Dose-related Effects of Psoralen and Ultraviolet Light on the Cell Cycle of Murine Melanoma Cells

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ABSTRACT

Cloudman (S91) murine melanoma cells were treated with 4'-hydroxymethyltrioxsalen (HMT), a bifunctional psoralen and exposed to long-wavelength (365 nm) ultraviolet light. DNA content of the cells stained with propidium iodide was measured by flow cytometry, and cell cycle phases were delineated from the DNA histograms by using a curve-fitting routine. We found that HMT in combination with long-wavelength (365 nm) ultraviolet radiation blocked melanoma cells in different phases of the cell cycle, depending on the dose of long-wavelength (365 nm) ultraviolet light and the concentration of HMT. The binding of [3H]HMT to DNA was measured parallel with cell cycle analyses. Treatments with HMT at concentrations corresponding to about 1 HMT bound per 10^6 base pairs of DNA led to the accumulation of cells predominantly in G_2 phase. At higher concentrations (2 to 3 HMT/10^6 base pairs), the cells were blocked in the S and G_1 phases. In conclusion, we have shown that extremely sparse substitution of HMT to DNA blocks melanoma cells in the G_2 phase or other phases of the cell cycle in a dose-dependent manner.

INTRODUCTION

Derivatives of psoralen (e.g., trimethylpsoralen, trioxsalen) in combination with UV irradiation (UV-A, 365 nm) are used therapeutically (PUVA therapy) in proliferative skin disorders such as psoriasis (13). The inhibition of cellular proliferation by PUVA treatment has been attributed to the inhibition of DNA synthesis (2, 16, 19, 23). On a molecular level, formation of covalently linked mono- and diadducts between psoralens and DNA have been demonstrated (4, 10).

Another effect of PUVA treatment is the enhancement of cutaneous pigmentation (7). We have investigated a murine melanoma cell line as a model to study the mechanism of hormonally induced pigmentation (11) and found that treatment of these cells with trioxsalen plus UV-A increased tyrosinase activity [the enzyme responsible for melanin synthesis (3)]. In addition, cell growth was inhibited and cells with high DNA activity [the enzyme responsible for melanin synthesis (3)]. In a series of filters into the photomultiplier. Signals from each cell were processed electronically and displayed as histograms of pulse-amplitude frequency distribution on a multichannel pulse-height analyzer. The data were stored and processed by a Sigma-2 computer (Xerox Co., Rochester, N. Y.). Further analysis of the histograms was carried out by means of a program based on the approach, according to the method of Fried (8).

We have investigated the effects of a bifunctional psoralen (HMT) by flow cytometry of melanoma cells stained with propidium iodide, and in parallel experiments we have determined the binding of [3H]HMT to DNA. Our studies showed that at extremely low levels of binding to DNA, HMT caused a G_2 block. However, as the number of substitutions increased, cells became arrested in the S phase and then throughout the cell cycle.

MATERIALS AND METHODS

Cells and Culture Conditions. The PcIA subclone (22) of the murine S91 Cloudman melanoma cell line was cultured in Ham’s F-10 medium containing 12.5% horse serum, 2.5% fetal calf serum, and gentamicin (50 µg/ml) [11, 21, 22]. Tissue culture materials were obtained from Grand Island Biological Co., Grand Island, N. Y. Viability was determined by the dye exclusion test (14). Cells were counted in a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.).

Treatment with Psoralens Plus UV-A. The culture medium of cells in the logarithmic phase of growth was replaced with fresh medium containing HMT. After incubation in the dark for 15 min, the cells were exposed to UV-A from a Black Light (Spectroline, XX-15; Spectronics Co., Westbury, Long Island, N. Y.). The dose of UV irradiation was measured by ferrioxalate chemical radiometry as described earlier (3). Immediately following irradiation, the medium was removed, the cultures were rinsed, fresh medium containing no psoralens was added, and the cultures were further incubated at 37°C for 24 hr.

