Erythrocyte and plasma protein modification in alcoholism: A possible role of acetaldehyde

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Abstract

Analysis of the oxidative modification of plasma and erythrocyte ghost proteins of chronic alcoholic subjects and healthy non-alcoholics has been performed. It was found that increased levels of protein carbonyls in both plasma and erythrocyte ghosts from alcoholic subjects occurred in comparison to the levels found in preparations from non-alcoholics. Plasma proteins from alcoholic subjects did not show evidence of cross-linking, although plasma protein concentration and composition were changed. In alcoholic subjects who displayed no evidence of abnormal erythrocyte morphology no cross-linking of erythrocyte ghost proteins was detectable, whereas the ghosts obtained from alcoholic subjects who displayed morphologically abnormal erythrocytes contained cross-linked proteins. The in vitro treatment with acetaldehyde of erythrocytes from non-alcoholics caused increased levels of protein carbonyls and cross-linking products in erythrocyte ghost preparations which were similar to those found in severe alcoholics. It is concluded that chronic alcohol consumption can cause abnormal erythrocyte morphology and increased erythrocyte fragility as a result of oxidation and cross-linking of erythrocyte ghost proteins. These effects can be ascribed, in part, to exposure of erythrocytes to circulatory acetaldehyde which is a product of ethanol metabolism.

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1. Introduction

The chronic oxidative stress in alcoholism can lead to oxidative modification of proteins [1–3]. Such modification occurs principally by formation of protein carbonyls [4], and it has been shown that the levels of protein carbonyls in blood and other tissues are increased in experimental animals treated with ethanol [5–7].

An immediate product of ethanol metabolism is acetaldehyde, and unlike ethanol, this is a highly reactive and extremely cytotoxic compound [8] which is involved in a number of non-enzymatic modifications of proteins, including formation of semialdehydes, mercaptosialdehydes and Schiff bases with consequent protein modification and inhibition of their biological functions [9]. The chronic administration of ethanol to experimental animals has been shown to cause formation of acetaldehyde–protein adducts [1] and antibodies to malondialdehyde–acetaldehyde protein adducts [10].

The principal site for acetaldehyde production from ethanol is in the liver, and it is known that after copious ethanol consumption, acetaldehyde (at sub-millimolar concentrations) and ethanol appear in the circulatory system from where they can then be taken up in peripheral tissues [11,12]. In addition to the known toxic effects of ethanol consumption on tissues such as muscle [13] and the nervous system [14,15], significant damage can occur in the circulatory system itself in alcoholism, including T-lymphocyte immunosuppression [16], platelet function abnormalities [17,18], and abnormal erythrocyte morphology and stability [19–21].
Although acetaldehyde may be responsible for much of the damage that occurs in the circulatory system in alcoholism, where it has a lifetime of about 3 hours \textsuperscript{[12]} and can reach concentrations of up to 550 μM \textsuperscript{[22]}, there is relatively little specific recent information about the action of acetaldehyde on blood cells and proteins \textsuperscript{[23]}. The initial work on blood protein modification by acetaldehyde \textsuperscript{[24]} showed that the in vitro treatment of isolated erythrocyte ghosts by acetaldehyde (5–10 mM) for 2 h at 4 °C produced cross-linked proteins, and that exposure of normal human erythrocytes to 1 mM acetaldehyde for 2 h at 4 °C caused poikilocytosis and stomatocytosis. Later work \textsuperscript{[25]} showed that in vitro incubation of plasma, erythrocytes and erythrocyte ghosts with 40 μM acetaldehyde increased the protein carbonyl contents, and that this effect did not occur with ethanol. In our more recent studies, we have shown that erythrocytes from alcoholics have abnormal morphology and decreased resistance to hemolytic agents \textsuperscript{[20]}. A high concentration (55 mM) of acetaldehyde was found to decrease erythrocyte resistance to hemolysis \textsuperscript{[21]}, but in vitro exposure of normal human erythrocytes to 2 μM and 3.6 mM acetaldehyde for up to 16 hours did not induce any changes in cell morphology, vulnerability to oxidation, or elevated rates of hemolysis \textsuperscript{[26]}.

In the present studies, we have examined the long term in situ effects of pathological ethanol consumption on both plasma and erythrocyte proteins using blood samples obtained from chronic alcoholics. We have also investigated if the damage to erythrocytes of alcoholics which was found in these in situ studies could be produced by in vitro treatment of normal erythrocytes with acetaldehyde.

