Red cell membrane abnormalities in chronic myeloid leukaemia

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Chronic myeloid leukaemia (CML) is a clonal neoplasm that arises in a stem cell common to granulocytes and erythrocytes[1–3]. Several abnormalities have been identified in the plasma membranes of granulocytes of CML patients[4–6], but to our knowledge no studies have been done on CML erythrocytes. We report here that CML erythrocyte spectrin becomes abnormal due to cross-linking of its two subunits via disulphide bonds. In addition, we show that this cytoskeletal defect in the erythrocytes is associated with loss of transmembrane phospholipid asymmetry. These observations, apart from demonstrating membrane abnormalities in CML erythrocytes, also provide strong support for the view that the asymmetric organization of phospholipids in the red cell membrane is maintained mainly by interactions between spectrin and aminophospholipids[7–10].

Blood from leukaemic male patients (aged 30–50 yr) and from healthy adult donors was obtained from King George's Medical College, Lucknow, and drawn by venepuncture into heparinized glass tubes. All the patients studied here were established cases of CML as shown by their haematological and clinical analyses. After removal of plasma from blood cells, the cells were washed three times with phosphate-buffered saline (PBS, *p*H 7.2). Leukocytes, platelets and immature cells were separated from erythrocytes by using a Ficoll–Hypaque gradient[11]. Red cells, obtained after the second gradient, were contaminated with <0.2% leukocytes and immature cells.

Membranes of CML and normal human erythrocytes were analysed for their protein compositions by SDS-polyacrylamide gel electrophoresis[12] in non-reducing conditions. The gels were stained for protein with Coomassie blue and scanned at 530 nm. Figure 1 shows typical densitometer scans of the gels. The gel profiles of CML erythrocyte ghosts contained one additional high-molecular weight protein band (H_p, Fig. 1*C*) which was absent from the gel patterns of normal red cell ghosts (Fig. 1*A*). Apart from this difference betweeen the two types of cells, the CML erythrocyte membrane also contained smaller amounts of spectrin (Fig. 1*C*). Treatment of CML erythrocyte ghosts with β-mercaptoethanol (β-ME) at 37 °C for 15 min before their extraction resulted in the disappearance of the H_p band and consequently an increase in the intensities of spectrin bands (Fig. 1*D*). No effect on the gel profiles of normal erythrocyte ghosts was observed after treatment with β-ME in identical conditions (Fig. 1*B*). These findings clearly indicate that the H_p protein in CML erythrocyte membrane has originated from cross-linking of spectrin via disulphide bonds. This is further supported by our observation that the glutathione (GSH) levels in CML erythrocytes were considerably lower than in normal cells (Table 1).

Similar spectrin oligomers have recently been observed in some types of glucose-6-phosphate dehydrogenase-deficient red cells[13]. This abnormality was found to be associated with other membrane changes such as increased bulk lipid fluidity and decreased cholesterol:phospholipid ratio. Cross-linked spectrin has also been detected in red cells after treatment with sulphydryl oxidizing agents[7,14–19]. These agents, in controlled conditions, exclusively cross-linked spectrin via disulphide bonds, which in turn has been shown to be associated with a decrease in erythrocyte deformability[15], inhibition of diskocyte–echinocyte shape changes[16], and reduced lateral diffusion of band 3 polypeptides[17]. Prolonged treatment of red cells with these oxidizing agents, however, resulted in extensive cross-linking of membrane proteins and consequently aggregation of intramembrane particles[18] and enhanced rate of transbilayer movement[19] of phosphatidylcholine (PC).

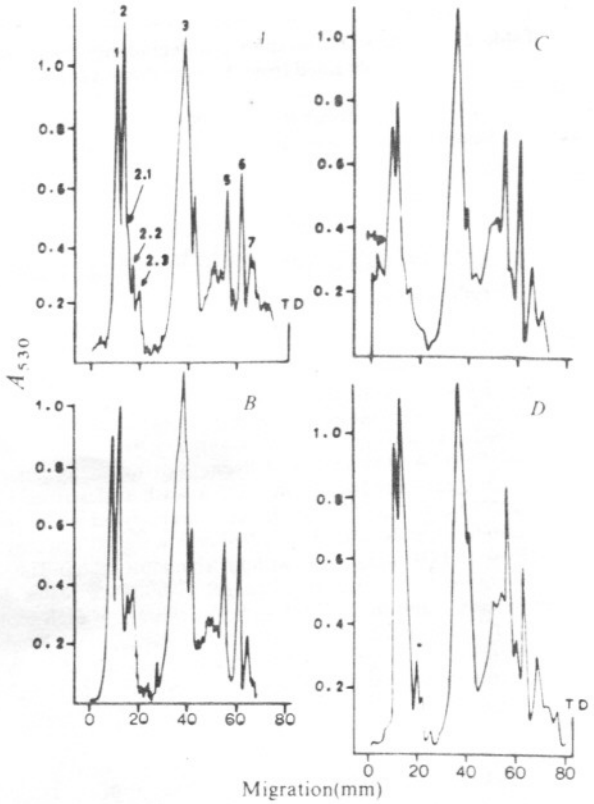

Fig. 1 Densitometer scans of gels stained for proteins with Coomassie blue after electrophoresis of red cell ghosts on SDS-polyacrylamide[12]. *A*, Normal human erythrocyte ghosts; *B*, β-ME-treated normal erythrocyte ghosts; *C*, CML erythrocyte ghosts; *D*, β-ME-treated CML erythrocyte ghosts. The protein bands of the normal human erythrocytes are numbered according to the nomenclature of Fairbanks *et al.*[12]. Similar gel patterns were obtained for erythrocytes of five healthy adults and six CML patients. TD, Tracking dye.

