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MEMBRANE DAMAGE CAUSED BY 8-MOP AND UVA-TREATMENT OF CULTIVATED CELLS

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Abstract. Treatment of red blood cells with a combination of 8-MOP and UVA did not cause any significant hemolysis under the experimental conditions used, and the deuterium test for identification of singlet oxygen mediated reactions was negative. However, similar experiments performed on in vitro cultivated human glia cells caused conspicuous surface membrane alterations, as revealed by scanning electron microscopy. The alterations were seen at higher concentration levels than was expected and might be secondary to earlier appearing intracellular events seen at lower concentrations. The clinical significance of membrane alterations for the results obtained with PUVA therapy require further investigation.

Key Words: Photosensitization; 8-methoxypsoralen and UVA; Photohemolysis; In vitro cultivated cells, human; Electron microscopy, scanning

Oral photochemotherapy with 8-methoxypsoralen (8-MOP) and longwave ultraviolet irradiation (UVA), so-called PUVA treatment, has been found effective in the therapy of skin diseases showing a high proliferation rate, such as psoriasis (11, 19) and for cutaneous malignant lymphoma, viz. mycosis fungoides (5, 6, 7, 23).

8-MOP intercalates with native DNA and forms adducts with thymine bases of the DNA helix upon UVA irradiation, with subsequent inhibition of DNA synthesis both in vitro and in vivo (2, 3, 13, 20, 21, 27).

All therapeutics successfully used for psoriasis, such as coal tar, anthralin, PUVA, methotrexate and shortwave ultraviolet radiation (UVB) probably have in common an effect on DNA-synthesis and are all mutagenic in one system or another (24).

Cellular surface abnormalities have recently been proposed as being of importance in the pathogenesis of psoriasis and ultrastructural differences have been demonstrated between normal and psoriatic cells (17, 18). According to more recent views, malignant neoplasms, whatever their cause, have critically altered plasma membranes (18, 26). This suggests that a common growth control mechanism may be altered in tumour cells and in psoriatic epithelium and that an effective treatment may involve alterations in plasma membrane structure and function.

The aim of the present study was to investigate whether or not the combined psoralen and UVA therapy could inflict any significant membrane damage on erythrocytes when studied by the photohemolysis technique, or on human cultivated cells evaluated by scanning electron microscopy—a sensitive experimental model earlier standardized by us for the investigation of photosensitizing substances (28).

MATERIAL AND METHODS

Photohemolysis

The experiments were performed with the modified photohemolysis technique of Kahn & Fleishaker (8) as used and described by us elsewhere (14, 25).

Erythrocytes were obtained from healthy human adults, and only blood group O Rh+ was used.

8-methoxypsoralen in five final concentrations varying from 0.5×10^{-4} to 2.5×10^{-3} M dissolved in 1% ethanol and 0.1 M Na-Veronal (barbital) buffer at pH 8.0, or in 0.02 M phosphate buffer at pH 7.4, based either on H_2O or D_2O (99.8%, AB Atomenergi, Studsvik, Sweden) was used. For singlet oxygen reactions which occur in aqueous solution, a tenfold increase in efficiency of photo-oxidation of amino-acids and proteins may be seen by replacing H_2O by D_2O as solvent, depending on a corresponding increase in singlet oxygen lifetime—the so-called deuterium test (12, 14, 25).

Packed human red blood cells were washed three times in physiological saline and 0.1 ml was then added to 10 ml of each of the buffered solutions mentioned above, and poured into 2 mm quartz cuvettes. The H_2O content of red cell suspensions, with D_2O as solvent, was calculated to be less than 1%. Controls were incubated in the dark at 37°C.

