Photosensitizing Dyes for the Selection of Nontumorigenic Revertants from Human Lung Cancer Cell Lines

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ABSTRACT

Certain positively charged, lipophilic dyes have been noted by various authors to localize selectively in the mitochondria of carcinoma cells. Oseroff et al. (Proc. Natl. Acad. Sci. USA, 83:9729-9733, 1986) studied 10 carcinoma-specific mitochondrial photosensitizers and judged N,N'-bis(2-ethyl-1,3-dioxolane)kryptocyanine (EDKC) to be the most effective in selective carcinoma cell photolysis, a system where light-absorbing molecules accumulate only in carcinoma cells and on illumination initiate a reaction that kills or damages those cells. The present study duplicated the published EDKC retention result for the normal monkey kidney epithelial cell line CV-1. A series of nontumorigenic and tumorigenic human bronchial epithelial and human pleural mesothelial cells were assayed for EDKC uptake and retention, with the intent of using selective carcinoma cell photolysis to isolate nontumorigenic revertants of the tumorigenic lung cell lines. In addition, the uptake and retention of the fluorescent, mitochondria- and carcinoma-specific dye rhodamine-123 were surveyed in a series of hybrids between tumorigenic and nontumorigenic human bronchial epithelial cells. The half-life of dye retention ranged from 6 to 12 h in all the bronchial epithelial and mesothelial cells studied, with little or no dye selectivity for tumorigenic cells. When EDKC-retaining bronchial epithelial cells were illuminated with red light, significant reductions in short term viability and colony-forming efficiency were seen, which became more pronounced as light and dye doses were increased. However, these effects did not correlate with tumorigenicity within the cell series. The method, therefore, does not appear generally useful for the selection of nontumorigenic variants of human bronchial epithelial or pleural mesothelial cancers of the lung.

INTRODUCTION

The isolation of nontumorigenic revertants derived from a clonal population of tumor cells is of interest in the identification of genes which can inhibit the tumorigenic phenotype ("tumor suppressor genes"). The events occurring in such revertants are considered to fall into two broad categories: inactivation of the transforming oncogene and reversion to a normal phenotype despite full expression of the transforming oncogene, presumably due to a dominantly acting tumor suppressor gene.

The first successful methods for isolating nontumorigenic revertants employed cytotoxic agents in a negative selection procedure, in which transformed cells were preferentially killed (1). Revertants have also been isolated through the use of parvovirus (2) and through their increased resistance to ouabain (3) and paraquat (4). One drawback of such cytotoxic or long term methods is that clonal growth of the isolated revertants can be inhibited or slowed by the very agents which promote their selection. For this reason, nontoxic procedures which allow for the rapid identification of revertants have been of interest. One such approach is that of Zarbl et al. (5), who used the positively charged, fluorescent, mitochondria- and carcinoma-specific dye rhodamine-123 in combination with cell-sorting methods to isolate revertants of transformed murine fibroblasts.

Relatively unaddressed, however, is the question of selecting for nontumorigenic revertants through selective carcinoma cell photolysis, i.e., using a carcinoma-specific photosensitizing dye which on illumination initiates a reaction that kills or damages the carcinoma cells, leaving the revertants behind. The selective photolysis procedure is based on the work of Summerhayes et al. (6), who reported that 123Rh was selectively retained for prolonged periods (>2 days) in the mitochondria of many carcinoma cells but not of most normal cells. The mitochondrial specificity of 123Rh was reported to be due to the high potential (negative inside) across the mitochondrial membrane of living cells (7, 8). Oseroff et al. (9) tested 10 carcinoma-specific mitochondrial photosensitizers as candidates for the selective photolysis procedure and judged EDKC to be the most effective in its selectivity for carcinoma cells, its spectral properties, and its low toxicity in the absence of light. They reported that EDKC was preferentially accumulated in carcinoma cells, with >80% of the dye retained 2 h after a 1.0 μM EDKC incubation in human bladder, squamous, and colon carcinomas. In comparison, they observed much lower EDKC uptake levels in normal monkey kidney epithelial (CV-1) cells and normal human keratinocytes, with complete or nearly complete dye release within a few hours. In the carcinoma lines, 0.1 μM EDKC, combined with red light, caused marked in vitro photolysis of human bladder, squamous, and colon carcinomas, but the same protocol had only a minimal effect on human keratinocytes and on CV-1 cells. The cell-killing effect was postulated to be due to toxic or reactive products, generated by the photodecomposition of EDKC, that have the electron transport chain of mitochondria as their primary target (9).

