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Ethanol-Induced Alterations in Human Erythrocyte Shape and Surface Properties: Modulatory Role of Prostaglandin E_1

Ronald W. McLawhon, Yehuda Marikovsky†, Neil J. Thomas, and Ronald S. Weinstein
Department of Pathology, Rush Medical College, Chicago, Illinois 60612, and †Department of Membrane Research, Weizmann Institute of Science, Rehovot 76 100, Israel

Summary. Exposure of human erythrocytes to ethanol (1 to 20% by vol) in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline, pH 7.4, transformed biconcave discs into spiculated echinocytes within 3 min at 25°C. The effects of ethanol were concentration- and time-dependent, but reversible by washing in the incubation buffer system within 60 min of initial exposure to ethanol. After prolonged ethanol exposure (180 min), washing of cells resulted in the formation of stomatocytes (cup-forms). Ethanol-induced echinocytosis was also accompanied by a 30% enhancement in the agglutinability of erythrocytes by ligands with high affinity for negative surface charge (poly-L-lysine and wheat germ agglutinin, 20 μl/ml) without any alterations in surface charge topography. Concomitant exposure of erythrocytes to prostaglandin E_1 (100 nM) selectively prevented the enhancement of ligand-mediated agglutinability, but did not modify cell shape. These data indicate that certain erythrocyte surface properties may not be directly influenced by cell shape and suggest a unique modulatory action of prostaglandin E_1 on shape-transformed cells.

Key Words cell shape control · membrane skeleton · lipid bilayer · ligand-mediated agglutinability · surface charge topography

Introduction

Erythrocytes are believed to maintain the shape of smooth biconcave discs under normal physiological conditions through an endogenous, energy-dependent process [28]. Nonetheless, when subjected to various physical and chemical factors, erythrocytes can be rapidly transformed into a broad spectrum of transitional shapes that may alter the hemodynamic properties of blood and decrease cell survival in the circulation [4]. Most evidence suggests that the lipid bilayer of the erythrocyte membrane [24] or its underlying cytoskeletal protein network [22] may be directly responsible for the control of cell shape and specific rheologic properties. However, at this time, no single molecular mechanism has been established. In previous studies [19], several amphipaths and ions (e.g., dinitrophenolate, lysolecithin) that are capable of intercalating and expanding the lipid bilayer of the erythrocyte membrane have been shown to modify cell shape in a manner similar (spiculation) to that seen with ATP-depletion, hyperosmotic pressure, or inborn errors of lipid metabolism [15–18, 28]. Associated with erythrocyte shape transformations, it has been observed that certain cell surface properties (i.e., ligand-mediated agglutinability, negative surface charge topography, lateral mobility of integral membrane glycoproteins) may be markedly altered in a consistent and characteristic manner [16–18]. In this report we describe novel actions of two agents, ethanol and PGE_1[1], that are known to interact with a variety of biological membrane systems [13, 26], but have not been previously demonstrated to alter the morphological characteristics or specific surface properties of human erythrocytes.

Materials and Methods

Fresh, heparinized, whole blood specimens were obtained from healthy, adult human donors (blood groups: A, Rh^+; B, Rh^-; B, Rh^-; O, Rh^+) by peripheral venipuncture and centrifuged at 500 × *g* for 5 min. Plasma and buffy coat layers were discarded, and the packed erythrocytes were washed twice with 10 vol of CMF-PBS. The erythrocyte preparations were resuspended at 1:20 (for morphological studies) and 1:500 (for agglutination studies) dilutions in 10 ml of CMF-PBS, and incubated at 25°C in the absence or presence of ethanol (USP 200-proof, AAPER Alcohol and Chemical Co., Shelbyville, KY; final concentrations: 1 to 20% by vol; 160 to 3200 mM) for time periods ranging from 1 to 180 min. In certain experiments, aliquots of stock solutions of PGE_1 (Upjohn Diagnostics, Kalamazoo, MI) in ethanol/CMF-

