosed in a clinical routine based on validation of symptoms, cognitive test results, and brain imaging results (CT or MRI). However, we also conducted a systematic and thorough review of brain imaging scans, without knowledge of the clinical presentation of the individuals. This review revealed vascular changes in several patients with AD (20 of 45), which could explain the slight increase in HA in this patient category. In light of these findings, we cannot not exclude the possibility that HA may have diagnostic value for distinguishing vascular pathology rather than specific diagnostic patient groups. Our analysis demonstrated increased levels of HA in individuals with a vascular component compared with individuals without a vascular component. Our analysis also indicated increased levels of HA in individuals with a vascular component when HA levels were compared within each dementia group. However, this increase was not statistically significant, which may be due to lack of statistical power.

HA varies greatly in length and the properties of the molecule are determined by its size. The major form of HA in vivo has a molecular weight of about 1 million kDa and it is incorporated into the stabilizing glycocalyx [3]. In addition, soluble forms of high molecular weight (HMW) HA exist and experimental studies

demonstrate that this form has protective, antiinflammatory and antiangiogenic properties [19, 20]. However, in response to pathologic events such as inflammation or damage, HA is degraded into low molecular weight (LWM) chains (<500 kDa) [21]. LMW HA has proinflammatory and proangiogenic properties [19, 20, 22]. Our HA assay, like all the available immunoassays for determining HA, does not distinguish between LMW and HMW. This is undoubtedly a limitation of the present study, since we consequently can not specify whether the increased amount of HA is due to a higher amount of LMW or HMW or both. Moreover, a recent methodological study has shown that HMW HA tends to yield a higher signal in HA immunoassays compared with LMW HA, although the concentration of the two HA samples is the same [23]. It may thus be that the differences in the CSF HA concentration found in our current study is in reality even higher given the possibility that most HA in patients with VaD and AD is the degraded LMW version.

The role of HA in vascular changes is further supported by the correlation between HA levels and Q-albumin in patients with AD and VaD. Q-albumin (i.e., the CSF/serum albumin ratio) is commonly used as a crude indicator of BBB function, because increased influx of albumin from blood to the CSF indicates enhanced BBB permeability. Intactness of the BBB depends on a well-functioning neurovascular unit, where the endothelial wall, tight junctions, and transporters are considered to be the key components [24]. Under pathologic conditions (such as hypoxia/ischemia), inflammatory processes disturb the microvascular integrity by disrupting endothelial tight junctions and transporters, and thereby increase the permeability of the BBB [24]. As mentioned in the introduction, glycocalyx also plays a specific role in microvascular integrity and BBB intactness because it sieve larger proteins, including albumin, leaving mainly water and smaller molecules to pass through the BBB [3].

Experimental studies have shown that HA binds albumin and removal of HA reduces glycocalyx albumin absorption [25]. The positive association between HA and Q-albumin in patients with dementia may thus reflect a pathology-provoked increase in degradation and shedding of HA, causing a thinner glycocalyx mesh with less albumin bindings sites, which in turn increases the amount of albumin passing through the BBB.

We also found a significant negative correlation between MMSE and HA in patients with AD. However, this correlation was not found in the controls only; a tendency to correlation was noted in the patients with VaD. This finding was not reproduced in our previous study in either of the diagnostic groups investigated [10], which highlights the importance of further investigation of the link between HA, in particular LMW HA, and cognition in larger patients cohorts.

