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Acetylcholinesterase provides new insights into red blood cell ageing \textit{in vivo} and \textit{in vitro}

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\section*{Introduction}
During its life of 120 days in the circulation, functionality of the red blood cell (RBC) is maintained and cell removal is postponed by disposal of modified haemoglobin and damaged membrane components through vesiculation. When vesiculation capacity has reached its limits, the ongoing mechanical, osmotic and oxidative stress induces recognition and removal of the old RBC by the immune system\textsuperscript{1-3}. The same mechanisms that cause the decrease in deformability, adenosine triphosphate (ATP) production and maintenance of redox status, are also likely to be responsible for the generation of removal signals. This process revolves around band 3 and its partners\textsuperscript{1,2}.

Although considerable progress has been made in the characterisation of RBCs of various ages by comparative proteomics and metabolomics, the molecular details of the ageing mechanism(s) remain elusive\textsuperscript{2,4-6}. This is mainly due to the practical and ethical limitations of manipulating RBCs in healthy volunteers. Insights obtained from investigating patients with RBC-centred pathologies are obscured by disease-specific and systemic effects\textsuperscript{7,8}. Thus, storage in blood bank conditions may be the most valid model for the elucidation of RBC ageing processes, with the highest translational value\textsuperscript{9-11}.

In all conditions, the study of RBC ageing is hampered by the absence of an easily determined, unambiguous, general age marker. Acetylcholinesterase (AChE), linked to the RBC membrane through a glycosylphosphatidylinositol (GPI) anchor, might be such a marker for RBC ageing \textit{in vivo}\textsuperscript{12}. Determination of AChE characteristics may also yield information on the mechanism of ageing-associated vesicle formation. Microvesicles generated after an increase in intracellular calcium concentration or after mechanically-induced membrane deformation \textit{in vitro} have been described to be enriched in AChE as well as other GPI-anchored proteins such as CD55 and CD59, with a concomitant depletion of these proteins in the cellular membrane\textsuperscript{13-15}. In addition, we have found that the membrane distribution of AChE in vesicles generated during
storage in blood bank conditions changes with storage time\textsuperscript{16}. Therefore, we investigated the structural and functional changes that AChE undergoes during RBC ageing \textit{in vivo}, and compared this with ageing \textit{in vitro} during storage in blood bank conditions.

**Materials and methods**

**Red blood cell sampling**

Fresh RBCs were isolated from 5 mL whole EDTA-blood from healthy volunteers. Written informed consent was obtained from all donors. The study was performed following the guidelines of the local medical ethical committee and in accordance with the Declaration of Helsinki. Storage experiments were performed with RBCs from 5 leucodepleted red cell concentrates (3 blood group A RhD-positive donors, 1 blood group A RhD-negative donor, 1 blood group O RhD-positive donor), as described before\textsuperscript{17}. Samples of 10 mL were taken at 1, 7, 14, 21, 28 and 35 days after blood collection. RBCs were isolated from fresh blood and leukocytes and platelets were removed as described before\textsuperscript{17}. RBCs were extensively washed three times with Ringer (125 mM NaCl, 5 mM KCl, 1 mM MgSO\textsubscript{4}, 32 mM Hepes, 5 mM glucose, 2.5 mM CaCl\textsubscript{2}, pH 7.4) by repeated centrifugation for 15 min at 700 g.

**Red blood cell fractionation**

Red blood cells were fractionated according to cell volume followed by a fractionation according to cell density as described earlier\textsuperscript{18}, and fractions were combined to achieve 5 fractions ranging in cell age from young to middle-aged to old cells, as determined by their HbA1c content\textsuperscript{3}.

Red blood cell fractionation according to density was performed using a discontinuous Percoll gradient consisting of six layers ranging from 0% Percoll (1.060 g/mL) to 80% Percoll (1.096 g/mL) and the resulting layers were combined into top, middle and bottom layer fractions, basically as described before\textsuperscript{17,18}.

**Acetylcholinesterase activity assay**

Acetylcholinesterase activity of intact RBCs at a concentration of $2\times10^6$ cells/mL and RBC-derived vesicles was measured using a modified Ellman's method under $V_{\text{max}}$ conditions, basically as described before\textsuperscript{12-15}. The AChE activity of vesicles was measured using the supernatant obtained after centrifugation for 30 min at 2,880 g in the presence of the butyrylcholinesterase inhibitor tetraisopropyl pyrophosphoramide (0.1 μM, Sigma Life Sciences, Zwijndrecht, The Netherlands). One arbitrary AChE enzyme unit was defined as an increase in absorbance at 412 nm of 0.005 per minute, determined from the slope of the initial part of the absorption vs the time curve that was obtained by linear regression analysis.