[1] *Abbreviations used:* PGE_1, prostaglandin E_1; PLL, poly-L-lysine; WGA, wheat germ agglutinin; CMF-PBS, Ca^{2+} and Mg^{2+}-free phosphate-buffered saline; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

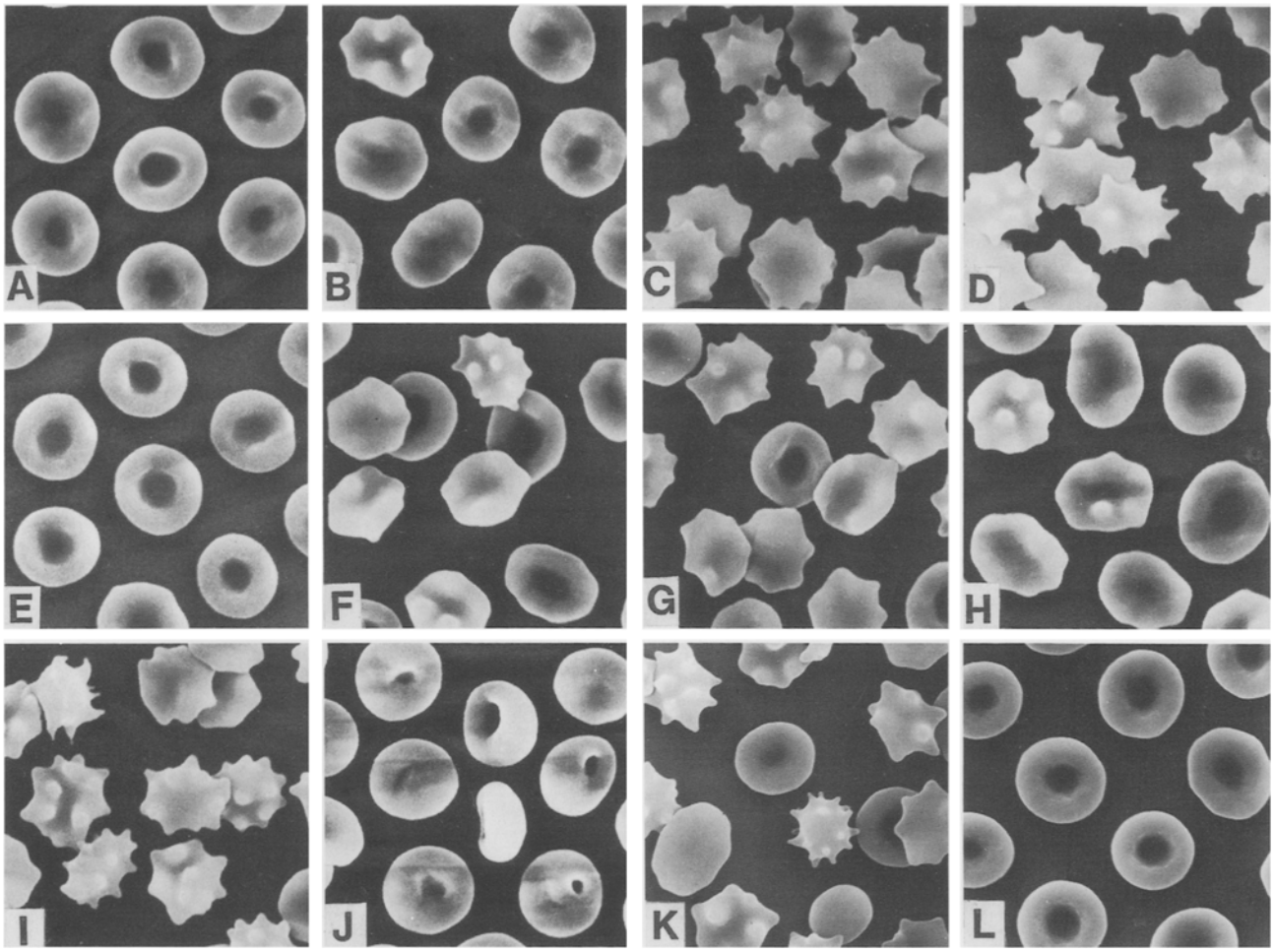

Fig. 1. Morphological appearance of human erythrocytes by SEM following exposure to ethanol and PGE_1. (*A*) Untreated cells; (*B*) 10% ethanol (by vol) for 1 min; (*C*) 10% ethanol (by vol) for 3 min; (*D*) 10% ethanol (by vol) for 15 min; (*E*) 10% ethanol (by vol) for 3 min, then washed immediately in CMF-PBS; (*F*) 2% ethanol (by vol) for 15 min; (*G*) 5% ethanol (by vol) for 15 min; (*H*) 20% ethanol (by vol) for 15 min; (*I*) 10% ethanol (by vol) for 180 min; (*J*) 10% ethanol (by vol) for 180 min, then washed immediately in CMF-PBS; (*K*) 100 nM PGE_1 + 5% ethanol (by vol) for 15 min; (*L*) 100 nM PGE_1 for 15 min

PBS (1 : 1) were also added to the erythrocyte suspensions in amounts not exceeding 2 μl/ml to yield a final PGE_1 concentration of 100 nM.

For morphological studies, 4-ml aliquots of the treated erythrocyte suspensions were added immediately to 2 ml of 1% glutaraldehyde (vol/vol) in CMF-PBS, or centrifuged at 600 × *g* for 5 min and washed in 10 vol CMF-PBS for up to 180 min prior to fixation. The fixed erythocytes were washed twice in deionized water, and monolayer smears were prepared on clean glass slides that were subsequently coated with gold-palladium (60 : 40). The smears were examined and