Carbohydrate induced modulation of cell membrane. VI. Binding of exogenous lectin induces susceptibility of erythrocytes to free radical damage: a spin label study

Krishnan Hajela*, Abhay H. Pande, Sumati

School of Life Sciences, Devi Ahilya Vishwavidyalaya, Vigyan Bhawan, Khandwa Road Campus, Indore 452001 (M.P.), India

Received 17 February 1997

Abstract The oxidation of erythrocyte membrane has been widely used as a model to study the damage of biomembranes by free radicals. Whether binding of lectin to erythrocytes has any effect on peroxidant injury has never been studied. This study reports for the first time that crosslinking of erythrocyte surface glycoprotein by an exogenous lectin significantly enhances the susceptibility to membrane damage by free radicals, as evidenced by the increase in membrane fluidity measured by EPR using spin label and the increase in the amount of oxyhemoglobin liberated due to cell lysis.

Key words: Lectin; Red blood cell membrane; Pyrogallol; Electron paramagnetic resonance

1. Introduction

The nonenzymatic free radical mediated oxidation of biological molecules, membranes and tissues is known to be related to a variety of pathological events, cancer and aging [1–5]. The peroxidation of membrane lipids has been implicated as one of the primary events in oxidative cellular damage. The oxidation of erythrocyte membrane serves as a model for the oxidative damage of biomembranes [6–8]. It is now well documented that asialoglycoprotein receptors present on the surface of hepatic cells, Kupffer cells and splenic and peritoneal macrophages mediate binding of galactose terminated glycoproteins, of desialylated erythrocytes and other blood cells [9–12]. After binding the blood cells are phagocytosed and lysed by oxidative process. Although the oxidative processes and free radical induced damage of erythrocytes membrane has been extensively studied [13,14], whether the binding of lectins to erythrocytes has any effect on peroxidant injury has never been studied. This study presents our results on the free radical induced damage of rabbit erythrocytes in the presence of hemagglutinating and subhemagglutinating concentrations of a lentil lectin. We report, for the first time, that crosslinking of cell membrane glycoproteins by an exogenously added lectin increases the susceptibility of cell lysis by free radicals.

2. Materials and methods

2.1. Reagents

Spin label, i.e. 16-doxyl stearic acid (16-DS), and spin trap, *N-t*-butyl-α-phenyl nitrone (PBN) were purchased from Sigma Chemical Co., MO, USA. Pyrogallol, trisodium citrate and other chemicals used were from Loba Chem., India. The glass capillaries used were from TOP Syringe Manufacturing Co., Bombay, India.

2.2. Methods

A blood sample from a healthy rabbit (adult, male, New Zealand white strain) was obtained by vein puncture, in 3.8% trisodium citrate solution as an anticoagulant used in a ratio of 4:1. The erythrocytes were then centrifuged at $3000\times g$ for 5 min and washed three times with 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). Finally a 1% erythrocyte suspension was made (1 ml of packed erythrocyte in 100 ml PBS).

Lectin was prepared from *Lens culinaris* seeds essentially as described by Tichy et al. [15]. The seeds were soaked in PBS overnight and then homogenized in PBS. The homogenate was then centrifuged at $3000\times g$ for 30 min to obtain a clear supernatant. The supernatant was then subjected to affinity chromatography on acid treated Sephadex G-200 column. The bound protein was then specifically eluted with 0.1 M glucose [15]. The eluted protein was then dialysed extensively in PBS to remove excessive glucose. The protein was further purified on a Biogel P100 column. The protein thus isolated was used as 'native' lectin. The hemagglutination activity was checked using untrypsinized rabbit red blood cells (RBC). The lectin solution was then adjusted to a hemagglutinating (1.0 mg/ml) and a subhemagglutinating (0.2 mg/ml) concentration.

The erythrocytes were exposed to oxyradical shock as described by Jain et al. [16]. From the cell suspension prepared as above, aliquots of 200 μl were made in different tubes. To these aliquoted cell suspensions was added 100 μl of either lectin or PBS and the mixtures were incubated for a period of 15 min at room temperature. After this, the cells were exposed to superoxide radicals ($O_2^{\bullet-}$) generated from a pyrogallol autoxidation system by adding 10 μl of pyrogallol solution (0.02 M freshly prepared in H_2O), and incubated for a period of 20 min at room temperature, and the erythrocytes were recovered after centrifugation of the sample at $3000\times g$ for 5 min. The cells were then washed thrice with PBS and the supernatant was collected for analyzing the hemoglobin released.