Flow Cytometry. The cells were lifted from the substrate by a treatment with 1 mm EDTA in Joklik’s medium (Grand Island Biological Co.). They were washed once with a saline:glucose medium (1.5 mM Na_2HPO_4:1.1 mM KH_2PO_4:1.1 mM glucose:0.14 M NaCl:5 mM KCl, pH 7.4) and were fixed for 1 hr in 25% ethanol in saline:glucose medium containing 15 mM MgCl_2. RNase (1 mg/ml, 5 times crystallized; Calbiochem, La Jolla, Calif.) was added, and the cells were incubated for 1 hr at 37°C. The RNase was removed by 2 washes with saline:glucose medium, and the cells were stained with propidium iodide (50 µg/ml) in saline:glucose medium for 15 min at room temperature. The cells were then washed once with saline:glucose medium, and the cell clumps were removed by filtration through a 37-µm mesh Nitex filter (Tetko, Inc., Monterey Park, Calif.). The stained cells were passed through a flow cytometer similar to that described by Stein-kamp et al. (18) but without the sorting mode of operation. The stained cells traversed the flow chamber at a rate of approximately 500 cells/sec and were illuminated by an argon ion laser beam tuned to 514 nm. The emitted light, collected at an angle of 90°, was passed through a series of filters into the photomultiplier. Signals from each cell were processed electronically and displayed as histograms of pulse-amplitude frequency distribution on a multichannel pulse-height analyzer. The data were stored and processed by a Sigma-2 computer (Xerox Co., Rochester, N. Y.). Further analysis of the histograms was carried out by means of a program based on the approach, according to the method of Fried (8). The data were processed by a CDC 6600 computer after the transformation of the DNA histograms to log scale.
functions were extracted from untreated control cell samples with prominent G2 populations (1).

Measurement of [3H]HMT Binding to DNA. Cells were treated with [3H]HMT followed by UV-A irradiation, or they were processed as "dark controls." The DNA was extracted and banded in a CsCl gradient (10); the syntheses of HMT and [3H]HMT were described earlier (9, 24).

RESULTS

Effects of HMT and UV-A Dose on the Cell Cycle. HMT (3 \(\mu M\)) without UV-A irradiation showed no significant effect on the DNA histograms obtained 24 hr after treatment (Chart 1A). By increasing the UV-A dose from 0.1 to 0.3 J/sq cm, the proportion of cells in G2 increased (maximum at 0.3 J/sq cm), and at higher doses the proportion declined. UV-A (0.3 J/sq cm) without HMT had no significant effect on the DNA histograms. At low (1 to 3 \(\mu M\)) HMT concentration, a predominant G2 population accumulated (Chart 1B). At concentrations above 30 \(\mu M\), HMT had no major effect on the DNA content of cells.

Cell Cycle Blockage and Growth Inhibition by HMT. The number of viable cells present 24 hr after treatment was determined parallel with measurements of the cell cycle following treatment with HMT and UV-A (Chart 2). By increasing the dose of UV-A at constant (3 \(\mu M\)) HMT concentration, 100% growth inhibition was attained by doses above 0.3 J/sq cm. At 0.3 J/sq cm, cells that were leaving G1 accumulated in roughly equal numbers in the S and G2 phases. Following higher UV-A doses (2 J/sq cm), the growth of cells was stopped at the phase in which they were at the time of treatment. Similar results were obtained when the HMT concentration was increased at constant (0.3 J/sq cm) UV-A dose. At 1 \(\mu M\) HMT concentration, growth was inhibited by 60%, and the cells were blocked primarily in the G2 phase. HMT (3 \(\mu M\)) caused a nearly complete inhibition of growth with cells accumulating in equal numbers in the S and G2 phases at the expense of G1. At higher HMT levels, the cell cycle phase distribution of the arrested cells is similar to that of the untreated cells.

At HMT concentrations that caused G2 and S blocks, the effects of PUVA treatment on growth and cell cycle were found to be temporary. Similarly to our observations made by using trioxsalen (3), cells treated with HMT recovered their ability to grow within 2 days after the blockage and become indistinguishable from untreated cells in terms of growth rate and cell cycle phase (data not shown).

Kinetics of Cell Cycle Changes. The distribution of cell cycle phases was analyzed by flow cytometry. A, untreated control; B, cells treated with 2 \(\mu M\) HMT plus UV-A irradiation (0.3 J/sq cm). This experiment has been performed 3 times with qualitatively similar results each time.

Chart 2. Comparison of growth inhibition and effects of HMT plus UV-A treatment on the cell cycle. Bottom, plot of values of cell cycle phases obtained from the curve-fitting analysis (1). Top curve, degree of growth inhibition, determined by counting the number of viable cells at 0 time and at the end of the experiment.

Chart 3. Kinetics of cell cycle phase distribution. The cell cycle was analyzed by flow cytometry. A, untreated control; B, cells treated with 2 \(\mu M\) HMT plus UV-A irradiation (0.3 J/sq cm). This experiment has been performed 3 times with qualitatively similar results each time.
tively, psoralen plus UV treatment could have stimulated cells to enter DNA synthesis. The kinetics of cell cycle phase distribution showed that while the G₁ population decreased from 65 to 19% during the first 24 hr (with a simultaneous increase in the S and G₂ populations), cell growth was arrested completely. By Day 2 following treatment, there were only 5% cells left in the G₁ compartment; the percentage of S phase decreased from 43 to 11, and 83% of the cells had G₂ DNA content. Parallel with these changes, the number of viable cells did not change significantly. Since these dramatic changes in the cell cycle occurred while the growth of cells was arrested, accumulation of cells with S and G₂ DNA content can be explained by an inhibition of cell cycle traverse by the treatment with psoralen plus UV.