This report describes the feasibility of achieving selective carcinoma cell photolysis for nontumorigenic revertant selection by applying the protocols for EDKC-mediated photolysis, as given by Oseroff et al. (9), to a series of human bronchial epithelial and pleural mesothelial cancer cell lines.

MATERIALS AND METHODS

Chemicals and Reagents. The cationic dyes EDKC and 123Rh were obtained from Molecular Probes (Eugene, OR). The media used for culture of the lung cells (LHC-8, RPMI 1640, LHC basal, PCS, and supplements to make LHC-MM and LHC-9) were purchased from Biofluids, Inc. (Rockville, MD). Other cell culture materials (0.02% w/v L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.02% w/v sodium pyruvate, 0.025% v/v fungizone, 0.05% w/v fungizone, and supplements to make LHC-MM and LHC-9) were purchased from Biofluids, Inc. (Rockville, MD). Other cell culture materials (0.02% w/v L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.02% w/v sodium pyruvate, 0.025% v/v fungizone, 0.05% w/v fungizone, and supplements to make LHC-MM and LHC-9) were purchased from Biofluids, Inc. (Rockville, MD). McCoy's 5A medium was obtained from Mediatech (Herndon, VA), Dulbecco's minimal essential medium from Biofluids, Inc., and minimal essential medium, α-modification, from Gibco (Grand Island, NY).

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2The abbreviations used are: EDKC, N,N'-bis(2-ethyl-1,3-dioxolane)-kryptocyanine; FCS, fetal calf serum; DPBS, Dulbecco's phosphate-buffered saline; NHBE, normal human bronchial epithelial; NHM, normal human mesothelial.
Vitrogen was from Collagen Corp. (Palo Alto, CA) and human fibronectin was from Upstate Biotechnology, Inc. (Lake Placid, NY). 1-Butanol (spectrophotometric grade) was purchased from Aldrich Chemical Corp. (Milwaukee, WI).

Cell Culture. NHBE cells were obtained as described in Ref. 10 and were maintained in LHC-9 medium (10). S6 cells [an immortalized nontransformed cell line obtained by transfection of SV40-large T-antigen into a plasmid containing the SV40 large T-antigen gene (11)] were grown in LHC-8 medium (10) supplemented with 0.5 μg/ml epinephrine. BZR-T33 cells [a tumorigenic cell line obtained by the transfection of SV40-immortalized bronchial epithelial cells with the v-Ha-ras oncogene (12, 13)] were also grown in LHC-8 medium with 0.5 μg/ml epinephrine. HuT292 cells [mucocoeipidermoid lung carcinoma (14) and HuT292DM cells [HuT292 cells which are 6-thioguanine and ouabain resistant (15)] were cultivated in either LHC-8 medium or RPMI 1640 medium, with 5–10 ml/100 ml FCS supplementation. 709C1 and 127T cells [nontumorigenic and tumorigenic hybrid cells, respectively, obtained from the fusion of SV40-immortalized bronchial epithelial cells with HuT292DM cells (15)] were grown in LHC-8 medium supplemented with 0.5 μg/ml epinephrine and 1 ml/100 ml FCS. NHM cells were obtained as described in (16). MET5A cells [an immortalized cell line obtained by transfection of NIH3T3 cells with SV40 large T-antigen (17)] were cultured in LHC-MM medium (16), as were AMT cells [mesothelioma cell line (18)]. T24 cells (human bladder transitional cell carcinoma) were purchased from American Type Culture Collection and cultivated in MyCoys 5A medium with 10 ml/100 ml FCS. FaDu cells (human squamous cell carcinoma of the pharynx) were also obtained from American Type Culture Collection, and were grown in minimal essential medium, α-modification, with 10 ml/100 ml FCS. CV-1 cells (normal monkey kidney epithelial cells) were a gift from Raji Padmanabhan of the Laboratory of Cellular and Molecular Biology, (National Cancer Institute, NIH, Bethesda, MD) and were cultivated in Dulbecco’s minimal essential medium (high glucose) with 10 ml/100 ml FCS.