2.3. Spin labelling of treated erythrocytes

The pelleted cells, obtained as above, were then resuspended in 400 μl PBS. After this, 20 μl of spin label 16-DS (2×10^{-4} M final concentration) was added and the suspension was incubated at 37°C for 30 min. Immediately after the incubation time, 50 μl $NiCl_2$ (500 mM final concentration) was added to each sample and incubation was continued for another 10 min. Then the cells were washed thrice with PBS to remove excess label. The pelleted cells were then resuspended in 100 μl of PBS. From these suspensions approximately 50 μl was transferred to glass capillaries and one end was sealed with plasticine, taking care not to trap air bubbles in it.

2.4. Spin trapping of superoxide radical

To detect that the superoxide radicals are generated by pyrogallol, 10 μl of pyrogallol solution was added to 80 μl of PBS, immediately followed by 10 μl of PBN (50 mM final concentration). The mixture was then incubated for 45 min and read in EPR.

The extent of cell lysis was monitored by calculating the amount of hemoglobin released in the supernatant after free radical ($O_2^{\bullet-}$) shock. The oxyhemoglobin (Oxy Hb.) concentration was measured by the method of Winterbourn [17], using absorbance measurements at

*Corresponding author. Fax: (91) (731) 472793.

Abbreviations: 16-DS, 16-doxyl stearic acid; PBN, *N-t*-butyl-phenyl nitrone; PBS, 0.01 M phosphate buffer pH 7.4 with 0.15 M NaCl; EPR, electron paramagnetic resonance

PII S0014-5793(97)00272-X

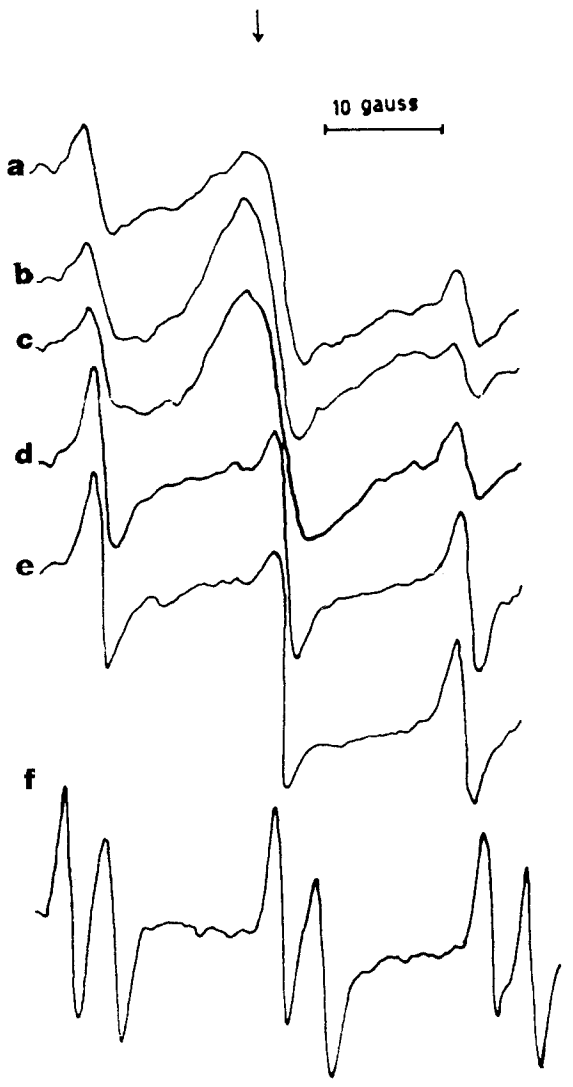

Fig. 1. Representative EPR spectra of erythrocytes labelled with 16-DS; (a) normal erythrocytes, (b) erythrocytes incubated with lentil lectin (0.53 mg/ml final concentration), (c) erythrocytes exposed to superoxide radicals, (d) erythrocytes exposed to superoxide radicals in the presence of a hemagglutinating concentration (0.53 mg/ml final concentration) of lentil lectin, (e) erythrocytes exposed to superoxide radicals in the presence of a subhemagglutinating concentration (0.06 mg/ml final concentration) of lentil lectin, (f) spectrum of superoxide radical-PBN adduct. Before labelling the erythrocytes with spin label (16-DS) the erythrocytes were washed thrice with PBS to remove excess pyrogallol. Instrument settings are given in Section 2.