photographed at ×1000 magnification in JEOL JSM-35C scanning electron microscope, operated at 15 kV with the stage at a 30° angle. Differential cell counts on 50 consecutive cells were performed in triplicate. Data are presented as mean values ± SD. Cells were classified as discocytes, echinocytes (Types I, II, III), spherocytes, and stomatocytes using the criteria established by Bessis [3].

For agglutination studies, 1.8-ml aliquots of ethanol and PGE_1-treated erythrocyte suspensions (nonfixed) were added to disposable plastic cuvettes containing 0.4 ml of 100 μg/ml stock solutions of PLL (M_r = 84,000; Miles-Yeda, Rehovat, Israel) or WGA (from Dr. J. Coon, Rush Medical College, Chicago, IL) in CMF-PBS. The mixtures were stirred continuously at 25°C with a Teflon-coated magnetic stirring bar, and erythrocyte agglutination was recorded automatically as a function of light transmission for 12 min using a Model D-2 Fragiligraph (Elron Electronics Industries, Tel Aviv, Israel) as described previously [8]. Osmotic fragility measurements were also performed using a Model D-2 Fragiligraph according to the methods of Danon [6]. Negative surface charge topography was visualized by membrane-labeling with cationized-ferritin using semithin-section TEM, according to procedures reported elsewhere [7, 17, 18].

Results and Discussion

Figure 1 shows, by SEM, the spectrum of morphological appearances of human erythrocytes following exposure to various concentrations of ethanol in