**Flow cytometry**

The presence of AChE on RBCs was examined by flow cytometry using goat anti-AChE ab34533 (biotin, 1:25; Molecular Probes, ABCAM, Uithoorn, The Netherlands), detected by a rabbit anti-goat labeled with Alexa 488 (1:200; Molecular Probes). RBCs were analysed with a flow cytometer (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA) and its accompanying software (CELLQUEST; Becton Dickinson). The results are expressed as percentages of AChE-positive RBCs and/or their mean fluorescence intensity (MFI). Vesicles were isolated and analysed by flow cytometry, as described previously\textsuperscript{19}.

**Microscopy**

Cells were suspended in Ringer and RBC morphology was assessed by classifying the cells in normocytes, echinocytes, cells of indistinct morphology (misshapen cells), stomatocytes, macrocytes, spherocytes, ovalocytes and echinocytes in random field pictures, using confocal laser microscopy, as described before\textsuperscript{20}. At least 100 cells were analysed per sample.

**Results**

In order to investigate the structural and functional changes in acetylcholinesterase upon ageing \textit{in vivo} and \textit{in vitro}, we started by determining enzymatic activity. RBCs isolated from whole blood and separated according to cell age by a double separation method showed a decrease in activity with increasing cell age

**Table I - Red blood cell (RBC) acetylcholinesterase (AChE) activity during ageing \textit{in vivo}.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>AChE activity ±SD</th>
<th>MFI anti-AChE</th>
<th>Layer</th>
<th>AChE activity ±SD</th>
<th>MFI anti-AChE ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>100 ±0.4</td>
<td>100</td>
<td>Top</td>
<td>100 ±0.4</td>
<td>100 ±0.4</td>
</tr>
<tr>
<td>Middle-aged</td>
<td>83.70±30.71</td>
<td>102.69</td>
<td>Middle</td>
<td>79.70±16.01</td>
<td>97.96±11.11</td>
</tr>
<tr>
<td>Old</td>
<td>68.73±18.34</td>
<td>99.78</td>
<td>Bottom</td>
<td>61.23±8.53</td>
<td>111.6±4.2</td>
</tr>
</tbody>
</table>

Acetylcholinesterase activity was measured for RBCs separated according to cell age using double separation, and on RBCs separated according to density using Percoll gradients (see "Materials and methods"). Enzyme activity, expressed as arbitrary units, and protein content, expressed as mean fluorescence intensity (MFI), were determined using a spectrophotometric assay and flow cytometry, respectively (see "Materials and methods").$^*$p<0.05, as determined by ANOVA, Tukey's multiple comparison test, as compared with the young fraction or top layer\textsuperscript{21}. SD: standard deviation; N: number; RBC: red blood cell.
(Table I). Similar differences were observed when cells were separated based on differences in cell density only (Table I). This loss of activity could be due to a selective loss of AChE-containing membrane by vesiculation. Quantitation of AChE content by flow cytometry, however, showed no differences between the various cell fractions (Table I). These data indicate that RBC ageing in vivo is not accompanied by a selective loss of AChE protein, but by a decrease in enzyme activity.

In contrast, during ageing in vitro, i.e. during storage as red cell concentrates in the blood bank, the mean AChE activity of RBCs did not undergo a statistically significant change with storage time (Figure 1A). However, there was considerable variation between the red cell concentrates (data not shown), probably due to biological, inter-individual variability in the response to the artificial storage conditions. Measurement of the AChE activities of RBCs in density-separated fractions did not show a consistent correlation between enzyme activity and density, although there was a significant decrease in the most dense cell layer after 21 days of storage, which was not observed at later time points (Figure 1B). These observations are in stark contrast to the differences between the activity of Percoll-separated RBCs of fresh samples, i.e. before they underwent the red cell concentrate production procedure. Already on the first day, the AChE activity of the least dense RBCs was significantly reduced compared with the activity of fresh RBCs (Figure 1C). In contrast to the RBCs, pronounced changes were observed in RBC-derived vesicles. During storage, the vesicle concentration increases from a vesicle/RBC ratio of 1.0 at days 7 and 14 to a vesicle/
RBC ratio of 1.5 at day 21, and a vesicle/RBC ratio of 4.3 on day 35, as has been shown before. The AChE activity of these vesicles, however, showed a strong, steady decrease during storage (Figure 1D).

CD59, another GPI-linked protein showed no change in cell content with storage time, neither in the whole population nor in the various cell fractions (Figure 2A). Similar data were obtained with an anti-AChE antibody and comparable data have been obtained for ageing in vivo. In addition to GPI-anchored proteins, we included the integral membrane protein CD235a (glycophorin A) in our analyses. Even though there were no storage-associated changes in the total cell population, there was a decrease in glycophorin A content in the higher density RBCs of the middle and bottom Percoll layers starting after one week of storage (Figure 2B). These data indicate a selective disappearance of integral membrane proteins upon storage.