Test suspensions were exposed to longwave ultraviolet irradiation after about 10 min, which was considered sufficient time for equilibration of the two types of water. The

ultraviolet source was a Blak-Ray B-100 A lamp (Ultra-Violet Products Inc.), giving a longwave ultraviolet radiation ranging from 350 to 380 nm, with its intensity maximum at 366 nm. Test suspensions were irradiated at a distance of 25 cm from the lamp in the quartz cuvettes placed against a black background. UVA doses used ranged from 5 to 15 J/cm². The UVA intensity of the lamp was estimated by a PUVA-meter (H. Waldmann Werk für Lichttechnik). Six independent experiments were performed for each 8-MOP concentration and the blood used in each experiment came from different blood donors.

After irradiation, the test suspensions were centrifuged at 2000 rpm and the optical density of the supernatant fluid at 540 nm was determined on a Beckman DB Spectrophotometer, after an incubation time of 2 h at 37°C. Results were compared with the dark control and with a total hemolysis control (8, 14, 25).

Cell lines and culture conditions

The experiments were performed on in vitro cultivated normal diploid human glia cells from the 787 CG line, established in culture by B. Westermark, The Wallenberg Laboratory, Uppsala, as described earlier (28). Cells were grown at 37°C in humidified air containing 5% CO_2, in Eagle's minimal essential medium supplemented with 10% calf serum and antibiotics (28) on 12 mm glass coverslips placed on the bottom of plastic Petri dishes. The cells were harvested 3 days after subcultivation, when they were actively dividing though not yet forming a confluent monolayer.

The following groups of cells were investigated: 1) Control cells, 2) cells growing in a medium with 8-MOP added in different concentrations: 0.5×10^{-4}, 2×10^{-4}, 4×10^{-4}, 8×10^{-4} and 1.6×10^{-3}M. 8-MOP was diluted in DMSO, the highest concentration of DMSO being 0.02% and this amount was also added to the controls. 3) Cells exposed to UVA solely, light dose 5 J/cm², 4) cells exposed to both 8-MOP and UVA in the concentrations and to the light dose as above.

The UVA light source was the Blak-Ray B-100 A lamp described above. The plastic Petri dishes were irradiated from below at a distance of 10 cm above the lamp. The cultures were cooled by an electric fan in order to avoid heating artefacts (28). 1 hour before irradiation, the normal medium was replaced with a medium containing 8-MOP at the final test concentration. After irradiation, the test medium was immediately replaced by fresh Eagle medium. The cells were harvested and examined 2 and 24 hours post treatment. All cells were maintained in a dark incubator except when irradiated. During irradiation, control cultures were kept outside the incubator for the same period of time as the irradiated cultures.

Scanning electron microscopy (SEM)

For SEM, cells were fixed in 2% glutaraldehyde in 0.1 M Na-cacodylate-HCl buffer with 0.1 M sucrose at 37°C for 60 min (28). The cells were postfixed in 2% OsO_4 in *s*-collidine buffer (pH 7.2) for 90 min at 22°C. A short rinse in 0.15 M cac. buffer (pH 7.2) at 22°C was interposed between the two fixations. Care was taken not to dry the cells during the replacement of medium with the aforementioned glutaraldehyde fixative, warmed to 37°C. After postfixation, dehydration was performed in a graded series of ethanol. The cells were then brought to acetone and critical-point dried from CO_2 in a Polaron E 3000 apparatus. After mounting on stubs with silver-conductive paint, the specimens were coated with a 300 Å thick layer of gold in a Polaron sputter apparatus. The specimens were studied in a Jeol JSM-S1 microscope operated at 10 kV; micrographs were taken with the specimens tilted 45°.

RESULTS

Photohemolysis

In the control experiments, no photohemolysis was seen for 8-MOP in the concentrations up to 2.5×10^{-3} M or for the maximum UVA light dose of 15 J/cm².

The combined treatment of red blood cells with 8-MOP and UVA, even when maximum experimental conditions were used concerning concentrations and light doses, were not found to induce any significant hemolysis when compared with the controls. No differences were noted when the barbital or phosphate buffer was used. The Deuterium test for identification of singlet oxygen reactions by using D_2O as solvent instead of H_2O revealed no additional photohemolytical capacity for 8-MOP in the concentrations investigated.

