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Dark effect of 8-methoxypsoralen on human erythrocytes

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Abstract—Furanocoumarin 8-methoxypsoralen (8-MOP) (1–100 μg mL^{-1}) in the dark showed a protective affect against hypotonic haemolysis of the erythrocyte membrane. However, the effect against heat-induced haemolysis was dependent on the concentration of 8-MOP; lower concentrations of 8-MOP showed an inhibiting effect, whereas higher concentrations caused acceleration of haemolysis. 8-MOP was not able to induce haemolysis in isotonic solution at 20 or 37 C. Reaction of erythrocytes with 8-MOP in the dark resulted in a shrinkage of the cells and alterations of their shapes. We conclude that modification of erythrocyte membrane by 8-MOP proceeds via reaction with membrane lipids and proteins. This indicates that the effect on the cell membrane plays an important role in the mechanism of the action of 8-MOP on the cells.

Furanocoumarin 8-methoxypsoralen (8-MOP) is used in combination with UV-A (PUVA) in the treatment of various skin diseases such as psoriasis, vitiligo, a disease of accelerated epidermal cell proliferation, and leucoderma (Dall'Acqua & Caffieri 1988). The photochemotherapeutic effects of furanocoumarins for the skin are thought to arise from their ability to form cyclobutane-type adducts with pyrimidine bases of nucleic acids after UV radiation. Recent investigations have shown that membrane constituents may be a biological target in the photosensitizing action of furanocoumarins. Damage to the membrane can be induced either by an oxygen-dependent mechanism (lipid peroxidation, formation of cross-links in ghost proteins) or an oxygen-independent mechanism, which is due to a direct photoreaction between furanocoumarins and unsaturated fatty acids (Midden 1988; Dall'Acqua & Caffieri 1988; Averbeck 1989; Dall'Acqua & Martelli 1991). The effect of furanocoumarins on the cell membrane without photoactivation is less known. It has been shown that various furocoumarins are able to cause haemolysis of red blood cells in the dark at relatively high concentrations (4·6 × 10^{-4} M) (Vedaldi et al 1988). Laskin et al (1985) detected specific binding of 8-MOP to the surface and cytoplasm of five human cell lines and Walther et al (1990) showed a time-dependent, reversible reduction of rosette-forming lymphocytes after a single in-vivo administration of 8-MOP. In our previous papers we reported an increased pro-

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liferation of phytohaemagglutinin-stimulated lymphocytes in the presence of 8-MOP (Gawron et al 1990; Górski et al 1991). The present studies carried out on human erythrocytes, give further evidence on the action of 8-MOP on the cell membrane in the dark.

Materials and methods

Chemicals. 8-Methoxypsoralen (8-MOP) (Sigma, USA) dissolved in dimethylsulphoxide (DMSO) (Merck, Germany) was the stock solution (10 or 20 mg mL^{-1}) used in the studies. The solutions were always kept in the dark.

Haemolysis in isotonic solution. Erythrocytes were obtained from peripheral blood taken by venipuncture. Sodium citrate was used to prevent clotting. Packed red blood cells were washed three times with phosphate-buffered saline (154 mM NaCl, 10 mM sodium phosphate, pH 7·4) and resuspended in the same saline solution at 50% v/v. Fifty microlitres of erythrocyte suspension was added to 2·5 mL 8-MOP solution (1–100 μg mL^{-1}) in phosphate-buffered saline in a siliconized glass tube. The control sample contained DMSO at the same concentration as that of 8-MOP. The highest concentration of DMSO was 0·5%. Each experiment was performed in triplicate. After 60 min of incubation (20 or 37°C) the reaction mixtures were centrifuged for 5 min at 1800 rev min^{-1} and the absorbance of the supernatant was measured at 540 nm. The relative haemolysis was determined by comparison with a sample showing 100% haemolysis.

All experimental procedures with 8-MOP were carried out in red light. Incubation took place in the dark.

Six independent experiments were performed with erythrocytes obtained from different blood donors.

