

Effect of photodynamic therapy combined with Celecoxib on expression of cyclooxygenase-2 protein in HeLa cells

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Abstract. The present study assessed the effect of photodynamic therapy (PDT) combined with Celecoxib (Cel) on cervical cancer HeLa cells. An MTT assay was performed to detect the inhibitory effects of Cel with different concentrations (10, 50, 100, 150, 200, 250 and 300 $\mu\text{g/ml}$) on the proliferation of HeLa cells. Subsequently, HeLa cells were divided into control group (group H), 50 g/ml Celecoxib group (group C), PDT group (group P), 50 g/ml Cel + PDT group (group P + C) and western blotting and immunohistochemistry were performed to detect the expression of cyclooxygenase-2 (COX-2) protein in the different groups. Cel inhibited HeLa cells proliferation 24 h following administration, among which 200 $\mu\text{g/ml}$ induced a 50% inhibition rate; the relative expression level of COX-2 protein in group P + C was significantly decreased compared with that in either group C or group P ($P < 0.05$). Cel inhibited the proliferation of human cervical cancer cells in a concentration-dependent manner, and combined PDT therapy may improve treatment outcomes.

Introduction

Cervical cancer (CC) is a common malignancy in females, with the second-highest mortality rate of cancer in females worldwide (1,2) and it affects mostly women of reproductive age; 14.0% of patients diagnosed with cervical cancer are between 20 and 34 and 25.9% between 35 and 44 years of age (3). Traditional treatment methods primarily include surgery, chemotherapy and radiotherapy and are supplemented by comprehensive therapies, including immunotherapy, biological therapy (targeting dendritic cells and cytokine induced killer cells) and genetic therapy (4-6). The presence of an isozyme of

cyclooxygenase (COX), cyclooxygenase 2 (COX-2), has been confirmed in a variety of human tumor tissues and cells (7). COX-2 inhibitors may participate in the occurrence and development of tumors through enhancing tumor nourishing angiogenesis, inhibiting immune surveillance, promoting cell proliferation, enhancing tumor invasion/metastasis or inhibiting apoptosis and therefore they are associated with the prognosis of patients with tumors (8). Studies have demonstrated that COX-2 is not expressed in normal cervical tissues, but that its expression in CC is significantly increased (9). Consequently, COX-2 inhibitors have become a focus of CC therapies (10).

Following clinical application, numerous studies have confirmed the efficacy of photodynamic therapy (PDT) in treating tumors (11,12), but the mechanism of action remains unclear; at present, potential mechanisms include inducing apoptosis, activating *in vivo* antitumor immunity reactions and injuring tumor-associated vasculature (13). Almeida *et al* (14) hypothesized that PDT induces apoptosis primarily through 2 signal transduction pathways: The death receptor-mediated external and the mitochondria-mediated internal pathway. Zhou *et al* (15) suggested that PDT may also increase the concentration of Ca^{2+} in the cytoplasm, activate the metabolism of certain lipids, including phospholipase C, ceramide, phospholipase A2 and arachidonic acid, and induce apoptosis. COX-2 inhibitors and PDT may act on COX-2, the rate-limiting enzyme via which arachidonic acid synthesizes prostaglandins (PGs), thereby decreasing the synthesis of PGs and inhibiting the action of COX-2.

The present study examined selective COX-2 inhibitor Cel combined with PDT and their inhibitory functions against the expression and proliferation of COX-2 in HeLa cells, aiming to additionally explore the potential mechanisms through which the apoptosis of CC is induced and consequently providing a theoretical basis for the clinical applications of Cel and PDT. At present, the associations between PDT and selective COX-2 inhibitors in treating CC have not been described and studies concerning the effect of Cel and PDT on the expression and proliferation of COX-2 in HeLa cells have not been performed, to the best of our knowledge. The present study will provide novel ideas for the clinical prevention and treatment of CC and is therefore of important practical significance in improving the survival rates and decreasing the mortality rates of CC. With advances from previous studies and clinical studies,

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this combined therapy may have broad application prospects toward the prevention and treatment of early and advanced CC (16,17).

Materials and methods

Cell lines and groups. HeLa cells (Obstetrics and Gynecology Department, the Fourth Affiliated Hospital of Harbin Medical University, Harbin, China) were co-cultured at room temperature with 50, 100, 150, 200, 250 and 300 $\mu\text{g/ml}$ Cel and then 5 mg/ml MTT in PBS was added for an additional 4 h incubation in room temperature prior to the measurement of optical density (OD) values at 492 nm; the inhibition rate of COX-2 in the HeLa cells was calculated as: Inhibition rate=(OD of the negative control group-OD of the sample)/(OD of the negative control group-OD of the blank group) $\times 100\%$.

