The peripheral blood of Aβ binding RBC as a biomarker for diagnosis of Alzheimer’s disease

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Abstract

Background: in vitro, it has been reported that amyloid β (Aβ) is bound to red blood cells (RBCs) and this process damages the red cell. Also, a possible relationship between RBCs and Alzheimer’s disease (AD) is supported by the findings of RBC impairment in AD. Therefore, Aβ fibrils binding RBC are of great interest as potential biomarkers.

Methods: in this study, we focused on Aβ amyloid fibrils and/or aggregation on the peripheral RBC from 50 subjects with AD and 50 healthy controls (HCs) through thioflavin T (ThT) staining followed by immunofluorescence assay to confirm the presence of Aβ amyloid fibrils and/or aggregation on the RBC. Then we optimised fluorescence staining and imaging conditions and analysed the images obtained by image processing software.

Results: we have analysed RBC morphology in blood from 50 subjects with AD and 50 HCs found that 16.8% of the RBCs are elongated as compared with 6.7% in normal controls (P<0.01), and there is a negative correlation between the two parameters (P<0.05). Our study showed that 98% of AD peripheral RBCs were amyloid binding-positive (ranging from 2 to 30%), while only 38% that of RBCs (ranging from 2 to 3.4%) were in HCs. We also found four modified morphologies of RBCs triggered by Aβ binding, which may serve as an ancillary investigation and indicate the progression of AD.

Conclusion: we first directly prove the existence of Aβ binding RBCs in peripheral blood. In addition, we observed new modified morphologies of RBC triggered by Aβ binding, all of those can serve as a biomarker for the diagnosis and progression of AD.

Keywords: Aβ fibrils, Alzheimer’s disease, red blood cells, thioflavin T, older people

Introduction

Alzheimer’s disease (AD) accounts for up to 60% of all diagnosed cases of dementia, an age-related condition which affected ~36 million people worldwide in 2010, will rise to 66 million by 2030 [1] and is characterised by progressive degenerative disorder of the central nervous system that ultimately results in the loss of cognitive function. One of the hallmarks of AD is the existence of amyloid plaques, which are composed primarily of amyloid-beta (Aβ) peptide, in a fibrillar conformation, within the post-mortem brain of disease victims [2, 3]; therefore, studies are focused on brain Aβ deposition for its pivotal role in triggering the inflammation [4], oxidative damage [5] or other pathogenic processes of neurons and is assessed by measuring of cerebrospinal fluid (CSF) Aβ42 and total and phosphorylated tau proteins and by related neuroimaging approaches. However, lumbar puncture is a relatively invasive procedure, and post-mortem brain dissection on large numbers of elderly infirm individuals in the community is challenging; on the other hand, neuroimaging approaches, such as structural magnetic resonance

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The peripheral blood Aβ binding RBC

imaging (MRI) of specific brain regions (i.e. hippocampus), amyloid tracer imaging (i.e. Pittsburgh compound B and florbetapir), 18C-fluorodeoxyglucose and functional MRI cost more and need special equipment. Therefore, the recognised and traditional biological markers obtained from CSF and neuroimaging are nearing and have reached clinical application and still have significant limitations. Consequently, blood, which is easy to obtain, and as ~500 µl of CSF is absorbed into the blood every day, may offer such a rich source for AD biomarkers for early and specific detection of AD.

The presence of Aβ amyloid fibrils in human peripheral blood has been established and it has been hypothesised that Aβ amyloid fibrils readily contact RBC and oxidatively impair RBC function [6–9]. In support of this hypothesis are changes of RBC in subjects with AD including increased levels of lipid peroxidation [10], perturbations in the physical state of membrane proteins [11, 12], irregular distortion of RBC [6, 8] and abnormal cellular ageing with increased IgG binding and breakdown of band 3 protein [13]. Given its proposed central role in the pathophysiology of AD, Aβ fibrils may serve as a more specific diagnostic biomarker.