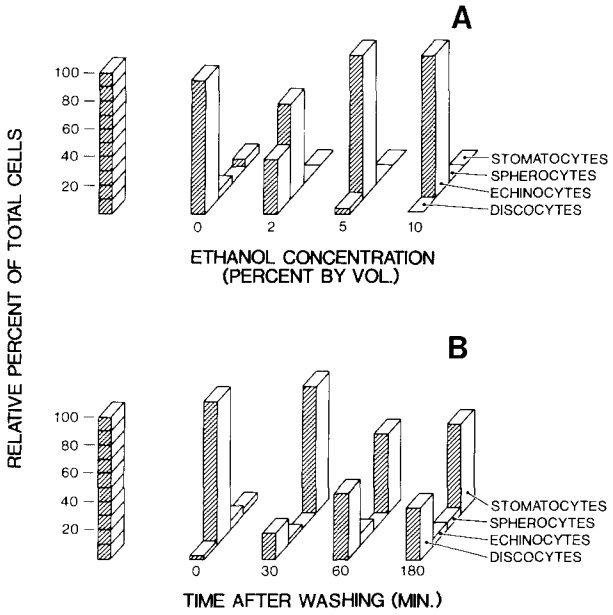

Fig. 2. Quantitative SEM analysis of ethanol action on human erythrocytes. (*A*) Concentration effect of ethanol on the morphology of erythrocytes following a 15-min exposure; and (*B*) time course effect of prolonged washing in CMF-PBS on the morphology of erythrocytes following prior exposure to 10% ethanol (by vol) for 180 min

vitro. Untreated discocytes (Fig. 1*A*) were transformed into spiculated erythrocytes (Fig. 1*C*) within 3 min of exposure to 10% ethanol (vol/vol) in CMF-PBS, pH 7.4. Short time periods of exposure (1 min or less) to ethanol had minimal effects on overall cell shape (Fig. 1*B*), whereas the degree of echinocytosis was found to increase progressively with the length of incubation (Fig. 1*C*, *D*, and *I*). In addition, the degree of echinocytic transformation observed was dependent on concentrations of ethanol included in the incubation buffer system (Fig. 1*D*, *F*, *H*), with maximal shape transformation occurring within 15 min of initial exposure to ethanol at all concentrations. Quantitative SEM (Fig. 2*A*) confirmed that the proportion of echinocytes increased from 64 ± 5% of total cells (Type I, 46 ± 4%; Type II, 14 ± 2%; Type III, 4 ± 2%) following a 15-min treatment with 2% ethanol (vol/vol) in CMF-PBS, to 98 ± 1% of total cells (Type I, 6 ± 3%; Type II, 24 ± 7%; Type III, 68 ± 9%) exposed to 10% ethanol (vol/vol) in CMF-PBS for the same time period.

The effects of ethanol on erythrocyte shape were readily and completely reversible by resuspending cells in fresh CMF-PBS within 60 min of initial exposure to ethanol. Comparison of Fig. 1*C* and *E* demonstrates that washing reverted crenated cells into discocytes following a 3-min treatment with 10% ethanol (vol/vol) in CMF-PBS. However, with prolonged periods of ethanol exposure (180 min), it was observed (Fig. 1*I* and *J*) that echinocytes were converted into both discocytes (64 ± 4% of total cells) and stomatocytes/cup-forms (32 ± 3% of total cells) immediately following washing in CMF-PBS. Maintenance of erythrocytes in the incubation buffer system (Fig. 2*B*) for an additional 30 min resulted in the formation of stomatocytes (82 ± 4% of total cells), with gradual transition back to a mixture of biconcave discs and cup-forms occurring over the next 90 min. While ethanol treatment had no significant effect on the osmotic fragility of erythrocytes, prolonged exposure to high concentrations of ethanol (20% by vol for 180 min) followed by washing in CMF-PBS resulted in a 20% increase in the susceptibility of cells to hemolysis. Untreated erythrocytes remained in the discocyte configuration throughout the 180-min incubation period of these experiments (*data not shown*).

Ethanol-induced alterations in human erythrocyte shape were accompanied by an enhancement in agglutination mediated by ligands with high affinity for negative cell surface charge. The effects of ethanol on PLL or WGA-mediated agglutinability were concentration dependent (Fig. 3*A*) and completely reversible by washing crenated cells in CMF-PBS (Fig. 3*B*). Moreover, concomitant exposure of erythrocytes to 100 nM PGE_1 and ethanol prevented the enhancement in ligand-mediated agglutinability (Fig. 3*B*), but did not modulate ethanol-induced echinocytic shape transformations *per se* (Fig. 1*K*). In contrast, PGE_1 alone did not significantly alter either cell shape (Fig. 1*L*) or agglutinability (Fig. 3*B*). Previous studies have shown [16–18] that a redistribution of negative cell surface charge may accompany alterations in human erythrocyte shape and ligand-mediated agglutinability induced by a variety of chemical agents and experimental conditions. However, we have not found any effect of ethanol or PGE_1 treatment on negative surface charge topography of erythrocytes, as assessed by TEM of cationized-ferritin labeled cell membranes (*data not shown*).

