Effects of Ultraviolet Irradiation on Cultured B-16 Melanoma Cells

Membrane Fluidity, Lipid Composition and Phospholipase Activity

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Summary: Changes of the plasma membrane during exposure to ultraviolet light were studied in cultured B-16 melanoma cells by the method of electron spin resonance with the spin labelling technique. The lipid peroxide content increased immediately after exposure to UV light and returned to the normal level, 3 hrs post-exposure. Plasma membrane fluidity increased significantly, 3-6 hrs after exposure, and remained at the increased level in the hydrophobic region of the membrane until 18 hrs. There were no significant differences in phospholipid content before and after exposure, except for cardiolipin.

Key words: B-16 melanoma cell—ultraviolet light—electron spin resonance—membrane fluidity—lipid peroxide—phospholipid—phospholipase

Introduction

Exposure of the skin to ultraviolet (UV) light is known to produce severe skin damage and changes of pigmentation. Melanocytes in the epidermis begin melanin synthesis, following UV irradiation. However, the process of melanocyte activation following UV irradiation is not sufficiently understood. Recently, the physiological importance of the plasma membrane in regulating cell functions has become widely recognized. The relationship between the physical state of the plasma membrane and the membrane associated enzyme activity has been explored. Hirata and Axelrod (1980) showed that adenylate cyclase activity is associated with an increase in membrane fluidity, which facilitates the coupling of β adrenergic receptors and adenylate cyclase. Sinensky (1974) showed that a decrease in temperature leads to a decline in the saturation of fatty acids in E. Coli. phospholipids, thereby maintaining the function of membrane proteins at an optimal level. It is important to study the changes of membrane fluidity to understand the regulatory systems responding to external stimuli such as ultraviolet light, hormones and chemicals in the target cells. In the present study, the plasma membrane fluidity, after UV irradiation, was studied using the method of electron spin resonance (ESR) with spin labelling techniques. Cultured B-16 melanoma cells were used as a model of the melanocytes. The phospholipid composition, lipid peroxidation, and phospholipase activity in the plasma membrane following UV exposure are also discussed.

Materials and Methods

Cell culture
The B-16 melanoma cell line was supplied by Shiseido Co., Ltd. (Tokyo). B-16 melanoma cells were cultured in Eagle's minimal essential medium (MEM, GIBCO) supplemented with 10% calf serum (GIBCO) at 37°C with a 5% CO2/95% air atmosphere. The cells were harvested using 0.02% EDTA Ca++-Mg++ free Dulbecco’s phosphate buffered saline (PBS (−))−0.25% trypsin/PBS (−) (9:1, v/v). The cell viability was determined with the trypan-blue exclusion test.

Cells (1×10⁶ cells) were plated in 60 mm dishes, usually 24 hrs prior to experiments. When the number of cells reached approximately 2.0×10⁶ cells/dish, the cells were used for UV experiment.

_Ultraviolet irradiation of cells_

Prior to irradiation, the culture medium was removed and the cells were washed twice with PBS (−). One ml of PBS (−) was added, and the cell monolayer was irradiated by four ultraviolet lamps (Toshiba, FL-20SE 30, wave length 290 nm -320 nm, UV-B) from a distance of 25 cm. The radiant energy was measured with thermocouple (Japan Spectroscopic Co. Ltd., AM 1001). The radiant energy under these conditions was 1.1×10⁵ erg/cm² per min. After irradiation, PBS (−) was replaced with fresh MEM, and the dishes were placed in a CO₂ incubator for the appropriate intervals.

_Spin labelling method and calculation of order parameter_

Two doxyl stearic acid (DSA) spin labels: 5-doxyl stearic acid (5-DSA) and 12-doxyl stearic acid (12-DSA) were obtained from the Aldrich Chemical Company. These stearic acid analogues have a nitroxide radical ring at the 5th and 12th carbon position of the acyl-chain, respectively. Ten μg of DSA in ethanol was placed in a small test tube, and dried to a thin film under a stream of nitrogen gas. The cell suspension (2.5×10⁶ cells in 3.5 ml) in Ca++-Mg++ free Hanks’ balanced salt solution (HBSS (−)) was put into a test tube, and incubated for 10 min at 37°C with gentle shaking. The DSA spin labels were incorporated into the B-16 melanoma cell membrane as described by Sugiyama et al. (1984) and Sakanashi et al. (1986). The cells were washed once to remove the free spin labels, and the pellet was then transferred to a disposable glass capillary tube. The ESR spectrum was obtained 37°C on a JOEL X-band spectrometer, Model JES-FE3X (micro wave power, 4 mW; modulation, 100 kHz, 2.0 G; response, 1 sec; JOEL Ltd., Tokyo, Japan).

