Laser photochemotherapy of rhodamine-123 sensitized human glioma cells in vitro

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The photochemotherapeutic effect of the mitochondria-specific dye rhodamine-123 (Rh-123) on human glioma cells in culture was studied. Cultured U-251MG glioma cells were incubated for 30 minutes in 10 μg/ml of Rh-123 and then exposed to blue-green light between 488 and 514.5 nm using a continuous-wave argon laser. Cells that were treated with Rh-123 and the argon laser at power densities less than 200 mW/sq cm demonstrated increasing tumor-cell killing with increasing time of exposure to laser light. Tumor-cell killing achieved with power densities of light less than 200 mW/sq cm was shown to be due solely to a photochemical effect and not to a direct (thermal) effect of the laser. The photochemical effect was dependent upon the intracellular concentration of Rh-123 and the length of light exposure, and not the intensity of light. The selective retention of Rh-123 by glioma cells and its exclusion from normal cells in conjunction with its photoactivated cytotoxicity suggest that Rh-123 may be a useful photosensitizing drug for the treatment of malignant gliomas in situ.

KEY WORDS • glioma • rhodamine-123 • laser • photochemotherapy • in vitro study

CURRENT techniques of treating locally invasive malignant tumors include surgery, radiation therapy, and chemotherapy. None of these modalities offers the possibility of selective annihilation of tumor that has invaded normal tissue. Photochemotherapy is a relatively new technique for the treatment of malignant tumors and depends upon the light activation of a photoreactive drug (a photosensitizer) that is selectively taken up and/or retained by tumor cells. Photochemotherapy has the advantage over routine chemotherapy of employing agents that have little or no systemic toxicity and that, in general, are specifically targeted to tumor tissue. Only in the presence of light is the photosensitizer converted into a cytotoxic agent able to kill tumor cells.

Photosensitizers that have been used in cancer therapy include fluorescein, eosin, tetracycline, acridine orange, and the porphyrins. Of these, the porphyrins, especially hematoporphyrin derivative (HPD), have been the most widely investigated agents to date. Clinical responses have been evident in some cancer patients treated with photochemotherapy utilizing HPD; but there has not been overall success with this agent, primarily because of problems with drug delivery to the tumor. Hematoporphyrin derivative is excluded by the intact blood-brain barrier because of its large molecular size and the fact that it is protein-bound. Preliminary work on HPD injected into animals and humans with malignant gliomas demonstrated inconsistency of HPD tumor staining and the questionable efficacy of this agent for photochemotherapy of malignant brain tumors.

Rhodamine-123 (Rh-123) is a lipophilic, cationic dye that is specifically taken up by mitochondria of living cells. Although Rh-123 is bound to all mitochondria of a wide variety of cells, different types of cells differ widely in their intensity of staining and their ability to retain dye after being placed in a dye-free medium. It has been suggested that mitochondrial staining by Rh-123 is related to the internally negative membrane potential of the mitochondria and the positive charge on Rh-123 that causes it to be drawn into the mitochondria in accordance with the Nernst equation. Carcinoma cells and muscle cells accumulate more Rh-123 and retain it longer than other cell types, probably because of an increased intramitochondrial negativity. The delocalization of the positive charge due to the ring structure of the Rh-123 molecule causes it to be highly...
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lipophilic, therefore enabling this agent to pass through the hydrophobic barrier of the plasma and mitochondrial membranes.24,26

In our laboratory, we have found Rh-123 to be retained for up to 24 hours in glioma cell lines derived from humans and animals, whereas the Rh-123 was expelled from normal human fibroblasts in culture within 4 hours of incubation in the same medium (unreported findings). Because of the specific staining of glioma cells offered by the selective retention of Rh-123 and the intracellular location of this dye it was our feeling that this would be an ideal drug to photosensitize malignant gliomas. This report discusses the use of Rh-123 in the presence of blue-green light from an argon laser for photochemotherapy of human glioma cells in culture.