Further, we found a weak tendency to a negative association between HA and the AD biomarker Aβ1–42 in the AD group. Although this correlation was not significant it may support previous studies describing Aβ1–42 as one of the culprits underlying vascular changes found in AD patients. Lowered levels of Aβ1–42 in CSF correlates with increased load of Aβ1–42 plaques in the brain [26], the hallmark of AD pathology. Hence the negative trend indicates increased HA shedding along with increased Aβ1–42 plaque load. Interestingly, preclinical *in vitro* studies, using the Tg2576 AD mouse model, have shown that accumulation of toxic Aβ1–42 is preceding hypervascularization, tight junction disruption and BBB dysfunction [27, 28] and that active Aβ immunization reverse these pathological events [29, 30]. Further, both *in vitro* and *in vivo* studies have demonstrated an direct impact of different Aβ species, including Aβ1-42 on endothelial and pericytes, cells that

constitute the vascular compartment [31] [32-34]. It may thus be that some of the vascular changes seen in the patients included in our study are due to increased Aβ1–42, but at the present time we are unable to differentiate whether the vascular changes is a consequence of Aβ1–42 load, hypoxia induced by risk factors (such as diabetes, atherosclerosis or cerebrovascular disorders) or a combination of both pathologies.

The link between HA and atherosclerosis mentioned previously [35] is interesting given that atherosclerosis is regarded as one of the major risk factors for dementia [36]. In addition, neuropathologic examinations have revealed atherosclerosis in patients with AD and VaD [37, 38]. From this perspective, it may be hypothesized that an alteration in HA production or degradation of glycocalyx, is an early event leading toward progression of dementia. In support, an interesting experimental in vivo study recently demonstrated accelerated atherosclerosis and severe damage in glycocalyx in rodents chronically treated with the HA synthesis inhibitor, 4-methylumbelliferone [7]. However, although this study suggests failure of glycocalyx function as a key event in the early stages of atherosclerosis, there is no direct evidence demonstrating increased HA degradation as the primary underlying cause of atherosclerosis. Our study demonstrates a link between increased HA degradation and vascular changes in the brain. We were unable to retrieve information on atherosclerosis in the individuals investigated in this study. Therefore we lack evidence of a direct link between this degradation and atherosclerosis. Further studies investigating the role of HA in the atherosclerotic process and VaD in the long term are therefore highly warranted.

We conclude that HA, as measured with the currently available HA immunoassays, does not fulfill the criteria of a valuable diagnostic biomarker for

VaD. Nevertheless, the significant increase in levels of HA in CSF in patients with VaD and in individuals with vascular changes as well as the correlations found with Q-albumin strongly suggest that HA plays an important role in vascular pathology–linked dementia. Our results thus encourage the development of new HA assays that specifically measure the proinflammatory and proangiogenic LMW HA molecules, in order to determine the full diagnostic potential of HA levels in CSF in patients with dementia.

ACKNOWLEDGMENTS

The work was supported by The Swedish Dementia Foundation (M.W.), ALF (K.N., L.M., M.W.), Swedish Brainpower (L.M.), Sigrud and Elsa Goljes Memory Foundation (M.W.), and Signe and Olof Wallenius Foundation (M.W.). The authors thank Camilla Orbjörn for technical support.

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Table 1

Demographic data

Variables	Control	AD	VaD
Men/women (*n*)	13/14	13/32	24/22
Age (years)	59±10	77±6***	76±7***
MMSE score	29 (26–30)	20 (6–29)***	21 (11–29)***
Aβ1–42 (ng/L)	688±237	350±96***	481±201***
T-Tau (ng/L)	296±148	829±327***	364±212
P-Tau (ng/L)	48±20	106±39***	47±17
Vascular changes, *n* (%)	1 (4)[a]	20 (43)[b]	31 (69) [c]
Increased CRP, *n* (%)	1 (4)	0 (0)	7 (15)
Head trauma, *n* (%)	3 (11)	5 (11)	0 (0)
Inflammatory diseases, *n* (%)	3 (11)	4 (9)	1 (2)
Tumors, *n* (%)	1 (4)	2 (4)	8 (17)

Data are presented as means±SD. ***Indicates a significant difference at the $p<0.001$ level compared with controls using ANOVA.

[a]Data missing (*n*=4).

[b]Data missing (*n*=10).

[c]Data missing (*n*=1).