Assay for Uptake and Retention of EDKC and 123Rh. Cells cultured in duplicate on 60-mm dishes at varying density (0.1–5.0 × 10^5/dish) were incubated with 1.0 μM EDKC or 0.1 μM 123Rh at 37°C for 30 min and then were washed with DPBS containing Ca^2+ and Mg^2+. The dishes were placed in complete medium and incubated at 37°C. After various incubation times (0–48 h), the cells were harvested with 0.02–0.05% trypsin/EDTA and washed twice with DPBS in successive centrifugation steps. The cells were counted and the final pellet was extracted with 1-butanol. After centrifugation to remove debris, the extraction mixture was read at 715 nm on a Beckman DU-65 spectrophotometer (for EDKC) or on a Perkin-Elmer MPF-66 spectrofluorometer with excitation at 516 nm and emission at 536 nm (for 123Rh). Cells were also incubated without dye, and the cells were extracted with butanol, in order to control for contributions of cellular debris to the spectrophotometric signal through nonspecific absorbance and light scattering. EDKC binding to plastic surfaces was studied using dishes containing medium (without cells) as a control. Nonspecific uptake not dependent on membrane potentials has previously been shown to account for 1% or less of the total dye uptake, as measured on cells killed by freeze-thawing (9).

Cell Survival Assay for EDKC Toxicity in the Absence of Light. Cells were plated at 4 × 10^5/60-mm dish. Duplicate dishes were incubated with EDKC at concentrations ranging from 0 to 10 μM for 30 min at 37°C, washed with DPBS, placed in complete medium, and incubated at 37°C. At 1 and 3 days after dye addition, dishes of cells were harvested and counted, and the cells’ replication curves were plotted. For this assay and the EDKC-mediated photolysis assay (see below), cell survival was defined by the presence of several factors: a normal translucent morphological appearance, ability to remain bound to plastic culture dishes, and, in select cases, the ability to exclude trypan blue stain.

Cell Survival Assay for EDKC-mediated Photolysis. From 0.6 to 1.0 × 10^6 cells/60-mm dish were plated. The dishes were incubated with 0.1–0.3 μM EDKC for 30 min. The cells were washed with DPBS and irradiated at varying light doses at 4°C with a xenon arc lamp filtered to deliver light between 600 and 800 nm. The irradiance was between 13.0 and 14.0 mW/cm², and irradiation times were 0–40 min, giving light doses of 0–32 J/cm². The medium was then replaced with complete medium and the cells were incubated at 37°C. After 2 days the cells were harvested and counted. For some of the dishes, the ability of adherent cells to exclude trypan blue stain was tested.

Light- and EDKC-mediated Reduction in Colony-forming Efficiency. Cells were seeded at a density of 1000/30-mm culture well. After incubation with 0.1 μM EDKC for 30 min, duplicate wells were washed with DPBS and irradiated with varying doses of light at 4°C, using the light source described above. The irradiance was between 13.0 and 14.0 mW/cm², and irradiation times were 0–20 min, giving light doses of 0–16 J/cm². An EDKC- and light-free clonogenic assay was done in triplicate to control for clonogenic inhibition by dye in the absence of light. Light alone has previously been shown to have negligible effects on the colony-forming efficiency of carcinoma and noncarcinoma cells (9). After irradiation, the cells were washed with DPBS and placed in complete medium. Colonies were permitted to form over 7 days, fixed with methanol, stained with crystal violet, and counted. The minimum colony size for the purposes of counting was 15 cells. The colony-forming efficiency was obtained by dividing the number of colonies by the number of cells inoculated and multiplying by 100.