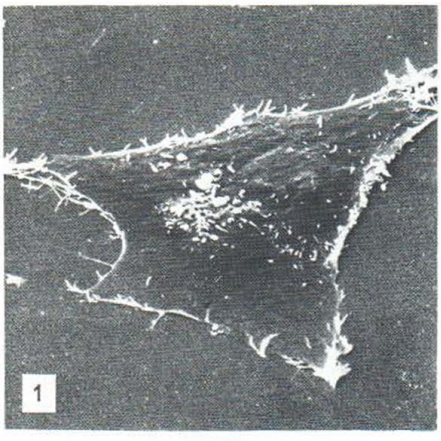
Light microscopy

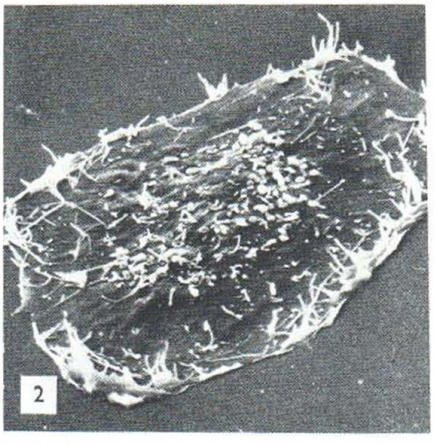
Control human glia cells treated with 8-MOP in the maximum concentrations used or UVA in a light dose of 5 J/cm² alone seemed quite normal when studied 2 and 24 hours post treatment.

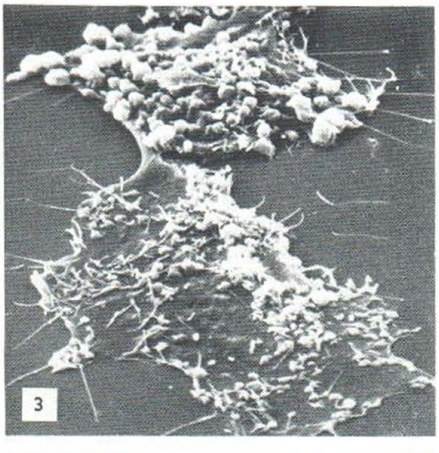
The combined treatment with 8-MOP and UVA evoked an increased pinocytosis visible 2 hours post irradiation for the concentration of 10^{-4} M, and 24 hours later some cellular shrinkage was observed. This cellular damage was more pronounced at the higher concentrations of 2×10^{-4} and 4×10^{-4} M and increasingly more cells were lost 24 hours post irradiation.

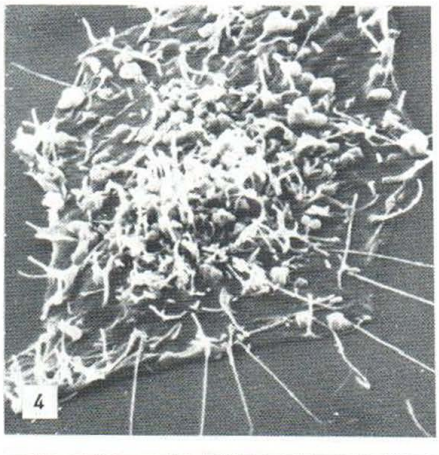
Scanning electron microscopy

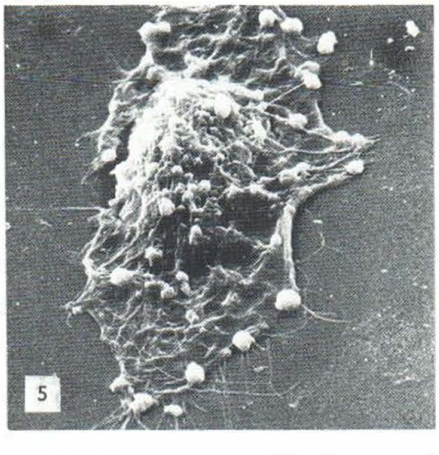
The glia cells in interphase in the control specimens were flattened and appeared to be firmly attached to the substratum. The upper cell surfaces were smooth except for a variable number of tiny microvilli and haphazardly distributed caveolae probably representing endocytotic invaginations. The cells showed leading lamellae with gracile ruf-

