THE INHIBITING EFFECT OF PHOTODYNAMIC THERAPY AND NOVEL RECOMBINANT HUMAN ENDOSTATIN ON THE IN VIVO GROWTH OF U251 HUMAN GLIOMA XENOGRAFTS

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Summary – Endostar, a novel recombinant human endostatin expressed in Escherichia coli, was approved by the State FDA in China. To investigate the effect of endostar and photodynamic therapy (PDT) on the in vivo growth of U251 glioma. Seven days after inoculation with U251 cells, nude mice with MRI-confirmed glioma were randomly assigned to 4 groups: PDT+endostar group; PDT group; endostar group and control group. In the PDT group, survival prolonged, accompanied by an increase in apoptosis, when compared with the control group. Furthermore, these changes were more pronounced in the PDT+endostar group. After PDT, hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF-A) expression was markedly increased and after endostar treatment, HIF-1α and VEGF-A expression was significantly reduced. PDT, in combination with endostar, can significantly inhibit the growth of U251 glioma. This approach may represent a promising strategy in the treatment of malignant tumors.

Glioma is a common intracranial malignancy accounting for approximately 40–50% of intracranial malignancies. The 5-year survival rate is approximately 30% in patients with astrocytoma [14]. Photodynamic therapy (PDT) has been an effective auxiliary strategy in the treatment of glioma [11]. PDT treatment is based on the presence of a drug with photosensitizing properties combined with visible or far red light and oxygen.

Following PDT, a state of hypoxia is induced within the tumor tissue as a result of rapid oxygen consumption [4]. Tissue hypoxia induces a plethora of molecular and physiological responses, including an adaptive response associated with gene activation. A primary step in hypoxia-mediated gene activation is the formation of the transcription factor complex (hypoxia inducible factor-1α – HIF-1α). A number of HIF-1α-responsive genes have been identified, including vascular endothelial growth factor (VEGF), erythropoietin, and glucose transporter-1. VEGF, also called vascular permeability factor, is an endothelial cell-specific mitogen involved in the induction and maintenance of the neovascularity in solid tumors [7].

A. Ferrario et al. [5] applied PDT in the treatment of mouse breast cancer. The results showed that HIF-1α and VEGF expression was increased after PDT but was markedly decreased after treatment with angiogenesis inhibitors (IM862 and EMAP-2). R. Bhuvaneswari et al. [2] revealed that subcutaneous PDT in an orthotopic model of prostate cancer increases not only VEGF secretion but also the fraction of animals with lymph node metastases. PDT followed...
by administration of an antiangiogenic agent, TNP-470, abolished this increase and reduced local tumor growth. Q. Zhou et al. [15] applied PDT in the treatment of mouse nasopharyngeal carcinoma. The semiquantitative RT-PCR results showed that the expression of HIF-1α and VEGF were increased in PDT-treated tumor samples collected 24 h post-PDT. SU5416 and SU6668 in combination with PDT, when used in the treatment, not only prolonged mouse survival but also inhibited cancer growth.

To sum up, in addition to an incomplete tumor cell kill, the ultimate failure of PDT as a treatment of cancer, might be attributed to enhanced angiogenesis powered by PDT induction of VEGF. PDT induction of VEGF could lead to angiogenesis which is associated with tumor growth and metastasis, therefore mitigating its cytotoxic and antivascular effects.

Endostar, a novel recombinant human endostatin expressed in Escherichia coli, was approved by the State FDA in China. Studies have reported that these antiangiogenic effect of endostatin were related with VEGF, which is a crucial regulator in angiogenesis.

In the present study, the effect of hematoporphyrin monomethyl ether mediated PDT and endostar on the growth of glioma was investigated in order to explore their anti-angiogenesis effect on glioma. We also studied HIF-1α and VEGF-α expression to better understand the mechanism of action of PDT on glioma with the hope that our results may demonstrate the clinical application of PDT.

Methods

The U251 glioma cell line was purchased from the Beijing Institute of Science, and cultured in RPMI 1640 medium containing 10% fetal bovine serum in an incubator containing 5% CO2 at 37°C. BALB/c (nu/nu) nude mice weighing 20–22 g (specific pathogen free; Beijing Vitalriver Experimental Animal Co., Ltd; License No. 021). Implantation technique of U251 cells were performed, as in previous studies [3]. Nude mice were anaesthetized with 5 mg/kg xylazine and then fixed in a stereotaxic instrument. After sterilization and skin incision, a hole was made on the skull at 1.0 mm anterior to the anterior fontanel, 2.0 mm lateral to the sagittal suture. The needle of a 50 μl microsyringe (Hamilton, Bonaduz, Switzerland) was inserted to a 3 mm needle through the center of the skull hole and 5×105 U251 cell in 3 μl PBS were injected intracerebrally during a 5 min interval and the syringe remained in the brain for 5 min followed by slow retraction. The hole was sealed with bone wax and the wound was closed.