It is difficult to detect Aβ amyloid fibrils because they are unstable and may disassemble or assemble during the analysis process. Sensitive and high-throughput enzyme-linked immunosorbent assay (ELISA) methods to measure Aβ fibrils have been described and generally comprise two approaches: (i) using conformation-specific antibodies [14–18] and (ii) using capture and (labelled) detection antibodies that recognise the same epitope [19–21]. However, it has demonstrated that application of the first strategy for the quantification of Aβ fibrils in body fluids has been limited [16, 22] and less of specificity [23], and the second method was efficient enough for the low level of RBC binding Aβ fibrils [7], it seems less sensitive. Fluorescence spectroscopy is, however, another mostly applied technique for the study of amyloid fibrils due to the inherent sensitivity and versatility of the method and the wide variety of probes available. Two classic probes for the detection of amyloid fibrils are thioflavin T (ThT) and Congo red (CR), both of which can recognise the common structure, cross-β architecture of amyloid fibrils. In this article, we selected ThT as a specific probe for the presence of amyloid fibrils on RBC, coupled with high sensitivity of emission techniques, laser scanning confocal microscope (LSCM), after optimising fluorescence staining and imaging conditions, and we established a new direct and facile method to monitor Aβ-associated RBC in AD peripheral blood. In addition, under this method, we found new modified morphologies of RBC induced by Aβ fibrils in AD peripheral blood, which may serve as an ancillary diagnosis and progression marker of AD.

Materials and methods

Materials

ThT was purchased from Sigma-Aldrich. All other reagents that include disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride were of chemical grade. The following antibodies were used in this study: mouse monoclonal anti-Aβ1-40 antibody [3H2], epitope within amino acids 1–13 (Abcam, Cambridge, UK), tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse Ig (DAKO, Glostrup, Denmark).

Optical imaging

Bright-field and fluorescence images were acquired using a Leica inverted LSCM (Leica TCS SPE), equipped with a ×60 Leica oil immersion objective. The fluorescence was measured with an excitation wavelength of 405 nm (ThT) and 532 nm (TRITC). To monitor ThT fluorescence, an emission wavelength of 480 nm was used, and for TRITC, an emission wavelength of 480 nm was used. Image analysis of the grey-scale distribution was performed using Leica Application Suite 2.0.1 and Igor Pro software version 6.02A (WaveMetrics) installed on Windows.

Blood samples from AD and healthy control

For this study, 50 AD patients (69.70 ± 4.70) and 50 non-demented (67.82 ± 3.76) controls were selected. AD cases were diagnosed based on accepted clinical criteria (National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer’s Disease and Related Disorders Association, NINCDS–ADRDA) and standard biomarker measurements [mini-mental state examination (MMSE) score/montreal cognitive assessment score: 21.12 ± 4.35/14.46 ± 3.43]. Subjects with a history of drug abuse, chronic systemic diseases, such as diabetes mellitus and hypertension, severe head injury or seizure disorders and those who were treated with electro-convulsive therapy were excluded from the study. Blood was collected in a tube containing ethylene-diamine tetraacetic acid disodium salt as an anticoagulant directly transported to our laboratory, made blood smear and examined within 6 h. For more results, see Supplementary data available in Age and Ageing online, Table Appendix S1.

The count of elongated cells

Cells were resuspended in Phosphate buffer saline (PBS) buffer to 5% haematocrit (hct) and cell morphology was recorded in an Olympus IX-70 using Delavision software. Microscopic images were saved as TIFF files, opened by Adobe Photoshop and elongated cells were counted manually in each frame. Then all the calculation and statistical analysis were performed using SPSS software.