Materials and Methods

Cultured human glioma U-251MG cells were grown in an antibiotic-free growth medium composed of a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s nutrient mixture F-12 supplemented to 20% with fetal bovine serum and to 4 mM with L-glutamine. This will be referred to as “the medium.” Twenty-four well plates (2 sq cm area per well) were painted along the sides of each well with black paint to prevent light scattering during laser light exposure. At 70% confluency, U-251MG cells were harvested from monolayer culture with trypsin (0.25%) and 10⁶ cells were plated into the prepared 24-well plates. The cells were incubated in 5% CO₂, 95% humidity overnight at 37°C. The cells were then incubated in the dark for 30 minutes (at 37°C in a 5% CO₂ atmosphere) in medium that contained 10 μg/ml of Rh-123. Experimental control wells contained cells that were incubated in medium without Rh-123. Cell wells were then washed with phosphate-buffered saline (PBS) and fresh medium was added. All work was performed in a darkened laminar-flow biological safety cabinet using indirect room lighting.

Laser-grade Rh-123* was supplied in powder form and prepared in stock solution at a concentration of 5 mg/ml. The solution was stored in the dark at 4°C and was diluted in medium as required.

Blue-green light from an argon laser† was coupled by a 200-μ quartz fiberoptic to a microlens assembly that gave a uniform mode of distribution of light intensity. A distal laser power meter‡ was used to determine the power output at the distal tip of the microlens. During laser irradiation the microlens was positioned 5 cm above each well such that the laser spot size would just cover the 2-sq cm area of each well being treated. Both Rh-123-sensitized and control U-251MG cells (not exposed to Rh-123) were exposed to laser energy and evaluated for cell viability at the end of each experiment. Cell wells were irradiated using variable power densities (10 to 300 mW/sq cm) and variable energy densities (0 to 200 joules/sq cm) in order to study direct thermal and photochemical effects of the laser light.

After laser irradiation, the plates were incubated for 24 hours in the dark at 37°C. The cells were then harvested from the individual wells and resuspended in fresh medium. Tumor cell viability was determined by a rapid colorimetric assay using 3-(4,5 dimethylthiazole-2-yl)-2,5 diphenyl tetrazolium bromide (MTT), a yellow dye which is reduced to a dark blue insoluble formazan by mitochondrial dehydrogenases that are present in viable cells. A 5-mg/ml solution of MTT was prepared in PBS and stored in the dark at 4°C. A 200-μl aliquot of MTT was added to each well and the plates were then incubated (at 37°C in a 5% CO₂ atmosphere) for 4 hours. After incubation, the medium containing unreacted dye in the supernatant solution was removed. The reduced MTT formazan was solubilized in 300 μl of acidified (0.04 NHCl) isopropyl alcohol solution. The optical density of the solubilized color product was quantified at 570 nm using a spectrophotometer calibrated to zero absorbance with acidified isopropanol. Results obtained with cells stained with Rh-123 and cells not exposed to Rh-123 were expressed in terms of OD₅₇₀ (optical density measurements at 570 nm). Preliminary experiments in our laboratories have established that there is a direct linear correlation between the number of live cells as assessed by trypan blue exclusion and the OD₅₇₀ values obtained from parallel cultures (data not shown), as previously reported by Green, et al.16

Results

Excellent granular intramitochondrial staining of all tumor cells was visualized by fluorescence microscopy after 30 minutes of incubation in medium containing Rh-123. There was a direct cytotoxic effect of Rh-123 toward the U-251MG cells that increased with stronger concentrations of Rh-123 in the incubation medium (Fig. 1). There was approximately a 15% cell kill following incubation of U-251MG cells in 10 μg/ml of Rh-123 for 30 minutes as determined by both direct cell counting using trypan blue exclusion of viable cells and the optical density measurements obtained with the MTT assay.

Figure 2 shows that, with increasing time of exposure in terms of increasing energy densities of the Rh-123-stained cells to blue-green light at a fixed power density of 100 mW/sq cm, there was a logarithmic decline in cell survival. Control cells (not stained with Rh-123), although exposed to equivalent energy densities of laser light, were not killed. Cell killing was seen when U-251MG cells not stained with Rh-123 were exposed to the argon laser light at power densities of 200 mW/sq
cm or greater for a total delivered energy of more than 75 joules/sq cm. However, no direct cell killing from laser light exposure was seen with power densities less than 200 mW/sq cm (Fig. 3). There was a logarithmic decrease in survival of Rh-123-stained U-251MG cells with increasing time of exposure that was independent of the intensity of the blue-green light (Fig. 4). Based upon these findings we conclude that it would be necessary to use low intensities of light energy to avoid nonspecific thermal injury due to light exposure in order to obtain selective tumor-cell killing using Rh-123 and blue-green light.