The HeLa cells were divided into 4 groups according to different treatment options: Blank control (Hela, group H); group Cel (group C, 50 $\mu\text{g/ml}$); group PDT (group P); and group PDT + Cel [group P + C, (Cel 50 $\mu\text{g/ml}$)].

PDT treatment. One DIOMED630 photodynamic laser treatment apparatus (Diomed Ltd., Hertfordshire, UK) was used based on Photofrine (American Cyanamid Company, New Jersey, USA) to spirally irradiate the culture plates for 25 sec, with the output power as 440 nm at the site 3 cm away from the light spot and the power density as 15 mW/cm²; the cells were then cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 15% fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37°C and 5% CO₂ for 24 h.

Western blotting. The total proteins were extracted from Hela cells using lysis buffer (P0013; Beyotime Institute of Biotechnology, Haimen, China) (radioimmunoprecipitation assay: Phenylmethane sulfonyl fluoride=100:1) for 30 min at 4°C and then 25 μg was separated using SDS-PAGE (10% gel) and transferred to a polyvinylidene fluoride membrane. Then the membrane was blocked with 5% skimmed milk in Tris-buffered saline with 20% Tween-20 for 2 h. The dilution of COX-2 primary anti-mouse antibodies (cat. no. aa584-598; Cayman Chemical Company, Ann Arbor, MI, USA) was 1:200, incubated at 4°C overnight; that of the rabbit anti-mouse immunoglobulinG secondary antibodies (cat. no. A-11059; Thermo Fisher Scientific Inc.) was 1:5,000. The analysis of the bands was used to detect the expression of COX-2 with β -actin (cat. no. MAB8969; Bio-Techne, Minneapolis, MN, USA), diluted by deionized water (1:200), as a reference and the ratio of gray between COX-2 and β -actin was defined as the relative expression of COX-2 by Quantity One (version 4.6.2; Bio-Rad Laboratories, Inc., Hercules, USA). The experiment was repeated ≥ 3 times.

Immunohistochemical staining. COX-2 protein expression was detected by 5 mg/ml DAB staining (room temperature for 4 min) with 50 μl primary (1:50, aa584-598; Cayman Chemical Company) and 50 μl biotin-labeled secondary antibodies (1:200, A-1,1059; Thermo Fisher Scientific, Inc.), which were both diluted with PBS contains 0.3% Triton x-100. The positive cells displayed brownish yellow granules on the surface and

cytoplasm. The expression grades of COX-2 were based on the ratio of positive cells among all the cells when calculated from 1,000 cells randomly from 10 high power fields: <5%, negative (-); 6-25%, weakly positive (+); 26-50%, positive (++); and >51%, strong positive (+++) by light microscope (magnification, $\times 400$, Olympus Corporation, Tokyo, Japan).

Statistical analysis. SPSS v11.5 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis and two-way analysis of variance with Turkey's test as the post hoc test for western blot results and the χ^2 test was applied to the immunohistochemical staining results. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Proliferation inhibition of COX-2. The OD values of HeLa cells at multiple concentrations of Cel were measured by MTT assay; subsequently, the cell survival curves were calculated with the OD value as the ordinate and the Cel concentration as the abscissa (Fig. 1A). The proliferation inhibition rate of COX-2 at different concentrations of Cel is demonstrated in Fig. 1B, which exhibited a dose-dependent trend. When the inhibition rate was 50%, the Cel concentration was 200 $\mu\text{mol/l}$. In group C, microscopy revealed that the membrane and cytoplasm of the HeLa cells were initially stained brown, followed by membrane and nucleus shrinkage, cracking and gradual formation of cellular debris.

Expression of COX-2 protein. The expression of COX-2 protein was highest in group H, while in PDT + Cel-treated HeLa cells it was significantly decreased ($P < 0.05$) (Table I). From the western blot, there was no statistically significant difference between groups C and H ($P > 0.05$) (Table I) observed, but the comparison between groups P and H indicated a statistically significant difference ($P < 0.05$) (Table I). Fig. 2 demonstrates the western blotting data and Fig. 3 presents the immunohistochemical staining results.

Discussion

COX-2 is the rate-limiting enzyme via which arachidonic acid synthesizes PGs (18) and, with the exception of red blood cells, all other body tissues possess the enzymes for the synthesis of prostaglandins (PGs). Platelets also have thromboxane A synthase 1, which has been confirmed by an inhibition experiment (19). Phospholipids in the cell membrane are rich in arachidonic acid so when the cells are stimulated by external stimuli, including bradykinin, thrombin, antigen-antibody complexes or other pathological factors, a number of which have not yet been characterized, the phospholipase A2 in cell membrane will be activated, consequently hydrolyzing the phospholipids and releasing arachidonic acid, which may then synthesize PGs under a series of enzymes including COX-2 and PGs synthetase (20). In the majority of normal tissues, PG is not expressed; however, under the stimuli of factors such as cytokines, growth factors, oncogenes or tumor-promoting agents, it may be upregulated and associated with the occurrence and development of tumors (21). Previous study has identified that COX-2 is upregulated in a number