2. Materials and methods

2.1. Materials

Hanks’ balanced salt solution (item H4891), BSA, heparin, primary rabbit anti-dinitrophenyl (DNP) antibodies and secondary goat anti-rabbit, Ig alkaline phosphatase conjugate were obtained from Sigma Chemical Co., St Louis, MO. Sodium hypochlorite was obtained from Aldrich Chemical Co, Milwaukee, and 2,4-dinitrophenylhydrazine was purchased from Chemapol (Czech Republic).

Table 1
Clinical profiles of subjects used in the study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control subjects (n=22)</th>
<th>All alcoholics (n=42)</th>
<th>Group I alcoholics (n=7)</th>
<th>Group II alcoholics (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>44.0±9.9</td>
<td>39.3±10.5</td>
<td>34.6±7.1</td>
<td>40.2±10.9</td>
</tr>
<tr>
<td>Duration of chronic drinking (years)\textsuperscript{a}</td>
<td>15.3±11.0</td>
<td>13.0±9.2</td>
<td>13.0±9.2</td>
<td>15.7±11.4</td>
</tr>
<tr>
<td>Ethanol intake (g/d)\textsuperscript{b}</td>
<td>250±90</td>
<td>270±80</td>
<td>270±80</td>
<td>240±80</td>
</tr>
<tr>
<td>Erythrocyte count (×10\textsuperscript{12}/l)</td>
<td>4.73±0.26</td>
<td>4.57±0.36</td>
<td>4.15±0.28\textsuperscript{b}</td>
<td>4.66±0.31</td>
</tr>
<tr>
<td>% Normal erythrocytes</td>
<td>87.2±5.0</td>
<td>66.8±20.9</td>
<td>38.3±9.6\textsuperscript{c}</td>
<td>80.1±4.6\textsuperscript{b}</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>15.00±0.63</td>
<td>13.99±1.26</td>
<td>11.97±1.13\textsuperscript{b,c}</td>
<td>14.40±0.82</td>
</tr>
<tr>
<td>Blood protein (g/dl)</td>
<td>7.44±0.49</td>
<td>8.29±0.53\textsuperscript{b}</td>
<td>8.22±1.03</td>
<td>8.31±0.40\textsuperscript{b}</td>
</tr>
<tr>
<td>Serum ALT (mmol/ml/l)</td>
<td>0.1–0.7\textsuperscript{d}</td>
<td>0.85±0.70</td>
<td>0.94±0.44</td>
<td>0.83±0.75</td>
</tr>
<tr>
<td>Serum AST (mmol/ml/l)</td>
<td>0.1–0.5\textsuperscript{d}</td>
<td>0.38±0.30</td>
<td>0.62±0.47</td>
<td>0.33±0.23</td>
</tr>
</tbody>
</table>

\textsuperscript{a} These values were self-reported by the subjects and may not be reliable. The values for ethanol consumption were approximated to the nearest 10 ml/day.

\textsuperscript{b} P<0.05 in comparison to control group.

\textsuperscript{c} P<0.05 in comparison to Group II alcoholics.

\textsuperscript{d} Normal range quoted in Russian open medical vade-mecum.

Fig. 1. Scanning electron micrographs of erythrocytes from a control subject and a Group I (anemic) alcoholic. Image A shows the control subject’s erythrocytes and image B shows those of the alcoholic subject. The scale bar in each micrograph represents 8 μm.

2.2. Erythrocyte sources and preparation

Blood was donated at the Mental Health Research Institute, Russian Academy of Medical Sciences, Tomsk. Institutional approval for the use of human subjects in this study and the prior informed consent of all donors were obtained. Blood samples were obtained from 22 adult male donors in normal health and from 42 adult male alcoholic patients who had abstained from alcohol consumption for between 1 to 5 days before blood donation. All of the subjects in the study were smokers. Heparin (25 units/ml of blood) was used to prevent coagulation of the drawn blood and erythrocyte purification was performed as
previously described [27] using Hanks’ buffered salt solution (HBSS) as the wash buffer.

Incubation of erythrocytes with various concentrations of acetaldehyde was performed in HBSS at 37 °C using filled sealed vials to prevent acetaldehyde evaporation. After incubation, the cells were collected by centrifugation at room temperature (600×g for 10 min) and then hemolysed by osmotic shock, after which erythrocyte ghosts were prepared [28].

2.3. Analysis of protein carbonyls and cross-linked proteins

Protein carbonyls in erythrocyte ghosts and plasma were measured immunochemically using slot-bLOTS after treatment of the proteins with DNPH. For the identification of the DNPH-labeled proteins, primary anti-dinitrophenyl (DNP) antibodies and a secondary anti-rabbit Ig alkaline phosphatase conjugate were used. Bovine serum albumin treated with 5 mM NaOCl for 1 hour at 25 °C was used as a standard for protein carbonyl-containing protein [29]. Quantitative analysis of slot-blot bands (triplicate samples) was accomplished using the OneDscan program (Scanalytics, USA).