Cells incubated in Rh-123 were exposed to variable power densities of laser light for a total exposure time of 15 minutes. There was no cell killing with blue-green light intensities of less than 5 mW/sq cm for light exposure times of less than or equal to 15 minutes (Fig. 5). The concentration of Rh-123 and its effect on phototoxicity in the presence of blue-green light was also explored. Figure 6 shows that only cells incubated in medium containing more than 1 μg/ml of Rh-123 and then exposed to 100 mW/sq cm of argon laser light for 15 minutes were killed. These data suggest that there is a minimum power density of light for a fixed intracellular concentration of Rh-123 and a minimum concentration of Rh-123 in the U-251MG cells for a fixed energy concentration of activating light that is necessary for phototoxicity to occur.
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FIG. 5. Effect of power density of blue-green light on the survival of U-251MG cells incubated in 10 μg/ml of rhodamine-123 for 30 minutes and exposed to light for 15 minutes. O.D. = optical density measurement.

Discussion

In 1903, Tappenier and Jesionek reported on the topical use of eosin and white light for photosensitization of skin tumors. Since that time, a wide range of photosensitizing drugs have been studied in vitro and in vivo for their photochemotherapeutic effect in the treatment of malignancy. In addition to the photosensitizer and light, oxygen is generally required for inactivation of the cell. It has been shown that photoinactivation of HPD occurs due to energy transfer from the triplet stage of the photosensitizer to oxygen, thus producing singlet oxygen which causes irreversible oxidation of essential cell components.

The effectiveness of a photosensitizer depends upon the degree of localization of the drug in the tumor, which in turn is related to its solubility, partition, and transport characteristics and the biochemical and biophysical properties of the tissue. Also, the effectiveness of the photosensitizer depends upon the photophysical parameters of the sensitizer which include the quantum yield, lifetimes, and energies of the excited singlet and triplet stages of the photosensitizer. A final consideration is assessment of light delivery based on tumor geometry and tissue absorbance of specific wavelengths.

Rhodamine-123, a vital dye which has been widely used in the study of living mitochondria by cell biologists, has been shown within the last 4 years to have selective affinity for certain types of tumor cells in vitro. Selective accumulation of Rh-123 appears to depend on its cationic charge and its consequent affinity for the mitochondrial membrane. Only positively charged rhodamines at physiological pH (Rh-123, rhodamine-6G, and rhodamine-3B) are able to stain mitochondria, whereas uncharged rhodamines (rhodamine-B, rhodamine-19, rhodamine-110, and rhodamine-116) and fluorescein (which is negatively charged) do not selectively stain mitochondria.

Rhodamine-123 is selectively retained by carcinoma cells either because the tumor cells contain Rh-123-binding sites which allow dye to accumulate more readily than untransformed cells or because the cells have a high electric potential (negative inside) which accounts for the attraction and accumulation of these positively charged molecules. If the latter condition is true, then carcinoma cells must have a higher mitochondrial and/or plasma transmembrane potential than normal epithelial cells such that positively charged dye molecules will preferentially accumulate and be retained by them. Malignant cells tend to exhibit an increased level of glycolysis when compared to their nontransformed cell counterparts and the degree of malignancy of the tumor. The mitochondria of transformed cells differ both structurally and functionally from those of normal cells. These mitochondrial abnormalities often parallel the growth rate and the degree of dedifferentiation of tumor cells such that the more rapidly proliferating and least differentiated cells show the most marked changes. It has been reported that cancer cells may have impaired respiratory capacity, which results in an elevated rate of glycolysis. Thus, the selective retention by Rh-123 that is seen in malignant glioma and in many types of carcinoma cell lines may be a function of a mitochondrial abnormality inherent in transformed cells that results in an increased mitochondrial negativity and thus an affinity for the cationic dye Rh-123.