560, 577 and 630 nm, with the following equation:

$$[\text{Oxy Hb.}] = 119\ A_{577} - 39\ A_{630} - 89\ A_{560}$$

2.5. EPR spectroscopy

EPR spectra were recorded on a Varian E-104 EPR spectrophotometer equipped with a Tm 110 cavity. Instrument setting was as follows: scan range 100 G; field set 3237 G; temperature 27°C; time constant 1 s; scan time 4 s; modulation amplitude 2 G; modulation frequency 100 kHz; microwave power 5 mW; microwave frequency 9.01 kHz; receiver gain 2.5×10^4. The rotational correlation time Tc, expressed in seconds, was calculated as described earlier [18].

The experiments were repeated four to six times and the data were analyzed statistically using the STAT-P-GW-BASIC-Rev. 1.02 program.

3. Results and discussion

The superoxide radical induced damage to the rabbit erythrocyte membrane in the presence or absence of the lectin was studied by measuring the fluidity changes in the membrane and by measuring the hemoglobin released. The stearic acid spin label 16-DS was used as a probe for the hydrocarbon portion of the membrane bilayer. The EPR spectra obtained were analyzed by computing the rotational correlation time Tc. Tc is a measure of the degree of immobilization of the spin label, hence a measure of local viscosity. The reciprocal of Tc (1/Tc) will thus denote the local 'fluidity', a higher 1/Tc value denotes a higher fluidity. The superoxide ($O_2^{\bullet-}$) radicals generated by autoxidation of pyrogallol were detected by trapping the radical as a PBN adduct. The results obtained are presented in Figs. 1–3.

Fig. 1 presents the EPR spectra of erythrocytes labelled with 16-DS after various treatments. The erythrocytes had a 1/Tc value of 0.079×10^{10} s^{-1}. It appears that on incubation with agglutinating concentrations of lentil lectin the membrane fluidity decreased to a 1/Tc value of 0.043×10^{10} s^{-1} ($P<0.01$), probably due to crosslinking of surface glycoproteins with the lectin. On exposure of erythrocytes to superoxide radicals, generated by pyrogallol autoxidation, the 1/Tc value (0.097×10^{10} s^{-1}) was not found to be significantly different from that of untreated erythrocytes. This was because the intensity of the free radical shock given was very low. Surprisingly, when the erythrocytes were exposed to an identical superoxide radical shock in the presence of an agglutinating concentration (0.53 mg/ml final concentration) of lentil lectin, the 1/Tc value increased to 0.364×10^{10} s^{-1}, thus exhibiting an approximately four-fold increase in membrane fluidity ($P<0.001$). When exposed to superoxide radicals in the presence of a subhemagglutinating concentration (0.006 mg/ml final concentration) of lentil lectin, then also fluidity increased approximately three-fold to a 1/Tc value of 0.271×10^{10} s^{-1}. The histogram (Fig. 2) shows clearly that

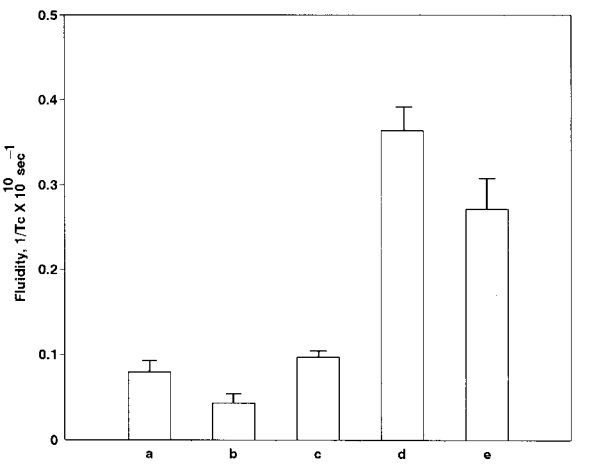

Fig. 2. Membrane fluidity (1/Tc value) of (a) erythrocytes only (control), (b) erythrocytes incubated with lentil lectin (0.53 mg/ml final concentration), (c) erythrocyte exposed to superoxide radicals, (d) erythrocytes exposed to superoxide radical in the presence of a hemagglutinating concentration (0.53 mg/ml final concentration) of lentil lectin, (e) erythrocytes exposed to superoxide radicals in the presence of a subhemagglutinating concentration (0.06 mg/ml final concentration) of lentil lectin. Bars represent means of four observations. The lines on the top of the bars represent S.D.