RESULTS

EDKC Uptake and Retention. EDKC retention curves from 0 to 48 h for S6 (SV40-immortalized), BZR-T33 (ras-transformed), and HuT292 (lung carcinoma) cells are shown in Fig. 1, upper left. These initial dye uptake studies were done at high density (~1 × 10^6 cells/60-mm dish for HuT292 and S6 cells, ~2 × 10^6 cells/dish for BZR-T33 cells) since the selection for nontumorigenic revertants, as a rare event, would be more likely in systems containing large numbers of cells. Two observations can be made from the graph. First, the lung carcinoma line HuT292 displayed higher levels of uptake than the ras-transformed (BZR-T33) and nontumorigenic SV40-immortalized (S6) bronchial cells. Second, the half-life of dye retention was approximately 10 h for all the cells, with no dye retention detectable at 48 h; thus, no correlation of dye retention with tumorigenicity was observed. HuT292DM cells also were assayed at high density (results not shown) and they displayed the same retention kinetics as the HuT292 cells.

The retention curve for NHBE cells is plotted in Fig. 1, upper right, along with curves for S6 and BZR-T33 cells. All cells here were tested at low density (1–2 × 10^5/60-mm dish) in order to parallel the NHBE data, whose primary culture into the dishes was carried out at the same low density. There was little difference between any of the curves in either slope or y-intercept.

In some systems (e.g., mouse keratinocyte), Ca^2+ ion has been found to be an important regulator and inducer of terminal squamous differentiation for normal cells, while allowing transformed cells to proliferate (19). For this reason, increasing the calcium ion concentration in LHC media was tested as a possible means of improving photosensitizing dye selectivity for transformed cells. Fig. 1, lower left, shows retention curves for the bronchial epithelial series (S6, BZR-T33, HuT292) in LHC-8 containing 1.9 mM Ca^2+ (the level found in LHC-MM) instead of the usual 0.08 mM. The curves for S6 and BZR-T33 duplicated those in Fig. 1, upper right, almost exactly. The HuT292 cells displayed the highest dye uptake, as in Fig. 1, upper left, but the half-life was 8–10 h for all the cell lines, with close to 0% retention at 48 h.

Fig. 1, lower left, shows EDKC retention curves for the human mesothelial cell series. The experiments, at a density of 2–4 × 10^5 cells/60-mm dish, showed a leveling off of the VAMT curve and a 48-h retention value of 8% for this tumor cell line.
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Fig. 1. EDKC retention for human lung cancer cell lines after 30-min incubation with 1.0 μM EDKC. Data points represent the average of duplicate experiments; agreement was within 10% except where indicated. In all cases, nonspecific absorbance and light scattering, as measured on the dye-free control, were negligible. For each graph, the horizontal dotted line represents the lower detection limit for mol ODKC/cell, which was obtained by dividing the lowest readable absorbance value of the spectrophotometer by the number of cells/plate harvested in each study and converting this value to 10^11 x (mol EDKC/cell). Because the number of cells/plate harvested in each experiment varied, the lower detection limit (dotted line) of spectrophotometric sensitivity also varied. The y-axis values can be converted from 10^11 x (absorbance/number of cells) to 10^11 x (mol EDKC/cell) by multiplying by 5.9. Upper left, bronchial epithelial cells at high density (1 x 10^6 cells/60-mm dish for HuT292 and S6 cells; 2 x 10^6/dish for BZR-T33 cells). Upper right, bronchial epithelial cells at low density (2-4 x 10^5 cells/60-mm dish). NHBE cells had no detectable retention at 48 h (results not plotted). Lower left, bronchial epithelial cells at low density (2-4 x 10^5 cells/60-mm dish) and added calcium (to 1.9 mM). Lower right, pleural mesothelial cells at low density (2-4 x 10^5 cells/60-mm dish).