Heat-induced haemolysis. The inhibition of heat-induced haemolysis was carried out as for haemolysis in isotonic solution. The erythrocytes were incubated with 8-MOP solutions in isotonic phosphate-buffered saline at 54°C for 30 min in a water bath in the dark.

Haemolysis in hypotonic solution. The extent of protection of erythrocytes was measured after 30-min incubation of erythro-

cytes (20°C) with 8-MOP solutions in hypotonic solution (56 mM NaCl in 10 mM sodium phosphate buffer, pH 7·4).

The haemolysis rate was calculated in relation to the haemolysis in the control which was taken as 100%. The actual value of haemolysis in this control was 62·3 ± 15·9% in hypotonic solution and 72·2 ± 16·8% for heat-induced haemolysis in comparison with that in water.

Measurement of erythrocyte diameter. After incubation of erythrocytes with 8-MOP solutions in isotonic buffered saline (20°C, 60 min, in the dark) cells were fixed with 4% glutaraldehyde (30 min) in isotonic saline solution. The diameter of 200 cells were measured using a microscope. Only non-deformed erythrocytes were measured.

Scanning electron microscopy. For scanning electron microscopy red blood cells were fixed with 4% glutaraldehyde and 1% osmium tetroxide in isotonic buffered saline. The erythrocytes were dehydrated in a graded acetone series, dried to the critical point with liquid CO_2 and then coated with gold. The samples were observed with a Tesla BS 300 microscope.

The results were analysed by Student's *t*-test.

Results

Incubation of erythrocytes suspended in isotonic solution containing 1–100 μg mL^{-1} 8-MOP at 20°C did not show a haemolytic effect of the compound. Neither did an increase of the incubation temperature to 37°C cause a haemolytic action of 8-MOP. However, incubation of erythrocytes with 8-MOP at 54°C caused haemolysis in all samples studied (Table 1). In solutions with 8-MOP at a concentration of 1, 10 and 20 μg mL^{-1} the haemolysis rate was less than in control and did not show a dependence on the compound concentration. In samples with 50 μg 8-MOP the haemolysis rate was close to that of control; whereas haemolysis in the solution containing 100 μg 8-MOP was over 30% higher.

8-MOP concentration-dependently decreased the rate of haemolysis in hypotonic solutions (Table 2). 8-MOP also caused changes in the size and shape of cells. Measurements made after 1 h incubation of erythrocytes with 8-MOP in isotonic solution

Table 1. The effect of 8-methoxypsoralen on heat-induced haemolysis.

Concn (μg mL^{-1})	Haemolysis rate (% ± s.d.)
1	75·9 ± 13·8*
10	72·6 ± 18·2*
20	71·1 ± 15·4*
50	97·3 ± 16·1
100	125·8 ± 17·2*

n = 6; *P < 0·001; s.d., standard deviation.

Table 2. The effect of 8-methoxypsoralen on hypotonic haemolysis.

Concn (μg mL^{-1})	Haemolysis rate (% ± s.d.)
1	80·7 ± 12·2*
10	70·1 ± 10·9*
20	60·1 ± 15·8*
50	52·8 ± 14·2*
100	40·3 ± 10·5*

n = 6; *P < 0·001; s.d., standard deviation.

Table 3. The effect of 8-methoxypsoralen on the size of the red blood cells.

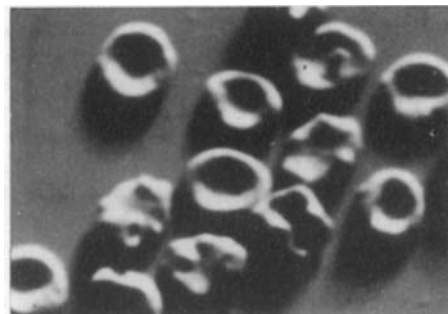| Concn (μg mL^{-1})	Cell diameter (μm ± s.d.)
control	8·12 ± 0·69
10	7·33 ± 0·57*
50	7·08 ± 0·49*
100	6·46 ± 0·63*

n = 200; *P < 0·001; s.d., standard deviation.