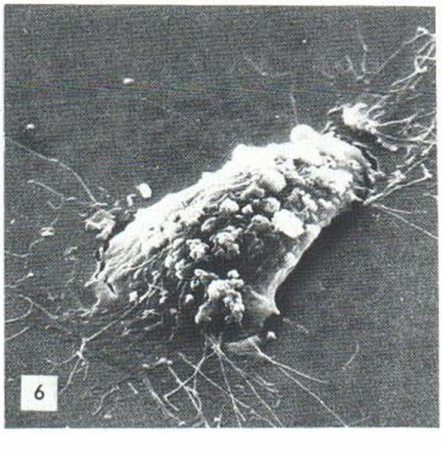










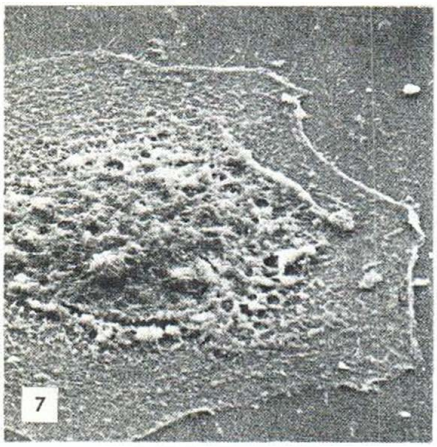


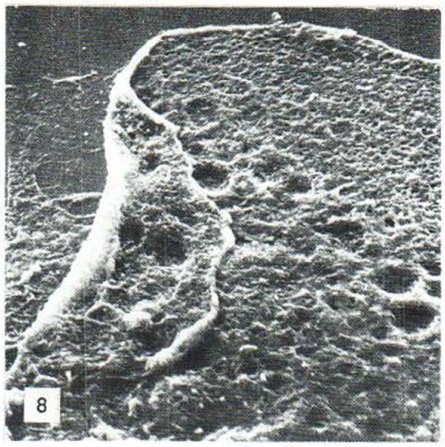
Figs. 1–6. Figs. 1–6 show cell surface alterations induced by UVA 5 J/cm² and increased concentrations of 8-MOP as observed in the scanning electron microscope.

Fig. 1. 0.5×10^{-4} M (×1 200). Fig. 2, 2×10^{-4} (×1 200). Figs. 3 and 4, 4×10^{-4} M (×1 200 and 1 500). Figs. 5 and 6, 8×10^{-4} M (×1 500).

fling membranes displaying great differences in length and width. No blebs or blunt extrusions of any significant kind were seen on the cells in the extended non-mitotic state.

Cells incubated with 8-MOP at the maximum concentration but not irradiated were still thin, flattened and attached to the substrate with microvilli and other structures were well preserved and only





Figs. 7–8. 'Fried' cells after treatment with 8-MOP at a concentration of 1.6×10^{-3} M and UVA 5 J/cm² when investigated 2 hours subsequently. Fig. 7 (×2000). Fig. 8 (×2000).

occasionally found with few very small and discrete blebs at the surface, when studied 2 and 24 hours post treatment. Similarly, the cells exposed to UVA irradiation only at up to 5 J/cm² showed no external fine structural alterations at all, 2 and 24 hours post irradiation.

