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Lipid peroxidation and platelet membrane fluidity—implications for Alzheimer's disease?

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IN humans, the fluidity of cell membranes generally decreases with age. Unexpectedly, several laboratories have found increased fluidity of platelet membranes (mainly endoplasmic reticulum) in patients with Alzheimer's disease (AD) compared with controls. In the present study, free radical induced lipid peroxidation was found to increase the fluidity of platelet membranes. Hydroxyl radicals were generated in the presence of Fe^{2+} and EDTA at low concentrations of ascorbate. It is hypothesised that platelet membranes are unable to restore their microviscosity by incorporating cholesterol. There may be a link between the result obtained in this study, the recently discovered decreased cholesterol content of affected AD neuronal membranes, and the increased frequency of $\varepsilon 4$ apolipoprotein E (a cholesterol carrier) found in AD patients.

Key words: Alzheimer's disease; Platelet membrane fluidity; Lipid peroxidation, Free radicals; Ascorbate; Cholesterol; Apolipoprotein E

Introduction

Membranes play an important role in the integrity and function of cells. They comprise approximately 80% of the dry weight of cells, and apart from their structural role, membrane constituents participate actively in chemical reactions such as the transmission of impulses by brain cells. One parameter important for the viability of a cell is the fluidity of its membranes, which can be determined by fluorescence polarisation spectroscopy.[1] In humans, the fluidity of cell membranes generally decreases with age, as a result of increased cholesterol content,[2] but unexpectedly several laboratories have found platelet membranes of patients with Alzheimer's disease (AD) to be more fluid than those from age-matched controls.[3] In a family study, Zubenko *et al*[4] found increased platelet membrane fluidity (PMF) to be a stable familial trait, raising hopes that a marker for AD had been found. However, the fluidity changes could only be demonstrated in 55% of patients with AD and, further, could not discriminate between AD patients with and without a family history of AD.[3] No biochemical explanation for this finding in half of all AD patients has been put forward, although Zubenko *et al*, suggested that an aberration of the endoplasmic reticulum (ER) may be the cause of the increased fluidity.[5]

The discovery of an increased frequency of the genetic variant transferrin (Tf) C2 in a population of AD patients,[6] which implicated free radicals in the aetiology of AD, prompted an investigation into the effect of lipid peroxidation caused by hydroxyl radicals on PMF. The iron-binding capacity of Tf C2 has previously been shown to be decreased,[7] which would render the iron (or other transition metal ions) carried by this Tf variant more available for free radical reactions.

Previously, paradoxical results were obtained regarding the effect of lipid peroxidation on membrane fluidity. Ghosh *et al*[8] found that membrane fluidity decreased when cell membranes from rat frontal cortex were subjected to lipid peroxidation. Jain *et al*, on the other hand, found that the production of free radicals in the uteri of mice caused the membranes of the uterine cells to become more fluid, and that this was a natural process which facilitated the implantation of the fertilized ovum.[9] These authors proposed a 'kinked fatty acid' theory to explain the increased fluidity.

One mechanism by which lipid peroxidation can be initiated in a biological system is through the generation of hydroxyl radicals, which requires the simultaneous presence of a transition metal ion, superoxide and hydrogen peroxide. In certain cases, ascorbate can replace superoxide as reducing agent in this system.

In the present study, the focus of the investigation was to determine whether platelet membranes from healthy volunteers, subjected to lipid peroxidation, would become more fluid, thus resembling the PMF observed in AD patients.

Materials and Methods

Diphenylhexatriene (DPH), 2-thiobarbituric acid (TBA) and butylated hydroxytoluene (BHT) were obtained from Sigma.

Preparation of platelet membranes: Platelets suspended in citrate-dextrose buffer, isolated as described previously,[10] were obtained from the blood bank at Tygerberg Hospital. Erythrocytes were removed by centrifugation at 800 *g* for 5 min. Platelets were then collected by centrifugation at 3500 *g* for 15 min. The pellets were resuspended in 0.01 M phosphate buffered saline (PBS) containing 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, and 0.2g KH_2PO_4 per litre, pH 7.4. Cell counts were done by a Technicon H2 Blood Analyser, which showed that there were no erythrocytes or white blood cells present. Membranes were prepared by sonicating the suspension with an MSE sonicator for 10 s with an amplitude of 5 μm. The protein concentration of the suspension was then determined by the Lowry method,[11] and adjusted with PBS to 6 mg/ml.

Lipid peroxidation: Of the 6 mg ml^{-1} membrane suspension, 100 μl aliquots were pipetted into silicon glass test tubes (Kimble). The following reagents were then added in sequence: Ascorbate in concentrations ranging from 80 μM to 1200 μM, EDTA 20 μM; $FeSO_4$ 80 μM; H_2O_2 500 μM. The total volume per tube was adjusted to 1000 μl with PBS (pH 7.4). The concentrations of ascorbate used were chosen to have the following concentration ratios of Fe^{2+} to ascorbate: 1:0, 1:1, 1:2.5, 1:5, 1:10, and 1:15.