Magnetic resonance imaging (MRI) scanning was carried out using the Signa 3.0 T MR System. The conditions for scanning were performed as previously described [10]: T1WI and T2WI coronal and horizontal images were acquired from both unenhanced and enhanced scans. Scanning parameters were slice thickness 3 mm, interval 0.3 mm, FOV 8x8 cm, matrix 196x160, 2 NEX. T1WI: SE array, TR450, TE8.6, SL: 3 mm; T2WI: TR5020, TE100, SL: 3 mm. For enhanced scan, Gd-DTPA (Schering AG, Germany, 0.2 mmol/kg) was injected peritoneally. The largest enhancing areas in the horizontal and coronal planes were analyzed and the maximum anteroposterior diameter (L), width (W) and height (H) determined were used for the calculation of tumor volume (V), as follows:

\[ V = \frac{4}{3} \pi \times L \times W \times H \times \frac{1}{8} \text{ (mm}^3\text{)} \]

One week after inoculation, MRI was employed to detect cancer in the brains of the rats. A total of 60 male nude mice with glioma were randomly assigned into 4 groups (n=15 per group): PDT+endostar group, PDT group, endostar group and control group. Eight days after inoculation, PDT was carried out and 0.5 ml of endostar (corporation of Simcere, Nanjing, China; Concentration: 5 mg/ml; doses 20 mg/kg) was given every day intraperitoneal injection for 14 days, as in previous studies [9]. Nude mice in the control group and the PDT group received equivalent normal saline. On day 21, 5 nude mice in each group were sacrificed followed by transcardial perfusion with 4% paraformaldehyde and gliomas were surgically removed using a microsurgical technique. The tumor was harvested for immunohistochemistry and ELISA. The remaining 10 nude mice in each group were used to determine survival rates. MRI was performed at 3 weeks after inoculation to detect tumor volume. All experimental procedures were approved by the Institutional Animal Care and Use Committee. Gliomas were surgically removed using a microsurgical technique.

PDT was carried out, as previously described [8]. HMME (Fudan Zhangjiang Biotech Co., Ltd, CHINA; 5 mg/kg) was injected peritoneally, and 3 h later animals were anesthetized with 10% chloral hydrate and fixed in a stereotoxic device, the skull was exposed, and a 1 mm diameter craniotomy was drilled over the right hemisphere 2.5 mm to the midline and 2.0 mm anterior to the bregma. Three minutes later, the inoculated tumor was exposed with microneurosurgical method. The optical fiber of the PDT instrument (DIOMED 630 Limited, British, wavelength: 630 nm) was placed on the tumor followed by photodynamic treatment for 10 min. The treatment dose rates were 120 J/cm2. After PDT, the hole was sealed with bone wax and skin was sutured. Nude mice in the antibody group and the control group did not receive HMME, but were administered all other procedures.

Tissues were embedded in paraffin and cut into 5-μm sections followed by hematoxylin and eosin staining. Sections were observed under a light microscope. For immunohistochemistry, sections were deparaffinized and hydrated followed by antigen retrieval. Endogenous peroxidase was inactivated with hydrogen peroxide. These sections were treated with rabbit anti-human HIF-1α antibody (1:200) or with rabbit anti-human VEGF-A antibody (1:100); rabbit anti-human GFAP antibody (1:200). Immunohistochemistry was performed according to the manufacturer's instructions. Evaluation of sections was performed based on the proportion of positive cells: <10%, negative (-); 11–30%, weakly positive (+); 31–50%, positive (++); >50%, strongly positive (+++). Sections were independently evaluated by two experienced pathologists blinded to the study. When the scores were different, a consensus was reached through consultation.
Extraction of total protein was performed using 200 mg of frozen tissues and the protein concentration was determined. The protein was stored at –70°C before use. Then, 20 μg of total protein thawed in 1 mL/mg lysis buffer containing protease inhibitors (1% PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/mL phenylmethylsulfonyl fluoride, 100 mmol/L sodium orthovanadate, protease inhibitors). The protein concentration was determined using a standard Lowry method. A human HIF-1α and VEGF-A DuoSet ELISA Development System (R&D) was used to quantify human HIF-1α and VEGF-A levels. Results were normalized to proteins.

The paraffin-embedded tissues were cut into sections and TUNEL assay was performed according to the manufacturer’s instructions (Roche). TUNEL positive cells demonstrated karyopyknosis, chromatin aggregation and dark nucleus. Negative cells were blue and had a normal nucleus. A total of 20 fields were randomly selected from each section at a magnification of 400× and positive cells were counted followed by calculation of the apoptotic index (AI) as follows: AI = (number of positive cells/total number of cells) × 100 %.

Data were presented as mean and standard deviation. Statistical analyses were performed using SPSS 10.0 software. Statistical comparison of tumor volume, HIF-1α and VEGF-A expression, as well as numbers of apoptotic tumor cells between two different treatment groups, were made using t-tests.