Ageing in vivo as well as ageing in vitro have been described to be accompanied by changes in cell shape. We performed a semi-quantitative analysis of RBC morphology during storage, both in unseparated samples and in the RBC fractions obtained after density separation. Our data confirm previous data, showing an overall decrease in the fraction of normocytes when storage time increases, with a concomitant increase in the numbers of echinocytes and otherwise misshapen cells (Figure 3). Morphological analysis of the various Percoll...
layers showed considerable variation, even within one red cell concentrate during storage (data not shown), but in general this change occurred in all layers (Figure 3A and B), and the most dense layers are enriched in echinocytes already after one day of storage (Figure 3A). We found no statistically significant correlations between cell morphology, AChE activity or AChE content.

Discussion

Flow cytometry measurements of protein expression show no alterations in cellular AChE content with ageing in vivo (Table I). Together with previous data on other GPI-linked proteins such as CD55 and CD59, as confirmed here, these findings suggest that there is no specific loss of GPI-linked proteins from the RBCs by vesiculation in physiological circumstances. Thus, previous findings suggesting selective sorting of GPI-linked proteins and microdomain-associated proteins during RBC vesiculation13,16,22 are likely to be related to the method that was employed to generate such vesicles in vitro16. This conclusion is supported by proteomic data that show selective membrane and cytoskeletal protein sorting during vesicle formation, but also indicate that different mechanisms underlie vesicle generation in vivo and in vitro5. In contrast, AChE enzyme activity clearly decreases with ageing in vivo. Such a decrease was already visible after Percoll separation only (Table I). Since this density-based separation generates fractions that are only moderately enriched with young but not with older cells20,23, this finding strongly suggests that the first and major loss of enzyme activity in vivo occurs relatively early in RBC life. Thus, AChE activity, but not AChE amount, constitutes a sensitive biomarker of RBC ageing in vivo. This apparent divergence between changes in expression and activity of one GPI-linked protein indicates that the absence of a clear ageing-associated decrease in CD55 and CD59 content, as has been reported previously3, does not necessarily imply sustained protection against complement activation-associated opsonisation during ageing24. Thus, during ageing in vivo, CD59 may very well undergo conformational changes that decrease its capacity to shield the RBC from the complement system. This implication of our findings warrants further investigation of the role of complement in RBC removal in physiological conditions.

The conspicuous discrepancy between AChE protein content and enzymatic activity becomes even more pronounced in the vesicles that are produced with increasing storage time (Table I and Figure 1D). This apparent enrichment of inactivated AChE in vesicles confirms previous proteomic and immunological data showing a removal of damaged membrane components by vesiculation, both in vivo and in vitro3-5,25-27. Thus, vesicles in red cell concentrates may constitute a rich and relatively pure source of damaged AChE, which may facilitate a molecular identification of the processes that cause the underlying structural alterations during ageing in vivo and in vitro. Such an identification will help in understanding the unwanted side effects of transfusion, especially with regard to complement-mediated haemolysis in susceptible patients.

The data obtained by determination of AChE expression and activity during RBC storage in blood bank conditions emphasise the differences between ageing in vivo and ageing in vitro. In general, neither AChE expression nor enzymatic activity decreased significantly with storage time (Figure 1A). This result is in agreement with previous data showing no significant or only small alterations in AChE activity after prolonged storage times26. Also, there were hardly any consistent differences between the RBCs of the different Percoll layers (Figure 1B). Many other observations show storage-associated changes in morphology (Figure 39), metabolism and membrane protein composition5,12.
A large inter-individual variability, together with an altered osmotic behaviour in response to the storage medium\textsuperscript{16}, are likely to obscure any ageing-related changes occurring in blood bank conditions. Data on phosphatidylserine exposure support these hypotheses, as does the absence of a decrease in cell volume during storage, in spite of the large increase in vesicles\textsuperscript{2,16,25,29}. These phenomena also hamper the interpretation of the flow cytometry data on CD59 and glycophorin A content with storage (Figure 2). A comparison of the various density fractions, however, suggests that during storage integral and lipid-linked membrane proteins undergo different fates when the RBCs change from discocytes to echinocytes (Figures 2 and 3). Such fates are likely to be dependent on selective removal by vesiculation.

The relatively large difference in AChE activity between fresh and 1-day old RBCs (Figure 1C) is likely to be caused by loss of AChE in vesicles that are generated during the mechanical stress that the RBCs undergo during the production of the red cell concentrates. The small, but significant increase in phosphatidylserine-exposing RBCs during blood bank processing and/or in the first week of storage supports this theory\textsuperscript{21}.

Conclusions

The physiological role of RBC-bound AChE is not clear, but our findings may provide new tools for studying the putative role of acetylcholine on RBC physiology, and the impact of transfusion on acetylcholinesterase-mediated RBC homeostasis and survival, and on the circulation\textsuperscript{30-33}.

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Authorship contributions

JKFL and GJCGMB performed the experiments, JKFL, MJWA-H, RB and GJCGMB designed the experiments. All Authors discussed the results and wrote the manuscript.

The Authors declare no conflicts of interest.

References


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