The Effect of Malonyldialdehyde on Erythrocyte Deformability

By C. Pfafferott, H. J. Meiselman, and P. Hochstein

The addition of malonyldialdehyde (5–20 μM) to human erythrocytes results in a marked decrease in cellular deformability as measured with a counter-rotating, cone plate Rheoscope when low shear forces (2.5–25 dynes/sq cm) are applied. At high shear forces (125–500 dynes/sq cm), malonyldialdehyde at 5 μM had no effect on deformability, although at concentrations of 10 and 20 μM a small but statistically significant decrease was evident. These effects of this crosslinking agent are observed in the absence of alterations in cell volume and intracellular viscosity. The results obtained are in accord with the view that the polymerization of membrane constituents may contribute to the events that lead to the removal from the circulation of either aging cells or cells exposed to peroxidation-initiating agents.

The capacity of erythrocytes to survive in the circulation is generally thought to be a consequence of factors that affect their mechanical properties (i.e., cellular deformability). Determinants of cellular deformability include extrinsic properties, e.g., cell shape and the surface area/cell volume ratio, and intrinsic properties, e.g., internal viscosity and membrane mechanical behavior. Among the biologic processes likely to affect the mechanical behavior of the plasma membrane are those which involve the peroxidation of endogenous membrane phospholipids. We have previously shown that the exposure of human erythrocytes to low concentrations of tertiary butyl hydroperoxide, an agent that induces the peroxidation of unsaturated fatty acids in membrane phospholipids, markedly increases membrane rigidity and decreases cellular deformability. Phenylhydrazine is another agent known to induce lipid peroxidation, to decrease erythrocyte survival, and to cause decreases in bulk lipid fluidity. Alterations in bulk lipid fluidity consequent to membrane lipid peroxidation may be associated with the formation of fluorescent aminophospholipid complexes and high molecular weight protein polymers derived from spectrin. An agent that might be responsible for such polymerization reactions during lipid peroxidation is malonyldialdehyde (MDA). This article describes experiments in which the addition of malonyldialdehyde to erythrocytes, in the absence of lipid peroxidation, resulted in decreased cellular deformability. These experiments are consistent with a view that the polymerization of membrane components may alter cellular deformability and survival through effects on membrane mechanical behavior.

MATERIALS AND METHODS

Venous blood was obtained from healthy donors and mixed with heparin (0.05 mg/ml). The blood was centrifuged and the plasma and buffy coats removed. The cells were washed twice in isotonic saline solutions (PBS) consisting of 0.121 M NaCl, 0.03 M KH2PO4/Na2HPO4, and 200 mg/100 ml human serum albumin (pH 7.4, 292 ± 2 mosmole/kg).

The washed cells were resuspended to a 5% hematocrit in PBS, which contained no serum albumin, and were incubated in siliconized flasks for 30 min at 37°C with varying concentrations of malonyldialdehyde (MDA). MDA was prepared from a solution of 1,1,3,3-tetramethoxypropane (Aldrich Chemical Co, Milwaukee, Wis.) exposed to 0.01 N HCl for 30 sec and then neutralized. Since acid hydrolysis of this compound yields methanol in a 1:4 ratio (MDA:CH3OH), this alcohol was also incubated with cell suspensions in each experiment. Moreover, since it is possible that the oxidation of methanol in erythrocytes may give rise to formaldehyde, this latter substance was also included in the incubation medium during certain experiments.

After incubation the cells were immediately washed twice in PBS containing human serum albumin. For the analysis of cell volume the cells were resuspended in Isoton-II (Coulter Diagnostics, Hialeah, Fla.). Mean cell volumes were determined with an Electrozzone-Celloscope (Particle Data Inc., Elmhurst, Ill.) equipped with a 76 μm orifice and operating with a 20% rejection level to eliminate the artifact caused by nonaxial transit of the RBC. The viscosities of the hemoglobin solutions were determined at 25.0°C with a Wells-Brookfield cone-plate viscometer, previously calibrated with standard oils of known viscosity. Hemoglobin concentrations were determined spectrophotometrically by the cyanmethemoglobin method.