While long-chain aliphatic alcohols (e.g., stearyl alcohol) have been shown to produce stable, rod-shaped membrane projections in human erythrocytes in vitro [12], the direct effects of ethanol (at the concentrations used in this investigation) on cell shape have not been reported previously. Ethanol expands and increases the fluidity [5, 26] of the lipid bilayer of the erythrocyte membrane. Thus, ethanol may govern the cell shape changes in a manner similar to that observed with other amphipaths and ions [15–17, 19, 24], as provided for under the bilayer-couple hypothesis [24] which attributes shape con-

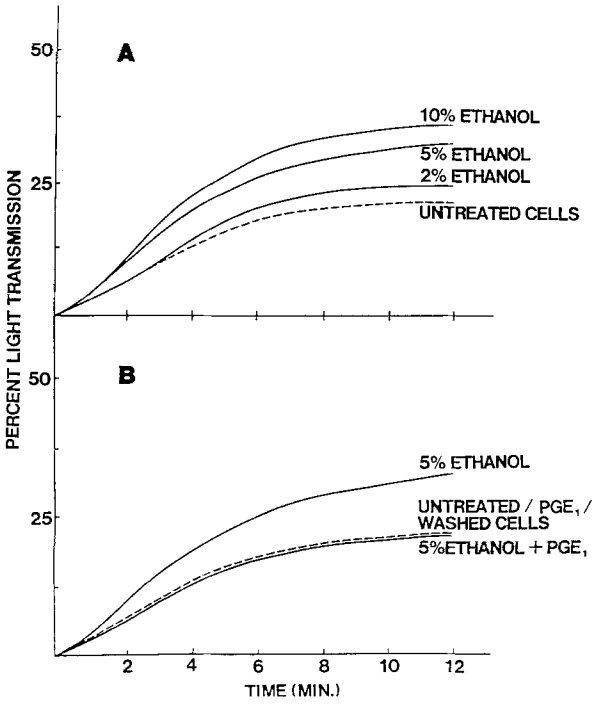

Fig. 3. Ligand-mediated agglutinability of human erythrocytes by PLL following exposure to ethanol and PGE_1. (*A*) Concentration effect of ethanol on erythrocyte agglutinability following a 15-min exposure (zero time in the agglutination assay); and (*B*) comparison of effects of exposure of erythrocytes to 100 nM PGE_1 in the absence or presence of 5% ethanol (by vol) for 15 min, with the effect of washing cells immediately in CMF-PBS after 15-min treatment with 5% ethanol (by vol)

trol to the unequal surface area of the two leaflets of the lipid bilayer. Alternatively, the membrane disordering actions of ethanol are known to have profound effects on Ca^{2+}-ATPase activity and intracellular Ca^{2+} levels in human erythrocytes [30], which may further influence cell shape through the regulatory actions of calcium on the phosphorylation of major cytoskeletal proteins such as spectrin [20, 22]. Several investigators have suggested an additional role for calcium in maintaining erythrocyte cytoarchitecture through the regulation of polyphosphoinositide metabolism; specifically, spiculation of erythrocytes under a variety of experimental conditions (including ATP-depletion and treatment with the ionophore A23187 and high concentrations of extracellular Ca^{2+}) appears to be associated with the accumulation of phosphatic acid and 1,2-diacylglycerol [1, 11]. Therefore, calcium and polyphosphoinositides may be important determinants of ethanol-induced shape transformations, particularly since previous studies [21] have shown that ethanol (at lower concentrations than those used in this study) may enhance echinocyte formation induced by the ionophore A23187 and extracellular Ca^{2+}. Moreover, it has been recently reported [2] that phophatidylinositol-4,5-biphosphate (triphosphoinositide) may specifically modulate the association of protein 4.1 (a cytoskeletal protein that links spectrin to the cell membrane) with the major erythrocyte membrane glycophorin and band 3. Triphospoinositide also increases the lateral mobility of these integral (WGA-binding) membrane glycoproteins [23] which have been implicated in the control of cell shape and deformability, and may partially explain the enhancement of ligand-mediated agglutinability in association with ethanol-induced echinocytosis. The molecular basis for the observations made in this paper are currently under investigation.