Fig. 1. (A) HA levels in CSF from controls without dementia (Ctrl), patients with AD, and patients with VaD. (B) HA levels in CSF from individuals without vascular changes (No VC) compared with individuals with vascular changes (VC). Significant difference compared with control is indicated by $*P<0.05$. Data are presented as means±standard deviation.

Fig. 2. (A) Scatter plot demonstrating the correlation between HA levels and Q-albumin in CSF from patients with AD. (B) Correlation between HA levels and Q-albumin in CSF from patients with VaD. (C) Correlation between HA levels in CSF and MMSE from patients with AD.

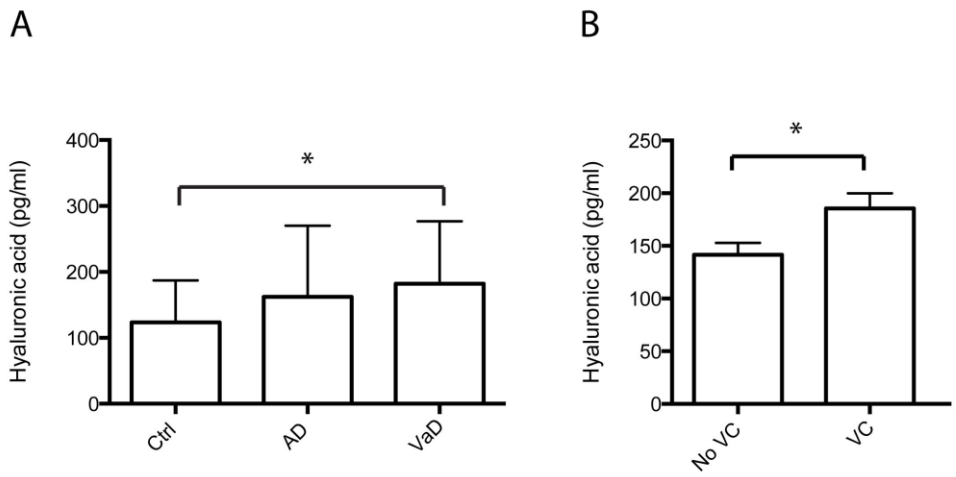
A
B
Hyaluronic acid (pg/ml)
400
300
200
100
0
*
Ctrl
AD
VaD
Hyaluronic acid (pg/ml)
250
200
150
100
50
0
*
No VC
VC

Figure 1.

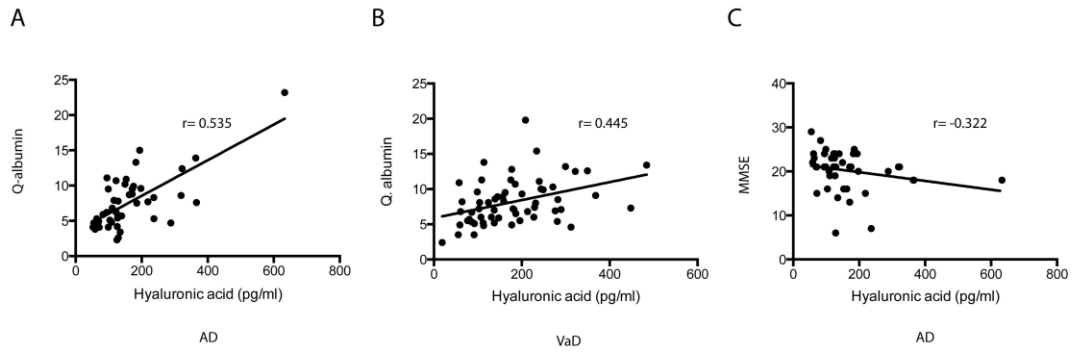
A
B
C
Q-albumin
r= 0.535
Hyaluronic acid (pg/ml)
AD
Q. albumin
r= 0.445
Hyaluronic acid (pg/ml)
VaD
MMSE
r= -0.322
Hyaluronic acid (pg/ml)
AD

Figure 2