Erythrocyte deformability measurements were carried out after resuspending the incubated and washed cells at hematocrits of 1%–2% in PBS containing 20 g/100 ml of dextran T-70 (Pharmacia, Uppsala, Sweden). The osmolality of these dextran solutions was 290 ± 5 mosmole/kg and the viscosity was 21 ± 0.5 cP at 25°C. The deformability of single cells was observed and measured using a counter-rotating, cone-plate Rheoscope, which was mounted on an inverted microscope (Leitz Diavert) equipped with a 40× (NA 0.70) phase-contrast objective. The cell suspensions placed in the gap between plate and cone were subjected to 10 different shear stresses, which were increased, in a step-wise fashion, from 0 to 500...
Erythrocytes aligned in the stationary layer of the shear field were observed at a radial distance of 1200 μm to 1500 μm from the center of rotation. Photomicrographs of the cells were taken at the various shear stresses using high contrast film (Kodak 2415) and short duration flash illumination. At each shear stress, the deformation index, $D$, of 25–30 cells was determined. $D$ is defined as the ratio $L - W/L + W$, where $L$ is the projected length of the cell in the direction of flow and $W$ is its width perpendicular to the direction of flow. $D$ values increase with increasing cellular deformation and approach a limiting value at high shear stresses. The value of $D$ is zero in the absence of deformation.

**RESULTS**

At the maximum concentrations utilized, the exposure of erythrocytes to MDA (20 μM), methanol (80 μM), and formaldehyde (40 μM) resulted in no change in their resting morphology from the normal biconcave shape. However, MDA-treated cells exhibited striking decreases in their capacity to deform when subjected to fluid shear stresses. This is illustrated in Fig. 1, where for clarity relative cell deformation ($D/Do$) is plotted versus the log of the shear stress. $D$ is the deformation of the treated cells and $Do$ is the deformation of the control (incubated but untreated) cells; a value of $D/Do$ equal to 1.0 indicates no change from control cell behavior. For the cells treated with 5 μM MDA, a 10%–20% reduction in cellular deformability was observed at lower shear stresses (2.5–25 dynes/sq cm); behavior coincident with the control population was observed for all higher stress levels. Treatment of cells with either 10 or 20 μM MDA further decreased their deformability at the lower stresses and, in addition, depressed their deformability at the higher stress levels. At these higher stress levels (125–500 dynes/sq cm), the 5%–7% decrease in $D/Do$ for 10 and 20 μM MDA was found to be statistically significant (two-tailed t test, $p < 0.01$).

We have previously shown that although glutaraldehyde affects cellular deformability at high concentrations, it has minimal, if any, effects at the concentrations of MDA utilized in these experiments. However, since methanol and formaldehyde might also be expected to affect deformability, it was of interest to determine whether these agents had such effects at low concentrations. Methanol is produced during the acid hydrolysis of tetramethoxypropane to form MDA, and formaldehyde might be formed in erythrocytes by the oxidation of methanol during the 30-min incubation period with MDA solutions containing methanol. Figure 2 shows the results of experiments in which these substances were incubated with erythrocytes and the deformability of the treated cells subsequently determined. $D/Do$ values for the incubated cells are again plotted against the log of the shear forces applied. It may be seen that only minimal changes were noted in cells treated with methanol (40 and 80 μM) or formaldehyde (40 μM) at low shear forces (less than 12.5 dynes/sq cm); these changes from control were not statistically significant. At higher shear stresses (above 12.5 dynes/sq cm), the deformability of cells treated with these agents was...
identical to control cells. Note that the concentrations of methanol and formaldehyde correspond to those that might be attained at the highest levels of MDA used in this study. These experiments indicate that the observed effects of MDA are not attributable to methanol formed during its preparation or to formaldehyde that might be derived from the oxidation of methanol in erythrocytes.