The typical ESR spectrum for the 5-DSA spin label embedded in the cultured B-16 melanoma cell membrane is shown in Fig. 1. The observed values of the outer 2T∥ and inner 2T⊥ hyperfine splitting were measured in gauss. The order parameters of S were calculated according to the formula of Gaffney (1976). In the calculation of the order parameter S (T∥) in 12DSA, the values of T∥+2T⊥ and C were taken as 44.5 G and 0.8 G, respectively, because the outer hyperfine splitting is not measurable. The equations used in the calculation are:

\[
S = \frac{T∥-T⊥-C}{T∥+2T⊥+2C} \times 1.723
\]

where \( C = 1.4 - 0.053 \times (T∥-T⊥) \)

\[
S(T⊥) = \frac{43.7 - 3T⊥}{46.1} \times 1.723
\]

The order parameter S or S (T⊥) indicates the degree of motion of a spin probe which has been intercalated into a phospholipid acyl chain, and reflects the dynamic state of the phospholipid bilayer, i.e. membrane fluidity. The range of S or S (T⊥) is from 0 to 1. As membrane fluidity increases, S or S (T⊥) approaches 0. In the 12-DSA spin labelling experiment, the ESR spectrum reflects the dynamic physical state in the hydrophobic region of the bilayer, in which a large proportion of the fatty acid double bonds...
Determination of lipid peroxide content

The lipid peroxide content of cells was measured with the thiobarbituric acid (TBA) reaction established by Yagi (1982). The lipid peroxide content (TBA value) was expressed as nanomoles of malondialdehyde per $1.0 \times 10^6$ cells.

Lipid analyses

Total lipids were extracted according to the method described by Folch et al. (1957). The lipids extracted were analyzed by thin-layer chromatography (TLC) with precoated silica-gel GF (Merck). The solvent system used for resolution of the phospholipids was chloroform/ethanol/water/triethylamine (4:5:1:4, v/v) (Chakravarthy et al. 1985). Identification of the phospholipids on TLC was carried out with authentic samples. The phospholipid content was quantified by the fluorescent quenching degree (Touchstone et al. 1971) with a TLC chromatoscanner (Shimazu).

Assay of phospholipase A$_2$

Phospholipase A$_2$ activity was measured according to the method described by Okazaki et al. (1978) in an incubation mixture (0.6 ml) containing Tris-HCl buffer (10 mM, pH 8.5), EDTA (0.17 mM), CaCl$_2$ (1 mM), 1-palmitoyl-2-[14C]-oleoyl-phosphatidyl choline (86.5 μM, 0.173 μCi, Amersham International plc), 0.05% bovine serum albumin and the pellet fraction after centrifugation at 105,000 × g (microsome enriched, 20-40 μg protein). The incubations were carried out at 37°C for 60 minutes and were terminated by the addition of 4 ml chloroform/methanol (2:1, v/v) and 0.4 ml water. After mixing, the two phases were separated by centrifugation at 750 × g for 10 min. An aliquot of the lower phase was evaporated and developed by paper chromatography. The developing solvent system was heptane/diisobutylketone/acetic acid (85:15:1, v/v). The spot of fatty acid on the paper chromatogram was developed with Rhodamine 6G, and the radioactivity was determined in a scintillation solution.

Assay of phospholipase C activity

Phospholipase C activity was measured according to the method of Sagawa et al. (1983) in an incubation mixture (0.25 ml) containing Tris-HCl buffer (20 mM, pH 7.0), 2-mercapto-ethanol (4 mM), CaCl$_2$ (4 mM), L-3-phosphatidyl-[2-3H] inositol (2 mM, 0.025 μCi, Amersham International plc) and the supernatant fraction after centrifugation at 105,000 × g (20-40 μg protein). The incubations were carried out at 37°C for 60 minutes and were terminated by addition of 1.0 ml chloroform/methanol (1:2, v/v). After mixing, 0.3 ml chloroform and 0.3 ml KCl (2 M) were added. After further mixing, the two phases were separated by centrifugation...
at 750 × g for 10 min. An aliquot (0.75 ml) of the upper phase was evaporated in a counting vial, and the radioactivity was measured employing a Triton X-100 scintillation mixture.