Immunofluorescence assay

For determination of Aβ fibrils on RBC, smears were made with fresh whole blood and fixed in methanol. Mouse monoclonal anti-Aβ diluted 1:2,500 in blocking buffer and a 1:100 dilution in blocking buffer of TRITC-conjugated goat anti-mouse IgG antibody were used as the primary and secondary antibodies, respectively. After the immunofluorescence assay the
same smears were stained with ThT (200 µM, pH 8.4, dissolved with PBS) for 5 min and examined under a microscope quickly.

**ThT staining with RBCs in or without the presence of plasma**

For ThT staining with whole blood, the whole blood was adjusted to 5% hct in ThT solutions (100 µM, pH 8.4, dissolved with PBS) and incubated at 37°C for 5 min. For ThT staining with RBCs without plasma, blood sample was centrifuged at 1000 × g for 10 min at 4°C. After plasma and buffy coat were removed, the RBCs were washed three times with PBS (pH 7.4) to prepare packed cells. The packed cells were adjusted to a 5% hct with ThT solutions (100 µM, pH 8.4, dissolved with PBS) and incubated at 37°C for 5 min. Bright-field and fluorescence images were acquired using a microscope.

**Results and discussion**

In our study, when we viewed the RBC with a counting chamber in the optical microscope, we found the differences of RBC in morphology and size. For more results, see Supplementary data available in *Age and Ageing* online, Figure Appendix S1. Consistent with our study, an in vitro study has already showed that in AD >15% of the RBCs are elongated as compared with 5.9% in normal controls [7]. As the percentage of RBC in AD patients is significantly higher than in age-matched control individuals, and there is a negative correlation between the two parameters (P < 0.01), it was suggested that the morphology and size of peripheral RBC may represent a risk factor for AD and may contribute to the development of dementia. It was hypothesised that the observed changes could be due to changes in the level of the protein components of the cytoskeleton and those linked to the RBC membrane and in phospholipid composition of erythrocyte membrane [11, 12]. These changes are likely to originate from modifications caused by Aβ interactions with RBCs, which need to be further studied.

**ThT can recognise Aβ amyloid fibrils and/or aggregation associated with RBC specifically**

In this article, to determine whether these morphology changes can ascribe to the binding of Aβ fibrils and/or aggregation, we selected ThT, which have been historically used for the detection of amyloid deposits in biopsies and in ex vivo post-mortem samples, as well as for kinetic studies of amyloid formation in vitro owing to its characteristics of strong increase in fluorescence upon binding to amyloid fibrils [24, 25], to directly probe fibrils and/or aggregation on RBC in the complex environment of living AD peripheral blood, coupled with the anti-Aβ peptide, we eventually achieved visualised, rapid and highly parallelised analytical determinations of amyloid fibrils and/or aggregation localised on RBC specifically (Figure 1, arrows). This study suggests that the changes of RBC in morphology and size are likely to originate from modifications caused by Aβ interactions with RBC.

The immunofluorescence assay also showed that anti-Aβ peptide is positive, while ThT-β-sheet rich fibrils are negative; it can be explained by the fact that RBC themselves contain Aβ peptide immunoreactivity [26] and increase with age [27].

We studied with 50 AD and 50 age-matched healthy controls (HCS), showing that 98% of AD peripheral RBCs were amyloid binding-positive (ranging from 2 to 30%), with only 38% that of RBCs (ranging from 2 to 3.4%) in HCs. For more results, see Supplementary data available in *Age and Ageing* online, Table Appendix S2. In addition to other factors, Aβ amyloid fibrils and/or aggregation binding may be the main reasons for the changes of RBCs.