Proteins (20 μg total) in erythrocyte ghost and plasma preparations were separated using SDS-PAGE with a 7.5% running gel and a 4% stacking gel, and then visualized by staining with Coomassie Brilliant Blue [30]. Thyroglobulin (330 kDa), phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), lactate dehydrogenase (36 kDa) and carbonic anhydrase (30 kDa) were used as molecular weight markers.

2.4. Statistical analysis

The data in the tables and figures are presented as means±SD. One-way ANOVA using the Dunnett method was performed to determine significant differences between data sets. Data sets with \( P < 0.05 \) were considered to be significantly different.

3. Results

3.1. Clinical profiles of the donor groups

Analysis of blood samples from alcoholics revealed that 7 of the 42 subjects were anemic as evidenced by a decreased hemoglobin concentration and a lowered erythrocyte count (Table 1), although no reticulocytosis was evident by light microscopy. As shown in Table 1 and Fig. 1, these subjects also had a large proportion of morphologically abnormal erythrocytes showing acanthocytosis, stomatocytosis, anisocytosis and poikilocytosis. Accordingly, two groups of alcoholic subjects were designated; Group I which consisted of those subjects who had anemia and erythrocyte populations with extensive abnormal morphology, and Group II which was comprised of non-anemic subjects with proportions of undistorted erythrocytes that were not significantly different from those of the control subjects (Table 1). Other relevant clinical parameters for the control and alcoholic groups are shown in Table 1. Plasma protein levels in all alcoholics and Group II alcoholics were significantly higher than in control subjects, but although the Group I alcoholics also showed an increase in mean protein levels, this figure was not significantly different from the control mean. There were also elevations above the normal range in serum alanine aminotransferase (ALT) activities in both Group I and Group II alcoholics and for serum aspartate aminotransferase (AST) for Group II alcoholics compared to control subjects, which are indicative of liver damage in the alcoholic subjects.

Table 2

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Protein carbonyl content (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythrocyte ghosts</td>
</tr>
<tr>
<td>Controls</td>
<td>0.131±0.096</td>
</tr>
<tr>
<td>All alcoholics</td>
<td>0.669±0.141\textsuperscript{a}</td>
</tr>
<tr>
<td>Group I alcoholics</td>
<td>0.760±0.090\textsuperscript{a}</td>
</tr>
<tr>
<td>Group II alcoholics</td>
<td>0.623±0.102\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Indicates a significant difference (all \( P < 0.001 \)) between means for the control group (\( n=6 \)) compared to all alcoholics (\( n=10 \), alcoholics with anisocytosis and poikilocytosis (Group I, \( n=3 \)), and alcoholics with no anisocytosis or poikilocytosis (Group II, \( n=7 \)).

Fig. 2. SDS-PAGE analyses of plasma and erythrocyte ghost proteins. Arrows show the positions of molecular weight standards with \( M_r \) values in kDa. Plasma protein (A) and erythrocyte ghost preparations (B) from control subjects and alcoholics were prepared and analyzed as described in Materials and methods. Sample identities in A and B are: 1 and 2, control (healthy) subjects; 3 and 4, Group II alcoholics; and 5 and 6, Group I alcoholics. Asterisks by gels A and B show the respective positions of the 52 kDa and 220 kDa bands whose levels are elevated in samples from alcoholics. In C, the effect of acetaldehyde on the protein composition of erythrocyte ghosts from a non-alcoholic control subject is shown. Samples were incubated with various concentrations of acetaldehyde for 5 h at 37 °C and then analyzed as described in Materials and methods. Sample identities are: 1, erythrocyte ghosts not treated with acetaldehyde; 2, erythrocyte ghosts treated with 11 mM (0.05%) acetaldehyde; 3, erythrocyte ghosts treated with 22 mM (0.1%) acetaldehyde; and 4, erythrocyte ghosts treated with 55 mM (0.25%) acetaldehyde.
3.2. Determination of protein carbonyl levels in erythrocyte ghosts and plasma

The results of quantitative analysis of protein carbonyl contents in erythrocyte ghost and plasma samples from the two groups of alcoholics and healthy donors are shown in Table 2. These results show that erythrocyte ghost and plasma preparations from all alcoholics and from both Group I and II alcoholics have significantly increased amounts of oxidized proteins in comparison to control subjects. The protein carbonyl contents in erythrocyte ghosts and plasma were higher in Group I than in Group II alcoholic samples, although the P values for the differences were above 0.05.