Results and Discussion

In an earlier study (Sugiyama et al. 1984), the localization of the 5DSA spin probe embedded in the B-16 melanoma plasma membrane was confirmed using a method with potassium ferricyanide, which can not penetrate the plasma membrane (Kaplan et al. 1973). The center band amplitude before and after the addition of ferricyanide were measured directly from the ESR spectra. The spontaneous decay of the ESR signal in the B-16 melanoma was observed after 3 hrs. The cells were then incubated with 1 mM K₃Fe(CN)₆ for 10 min, and the signal intensity reversed to 90.1 ± 6.8 % of the initial value. From these results, the 5DSA probe was found to remain predominantly in the plasma membrane. Therefore, the ESR spectra of spin labels indicate the dynamic physical states of the lipid bilayer of the plasma membrane. The cell viability decreased with increasing doses of UV exposure, and dropped to less than 70 % at UV doses greater than 3.2×10⁶ erg/cm², as shown in Table 1. The maximum UV dose at which 90 % cell viability could be maintained was 1.6×10⁶ erg/cm². This level of radiant energy was used throughout the following experiments.

As shown in Fig. 2, the TBA value increased immediately after UV exposure and returned to the normal level within 3 hrs after exposure. However, 6 hrs after UV exposure, a decrease in the TBA value was observed. Fig. 3 shows the alterations of order parameters of plasma membranes exposed to UV, expressed as percent deviation from control. In the 5DSA labelling experiment, the order parameter S decreased at 3 hrs post-exposure and returned to the normal level by 18 hrs post-exposure. This indicates that 3 hrs after UV exposure, an increase in membrane fluidity occurs in the hydrophilic region (5DSA). In the 12DSA labelling experiment, the order parameter S (T⊥) did not change at 3 hrs post-exposure. However 6 hrs after exposure, a decrease in the S (T⊥) value was observed. After that, the

<table>
<thead>
<tr>
<th>UVdose (erg/cm²)</th>
<th>viability (%)</th>
<th>number of experiments</th>
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<tbody>
<tr>
<td>0</td>
<td>94±2</td>
<td>4</td>
</tr>
<tr>
<td>1.6×10⁶</td>
<td>88±4</td>
<td>4</td>
</tr>
<tr>
<td>3.2×10⁶</td>
<td>67±3</td>
<td>4</td>
</tr>
<tr>
<td>4.8×10⁶</td>
<td>58±3</td>
<td>4</td>
</tr>
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B-16 melanoma cells were irradiated by UVB as described in Materials and Methods.
* Mean±S.D.

![Fig. 2. Effect of UVB exposure on lipid peroxide content.](image)

The absolute values of lipid peroxide content (TBA value) in the controls were 0.15±0.01 (0 hr), 0.15±0.02 (3 hr) and 0.14±0.02 (6 hr) nanomoles of MDA per 1.0×10⁶ cells.

The number of experiments was six.
The absolute values of the order parameter $S$ were 0.564±0.003 (0 hr), 0.573±0.006 (3 hr), 0.586±0.009 (6 hr) and 0.562±0.007 (18 hr). The number of experiments was six.

The absolute values of the order parameter $S (T_m)$ were 0.388±0.018 (0 hr), 0.364±0.010 (3 hr), 0.386±0.009 (6 hr) and 0.380±0.011 (18 hr). The number of experiments was six.

$S (T_m)$ value remained under the control level. Thus 6 hrs after UV exposure, the change in membrane fluidity had spread to the hydrophobic region (12 DSA); and the membrane fluidity remained at increased levels until 18 hrs after UV exposure.

It is of interest that the change of TBA values after UV exposure did not parallel the alterations in membrane fluidity. The TBA values increased immediately after UV exposure; but the plasma membrane fluidity increased 3 to 6 hrs after UV exposure, when the TBA values had returned to the control level. However 6 hrs after UV exposure, an increase in the membrane fluidity (12DSA) was observed; whereas the TBA values were less than the control levels by this time (Sugiyama, 1985; Sakanashi, 1986). Further studies on the early changes of membrane fluidity within 3 hrs are now in progress.

