Enhancement of Radiosensitivity in Human Malignant Glioma Cells by Hypericin * in Vitro*¹

Wei Zhang, Lars Anker, Ronald E. Law, David R. Hinton, Rayudu Gopalakrishna, Qian Pu, Usha Gundimeda, Martin H. Weiss, and William T. Couldwell²

Departments of Neurological Surgery [W. Z., L. A., R. E. L., D. R. H., M. H. W., W. T. C.], Medicine [R. E. L.], Pathology [D. R. H.], Cell and Neurobiology [R. G., U. G.], and Radiation Oncology [Q. P.], University of Southern California School of Medicine, Los Angeles, California 90033

ABSTRACT

Hypericin, an antidepressant and antiviral agent being evaluated in phase I and II trials for patients with HIV infection, is known to be a potent protein kinase C inhibitor. We have investigated its effects on cellular response to radiation via a tetrazolium-formazan cell growth rate assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and clonogenic assay in three human glioblastoma cell lines, U87-MG, A-172, and T98G, and a low-passage malignant glioma culture, 93-492. At a concentration of 5 μM, hypericin inhibited these cells slightly but caused significant radiosensitization (e.g., the cell survival rate after the radiation treatment was 50.2 and 26.0% in cells treated with 6 Gy and 6 Gy plus 5 μM hypericin in U87-MG cells, respectively; P = 0.0285). Hypericin also enhanced the radiosensitivity significantly in the low-passage glioma 93-492 cells. These findings suggest that hypericin represents a potential new agent in combination with radiation therapy of malignant gliomas.

INTRODUCTION

Malignant gliomas (WHO grades 3 and 4), the most common malignant primary tumors arising in the human brain, represent a formidable clinical challenge (1). Following surgical biopsy or gross resection (surgical cure is not possible), radiation therapy is the most common treatment. Unfortunately, therapeutic efficacy of radiation in these tumors is limited by either intrinsic or acquired cell resistance. For this reason, effort has recently been directed toward increasing cellular sensitivity to radiation therapy. Hypericin, a polycyclic aromatic dione isolated from plants, has been used clinically for many years for its antiviral activity (5). Moreover, recent work from our laboratory has revealed that hypericin inhibits malignant glioma cell growth *in vitro* and induces an apoptotic signal in these cells (7). Clinical trials of hypericin in the treatment of patients with recurrent malignant gliomas are currently being undertaken (8). As hypericin is a potent inhibitor of PKC (6), and because the radiosensitivity of glioma cells may be regulated via the PKC system, in the present report we investigate the effects of hypericin on the radiosensitivity of malignant glioma cells *in vitro*. We show here that hypericin sensitizes human glioma cells to ionizing radiation, suggesting that hypericin may be a useful drug in combination therapy for patients with malignant gliomas.

MATERIALS AND METHODS

Cell Cultures. Human glioblastoma (WHO grade 4) cell lines U87-MG, A-172, and T98G and human neuroblastoma cell line SK-N-SH were obtained from the American Type Culture Collection. These cells and a low-passage primary malignant glioma culture (93-492), which was established at our institution, as well as primary human fibroblasts provided by Dr. Tapscott (Fred Hutchinson Cancer Research Center, Seattle, WA) were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 units/ml; 100 μg/ml), and 10 μM HEPES buffered to a pH of 7.0 (all medium constituents were purchased from Life Technologies, Inc., Grand Island, NY). Cells were grown at 37°C in a humidified 5% CO₂ incubator. For radiation sensitivity study, the cells were cultured in 96-well plates (Corning Glass Works, Corning, NY) at a density of 2000 cells/well.

Drug Exposure and Irradiation. For radiation experiments, the cell lines grown exponentially in monolayer were treated with or without hypericin (L. C. Service Corp., Woburn, MA) or other test drugs for 1 h. The cells were then irradiated at room temperature with a 4-MeV linear accelerator at a dose rate of 2 Gy/min. Single-dose exposure ranged from 0 to 8 Gy. After irradiation, the medium was changed with fresh medium with or without hypericin. The cells were incubated for another 5 days (for MTT assay) or 7 days for clonogenic assay.