Table I. Expression levels of COX-2 protein in different groups.

| Expression | Groups | | | |
|------------------------|-----------------|-----------------|------------------------------|--------------------------------|
| | H | C | P | P + C |
| COX-2/ β -actin | 0.83 \pm 0.17 | 0.73 \pm 0.15 | 0.51 \pm 0.13 ^a | 0.36 \pm 0.14 ^{a-c} |
| Positive rate of COX-2 | +++ | ++ | - | - |

-, <5%; +, 6-25%; ++, 26-50%; +++, >51%; group H, blank control group; group C, Cel group; group P, PDT group; group P + C, PDT + Cel group. PDT, photodynamic therapy; Cel, Celecoxib; COX-2, cyclooxygenase 2. ^aP<0.05 vs. group H; ^bP<0.05 vs. group C; ^cP<0.05 vs. group P.

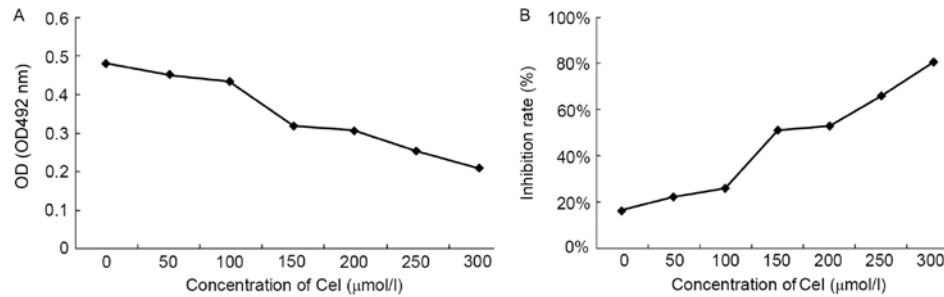


Figure 1. (A) Association between Cel concentration and OD of HeLa cells. (B) Inhibition rate of Cel against cervical cancer cells. OD, optical density; Cel, Celecoxib.

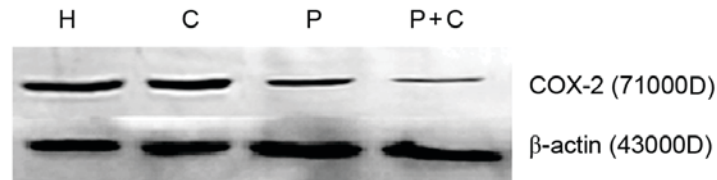


Figure 2. Expression levels of COX-2 protein in HeLa cells of different groups. COX-2, cyclooxygenase 2; PDT, photodynamic therapy; Cel, Celecoxib; group H, blank control; group C, Cel group; group P, PDT group; group P + C, PDT + Cel group.

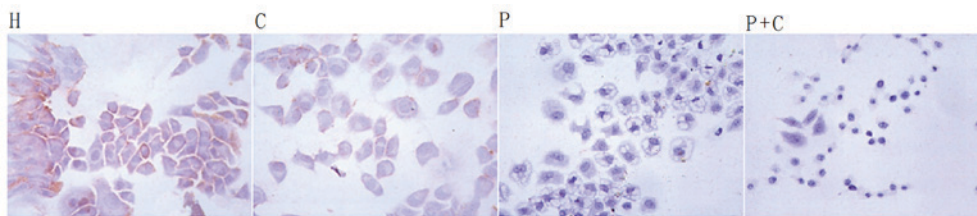


Figure 3. Immunohistochemical staining of cyclooxygenase 2 protein in different groups (magnification, $\times 40$). H for control group, C for 50 g/ml Celecoxib group, P for photodynamic therapy group, P + C for 50 g/ml Celecoxib + photodynamic therapy group.

of tumor tissues and is associated with tumorigenesis, malignant transformation, resistance or prognosis (22). It may participate in the occurrence and development of tumors via enhancing angiogenesis of neovascularization, inhibiting immune surveillance promoting cell proliferation, enhancing invasion/metastasis and inhibiting apoptosis (23). Chen *et al* (24) identified that COX-2 is not expressed in normal cervical tissues and is primarily expressed in CC cells and in the neovascular endothelial cells of CC, so it may be presumed that it participates in the angiogenesis of CC. Previous studies have also demonstrated that COX-2 is significantly associated

with the pathological grading, cytology and lymph node metastasis of CC, the expression rate of which is increased in adenocarcinoma compared with squamous carcinoma; COX-2 is highly expressed in well-differentiated tumors and it may be hypothesized that COX-2 serves functions in the early stages of CC (25), is associated with lymph node metastasis (26) and is associated with the occurrence and metastasis of CC (27). Gaffney *et al* (28) followed up patients with CC following radiotherapy and identified that the decrease of COX-2 level was the only factor to improve the survival rate: The 5-year overall survival rates in the patients with high and low COX-2

expressions were 75 and 35%, respectively, indicating that the upregulation of COX-2 may decrease the post-radiotherapy survival rate in patients with CC.