The tubes were incubated at 37°C for 30 min. The following were then added: 1 ml of a 1% (w/v) TBA solution plus 1 ml of 2.8% (w/v) trichloroacetic acid. BHT (0.01%) was also included to abolish metal-catalysed autoxidation of lipids during heating of the mixtures at 100°C for 10 min. After cooling, the formation of thiobarbituric reactive substances (TBARS) were determined by reading the chromogen at 535 nm in a Beckman DU 640 spectrophotometer. Appropriate corrections to the absorbance readings were made for the presence of $FeSO_4$-EDTA and ascorbate in the reaction mixtures.

Membrane fluidity: Aliquots of membrane suspension and free radical generating reagents were prepared as for the lipid peroxidation determinations (total volume 1 ml). After incubation at 37°C for 30 min, 5 μl DPH (1 mM in tetrahydrofuran), as well as 4 ml of PBS were added to each tube. The tubes were incubated in the dark at 37°C for 30 min. The change in fluorescence intensity was measured at 37°C using a Perkin-Elmer MPF 44A fluorescence spectrophotometer. Excitation and emission wavelengths were 360 and 428 nm respectively. Relative polarization (P) was obtained by determining the emission intensities through an analyser orientated parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the direction of polarization of the excitation light, and using the formula for fluorescence polarization[1] $P = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$. The reciprocal of P represents the membrane fluidity.[1]

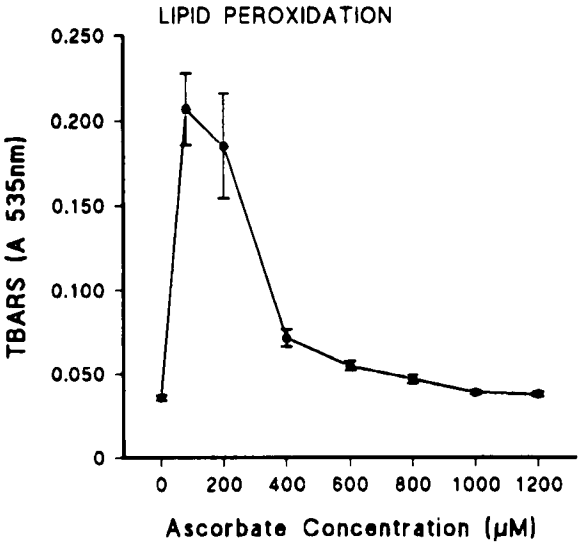

FIG. 1: Varying lipid peroxidation of platelet membranes at increasing ascorbate concentrations.

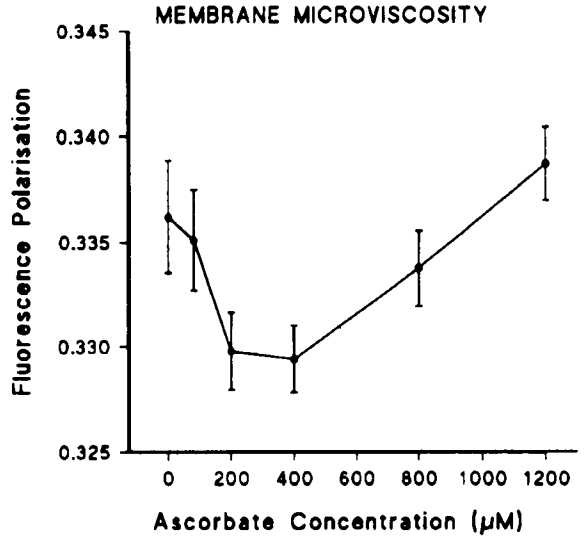

FIG. 2: Alteration of platelet membrane microviscosity (reciprocal of fluidity) during lipid peroxidation at increasing ascorbate concentrations.

Results

Lipid peroxidation: Figure 1 shows that lipid peroxidation was highest when the ratio of Fe^{2+} to ascorbate was between 1:1 and 1:5. At higher concentrations of ascorbate, less TBARS were formed, i.e. less lipid peroxidation occurred. At concentrations higher than about 1 mM ascorbate (ratio 1:10), levels of TBARS were similar to the control value in the absence of ascorbate.

Membrane fluidity: From Figure 2 it is evident that the fluorescence polarisation, which is directly proportional to membrane microviscosity, decreased (i.e. the fluidity increased) as the ascorbate concentration increased from 0 to 400 μM. At about 1 mM ascorbate the microviscosity was similar to the control value.

Discussion

In the above experiments it was found that lipid peroxidation increased the fluidity of platelet membranes. These results were similar to those obtained by Jain *et al*,[9] but contrary to the findings of Ghosh *et al.*[8] These workers found an increased cholesterol to phospholipid molar ratio in the neuronal membranes after peroxidation. Increased membrane cholesterol is normally not desirable, since it is associated with decreased learning ability.[12] When membranes are damaged by lipid peroxidation, however, specific enzymes are responsible for incorporating cholesterol into cell membranes in order to repair them.[2]

The microviscosity recorded in this study mainly reflected the microviscosity of internal (ER) membranes.[5] The lipid composition of ER differs from that of external plasma membranes in that ER membranes contain very little cholesterol.[13] Thus ER membranes probably do not have the enzyme systems necessary to assimilate cholesterol. In a situation where peroxidation occurs, ER membranes and neuronal membranes would therefore react differently, since neuronal membranes under normal circumstances would be able to protect themselves against the disruption of membrane integrity by the incorporation of cholesterol.