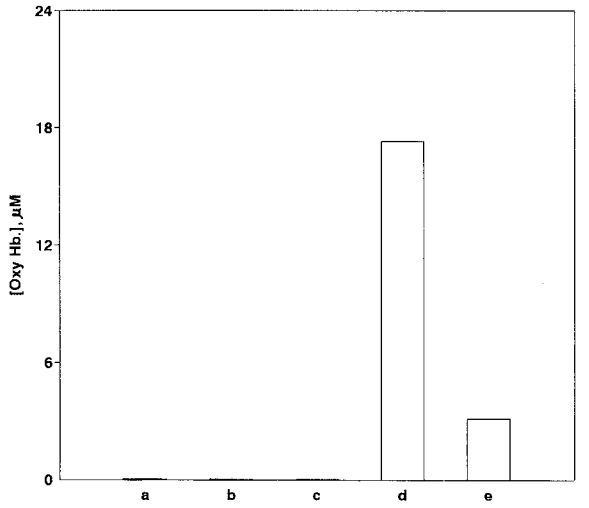

Fig. 3. Oxyhemoglobin concentration in the supernatant of (a) erythrocytes only (control), (b) erythrocytes incubated with lentil lectin (0.53 mg/ml final concentration), (c) erythrocytes exposed to superoxide radicals, (d) erythrocytes exposed to superoxide radicals in the presence of a hemagglutinating concentration (0.53 mg/ml final concentration) of lentil lectin, (e) erythrocytes exposed to superoxide radicals in the presence of a subhemagglutinating concentration (0.06 mg/ml final concentration) of lentil lectin. Bars represent means of four observations.

exposure of erythrocytes to superoxide radicals in the presence of either hemagglutinating or subhemagglutinating concentrations of lectin enhances their susceptibility to membrane damage in a statistically significant manner. The erythrocytes agglutinated rapidly (within 2 min) on addition of lentil lectin. Clumps were also found to settle down rapidly on addition of lentil lectin, even in the presence of pyrogallol. However, lysis of cells as evident by a red color due to hemolysis was also visible.

To further confirm this, the supernatant from the first three washes after superoxide radical exposure was collected and hemoglobin content was analyzed spectrophotometrically. It was found that on exposure to superoxide radicals in the presence of hemagglutinating and subhemagglutinating concentrations of lectin erythrocytes liberated 17.29 μM and 3.13 μM of oxyhemoglobin respectively. Erythrocytes exposed to superoxide anions in the absence of lectin did not liberate any oxyhemoglobin in the supernatant (see Fig. 3). This again confirmed that in the presence of lectin, the superoxide anions lysed the erythrocytes causing hemoglobin release in the supernatant but in the absence of the lectin the same concentration of superoxide anions could not produce enough damage to release hemoglobin in the supernatant. These results were obtained using a very low intensity of free radical shock generated by the autoxidation of pyrogallol at a final concentration of 0.665 mM. If two- and three-fold higher concentrations of pyrogallol are used then the damage produced in controls (i.e. erythrocytes without lectins) is also greater but the induction of susceptibility to free radical damage by lectin is still observed (data not shown).

Agglutination is the most easily detectable manifestation of the interaction of a lectin with cells. For agglutination to occur the bound lectin must form multiple cross-bridges between opposing cells. Lateral crosslinking may obviously also occur upon lectin-glycolipid interaction. This will eventually result in clustering of glycolipid lectin complexes within the lateral plane of the bilayer. With spin probe techniques such crosslinking has been demonstrated directly [19,20]. It can be readily envisaged that the bulky and strongly hydrated carbohydrate head group forming a protrusion on a membrane surface can act as a steric barrier which might be acting as a protective shield against peroxidant damage. Lateral phase separation or local clustering of glycolipids induced by glycolipid-lectin interaction can result in a substantial reorganization of the membrane component [21]. Such lectin induced reorganization may produce a perturbation or destabilization of the membrane making it more susceptible to free radical damage as is evident from experimental results. That changes in membrane integrity may render the red cells more susceptible to oxidative attack [22] is also proved by the fact that red cells from pathological conditions which have been reported to have either a primary or a secondary defect in membrane integrity (for example paroxysmal nocturnal hemoglobinuria, erythropoietic protoporphyria, β-thalassemia major, and sickle cell anemia) have all been shown to be susceptible to peroxidant injury [22]. Early experiments in humans and other animals have shown that injected desialylated erythrocytes are cleared rapidly into the liver and spleen where they are taken up by Kupffer cells and splenic macrophages respectively [23]. We speculate that the clearing is not only because of lectin mediated phagocytosis of the desialylated erythrocytes but also due to the increased susceptibility to free radical damage subsequent to lectin binding.

Acknowledgements: The authors thank Prof. S. Bharti for his help, cooperation and encouragement and for providing excellent research conditions without which this work would not have been possible. Financial assistance from DAE (BRNS Grant 4/12/95-R and D-II/702) and from C.S.I.R. to Dr. Sumati is gratefully acknowledged.

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