To assess the cytotoxic effect exerted on glioma cells, we chose to employ a new method, the MTT assay. As reported by Green, et al., and confirmed by preliminary studies in our laboratory, this assay provides a direct correlation between viable cell numbers as determined by direct cell counting and the amount of insoluble blue dye product obtained from given numbers of viable cells. Moreover, the assay is simple to perform, requiring only the addition of the unreduced (yellow) MTT dye 4 hours prior to termination. Further advantages include the facts that all experiments can be...
terminated simultaneously and that the insoluble dye product can be solubilized in a stable form for subsequent quantification in a semi-automated spectrophotometer at the convenience of the investigator. By comparison, both direct cell-counting and colony-forming assays are more labor-intensive and slower to perform. The fact that intact functioning mitochondria are required for reduction of the MTT dye to an insoluble product is especially relevant, since the photodynamic effect seen in studies with Rh-123 is presumed to be the result of destruction of normal mitochondrial structure and function.

We have shown that, with increasing concentration of Rh-123 in the incubation medium, there is decreased tumor cell viability particularly with concentrations greater than 10 \( \mu g/ml \). Previous studies have shown that Rh-123 is toxic to carcinoma cells in vitro with continuous exposure to Rh-123 in culture medium, whereas no toxicity has been noted for normal cells. There is a change in dye location from mitochondria-specific (seen at 10 minutes) to cytoplasm-nonspecific (seen at 4 hours) in carcinoma cells but not in normal cell lines which maintain mitochondria-specific staining. This change is noted to correlate with the cytidal activity of Rh-123. Cotreating carcinoma cells with 2-deoxyglucose has been shown to enhance inhibition of growth of carcinoma cells by Rh-123 as determined by clonogenic survival assays. It has been suggested that Rh-123 may disrupt the \( \text{R}_\text{h}\text{F}_\text{i} \) adenosine triphosphatase (ATPase) complex, electron transfer reactions, oxidative phosphorylation, and transport into the cell or mitochondria of malignant cells.

We evaluated the effect of Rh-123 as a photosensitizing agent by using much lower concentrations of this agent and a briefer period of exposure to dye-containing medium than was used in previous studies that examined direct tumor cytotoxicity of Rh-123. Our results show that Rh-123 can be activated by blue-green light to be cytocidal to malignant cells to which it is bound. Increased killing of cells is seen with increases in drug dosage and in the length of activating light exposure. Preliminary studies in our laboratory on rats bearing avian sarcoma virus-induced gliomas have demonstrated that Rh-123 readily crosses the blood-brain barrier and intracellularly stains gliomas without staining normal brain tissue (WC Beckman, et al., in preparation). Because of its selective retention by glioma cells and its exclusion from normal cell lines, Rh-123 has the potential of being a highly selective photosensitizing agent for the treatment of malignant gliomas.

The difficulty with using Rh-123 as a photosensitizing agent lies in the fact that it is only activated by blue-green light. In solution, Rh-123 has an absorption maximum of 502 nm, whereas the absorption maximum shifts to 512 nm upon interaction of Rh-123 with mitochondria. Hemoglobin and oxyhemoglobin have a strong affinity for visible-wavelength light in the blue-green range and thus readily absorb light at these wavelengths. Therefore, penetration of blue-green light through most biological tissues is significantly less than the longer wavelengths of red and near infrared light because of competitive absorption by hemoglobin and other tissue chromophores. Although Rh-123 may potentially be easily targeted into the tumor tissue, it may be difficult to deliver an adequate amount of light energy in order to activate it into its toxic form at a distance from the light source. Certainly, extremely vascular tumors would be poor candidates for this type of treatment because of the competitive absorption of the blue-green light by hemoglobin over the photosensitizer, Rh-123.

Studies are under way in our laboratory to investigate modification of the chemical structure of Rh-123 and utilization of similar compounds that are capable of absorbing longer wavelengths of light that would penetrate brain tissue more readily. In addition, techniques of improving light delivery to the tumor using implantable fiberoptics are being developed. At the present time, photochemotherapy of malignant brain tumors is still in its infancy. Future refinements in both light and drug delivery to tumor may offer a safe therapeutic method of dealing with the invading tumor margin of malignant gliomas.

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References

11. Figge FJH, Weiland GS, Mangiello LOJ: Cancer detection and therapy. Affinity of neoplastic, embryonic, and
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