Although we have observed that ethanol-induced echinocytosis was accompanied by a significant increase (30% maximal) in the agglutinability of erythrocytes mediated by PLL or WGA, as has been shown [16–18] with a variety of other shape-modifying chemical agents and experimental conditions, these changes occurred in the absence of any discernable alterations in other cell surface properties such as negative charge topography. In addition, PGE_1 was found to effectively dissociate the erythrocyte surface property of agglutination from ethanol-induced alterations in cell shape, without any effect of untreated discocytes. These findings are of considerable interest since they do not support the concept that specific cell surface properties are directly influenced by or coupled to erythrocyte shape, as had been proposed in previous studies [16–18]. In this respect, ethanol and PGE_1 may be useful as novel investigative tools for dissecting the complex molecular mechanisms that control cell shape and various surface properties.

Specific interactions between the human erythrocyte membrane and PGE_1 have been postulated for several years [26], as it is known that subnanomolar concentrations of PGE_1 not only increase the fluidity of the lipid bilayer of the erythrocyte membrane but also enhance cell deformability [13, 14]. Although such observations suggest the presence of selective sites (i.e., receptors) in the cell membrane that could mediate the actions of this potent autacoid, it was only recently [9] that a specific, high-affinity binding site for PGE_1 was identified in human erythrocyte membrane preparations. While this binding site is similar to PGE_1 receptors in human platelets and erythrocytes from nonhuman species in its affinity for prostacyclin and its metabolites, it differs in that it is not coupled to the effector enzyme adenylate cyclase or subject to regulation by guanine nucleotides. Consequently, the physiological significance of a PGE_1 binding site in human erythrocytes remains uncertain. Our observations that PGE_1 can effectively modulate ligand-mediated

agglutinability of erythrocytes may support the notion of its role as a physiologically relevant membrane receptor, as the effects of PGE_1 were concentration dependent (in the range 0.1 to 100 nM) and mimicked by prostacyclin (*unpublished results*). The physiological relevance of these observations may be further supported by other studies in this laboratory [29] which suggest that PGE_1 derivatives prevent the formation of intravascular macroaggregates of interadherent, shape-transformed erythrocytes (and relatively few trapped platelets) in association with ethanol-induced injury of gastric mucosa in rats.

PGE_1 has been classically recognized for its role in hemostasis and thrombosis, primarily through its modulatory actions on platelet aggregation and vascular smooth muscle contraction [13, 25]. Based on the results of the present study, it is tempting to speculate that the net effect of PGE_1 action on human erythrocytes in the circulation may be functionally similar to that of platelets, namely, the prevention of cell-cell interactions that can potentially alter and impede blood flow. However, it is clear that platelets and erythrocytes respond in an entirely different and opposing manner when exposed to other external stimuli and environmental factors such as ethanol. Ethanol acts [10] to inhibit aggregation of platelets induced by a variety of chemical agents (including thrombin and A23187), whereas it enhances the agglutinability and aggregation of erythrocytes mediated by specific ligands in vitro. In this context, erythrocytes have been previously proposed [27] to have a key role in the complex interactions that exist between physical and chemical mediators in the local environment and various intravascular components (i.e., endothelial cells, platelets) that are responsible for the maintenance of adequate blood flow to meet the metabolic demands of tissues and organ systems. Our data suggest that PGE_1 may have a unique modulatory action on nondiscoid erythrocytes that may also be of considerable biological and clinical significance in determining specific rheologic properties and maintaining normal hemodynamics in vivo.

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