Following combined treatment with 8-MOP and UVA irradiation, surface alterations were observed, increasing with the 8-MOP concentration used and the time elapsed post treatment (Figs. 1–8). The alterations consisted of gradual formation of cytoplasmic blebs, first visible around the edges and sparsely distributed over the cell surface, then becoming densely packed with simultaneous surface shrinkage, rounding up of the cells and formation of long slender cytoplasmic projections and retraction fibrils. For 8-MOP concentrations of 4×10^{-4} M the cells were gradually lost and, for the concentrations of 8×10^{-4} and 1.6×10^{-3} M, had totally disappeared when studied 24 hours post irradiation. The ruffling membranes were seriously damaged and converted into a collection of small bubbles. The ruffling activity was still observable for concentrations up to 4×10^{-4} but not identifiable at higher concentrations. Cells treated with 8-MOP at a concentration of 1.6×10^{-3} M looked 'fried' when observed 2 hours post treatment (Figs. 7–8).

DISCUSSION

The present experiments were performed on red blood cells and normal human glia cells. These cellular systems were used as they have been extensively studied in our laboratories with respect to morphology and reactions in investigations with other photosensitizing compounds where properly standardized experimental models were established (14, 25, 28). The cells seemed well suited for the investigation of membrane damage and fine structural surface alterations.

No significant photohemolysis was observed when the erythrocytes were treated with the combination of 8-MOP and UVA at fairly high concentrations and light doses. The deuterium test (12, 14, 25) for identification of singlet oxygen mediated reactions was negative. Photohemolysis is a photodynamic process probably representing oxidation of either protein and/or lipid components of the erythrocyte membrane. Several well known photosensitizing compounds are capable of inducing photohemolysis, others are not (4, 8). The system is a poor screening model for photosensitizing agents with avidity for desoxyribonucleic acid or other cellular constituents. Furthermore, photosensitizers which do not require molecular oxygen for their action probably lack photohemolytical capacity. Psoralens do not require molecular oxygen (15, 20) although conflicting findings recently were reported for 8-MOP in aqueous solution (22). It has been shown that some photosensitizers may induce delayed photohemolysis, not visible until at least 24 hours post irradiation (4, 8) probably representing some minor membrane alterations, but the mechanisms are poorly understood. In the present study, analysis was performed 2 hours post irradiation, which is normally sufficient for most photo-

sensitizers, though this consequently does not exclude the occurrence of minor alterations.

In the present experiments on cultivated cells it was observed that the combined treatment with 8-MOP and UVA not only resulted in a reduction of cell proliferation and a change in cell shape, but also had a marked influence on ruffling activity and pinocytosis of the cells. In actively growing cells cultivated in vitro the uptake of medium occurs partly by pinocytosis. It has been suggested that this process is intimately related to the occurrence of ruffles or ruffling membranes. The significance of pinocytosis for growth and multiplication of cells cultivated in vitro is not well known but pinocytosis may be an essential process for cell nutrition and turnover of plasma membrane components.

The threshold concentration for fine structural surface alterations seems to be about 10^{-4} M for 8-MOP, which closely parallels earlier findings where photocytotoxic effects on cultivated leukocytes were not obtained at lower concentrations (29). However, this concentration is at least 100 times greater than the plasma concentration in patients on PUVA therapy. Chromosomal aberrations have been noted for concentrations around 10^{-5} M (16, 24), nuclear mutations (24), inhibition of leukocyte chemotaxis (10) and effects on lymphocytes (1, 9) were observed for still lower concentrations.

From the present study, conclusive evidence of the capacity of combined 8-MOP and UVA treatment to induce conspicuous surface membrane alterations was obtained. The alterations were seen at higher concentration levels than was expected and may be secondary to earlier appearing intracellular events seen for lower concentrations. However, observed surface alterations must imply severe biochemical changes affecting the membrane-bound second messengers systems and the so-called metabolic code. The significance of these membrane alterations for the therapeutic effect must be confirmed by further experiments, including biochemical investigations.

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