**Figure 1.** Immunofluorescence assay of Aβ-rich amyloid fibrils using confocal microscopy, confocal microscopy images of Aβ-rich amyloid fibrils (F and B), anti-Aβ peptide immunofluorescence (G and C) and transmitted light (E and A) of RBCs from control subjects (E–H) and patients with AD (A–D). Scale bar: 10 µm.
Simple mental state examination (MMSE) is the most widely used cognitive screening scale, which was evaluated by the change of directional power, memory, attention, calculation, language ability and visual. In this article, we did not observe the relation between the percentage of amyloid binding-positive RBCs and MMSE score in AD, suggesting that the low level of Aβ binding RBCs, owing to short survival of Aβ binding RBCs in peripheral and to the transfer of Aβ on RBCs to unmodified RBCs and to endothelium of vascular, cannot result in those changes of cognitive behaviour used in MMSE evaluation. For more results, see Supplementary data available in Age and Ageing online, Figure Appendix S2.

It has been accepted that Aβ readily contacts RBC and oxidatively impairs RBC functions by binding to them, causing RBC phospholipid peroxidation and diminishing RBC endogenous carotenoids [9]. Our study showed the direct evidence of Aβ binding to RBC. Therefore, we hypothesised that this type of binding can result in RBC overoxidation, potentially reducing oxygen delivery to the brain and facilitating AD.

**ThT staining with RBCs in or without the presence of plasma**

The amphoteric and amphipathic characteristics of the Aβ peptides endow these molecules with a capacity to interact with a large number of plasma proteins such as albumin, α2-macroglobulin, α1-antichymotrypsin, amyloid P component, complement proteins, transthyretin, apoferritin, apolipoproteins and lipoproteins [28], so binding of Aβ fibrils to whole blood in vitro compared with binding of Aβ fibrils to RBC without plasma indicates a 5-fold increase in the number of binding sites in the absence of plasma [7]. To determine whether plasma in the circulation would prevent binding of ThT to amyloid fibrils on RBCs, the binding of Aβ amyloid fibrils in or without the presence of plasma was studied in vitro (Figure 2). Both the high contrast fluorescence images indicate that ThT can recognise Aβ amyloid fibrils and/or aggregation on RBC even if in whole blood.

A high affinity of ThT for amyloid fibrils and/or aggregation, leading to accumulation of the dye on the RBC and the concentration of ThT in the stained RBC, was higher than the concentration of ThT in the surrounding staining fluid.

**Figure 2.** ThT staining with RBCs in or without the presence of plasma. Confocal microscopy images of Aβ fibrils ThT immunofluorescence (B, E and H), and transmitted light (A, D and G) of RBCs from control subjects (G–I) and patients with AD with (A–C) or without (D–F) plasma. Scale bar: 10 µm.
Figure 3. Confocal microscopy analysis of morphologies for Aβ amyloid fibril binding RBCs. Confocal microscopy images of ThT-positive (blue) of RBCs from control subjects (A) and patients with AD (B–E), and image line profile analysis HC (F) and AD (G–J). ΔI represents the elongated diameter of RBCs from AD when compared with normal controls. Upper line, transmitted light; lower line, fluorescent. Scale bar: 7.5 μm.
solution. Therefore, a sample wash was not required after the staining and prior to imaging, which not only provided experience of this approach but also allowed for preserving the fibrils associated RBCs physiologically intact, which is essential for imaging and pathology analysis.

New modified morphologies of erythrocytes triggered by Aβ binding and discovered by ThT staining assay

In our study, we found the differences of RBC in morphology and size when we viewed the RBC with a counting chamber in the optical microscope. To determine whether the morphology abnormal triggered by Aβ binding, we did ThT staining assay (Figure 3), finding that RBCs elongated with a non-biconcave shape of AD subjects, such as crenated (Figure 3B), partial crenated (Figure 3C and D) and spherical (Figure 3E), were all ThT-positive. Therefore, we hypothesised that the observed abnormal morphology of RBCs from AD subjects was likely to originate from perturbation caused by Aβ binding, and all of those can serve as a biomarker for the diagnosis and progression of AD.

Conclusions

In this study, we first directly prove the existence of Aβ binding RBCs in peripheral blood. In addition, we observed new modified morphologies of RBCs triggered by Aβ binding, and all of those can serve as a biomarker for the diagnosis and progression of AD.


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