Selective COX-2 inhibitors may specifically act on COX-2, thus decreasing the synthesis of PGs and inhibiting tumors (29). A previous study indicated that Cel pretreatment radiosensitizes HeLa cells via a mechanism dependent on downregulating COX-2 (30), indicating that long-term and regular application of selective COX-2 inhibitors contributes to improving the rate of apoptosis and the sensitivity of radiotherapy and chemotherapy, consequently increasing the 5-year survival rate. A previous study demonstrated that the selective COX-2 inhibitor NS398 may inhibit the growth of epithelial cells of ovarian cancer in a concentration- and time-dependent manner and that the cells with and without the expression of COX-2 exhibited a statistically significant difference in prognosis, which suggests that the inhibitory functions of NS398 against the growth of ovarian cancer cells were mediated through the inhibition of COX-2 expression, resulting in the decrease of PG synthesis rate and a facilitation of apoptosis in a time- and dose-dependent manner (30).

In the present study, the MTT assay revealed that the selective COX-2 inhibitor Cel may inhibit the proliferation of human CC HeLa cells and exhibit an association with Cel, consistent with a previous study (31).

The principle of PDT is that, subsequent to intravenous or intraperitoneal administration, the photosensitizer may be taken up by tumor tissues so that, when a laser with a suitable wavelength is introduced, the photochemical effects may be induced to kill tumor cells, which is effective toward chemotherapy- or radiation-resistant tumor cells. Colussi *et al* (32) applied the photosensitizer metal phthalocyanine to nude mice with human ovarian OVCAR3 carcinoma xenografts, and identified that the tumor tissues exhibited early apoptosis and the overexpression of cyclin-dependent kinase inhibitor 1, but that the group without the PDT did not. Applying PDT to treat ovarian cancer incurs certain problems; as a small amount of photosensitizer is absorbed by intraperitoneal normal tissues and organs, large-area irradiation targeting the abdominal cavity will not only achieve effective irradiation against the tumor tissues and therefore affect treatment efficacy but may also damage normal peritoneal tissues and organs, causing intestinal perforation. Therefore, carrier systems such as liposomes or monoclonal antibodies are required so as to improve the selective accumulation of the photosensitizer (33). To assess the effect of Cel plus PDT on the expression of COX-2 protein in HeLa cells, western blotting and immunohistochemistry assays were used to examine the expression of COX-2, with Photofrine® as the photosensitizer. The results indicated that the expression of COX-2 protein in group P + C was significantly decreased compared with that in the other 3 groups, which was improved compared with group P and group C. The results of immunohistochemical analysis also supported the results obtained by western blotting, indicating that the selective COX-2 inhibitor Cel combined with PDT may exhibit inhibitory effects on the expression of COX-2 in HeLa cells, resulting in the downregulation of COX-2 protein. However, group P also exhibited inhibitory effects on the expression of COX-2 in HeLa cells, but the effects were decreased compared with those in group P + C; no statistically

significant difference between groups C and H was observed and this may be due to the concentration selected or decreased duration of exposure, meaning that the protein was not significantly downregulated. This result is not contradictory to that obtained in the MTT assay, since Cel inhibited the HeLa cells in a concentration- and time-dependent model; the higher the concentration and the longer the duration of the treatment, the more marked the inhibitory effects against COX-2 appeared. The cells in group P + C exhibited apoptotic bodies but no COX-2 expression according to the immunohistochemical analysis, indicating a possible pathway COX-2 inhibition, which may reduce the rate of PGs and promote apoptosis; however, other pro-apoptosis pathways should not be ruled out and this requires additional study.

The greatest obstacle of Cel plus PDT in current clinical applications is that the penetration distance of PDT is short, usually <1 cm, but the speed of promoting the apoptosis is fast. Since Cel is an oral drug, therefore it may only be used as a means of adjuvant therapy instead of a primary treatment, so as to help to improve the clinical prognosis of CC. For patients who are resistant to radiotherapy and chemotherapy, treatment sensitivity may be increased. Cel combined with PDT may be used as an early treatment of cervical intraepithelial neoplasia and as an adjuvant treatment for advanced CC, which is crucial for improving the 5-year survival and quality of life of patients. Therefore, with ongoing basic studies and clinical trials, the combination of Cel and PDT may be expected to become a novel approach for the prevention of CC.

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