Table 1 Concentration of GSH in erythrocytes

Sample	*n*	GSH (mg per 100 ml packed cells)
Normal erythrocytes	8	62 ± 5
CML erythrocytes	6	34 ± 4

The concentration of GSH in erythrocytes was determined by the procedure of Beutler *et al.*[27]. Values are expressed as mean ± s.d.; *n*, number of humans examined.

Haest *et al.*[7] observed that the cross-linking of spectrin achieved by treating human red cells with sulphydryl oxidizing agents leads to loss of transmembrane phospholipid asymmetry. Therefore, we studied the transbilayer distributions of diacylglycerophospholipids: PC, phosphatidylethanolamine (PE) and phosphatidylserine (PS), in the membranes of both normal and CML human erythrocytes using phospholipase A_2, from two different sources, as an external membrane probe[20,21]. Table 2 shows that *Naja naja* phospholipase A_2 degraded only PC in the normal red cells, whereas in CML erythrocytes this enzyme also hydrolysed PE (~27%) and PS (~30%). The degradation of PE and PS was not due to penetration of the enzyme into CML erythrocytes, because the extent of hydrolysis of these phospholipids remained almost constant even after prolonged incubation (2 h) of these cells with the enzyme (Table 2).

The presence of PS in the outer leaflet of the CML erythrocyte membrane bilayer was confirmed by using pancreatic phospholipase A_2 to probe the membrane phospholipid organization in the cells. The data in Table 3 indicate that this enzyme did

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Table 2 Erythrocyte phospholipid degradation by phospholipase A_2 obtained from *N. naja* snake venom

Sample	Incubation time (h)	PC (%)	PE (%)	PS (%)
Normal erythrocytes	1	56.43 ± 3.59	0	0
	2	56.82 ± 3.34	0	0
CML erythrocytes	1	41.10 ± 2.37	26.66 ± 1.62	30.41 ± 3.31
	2	42.08 ± 2.74	27.02 ± 0.76	30.33 ± 1.05

Erythrocytes were incubated with *N. naja* phospholipase A_2 for 1 and 2 h in conditions described previously[28]. The enzyme reaction was stopped by washing the cells three times with PBS containing 10 mM EDTA. The extent of haemolysis was determined at the end of each incubation before the EDTA wash as described by Roelofsen *et al.*[29]. Less than 5% of the cells were lysed in the experimental conditions. Lipids from the washed cells were extracted by the method of Rose and Oklander[30] but without a haemolysis step. The lipid mixture was chromatographed on silica gel G-60 TLC plates as described previously[28] and the total phosphorus content in each spot was determined[31]. The percentage of the total phospholipid hydrolysed after treatment of red cells with the enzyme was determined by measuring the ratio of remaining diacylglycerophospholipids to the corresponding lyso derivative. Values shown are the mean of five determinations ±s.d.

not attack the normal red cells, but readily hydrolysed PC (~40%), PE (~25%) and PS (~28%) in CML erythrocytes. This is quite consistent with the previous observation that pancreatic phospholipase A_2 hydrolyses only those red cells which contain PS on their outer surface[7,22].

As accessibility of different phospholipids to phospholipases in intact red cells has been correlated with their localization in the external leaflet of the membrane bilayer[20,21], we conclude that the typical asymmetric transbilayer phospholipid organization in the human erythrocyte membrane[23] is lost during CML. This conclusion is in accordance with earlier studies[7–10] which showed that the abnormalities in erythrocyte spectrin are invariably associated with abnormal membrane phospholipid organization, and therefore support the view that the aminophospholipid–spectrin interactions are the major determinants of transmembrane lipid asymmetry in red cells[7–10].

These observed abnormalities suggest that externalization of PS in CML erythrocytes could hyperactivate the blood coagulation system[24,25], and hence may cause thrombosis. Also, the defective erythrocyte cytoskeletal network would enhance destruction of these cells by the spleen[26], making the patients severely anaemic.

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Table 3 Erythrocyte phospholipid degradation by phospholipase A_2 obtained from hog pancreas

Sample	Incubation time (min)	PC (%)	PE (%)	PS (%)
Normal erythrocytes	45	2.91 ± 0.60	0	0
	90	3.57 ± 0.65	0	0
	135	4.71 ± 1.60	0	0
CML erythrocytes	45	30.72 ± 1.81	19.96 ± 1.57	28.76 ± 2.44
	90	38.07 ± 1.00	24.31 ± 1.36	28.45 ± 2.78
	135	39.77 ± 2.23	24.98 ± 1.04	27.94 ± 3.99

Treatments of erythrocytes with pancreatic phospholipase A_2 (Boehringer Mannheim) were carried out according to the method of Haest *et al.*[7]. The amount of enzyme used was 10 IU per 0.25 ml of packed cells. Incubations were for 45, 90 and 135 min at 37 °C. The extent of haemolysis was <1% in the experimental conditions. The enzyme reactions were stopped, cells extracted and amount of degraded phospholipids determined, as described in Table 2. Values are the mean of four determinations ±s.d.

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