Photodynamic Therapy with Photofrin Reduces Invasiveness of U87 Malignant Human Glioma Cells

Hye Kyung Woo, M.D., Kyung-Keun Cho, M.D., Hyung Kyun Rha, M.D., Kyung Jin Lee, M.D., Sung Chan Park, M.D., Jung Ki Cho, M.D., Hea Kwan Park, M.D., Joon Ki Kang, M.D., Chang Rak Choi, M.D.

Catholic Neuroscience Center, Department of Neurosurgery, College of Medicine, The Catholic University of Korea, Seoul, Korea

Abstract

Photodynamic therapy (PDT) with Photofrin reduces invasiveness of U87 human glioma cells using several in vitro assays to measure tumor invasiveness. The effects of PDT on cell growth, directional migration and cell invasion were investigated.

Material and Method

Tumor cells were treated with Photofrin at various doses and at a fixed optical (632 nm) dose of 100 mJ/cm². Cytotoxicity was tested using the MTT method. Invasion assays including the matrigel artificial basement membrane barrier migration and spheroid confrontation with confocal microscopic analysis were used to study the relationship between PDT and invasiveness.

Result

U87 cells showed a dose dependent cytotoxic response to increasing Photofrin dose. Data from the matrigel artificial basement membrane assay indicate that PDT inhibits the U87 cell migration dose dependently. Low doses of subcytotoxic PDT treatment, such as 2.5 μg/ml Photofrin dose, also appeared to significantly inhibit migration of U87 cells (p<0.05).

In cocultures between U87 cell spheroids and brain aggregates, progressive invasion with destruction of the brain aggregate occurs. The extent of tumor cell infiltration and proportion or intact brain aggregate remaining after 24 h differs in Photofrin PDT treated versus Photofrin only control, with changes suggestive of a dose- response effect.

Conclusion

Our data indicate that PDT with Photofrin significantly inhibits the invasiveness of U87 cells, and this inhibition is dose dependent.

KEY WORDS: Brain tumor, Tumor spheroid, Photodynamic therapy, MTT assay, Invasion, Laser confocal microscopy.

Introduction

Malignant gliomas, the most common of primary brain tumors, are highly aggressive tumors characterized by a recurrence rate of virtually 100%, even in the presence of aggressive treatment with surgery, radiation and chemotherapy. Despite significant advances in neuro-imaging and neurosurgical techniques, the median survival time of patients with glioblastoma multiforme has barely improved over the past 50 years and remains less than one year. Glioblastoma multiforme is the most common form of malignant brain tumor in adults and is characterized by extensive angiogenesis and invasiveness that cause massive tissue destruction...
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at both the tumor invading edges and in the areas that are not in direct contact with glioma cells. This invasion process consists of a complex series of steps, involving alteration of the tumor cells interaction with specific extracellular matrix (ECM) ligands, proteolysis of the matrix by hydrolytic enzymes, and subsequent migration of the cells through the degraded structures). Most failures of treatment occur because of local recurrence of the tumor, indicating that a more aggressive local anti-neoplastic therapy could be beneficial. During the past decade, it has been shown that photodynamic therapy (PDT) can be used in the treatment of gliomas13).

PDT utilizes a photosensitizer that is selectively taken up and/or retained by neoplastic tissue. When absorbing light of an appropriate wavelength, the photosensitizer produces cytotoxic oxygen products) causing direct cell death, and/or vascular shutdown. Photofrin has been employed as a photosensitizer for PDT in clinical and experimental animal studies) of brain tumor.

Typically, the in vitro sensitivity of cancer cells to PDT is determined by clonogenic survival or growth inhibition of treated cells. However, cells growing under different conditions, such as cell-cycle distribution, nutrition supply, oxygen tension and other environmental factors may differ in their sensitivity to PDT. Multicellular spheroids have quite different growth conditions for cells in different parts of the spheroid. Spheroids both from normal and from malignant tissues may maintain several biochemical and morphological characteristics similar to those of the corresponding tissue in vivo).