The VAMT cells also displayed the highest dye uptake, a finding analogous to that for the HuT292 cells in the bronchial series. NHM cells showed a half-life of 6 h and ~0% retention at 48 h, whereas METSA (SV40-immortalized) cells showed a half-life of 6 h and ~0% retention at 24 h.

In order to provide a non-lung cell control and to best match the previously published data for EDKC retention (9), T24 (transitional bladder carcinoma), FaDu (squamous cell carcinoma of the pharynx), and CV-1 (normal monkey kidney epithelial) cells were obtained, assayed for EDKC retention, and plotted (Fig. 2) against the published data. Our dye retention assay was reviewed to ensure that it agreed with the published procedure with regard to media type, method of dye addition, and incubator [CO2]. The results showed our T24 cells with 70% retention at 2 h and the FaDu cells with 90% at 3 h. Over 48 h, the half-lives of dye retention were 12 h for T24 cells and 15 h for FaDu cells (results not shown). For CV-1 cells, our results matched the previous data in showing a small barely perceptible dye uptake and complete dye release within a few hours.

123Rh Uptake and Retention. Fig. 3 shows 123Rh retention curves for the bronchial epithelial hybrid cells 709C1 (nontumorigenic) and 127T (tumorigenic). The experiment was done in order to observe the correlation with tumorigenicity of another cationic, mitochondria-specific dye besides EDKC at a minimally toxic dye concentration. As the figure shows, there was little difference between the two cell lines, with negligible retention at 24 h.

Cell Survival Assay for EDKC Toxicity in the Absence of Light. When the number of S6 and BZR-T33 cells harvested 3 days after EDKC treatment (log scale) was plotted against EDKC concentration (linear scale), both cell lines displayed a relatively linear decrease in cell number as the EDKC concentration was increased, starting from 4.1-4.5 x 10^6 cells harvested/60-mm dish at 0 μM EDKC (data not shown). There was little difference in the slopes of the curves for the two cell lines. At 1 μM, the cells were static, as shown by the recovery of about the same number of S6 and BZR-T33 cells as originally plated. In addition to slowing replication, EDKC was also able to kill cells directly; survival of S6 and BZR-T33 cells 1 day after treatment with 10 μM EDKC was 0%.

Cell Survival Assay for EDKC-mediated Photolysis. The 2-day survival of S6, BZR-T33, VAMT, and FaDu cells after treatment with 0.1 μM EDKC and red light is plotted in Fig. 4. Here, for many of the plates, the number of cells recovered exceeded the number originally plated (1 x 10^9), with successive
T292DM cells are plotted, with the EDKC dose increased to cells. The FaDu curve tracked significantly below those of the colony-forming efficiency than the tumorigenic BZR-T33 cells. The nontumorigenic S6 cells displayed only a slightly higher efficiency. Fig. 6A shows the effects of 0.1 \( \mu \text{M} \) EDKC and red light on the colony-forming efficiency due to the dye alone was <10%. A, reduction in colony-forming efficiency for S6 and BZR-T33 cells. B, reduction in colony-forming efficiency for 709C1 and 127T cells.

Fig. 6B shows the results of the same protocol for 709C1 and 127T cells. Qualitatively, the results are the same; the nontumorigenic 709C1 cells showed only a slightly higher colony-forming efficiency than the tumorigenic 127T cells.

**DISCUSSION**

The data indicate that the uptake and retention of the lipophilic, mitochondria-specific, photosensitizing dye EDKC does not correlate generally with immortalization or tumorigenicity within a homologous series of human bronchial epithelial and human pleural mesothelial cell lines. More limited studies done with the lipophilic fluorescent dye \( ^{125}\text{Rh} \) give the same result in a series of hybrids between tumorigenic and nontumorigenic bronchial epithelial cells. Two exceptions should be pointed out, however. First, EDKC uptake levels in the human tumor lines (HuT292 and VAMT) were higher than for the other cells in the bronchial and mesothelial series. Second, VAMT cells were unique among all lung cells tested in that there was a detectable (although small) 48-h dye retention.