3.3. SDS-PAGE analysis of plasma and erythrocyte ghost proteins

As shown in Fig. 2A, the plasma protein profiles of alcoholics from both groups showed no evidence of higher molecular weight components in comparison to the control group, and the only difference between the alcoholic and control samples was an increased relative amount of a 52 kDa protein which migrates in front of the predominant albumin band. It appears unlikely that this band is a cross-linked product caused by ethanol intoxication as it was present in the plasma of control subjects and because there were no major changes in the lower molecular weight bands of the plasma samples of the alcoholics.

In contrast, the erythrocyte ghost profiles of the Group I and II alcoholics (Fig. 2B) showed increases in the amounts of a 220 kDa protein band in comparison to the control, and the Group I alcoholics also showed increased amounts of high molecular weight proteins at the top of the running gel and of very high molecular weight protein at the top of the loading gel. A further difference in the erythrocyte ghost profiles was that Group I alcoholic samples had lower relative levels of protein bands below 94 kDa in comparison to the Group II alcoholic and control samples, suggesting that in alcoholics the increases in the 220 kDa band and other higher molecular weight proteins are cross-linked products derived from the lower molecular weight components.

3.4. Analysis of the in vitro effects of acetaldehyde on proteins of erythrocyte ghosts

Because we found changes in both oxidized protein levels and cross-linking effects in the erythrocytes of Group I alcoholics, experiments were performed to determine if in vitro acetaldehyde treatment could cause these effects in erythrocytes of control subjects.

In experiments to determine oxidized protein levels, incubation of normal erythrocytes with 55 mM (0.25%) acetaldehyde was performed for 1, 2.5 and 5 h, after which protein carbonyl contents were measured by the immunochemical slot-blot procedure. As shown in Table 3, protein carbonyl levels in the ghosts were significantly increased in a time-dependent manner at this non-physiological level of acetaldehyde, suggesting that lower chronic in vivo levels of circulatory acetaldehyde could also give rise to this phenomenon over a longer period of time.

These studies on chronic alcoholics clearly show that there is an increase in oxidized proteins in their plasma and erythrocyte membranes, but only an increase in higher molecular weight proteins in their erythrocyte membranes. The higher molecular weight proteins appears to result from cross-linking reactions, and not from de novo protein synthesis, as very similar patterns were not assessed. We also found that there was a significant increase in plasma protein concentration in the Group II (but not the Group I) alcoholics. It is possible that this increase may be the result of dehydration caused by ethanol inhibition of anti-diuretic hormone effects, even though the alcoholic subjects were abstinent for at least one day before blood sampling. An increased amount of a plasma protein of approximately 52 kDa was seen in the alcoholics which does not appear to be a cross-linked product. This change might represent a difference in protein synthesis levels in the alcoholic subjects, and may also contribute to the elevation of plasma protein concentration in the alcoholics.

These studies on chronic alcoholics clearly show that there is an increase in oxidized proteins in their plasma and erythrocyte membranes, but only an increase in higher molecular weight proteins in their erythrocyte membranes. The higher molecular weight proteins appears to result from cross-linking reactions, and not from de novo protein synthesis, as very similar patterns were observed when erythrocyte ghosts prepared from non-alcoholic donors were subjected to in vitro exposure to acetaldehyde. Increases in protein damage were related to the severity of the alcoholism as protein cross-linking was observed only in erythrocytes from Group I alcoholics whose erythrocytes were morphologically abnormal. A further indication that the degree of protein damage is related to the extent of the alcoholism was that both Group I erythrocyte ghost and plasma mean protein carbonyl levels were elevated over the Group II

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Relative protein carbonyl content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.82±0.05</td>
</tr>
<tr>
<td>2.5</td>
<td>3.59±0.08</td>
</tr>
<tr>
<td>5</td>
<td>6.79±0.13</td>
</tr>
</tbody>
</table>

Erythrocytes from control (healthy) donors were incubated in HBSS containing 55 mM (0.25%) acetaldehyde for different time periods and protein carbonyl contents of erythrocyte ghost preparations were determined as described in Materials and methods. Results are expressed relative to a parallel time control without acetaldehyde. The protein carbonyl content for each incubation period was significantly different (P<0.01, n=8 for each incubation time).