MTT Assay. The effects of radiation and hypericin on cell growth inhibition were evaluated by MTT assay (9), with some modifications. In brief, the cells were seeded into 96-well

¹The abbreviations used are: PKC, protein kinase C; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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²To whom requests for reprints should be addressed, at Department of Neurological Surgery, Trinity Medical Center, University of North Dakota, One Burdick Expressway West, Minot, ND 58701.
cell culture plates at a density of 2000 cells/well in 100-μl volumes of 10% fetal bovine serum-DMEM and incubated at 37°C. Four to six wells were used for each group. After a 24-h incubation, 100 μl medium with or without test drugs were dispensed in appropriate wells 1 h before irradiation. MTT (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile PBS at 5 mg/ml, filtered through a 0.22-μm filter to remove formazan crystals, and stored for no more than 3 weeks in the dark at 4°C. MTT was prepared at 1 mg/ml in DMEM, and 50 μl of that was added to each well. After a 4-h incubation at 37°C, all of the medium was removed, and 150 μl of 100% DMSO was added to solubilize the MTT-formazan product. The absorbance measurements carried out using a multiple spectrophotometer (Thermo, Molecular Devices Corp.) at 550 nm. The surviving fraction was obtained by comparing the irradiated absorbance with control absorbance.

Clonogenic Assay. Clonogenic assays were performed using a standard colony formation technique as described previously (10), with some modifications. Briefly, U87-MG and 93-492 cells were seeded into 96-well plates (2000 cells/well) and treated with or without hypericin and/or radiation on the next day. After a 7-day incubation, the cells were fixed with 10% formalin and counterstained with 0.5% crystal violet. Only colonies containing 50 or more cells were scored under a microscope (Olympus, Tokyo, Japan). The radiation survival fraction (SF) was then calculated as:

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SF = \frac{\text{Number of colonies in radiated cells}}{\text{Number of colonies in control}} \times 100
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PKC Assay. The PKC activity assays were performed as described previously (11). Briefly, the cells cultured in dishes 10 cm in diameter were treated with or without hypericin (5 μM, 1 h). The cells were rinsed with ice-cold PBS, then with a homogenizing buffer containing 50 mM Tris-HCl, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 2 mM EDTA. The cells were subsequently scraped from the culture dishes, suspended in 2 ml of the solution, and homogenized; the homogenate was centrifuged at 100,000 g for 60 min and designated as the cytosolic fraction. The pellet was resuspended in 2 ml of the buffer containing 1% Triton X-100, homogenized, gently mixed for 30 min, centrifuged at 100,000 g for 60 min, and designated as the particulate fraction. All of the preceding was done at 4°C. The method used for PKC activity measurement (ATP transfer into lysine-rich histone) has been published previously (11). For each cell culture condition, four separate cultures were assayed, and mean and SE values from the independent experiments were then calculated.

Statistical Analysis. Every assay was performed in quadruplicate and repeated at least twice. Representative data from these experiments are presented in “Results and Discussion”; the significance of results was assessed with a one-way ANOVA and Duncan’s multiple comparison.

RESULTS AND DISCUSSION

Despite advances that have been made in conventional surgical, radiotherapeutic, and chemotherapeutic modalities, the prognosis of patients with malignant gliomas still remains poor, with the median survival of patients harboring glioblastoma remaining at less than 12 months (12). Radiotherapy is a treatment that has been consistently shown to extend survival after surgery (13), but most malignant gliomas show resistance to radiation at dosages tolerated by normal brain tissue. An important goal in the management of malignant gliomas is, therefore, to increase cellular sensitivity to radiotherapy. The results of the present study demonstrate that hypericin enhances radiosensitivity in human glioblastoma cells, raising the possibility that this drug could be used in combination therapy for malignant glioma.