**Binding of [³H]HMT to DNA.** Cells were exposed to [³H]HMT under conditions identical to those used in the cell cycle studies. DNA was extracted and fractionated, and the bound [³H]HMT was determined as described in "Materials and Methods." "Dark-binding" was measured, and it was found to be in the range of 1.5 to 9.3% of the values obtained following UV-A treatment. The uptake of [³H]HMT into DNA increased linearly from about 1 to 5 molecules of [³H]HMT per 10⁶ base pairs as the free [³H]HMT concentration increased from 0.7 to 3.5 μM (Chart 4). In parallel experiments, the effect of [³H]HMT on the cell cycle was investigated and found to be the same as that of cold HMT (data not shown).

**DISCUSSION**

The cytostatic effects of furocoumarins are explained by their intercalation with DNA in a "dark reaction" (6) followed by the formation of covalently linked mono- and diadducts upon absorption of UV-A (4, 10). We have shown that treatment of melanoma cells with HMT in combination with UV-A irradiation causes the accumulation of cells in the G₂, S, or all phases of the cell cycle, depending on both the dose of UV-A irradiation and the concentration of HMT. Parallel with cell cycle analysis, we have determined the amount of [³H]HMT bound to DNA and found that at a low level of HMT binding (corresponding to about one molecule bound per 10⁶ base pairs) cells accumulate in the G₂ phase. We explain the emergence of G₂ block by assuming that an extremely sparse substitution of HMT to DNA (corresponding to approximately 1 molecule bound through the length of 200 replicons; Ref. 17) may allow DNA replication to proceed but inhibit cells to enter mitosis by preventing the separation of DNA strands cross-linked by HMT. The fact that angelicin, a monofunctional angular psoralen, had no similar effect on the cell cycle (data not shown) points to the importance of diadduct formation by HMT. Also, in another system, Chinese hamster cells were treated with trioxsalen under similar conditions as used in our studies, and 11% of the DNA-bound psoralen was found to be in the form of diadducts (2). By increasing the number of substitutions, DNA replication is hindered and cells accumulate in the S phase. It is interesting to note that a relatively small increase in HMT concentration (i.e., 1 to 3 μM) changes the proportion of cells in G₂ relative to S from 2:1 to 1:1 (see Chart 2B). The dose dependence of cell cycle blocks could then explain the dichotomy between earlier data, that showed DNA synthesis inhibition as the major effect of psoralens (2, 19, 21), and our own observation, that showed G₂ block when low concentrations of psoralens were used (3, 5).

We hypothesized earlier that enhanced cutaneous pigmentation, following the exposure of skin to psoralens plus UV light, was due to cell cycle modulation of normal skin melanocytes (3). Since activation of tyrosinase and formation of melanized granules take place in the G₂ phase (20), blocking the cells in the same phase could have had a positive effect on pigmentation. Indeed, melanoma cells treated with 0.2 μM trioxsalen plus UV-A accumulate in G₂ (5). However, as we have now shown, whether cells are blocked in the G₂, S, or G₁ phases depends on a narrow margin of the dose of PUVA treatment. For example, treatment of cells with 1 μM HMT plus 0.3 J/sq cm UV-A blocked cells in G₂. However, when the dose of UV irradiation was increased 3-fold, cells were arrested in G₁ plus S (Chart 2A). From these dose-related effects, we can anticipate that there is an optimal dose of PUVA treatment to enhance pigmentation. Such an UV dose optimum has been found earlier for the stimulation of tyrosinase enzyme (3).

The concentration-dependent effects of psoralens may provide a tool to modulate the cell cycle to increase the effectiveness of cell cycle-dependent anticancer drugs. As an example, melanoma cells of the same strain that were used in these studies can be destroyed selectively by an MSH:daunomycin conjugate that is recognized by MSH receptors present on cells in the G₂ phase of the cell cycle (22). However, the cell cycle dependence of MSH receptors permits cells to escape from chemotherapy, thereby reducing the therapeutic effectiveness of the conjugate. The use of MSH:daunomycin in combination with PUVA treatment, which blocked melanoma cells in the G₂ phase, has increased the cytotoxic effectiveness of the conjugate (21).

In conclusion, we have correlated the effects of HMT on the cell cycle of melanoma cells with the amount of radiolabeled HMT bound to the DNA of the same strain of cells. At a very
low level of binding, HMT blocks cells in the G2 phase of the cell cycle. Numerous other drugs and treatments have the same effect (15). The interesting feature of the effect of HMT on melanoma cells is that by a moderate increase in the number of HMT molecules bound to DNA, cells accumulate in the S plus G1 phases. In this respect, the effect of HMT plus UV-A treatment resembles the effect of radiation (12).

REFERENCES
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