Results

Glial fibrillary acidic protein expression and H&E staining: Immunohistochemistry for Glial fibrillary acidic protein revealed positive staining (brown granules) in a variety of cells suggesting glioma. H&E staining showed nuclear atypia and cell pleomorphism in tumor (Fig. 1).

Tumor volume and survival time: Tumor volume in the PDT group was markedly decreased compared to the control group. In the endostar group, cancer growth was significantly slower compared to the control group. In the PDT+endostar group, cancer growth was significantly slower than that of the PDT group or endostar group (Fig. 2).

Kaplan-Meier survival analysis showed that the mean survival was 17.100±1.120 days in control group, which was significantly shorter than that of the PDT group (31.700±1.647 days) or endostar group (24.900±1.464 days). The survival in the PDT+endostar group was the longest at 43.200±1.679 days (Fig. 3).

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Detection of protein expression of HIF-1α and VEGF-A by ELISA: HIF-1α levels, measured by ELISA, in control group, PDT group, endostar group and PDT+endostar group was 223.56±22.39, 769.98±53.38, 109.24±35.21 and 369.87±36.94 pg/mg. VEGF-A levels, measured by ELISA, in control group, PDT group, endostar group and PDT+endostar group was 919.39±63.31, 1860.39±163.58, 369.87±63.35 and 1058.39±83.38 pg/mg.

HIF-1α and VEGF-A expression in the PDT group was markedly higher compared to the control group, but was lower in the endostar group compared to the control group. Moreover, VEGF-A expression in the PDT+endostar group was markedly lower compared to the PDT group (Fig. 4).

Immunohistochemistry for HIF-1α and VEGF-A: HIF-1α positive cells had brown granules. HIF-1α was mainly expressed in the cytoplasm and nucleus of cancer cells. VEGF-A positive cells had yellow granules. VEGF-A was mainly expressed in the cytoplasm and membrane of cancer cells and endothelial cells. HIF-1α and VEGF-A expression in the PDT group was markedly higher compared to the control group, but was lower in the endostar group compared to the control group (Fig. 5, Fig. 6)

Apoptotic index: In the PDT group, endostar group and PDT+endostar group, TUNEL assay showed that some cells had shrinkage and chromatin margination...
characterized by crescent shape or lobular shape. Apoptotic bodies were noted. The apoptotic index was 39.3±3.4, 25.4±2.8 and 61.7±4.9 % in the PDT group, endostar group and PDT+endostar group, respectively. In the control group, the cells had integral morphology and scant apoptotic cells. The apoptotic index in the control group was 7.8±0.5 % (Fig. 7).

**Discussion**

In the present study, we demonstrated that PDT combined with endostar significantly reduced tumor volume and prolonged survival time of nude mice bearing glioblastoma, compared with either no treatment or monotherapies.

Hematoporphyrin monomethyl ether is a novel photosensitive reagent developed in China. *In vitro* and *in vivo* studies have shown that Hematoporphyrin monomethyl ether is a promising photosensitizer. S.S. Hu et al. [8] have shown, in their in vitro study, that Hematoporphyrin monomethyl ether mediated PDT could induce the...
apoptosis of C6 cells and that calcium overload played an important role in extensive ultrastructural damage. In the present study, in the PDT group was significantly reduced tumor volume and prolonged survival time, which further confirmed that PDT can inhibit the growth of glioma in vivo. However, in the PDT group residual cancer cells highly expressed HIF-1α and VEGF-A, which is beneficial for angiogenesis. Thus, cancer cells can escape from the attack, resulting in the proliferation of residual cancer cells and subsequent recurrence and metastasis, which are the main cause of unfavorable long-term efficacy of PDT.

In 1970s, professor J. Folkman et al. first proposed the theory of antiangiogenic as an anti-tumor therapy [6]. In 1997, M.S. O’Reilly et al [12] found endostatin. Endostatin is considered as the most active inhibitor of angiogenesis. F.H. Barnett et al. [1] applied intra-arterial delivery of endostatin gene to 9L glioblastoma model prolongs survival and tumor volume reduction. N.O. Schmidt et al. [13] demonstrated that local i.c. administration of endostatin at a total daily dose of 2 mg/kg via osmotic minipumps inhibited the growth of intracranial U87 human glioblastoma xenografts in nude mice by 73.6 % and was associated with a decrease in blood vessel density and a significant increase in apoptosis. Our results showed that, in the endostar group was significantly reduced tumor volume. Endostar could inhibit not only the endogenous VEGF-A expression in normal U251 glioma cells but also the increased VEGF-A expression induced by photodynamic therapy.

In short, endostar in combination with PDT could markedly decrease PDT induced HIF-1α and VEGF-A expression, inhibiting new angiogenesis and simultaneously increase the apoptosis of cancer cells, which may be the primary way endostar enhances the efficacy of PDT in glioma. PDT, in combination with endostar, can more effectively prolong survival, and increase the apoptosis of cancer cells compared to either PDT or endostar alone. This finding may be a promising strategy in the treatment of malignant tumors.

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