As has been mentioned, several authors have demonstrated preferential uptake and retention of certain lipophilic cationic dyes in the mitochondria of carcinoma cells (6, 9, 20). This effect has been postulated to result, at least in part, from differences in mitochondrial membrane potential between carcinoma and normal cells (9, 21–23). Whatever the basis for dye selectivity in other systems, dye uptake levels and retention kinetics for our lung cells appear to be governed by different mechanisms than those which produce the tumorigenic phenotype. It is of interest that immortalization with SV40 T-antigen and transformation with the v-Ha-ras oncogene had little effect on dye half-life or uptake levels for bronchial epithelial cells; thus, these processes by themselves are not capable of inducing selective dye retention. Also, the very similar retention curves displayed by nontumorigenic and tumorigenic human bronchial epithelial hybrid cells are consistent with the idea that tumorigenicity and dye retention may be governed by unlinked genetic factors.

The EDKC-mediated photolysis protocol was undoubtedly effective in reducing both cell survival and colony-forming efficiency for all the dye-retaining cells we studied. The major intracellular targets and damage mechanisms of photosensitizing cationic dyes are not fully understood, but mitochondria are likely to be a principal site of damage (9). The ability of cells with damaged mitochondria to produce ATP from oxidative phosphorylation is typically impaired; however, such cells

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**Fig. 4.** Cell survival assay for EDKC-mediated photolysis. All cells were plated at \( 1 \times 10^6/dish \). Photolysis was carried out with 0.1 \( \mu \text{M} \) EDKC plus light, with harvesting and counting 2 days later. Dye without light had no appreciable effect, as indicated by the control without dye or light (measured for FaDu cells).

**Fig. 5.** Cell survival assay for EDKC-mediated photolysis. 709C1 and 127T cells were plated at \( 6 \times 10^6/60-\text{mm dish} \) and HuT292DM cells at \( 1 \times 10^6/dish \). Photolysis was carried out with 0.3 \( \mu \text{M} \) EDKC plus light; all cells were harvested and counted 2 days later. Dye without light (measured on HuT292DM cells) resulted in 30% inhibition of survival.

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Fig. 6 shows the effects of 0.1 \( \mu \text{M} \) EDKC and red light on the colony-forming efficiency of S6 and BZR-T33 cells. The nontumorigenic S6 cells displayed only a slightly higher colony-forming efficiency than the tumorigenic BZR-T33 cells.
may manage for a time on glycolysis, although their replication is inhibited. This is likely to explain the static behavior of S6 and BZR-T33 cells induced by 1.0 μM EDKC in the survival assay previously described.

The flattening of the photolysis curves at higher light doses may result in part from simple dye photodecomposition, i.e., as most of the dye molecules become photolyzed, each successive increment of light has a decreasing marginal effect. However, increasing the dye concentration did not increase the threshold for the plateau effect; thus, a biochemical mechanism based on selective phototoxicity for certain subpopulations of cells may be important. For example, cells at certain stages in the cell cycle may be less affected by the photolysis procedure, resulting in their persistent 2-day survival in the assays previously described.

EDKC-mediated photolysis has been reported to produce significant selective killing of carcinoma cells in vitro in other systems (9). For the lung cells, however, the photolysis results followed the same pattern as the dye uptake results, in that there was no selectivity for tumorigenic cells. The 2-day survival assays and the clonogenic assays together provide evidence that the nonsensitivity of EDKC-mediated photolysis for lung cells is likely to be both a short and long term phenomenon. The procedure thus appears to have little value in the selection of nontumorigenic revertants of lung cancer cells even if combined with other selection methods. The roughly equivalent avidity of cationic dye for normal and transformed bronchial epithelial and mesothelial cells presents a fundamental difficulty for the use of a mitochondrial dye-dependent photolysis procedure to select nontumorigenic revertants from human bronchial epithelial or mesothelial cancers of the lung.

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