The aim of the present study is to measure the anti-invasion effect of PDT with Photofrin on U87 human glioma cells in a multi-cellular spheroid tumor model system

Materials and Methods

1. Cell culture

Human glioma cell lines (U87) were obtained from American Type Culture Collection. The cells were cultured in a standard tissue culture incubator (37°C, 5% CO2, 95% air, 100% humidity). Cells were regularly passaged by treatment with trypsin (0.05%) and were grown in feeding medium of Eagle’s minimum essential medium supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin, nonessential amino acids, 1mg/ml glucose, and 1mM pyruvate (Grand Island Biological Co., Grand Island, NY).

2. Tumor spheroids

Tumor spheroids were generated from trypsinized monolayers by seeding 5 × 10^6 cells in 15ml of the medium into 75cm² medium agar-coated tissue culture flask. Spheroids were formed after 7 days with a diameter of 200–400µm.

3. Brain spheroids

Rat brain spheroids were formed by dissociating fetal rat brain cells from E 18 fetuses and maintaining them in agar overlay culture for 21 days. During this time, the cells aggregate and differentiate into normal adult rat brain elements in 3D spheroids, containing functional neurons, cortical lamination and functional synapses, glial cells and an ependymal core, often with a micro ventricle.

4. PDT of cells

U87 cells and U87 spheroids were loaded with Photofrin by adding an aliquot of the stock solution to the culture medium 24h before irradiation. An argon-dye laser system (Coherent, model INNOVA-70 and CR-500, Palo Alto, CA) provided the light (632nm) for treatment and optical measurement. The light was coupled into a 400µm optical fiber with a distal micro lens (PDT, Santa Barbara, CA) for an 8mm diameter, uniform spot for superficial irradiation. The power at the distal end of the fiber was adjusted to 10mW and was measured before and after each treatment with a power meter (Photodyne, Westlake Village, CA) with a 1 inch integrating sphere detector head. The irradiation power proved constant in all experiments.

5. MTT assay

The cell killing efficacy of PDT with Photofrin was determined using the 3-(4,5-dimethyl-2-yl-2,5-diphenyltetrazolium (MTT) assay. The U87 cells were plated out in 96-well cell culture plates at a density of 1000 cells per well. Following attachment overnight, the cells were incubated with different concentrations of Photofrin for 24h in normal medium. The cells were then exposed to the light at the optical dose of 100mJ/cm². Dark controls for every experimental condition were included. The plates were then returned to the incubator for 24h, at which time MTT assay was performed. Following solubilization of the blue formazan crystals in acidified isopropanol, the plates were read at 570nm on a Titretek Multiscan plate reader.

6. Matrigel artificial basement membrane invasion assay

The invasive potential of PDT treated U87 cells were
tested using the artificial basement membrane constituent polycarbonate filters (polyvinyl pyrrolidone-free, 13 mm diameter 12 μm pores; Poretics, Corp., Livermore, CA) precoated with 1% gelatin and dried overnight. Matrigel stock (11-12 mg/ml) (Collaborative Biomedical, Bedford, MA) was diluted to 0.5 mg/ml with ice-cold double-distilled water, and 100 μl of this solution was spread over the entire surface of the gelatin-coated filter, yielding a 50 μg barrier per filter and a matrigel density of 0.36 μg/mm. The matrigel was then dried at room temperature to a thin film under a laminar flow hood. The matrigel assay, as described by Albini et al.17 and Amar23 was adapted to the multi well Boyden chambers. Briefly, the lower wells of the Boyden chamber were loaded with conditioned medium and the filters were placed coated-side up between the lower and upper wells. Exponentially growing U87 glioma cells were harvested with phosphate-buffered saline with 0.04% versene and suspended in modified Eagle’s medium with Earle’s balanced salt solution supplemented with 0.1% bovine serum albumin. 50,000 cells were added to each upper well. The chambers were placed in a humidified incubator at 37°C in 5% CO2 for 5h, after which the filters were removed, air dried, and stained with Diff-Quik staining system (Baxter Scientific, McGaw Park, IL.). The matrigel and the cells from the top surface were removed with a cotton swab, leaving only cells that had traversed the matrix barrier on the filter. Invasion was quantified by blindly counting cells in 10 fields under an ocular microscope at 200× magnification. Each of 4 replicate filters was counted and data were presented as percent of invading cells. For each experimental condition, the assay was performed at least in triplicate (Fig. 1).

7. Tumor spheroid and normal fetal rat brain aggregate confrontation assay

Aggregates and spheroids with a diameter of 300±50 μm were selected for the experiments.