Interestingly, X-ray diffraction analysis of the structure of brain membranes in AD, has revealed a reduction in the lipid bilayer width in membranes of affected (cortical) neurones, owing to a cholesterol deficit in these membranes.[14] These results may provide a link with the finding that an increased frequency of the type 4 allele of apolipoprotein E, a carrier of cholesterol, occurs in AD patients.[15]

In the current study, the increase in platelet membrane fluidity followed the pattern of TBARS-formation; i.e. increased lipid peroxidation at low concentrations of ascorbate caused the platelet membranes to become more fluid, while control values for membrane fluidity were regained at higher ascorbate concentrations. This dual effect of ascorbate is well known. At low concentrations ascorbate promotes free radical formation (at least *in vitro*) by reducing Fe^{3+} to Fe^{2+}. Conversely, at higher concentrations, by scavenging any superoxide and hydroxyl radicals produced, ascorbate terminates lipid peroxidation.[16]

If lipid peroxidation were to be held responsible for the increased PMF found in AD, reduced transition metals such as Fe^{2+} must be present. Fe^{2+} is normally not available in the body, since virtually all iron is bound to proteins such as haemoglobin, Tf and ferritin, as well as to other low-molecular weight compounds. The following may be the mechanism.

Fe^{3+} attached to Tf is transported into cells by the process of endocytosis.[17] The pH inside the endocytic vesicles is low, causing the release of iron which is then bound by an unidentified ligand[18] and transported into the cell for utilization or storage in ferritin. If the transfer of Fe^{3+} to the ligand were impaired due to genetically defective Tf, the Fe^{3+} might linger in solution long enough to be reduced by an electron donor, and lipid peroxidation would then be initiated. When aluminium is transported into the endocytic vesicles together with iron, lipid peroxidation is enhanced.[19] Aluminium does this by causing a subtle rearrangement of membrane lipids, thereby facilitating the peroxidative action of iron salts.[19]

Recently, an inflammatory process has been detected in the brains of AD patients.[20] In the area of inflammation, phagocytes produce superoxide radicals which are capable of reducing iron. Some of the superoxide radicals may in addition be converted to hydrogen peroxide by superoxide dismutase, thus providing the substrate for the production of hydroxyl radicals.[21]

Ascorbate is another possible electron donor. In the gut, Fe^{3+} needs to be reduced to Fe^{2+} by ascorbic acid to allow its absorption into the blood stream. Cell membranes in the body, however, are protected against the effects of Fe^{2+} by the rapid conversion of these ions back to Fe^{3+} and their immediate chelation by Tf. It must be emphasized that it is not the actual concentration of ascorbate present that promotes free radical formation, but rather the ratio of free iron to ascorbate. In the blood, where very little free iron exists and cells are constantly recycled, the normal blood concentrations of 25–70 μM ascorbate suffice to protect membranes against free radical damage. In the brain, however, where cells are not recycled, the elimination of free radicals becomes very important. This may account for the very high ascorbate levels (up to 10 times higher) found in the brain, where special transport systems exist to concentrate ascorbate in neurones.[15]

Lipid peroxidation could thus occur in platelets as a result of iron being either carried or released in its reduced state by genetically defective Tf. The question then arises as to whether the reduction of iron occurs as a result of low ascorbate concentrations inside the platelets. That is, could AD patients—apart from their significant deficiencies of vitamins A, E and carotenoids;[22] vitamin B12;[23] zinc, selenium, and essential fatty acids;[24] as well as certain amino acids[25]—also be suffering from a vitamin C deficiency?

It would be of interest if an increased frequency of Tf C2 were found to correlate with increased PMF in patients with AD. It is possible, however, that increased PMF may not be demonstrable in all patients with AD because environmental factors, such as

adequate intake of antioxidants in the diet, could determine whether the genetic contribution of Tf C2 were to be expressed. This hypothesis would be in keeping with a multifactorial aetiology of AD, which already involves more than one gene, and implicates environmental contributions.

Conclusion

The increased fluidity of platelet membranes, as well as death of neurones in AD, may be caused by lipid peroxidation of cell membranes resulting from free radical damage. In order to restore viscosity, one method would be to increase the content of cholesterol in the membranes. Should cholesterol or any other rigidifying agent not be available, injury to the membranes could result in cell death. In cells that are constantly renewed, such as platelets, a fluid state of the membranes would possibly be of little consequence. In neurones, however, which are not recycled, such changes could eventually be lethal. It would thus be imperative to prevent free radical damage to neurones by providing these cells with the appropriate protective essential metabolic factors.

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