FIG. 1. Scanning electron micrograph of erythrocytes treated with 8-methoxypsoralen (40 μg L^{-1}).

(37°C) showed a reduced diameter of red blood cells (Table 3). The diameter of erythrocytes incubated with 8-MOP at a concentration of 10, 20, and 50 μg mL^{-1} was reduced by 9·7, 12·8 and 20·4%, respectively, compared with control cells.

Light microscopy and scanning electron microscopy also showed changes in the shape of cells. Erythrocytes treated with 8-MOP at a concentration of 10 μg and higher showed irregular cells with numerous extrusions on their surfaces, or discoidal cells with ruffled edges (Fig. 1). The number of deformed erythrocytes in a suspension containing 50 μg 8-MOP was 42·0%, whereas in control 9·1% of cells showed a changed appearance.

Discussion

The finding that 8-MOP in isotonic solution at 20 or 37 C did not induce haemolysis at the concentration used is compatible with the findings of Vedaldi et al (1988) and Meffert et al (1980). However, lack of haemolytic effect of 8-MOP does not mean that this compound has no effect on the cell membrane as it was found effective in conditions denaturating proteins (54°C) or in solutions with a low osmotic pressure. The effect of 8-MOP on haemolysis in hypotonic solution was unambiguous. The protective effect increased with increasing concentration of the compound. A possible explanation of inhibited hypotonic haemolysis is changed permeability of the membrane or increased surface area/volume ratio of the cell (Abe et al 1991). The increased proportion of the cell surface for its volume may be caused by intercalation of 8-MOP into the membrane or by cell shrinking. Simultaneous occurrence of both phenomena is also possible. The observed reduction of the diameter of red blood cells as well as changes in the configuration of their surface suggest that increased resistance to the effect of hypotonic solution is caused by increased surface area of the erythrocyte to its volume. A comparison of the magnitude of the protective effect with the decrease in the diameter indicates, however, that another mechanism causing the observed changes may occur.

The extrusions on the surface of erythrocytes effected by 8-MOP are similar to the changes observed by other investiga-

tors. Wennersten (1979) described the formation of blebs on the surface of glia cells, whereas Malinin et al (1990) reported the emergence of plasma-membrane-associated spherical bodies in HUT 102 lymphoblast and a modifying action of 8-MOP on the structure of the erythrocyte ghost (Malinin et al 1986b). Proteins of the cytoskeleton and integral membrane proteins determine the shape of red blood cells (Sato et al 1986; Kwiatkowska 1989). Therefore, interaction of 8-MOP with membrane proteins may lead to changes of the shape and size of erythrocytes, causing its altered reaction to haemolytic agents.

In the search for the mechanism of erythrocyte protection by 8-MOP, membrane permeability should also be taken into consideration as increased permeability of the cell membrane for 5-iodo-2-deoxyuridine and trypan blue after 8-MOP treatment has been observed (Hornicek et al 1989; Malinin et al 1990).

An evaluation of haemolysis at 54 C has shown a concentration-dependent effect of 8-MOP. At lower concentrations 8-MOP decreased the haemolysis rate. The protective effect in the case of heat-induced haemolysis is interpreted as a stabilization of membrane proteins (Benga 1989). Malinin et al (1986a) found that photoactivated 8-MOP reacted with erythrocyte ghost protein causing formation of cross-linked protein. However, such links are not known to be formed after thermal activation. Therefore, it appears that decreased haemolysis can be caused by the influence of 8-MOP on membrane lipids.

The mechanism of photohaemolysis of erythrocytes by psoralens involves the formation of photo-oxidized derivatives and free oxygen radicals. Photolysis products are covalently linked to membrane components, while oxidized derivatives and oxygen radicals cause oxidation of non-saturated fatty acids and amino acids, damaging the erythrocyte membrane (Carrell et al 1975; Fridovich 1986; Potapenko et al 1986a, b, 1988; Dall'Acqua & Caffieri 1988). The results of our studies show that the haemolysis of erythrocytes without UV irradiation is also possible by using a high concentration of the compound at increased temperature (54 C).

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