To clarify the relationship between lipid peroxidation and membrane fluidity, Sakanashi et al. (1986) determined the direct effect of tert-butyl hydroperoxide on B-16 melanoma cells. 100 μM tert-butyl hydroperoxide was added to the tissue culture medium. The order parameter $S (T_m)$ of 12DSA was determined after 1 hr and 4 hrs of incubation with B-16 melanoma cells. After 1 hr incubation, no change of $S (T_m)$ was detected; however after 4 hrs incubation a decrease of $S (T_m)$ was observed, indicating an increase of membrane fluidity. These results suggest that the membrane fluidity is not influenced directly by lipid peroxide itself, but that the metabolites of lipid peroxide lead to increased membrane fluidity.

Dawson (1976) has shown that phospholipase may be involved in the repair of phospholipids damaged by UV exposure. Table 2 shows the phospholipase $A_2$ and $C$ activities after UV exposure. No significant differences in the activities were
TABLE 2
Effect of UV-B irradiation on phospholipase A2 and C of cultured B-16 melanoma cells

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>UV-B exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>phospholipase A2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n mole [14C] oleic acid release/mg protein/hr</td>
<td>12.3± 5.38</td>
<td>7.14± 4.11</td>
</tr>
<tr>
<td>phospholipase C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n mole [2-3H] inositol phosphate release/mg protein/hr</td>
<td>200±22.9</td>
<td>163±30.3</td>
</tr>
</tbody>
</table>

Values are means±SE

found. To obtain a definite conclusion, the release of [3H]-arachidonic acid from B-16 melanoma cells after UV exposure was investigated. An immediate release of arachidonic acid was observed after UV exposure (data is not shown). The interaction of calcium with the release of arachidonic acid will be discussed in the following paper. De Leo et al. (1985) observed in a mouse embryonal fibroblast cell line that UV exposure resulted in the release of arachidonic acid from membrane phospholipids, through phospholipase A2.

It has been established that membrane fluidity is influenced by the composition of phospholipids, length of the acyl chains in the fatty acids, proportion of unsaturated double bonds, content of cholesterol, lipid peroxidation products, etc. (Parkers, 1982; Yamauchi, 1982; Kameda, 1983a, 1983b). The composition of phospholipids in B-16 melanoma cells exposed to UV light was examined. The total lipid extracted from B-16 melanoma cells was analyzed by the TLC method, 6 hrs after UV exposure; because the change of membrane fluidity had spread throughout the plasma membrane by this time. Fig. 4 shows a typical TLC chromatoscanner record and Table 3 indicates the content of phospholipids calculated from the chromatograms. There were no significant differences in phospholipid content before and after UV exposure, except with cardiolipin. Mitochondria are known to be rich in cardiolipin which influences mitochondrial respiration (Mitchell and Green, 1980). The physiological significance of cardiolipin in plasma membranes exposed to UV light has not been elucidated. The ratio of PC/PE tended to increase after exposure, however a significant difference was not detected in the present experiments. Further studies of phospholipid
TABLE 3

| Phospholipid analysis of B-16 melanoma cells |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | SM             | PC             | PS             | PI             | PE             | CL             | PC/PE           |
| control         | 0.2±0.1        | 31.0±1.3       | 0.6±0.3        | 14.8±1.4       | 48.2±1.2       | 5.3±0.6        | 0.64±0.03       |
| UV B            | 0.1±0.1        | 32.2±2.7       | 0.5±0.1        | 15.6±0.6       | 48.0±3.1       | 3.7±1.3*       | 0.68±0.10*      |

duplicated data, n = 4, *p < 0.01
data indicates quenching ratio (%), Mean±S.D.

components is to be discussed relating to the lipid peroxidation.

This paper is a preliminary report to observe changes of the plasma membrane induced by exposure to ultraviolet light. This model experiment will provide the basis for elucidating the mechanisms of membrane responses to oxidative stress, such as ultraviolet light. Further detailed studies on membrane fluidity perturbated by UV exposure are in progress. The mechanism of chemical transmission in the cell membrane and its relationship to intracellular melanin formation will also be explored.

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References