To evaluate the effect of hypericin on the cellular response to radiation, a MTT assay was performed, in which the cell number was quantified by tetrazolium dye reduction. Carmichael et al. (9) evaluated the application of MTT assay as a radiosensitivity test and demonstrated that the MTT assay is a rapid, simple method for the assessment of radiation sensitivity in selected cell lines, with the potential for use in the screening of compounds for this purpose. In this regard, the MTT assay has been successfully used for radiosensitivity testing in human malignant gliomas (14). In the present study, radiation dosage-
dependent cell killing in both neuroblastoma and malignant glioma cell lines was observed using the MTT assay. As shown in Fig. 1, significant radiation-induced cell killing was found in SK-N-SH cells, a well-characterized radiosensitive human neuroblastoma cell line (15), whereas the human glioblastoma cell lines were relatively radioresistant. After treatment of these glioma cells with hypericin, the radiation-induced cell death was significantly increased (Fig. 1, A, B, and C). Similarly, hypericin enhanced the radiation effect on 93-492, a low-passage malignant glioma cell culture. Furthermore, the clonogenic assay also demonstrated similar radiosensitization effects of hypericin on U87-MG and 93-492 cells, which form colonies in vitro (Fig. 2). The effect of hypericin on glioma cellular response to irradiation is dosage dependent; low dosages of hypericin caused little or no growth inhibition in both established and low-passage malignant glioma cell lines, but markedly enhanced the radiation-induced cell killing in these cells, suggesting a potent role for hypericin as a radiosensitizer for malignant gliomas. However, at a concentration of 10 μM, hypericin alone inhibited the growth of malignant glioma cells in vitro, which is consistent with our previous results, obtained with the [3H]thymidine uptake method (7). Neither growth inhibition nor radiosensitization was seen in normal human fibroblasts at this dosage (Fig. 3).

The cellular events that increase the radiosensitivity of hypericin-treated malignant glioma cells are probably multifold, as multiple actions of the drug include membrane damage (16), generation of singlet oxygen and free radicals (17), and inactivation of mitochondrial enzymes with impairment of mitochondrial function (18). It has been postulated that one of the antitumor mechanisms of hypericin is via inhibition of PKC (6). PKC is a family of phospholipid-dependent kinases that is known to play an important role in a wide variety of fundamental cellular processes, including proliferation and differentiation in a variety of cell types. Furthermore, the recent work of other groups has demonstrated an important role of PKC in cell survival after irradiation (19, 20). Kim et al. (19) used the PKC inhibitor staurosporine to decrease PKC activity in lung cancer cell lines and found that staurosporine sensitized cells to killing by ionizing radiation. More recently, Haimovitz-Friedman et al. (20) reported that PKC mediated basic fibroblast growth factor protection of endothelial cells against radiation-induced apoptosis, suggesting that PKC activation may provide an antiapoptotic mechanism in vitro and in vivo and may constitute a generic mechanism of radiation resistance. Malignant gliomas possess very high PKC activity when compared to nontrans-
formed glial cells, in both human and rat systems, which correlates strongly with the proliferation rates of these tumors in vitro; tamoxifen, another potent PKC inhibitor, has been used in the treatment of patients with malignant gliomas with some success (11, 21). Furthermore, our previous in vitro studies have demonstrated that the radiosensitivity of glioma cells has been enhanced by depletion of PKC (10). In this study, the radiosensitization activity of hypericin is within the same concentration range as its PKC inhibitory activity (IC50 for PKC, 3.3 μM; IC50 for cAMP-dependent protein kinase, >80 μM; Ref. 6), which is also consistent with our PKC assay data (Fig. 4). Treatment of the human malignant glioma cells with 5 μM hypericin for 1 h in the present study resulted in a significant inhibition of classical (calcium-dependent and phospholipid-activated) PKC activity: 43.6% in U87 cells (P < 0.05) and 64.8% in 93-492 cells (P < 0.01), respectively. These findings suggest that the effects of hypericin on cell proliferation and radiosensitization may depend in part on inhibition of PKC, although the precise mechanism remains to be determined.

Clinical trials of hypericin for the treatment of recurrent malignant gliomas following standard therapy are in progress.4 The drug readily crosses the blood-brain barrier and may be administered p.o. or parenterally (8). The results of the present study, taken together with previous observations of the growth inhibition and apoptosis induction by the drug, suggest that hypericin may play a useful role in combinational therapy of malignant glioma.

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