Confrontation in culture of a tumor aggregate and a normal brain aggregate allow the observation and manipulation of the glioma invasion process in vitro. Briefly, mature brain aggregates with similar diameters of approximately 300 μm were transferred individually into agar-coated 96 well culture dishes (Nunc) containing the medium (200 μl). The exact diameter of each aggregate was subsequently determined. Thereafter, tumor spheroids without treatment as well as with PDT treatment at different Photofrin doses were transferred to the wells containing aggregates. The tumor spheroids used in this experiment were the same size as brain aggregates, and the tumor spheroids as well as

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**Fig. 1.** Matrigel migration assay. A total of 50,000 cells in suspension is added to the upper chamber of modified Boyden chambers. A gelatin coated nucleopore filter with a pore diameter of 12 μm is coated with 50 μg Matrigel. NIH3T3 cell-conditioned medium is placed in the lower chamber. After 5 hours, the filter is removed, inverted, and the cells stained and counted. This time period results in minimal cell proliferation yet allows a significant proportion of the cells to migrate.

**Fig. 2.** Fetal rat brain spheroid confrontation model. Dissociated fetal rat brain cells are allowed to reaggregate in three-dimensional culture and are confronted with a similar spheroid composed of dissociated tumor cells or primary tumor fragment explants. Their interaction at the confrontation zone is allowed to proceed for 48 to 72 hours, at which time the tissue is fixed and embedded, and semi thin sections are prepared. This assay allows the discrimination of the confrontation zone between the spheres as well as of single cell infiltration into normal brain tissue.
aggregates were brought into proximity using a sterile syringe (Fig. 2).

8. Laser confocal microscopy analysis

We applied laser confocal microscopy to analyze the invasiveness of tumor spheroid to normal brain aggregate. The lipid-binding fluorescent carbocyanine dyes 3,3'-dioctadecyl oxacarbo-cyanine perchlorate (DiO) and 1, 1 dioctadecyl 3,3,3',3'-tetramethylino carbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR) were used as tracers to study cell movement. The confrontation pairs in culture were imaged after 5 days using continuous optical sections with a Bio-Rad MRC 1024 laser scanning confocal microscope using photomultiplier tube (PMT) detectors and filter sets for rhodamine (DiI) and fluorescein isothiocyanate-conjugated (DiO). Pseudocolored 8-bit planes were merged together and the distribution of DiI-labeled tumor cells within the DiO-labeled brain aggregates was plotted.

Results

Fig. 3 shows the results of PDT cytotoxicity of U87 cells with different doses of Photofrin at the optical dose of 100 mJ/cm². No significant cytotoxicity was detected at the Photofrin doses of 1 μg/ml and 2.5 μg/ml. However, with Photofrin doses of 5 μg/ml and 10 μg/ml, significant cytotoxic response was present 25.8% (p<0.05) and 60.35% (p<0.05) cell death, respectively.

Fig. 4 shows the results of invasion of U87 cells through matrigel. Our data indicate that PDT inhibits the U87 cell invasion. The invasion rates of U87 cells with the PDT pretreatment with Photofrin dose of 1 μg/ml, 2.5 μg/ml, 5 μg/ml, and 10 μg/ml were 84.5±13.8%, 25.9±11.1%, 13.1±3.7% and 11.3±5.2%, respectively. Thus, even at very low doses of subtotoxic PDT treatment, such as at the 2.5 μg/ml Photofrin dose, significantly inhibits invasion of U87 cells (Photofrin 2.5 μg/ml) p<0.001, 5 μg /ml p<0.001, 10 μg/ml p<0.001).
μ g/ml and 10μ g/ml were 84.5±3.8 %, 25.9±11.1%, 13.15±3.7%, 11.3±5.2% respectively. Thus, even very low doses of subcytotoxic PDT treatment, such as 2.5μ g/ml Photofrin dose, significantly inhibits invasion of U87 cells (p<0.05) (Fig. 5).