4. Discussion

The clinical parameters of the alcoholic subjects used in this study reveal that there are changes in blood chemistry indicative of anemia, abnormal erythrocyte morphology and possible liver damage, although other parameters which are affected in alcoholism such as MCV [31] and erythrocyte osmotic stability [32] were not assessed. We also found that there was a significant increase in plasma protein concentration in the Group II (but not the Group I) alcoholics. It is possible that this increase may be the result of dehydration caused by ethanol inhibition of anti-diuretic hormone effects, even though the alcoholic subjects were abstinent for at least one day before blood sampling. An increased amount of a plasma protein of approximately 52 kDa was seen in the alcoholics which does not appear to be a cross-linked product. This change may represent a difference in protein synthesis levels in the alcoholic subjects, and may also contribute to the elevation of plasma protein concentration in the alcoholics.
values, even though the differences did not appear to be statistically significant. In this case, it is possible that the relatively small number of alcoholic subjects used for these analyses could account for the P values for these differences being larger than 0.05.

Our results confirm the findings of previous in vitro and in situ studies [6,7,24,25] in which it was shown that oxidation and cross-linking of proteins is a consequence of alcohol consumption or acetaldehyde treatment. Furthermore, we have been able to demonstrate that some of the protein oxidation and cross-linking changes in erythrocytes from alcoholics could be produced under in vitro conditions when erythrocytes from non-alcoholics were exposed to acetaldehyde. These findings support the concept that oxidative damage in the circulatory system and peripheral tissues may result from the chronic circulation of acetaldehyde at low concentrations as a result of prolonged high levels of ethanol metabolism to acetaldehyde in the liver [15,33].

A further important finding of this work was that formation of oxidized and cross-linked protein in erythrocyte ghosts from alcoholics was seen only in alcoholic subjects who displayed anemia and extensive erythrocyte abnormal morphology. In contrast, in those alcoholics who were not anemic and showed no significant differences from control subjects in terms of abnormal cell morphology only protein oxidation was observed. These results indicate that the abnormal erythrocyte morphology of alcoholism in Group I alcoholics may be related to protein cross-linking at the membrane–cytosol interface of the cell, and that this phenomenon is dependent on ethanol dosage. In those cases (Group II alcoholics) where no increased abnormal morphology is seen, although acetaldehyde must be able to penetrate the erythrocyte and cause some oxidative damage to proteins, this level of damage (approximately 80% of that seen in Group I alcoholics) does not lead to detectable protein cross-linking. It is possible that under such lower loads of acetaldehyde, this compound can be oxidized to acetate by erythrocyte aldehyde dehydrogenase at a rate which limits protein damage, whereas in more severe alcoholism, the capacity of this enzyme to degrade acetaldehyde is exceeded, permitting further oxidative damage to proteins which leads to cross-linking.

Although erythrocytes from severe alcoholics were found to contain oxidized cross-linked proteins, no evidence was found for cross-linking of plasma proteins from these subjects, although protein oxidation occurred. There are several possible explanations as to why the damage to plasma proteins is apparently less severe than that for erythrocyte proteins. One possibility is that the much shorter half-lives [34–36] of plasma proteins in comparison to those of erythrocyte proteins would prevent the cumulative damage leading to cross-linking that occurs in the erythrocyte. Another factor may be that the much higher protein concentration inside the erythrocyte than in plasma would favor protein cross-linking. A further issue may be that the higher concentrations of free radicals within the erythrocyte [37] could favor more protein cross-link formation. It has also been suggested that erythrocyte catalase can oxidize ethanol to acetaldehyde, thereby increasing the concentration of acetaldehyde within the erythrocyte to levels greater than that in the plasma [38,39]. Although this remains a possibility as acetaldehyde levels have been reported to be about 50% higher than those in plasma [40], our previous studies [26] have failed to detect any significant in vitro metabolism of ethanol in erythrocytes which suggests that intracellular generation of acetaldehyde from ethanol is probably not responsible for the elevated levels of protein damage observed in severe alcoholics.

In summary, our studies have shown that chronic alcohol consumption which causes abnormal erythrocyte morphology is associated with oxidation and cross-linking of protein in the erythrocyte membrane proteins. These changes could also be produced by in vitro exposure to acetaldehyde of erythrocyte ghost preparations from subjects who were not alcoholics. The conclusion from these findings is that acetaldehyde is responsible, at least in part, for erythrocyte and plasma protein damage in vivo, and that the protein cross-linking effects in erythrocytes cause abnormal morphology of the erythrocytes and decrease their normal life span. Current studies in our laboratories are in progress to determine if other blood cell types (leucocytes and platelets) also manifest similar types of damage in alcoholism and to investigate the effects of such modifications on the stability and function of these cell types.

Acknowledgements

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References