The peroxidation of membrane phospholipids and the accumulation of MDA may cause inhibition of membrane \( \text{Na}^+ - \text{K}^+ \) ATPases and the accumulation of intracellular \( \text{Na}^+ \). Such a series of events could result in an increase in cellular volume and a decrease in cellular deformability. It was, therefore, of interest to determine whether the alterations in cellular deformability of MDA-treated cells might be a consequence of increases in cell volume that took place even during the short (30 min) exposure to the crosslinking agent.

Table 1 shows the mean cell volumes of control and treated cells for three typical experiments. It is apparent that although the volumes of control cells differed from experiment to experiment (85.16–88.95 \( \text{cu \, \mu m} \)), there was no significant change in cell volume within a single experiment after treatment with any of the agents listed.

Viscometric analysis of membrane-free hemoglobin solutions prepared from MDA-treated cells also failed to demonstrate measurable viscosity changes from solutions prepared from incubated MDA-free RBC. For cells incubated with 20 \( \mu M \) MDA, hemoglobin solutions of 30.5 and 17.6 g/dl were indistinguishable, within the 2%–3% relative accuracy of the viscometer, from equal concentration control solutions.

DISCUSSION

We view the effects of lipid peroxidation in erythrocyte membranes and the experiments described in this article in terms of the following scheme:

Membrane Unsaturated Fatty Acids

\[ \xrightarrow{\text{Fatty Acid Hydroperoxides}} \]

\[ \downarrow \]

MDA

\[ \downarrow \]

Polymerization of Membrane Phospholipids (PE,PS) and

Polymerization of Membrane Protein (Spectrin)

\[ \downarrow \]

Increased Membrane Rigidity

\[ \downarrow \]

Decreased Cellular Deformability
The peroxidation of unsaturated fatty acids in erythrocyte membranes leads to the formation of fluorescent chromolipids derived primarily from phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) and high molecular weight protein aggregates derived from spectrin. Of special interest is the fact that these polymers of lipid and protein are both found to be concentrated in the membranes of older erythrocytes, and their formation can be mimicked by the addition of MDA to intact erythrocytes. The peroxidation of erythrocytes has also been found to be associated with decreased cellular deformability. Moreover, the peroxidation of erythrocyte membranes causes an increase in bulk lipid microviscosity as measured with the fluorescent probe diphenyl hexatriene. Such an increase may be the consequence of increased lipid–lipid interaction or decreased lipid–protein interaction. Either of these latter events might result directly from the formation of fluorescent chromolipid polymers or from the formation of spectrin aggregates with diminished interaction with phospholipids. The effects of MDA described in this paper suggest that the decreased deformability of cells subsequent to lipid peroxidation may, in fact, be a consequence of these polymerization reactions that lead to altered membrane mechanical behavior. It is reasonable to expect that such effects would be most patent at low shear forces. At high shear forces, the effects of increased membrane rigidity on cellular deformability may be obscured, and other factors (e.g., surface/volume relationships) may assume predominant roles. The amounts of MDA utilized in these experiments are probably more than an order of magnitude greater than those that might be formed from endogenous phospholipids. However, it also seems likely that MDA generated through the decomposition of endogenous peroxidized lipids would more effectively participate in polymerization reactions than exogenous MDA. In any event, these experiments clearly demonstrate that an agent that affects the intrinsic mechanical properties of the plasma membrane may, without altering cell shape, intracellular viscosity, or surface/volume ratios, also affect cellular deformability. Such a phenomenon may contribute to the complex series of events that results in the removal from the circulation of either aging cells or cells exposed to the variety of agents that induce peroxidation reactions.

ACKNOWLEDGMENT

The authors wish to thank Rosalinda Wenby and Silvia Montestruque for their technical assistance with the experimental procedures.

REFERENCES

1. LaCelle PL, Weed RK: The contribution of normal and pathologic erythrocytes to blood rheology. Prog Hematol 7:1, 1971
The effect of malonyldialdehyde on erythrocyte deformability

C Pfafferott, HJ Meiselman and P Hochstein