In co-cultures between U87 cell spheroids and brain aggregates, progressive invasion with destruction of the brain aggregate occurs. The extent of tumor cell infiltration and proportion of intact brain aggregate remaining after 5 days differs in Photofrin PDT treated versus Photofrin only without PDT treatment with dose-responseness. While quantitation of infiltration is difficult using this assay, these results in a more organotypic model of invasion correlates with the migration data (above), in which a dose response is more easily quantitated. Note the extent of tumor cells (red) infiltrating into the normal brain aggregate (green) and the brain aggregate signal having disintegrated and extended into the substance of the tumor aggregate (Fig. 6).

**Discussion**

Glioma in general, and more highly anaplastic gliomas in particular, infiltrate and spread great distances in the brain. Elegant studies have shown that tumor cells migrate from the primary site of malignant gliomas by the time of diagnosis in the majority of cases and tumor migration is responsible for the local recurrence and tumor progression seen clinically. The poor prognosis of patients with malignant gliomas is at least partially due to the invasive nature of these tumors. The tumors invade the surrounding brain tissue by massive destruction and by migration of single cells along vessels or white matter like corpus callosum.

Since the PDT of brain tumor has attracted increasing interest, both experimentally and clinically, the aim of this study was to evaluate the effect of PDT on inhibition of glioma invasion.

Several in vitro systems have been used to investigate different aspects of the invasion process. Commonly used methods of assessing brain tumor invasiveness in vitro involve measuring migration across an artificial basement membrane of brain extracellular matrix (ECM) components or through a chemotaxis chamber filter. Another model for measuring tumor invasiveness is confrontation assay with aggregates of dissociated embryonic cells or with fra-
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During the invasion process, tumor cells often break through the basement membrane in vivo, but this process has not yet been fully explored. The aim of this study was to evaluate the ability of U87 cells to invade through an artificial basement membrane, and to determine whether PDT could inhibit this invasion. U87 cells displayed a dose-dependent invasion through the matrigel layer. Low doses of PDT did not significantly inhibit invasion, while higher doses increased inhibition. In conclusion, this study demonstrates that PDT with Photofrin significantly inhibits U87 cell invasion, and this inhibition is dose dependent. Further anti-invasion investigations using in vivo PDT are therefore warranted.

Conclusion

In this study U87 cells showed a dose-dependent cytotoxic response to increasing photofrin dose and data from the matrigel artificial basement membrane assay indicate that PDT inhibits the U87 cell migration. Low doses of subcytotoxic PDT treatment such as 2.5ug/ml photofrin dose, also appeared to significantly inhibit migration of U87 cells (p<0.05). In normal fetal rat brain aggregate and tumor spheroid confrontation assay, progressive tumor invasion with destruction of the brain aggregate was present in control group and the extent of tumor cell infiltration and proportion of intact brain aggregate remaining after 24h shows dose response effect in photofrin PDT treated group. In conclusion, our data indicate that PDT with photofrin significantly inhibits the invasion of U87 cells, and this inhibition is dose dependent. Such information may be invaluable in the design of more selective therapeutics.
References


교모세포종 세포주 U87에서 Photofrin을 사용한 광역학 치료가 종양 침습성에 미치는 영향

목적

U87 세포주에서 Photofrin을 사용한 광역학 치료가 종양 침습성에 미치는 영향을 알아보기 위해 U87 세포주를 주사한 연장 척수 기관경 화학 실험에 이용하였다. 재료 및 방법

U87 세포주를 주사한 연장 척수 기관경 화학 실험에 이용한 재료로는 Photofrin(632nm 100mJ/cm²)을 이용하여 Matrigel artificial basement membrane assay와 tumor spheroid fetal rat brain aggregate(FRBA) confrontation assay가 사용되었다. 중앙 단어

Matrigel artificial basement membrane assay와 tumor spheroid fetal rat brain aggregate(FRBA) confrontation assay는 Photofrin 150mJ/cm²로 2.5ug/ml의 photofrin을 사용하고 실험 후 염증 반응과 영향이 감소한 (p<0.05) 종양 침습성에 영향을 미친 양상이 관찰되었다. 결과

PDT(Photofrin 150mJ/cm², photofrin 2.5ug/ml)를 치료한 경우 종양 침습성에 미치는 영향을 알아보기 위해 PDT를 도입한 실험 결과를 살펴보았다.

본 연구의 결과는 Photofrin과 PDT를 이용한 치료가 종양 침습성에 미치는 영향을 알아보기 위한 실험의 결과를 제공한다.