Anti-cancer effects of celecoxib on nasopharyngeal carcinoma HNE-1 cells expressing COX-2 oncoprotein

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Received: 17 April 2010 / Accepted: 29 July 2010 / Published online: 1 September 2010
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Abstract Celecoxib is a selective cyclooxygenase-2 (COX-2) inhibitor with antitumor and antiangiogenic activities. To investigate the effects of celecoxib on nasopharyngeal carcinoma (NPC), HNE-1 cells were treated with celecoxib at various concentrations. MTT assay, migration assay and invasion assay were performed to observe the inhibitory activity of celecoxib on HNE-1 cells. Additionally, VEGF-A expression and radiation survival of NPC cell were also examined after treatment with celecoxib. Celecoxib treatment presented an anti-proliferation function in a time and dose-dependent manner on HNE-1 cells which highly express COX-2 protein. Celecoxib also displayed an obvious inhibitory activity on invasive capacity of NPC cells. Moreover, the celecoxib's effects to suppress VEGF-A expression and enhance radiosensitivity were detected in HNE-1 cells. These findings implicate that application of celecoxib may be an effective strategy for NPC therapy.

Keywords COX-2 · Celecoxib · Anti-cancer drug · Nasopharyngeal carcinoma

Introduction

Cyclooxygenase (COX), a key enzyme that catalyzes the rate-limiting step in prostaglandin synthesis from arachidonic acid, exists in two isoforms of COX-1 and COX-2 (Wang et al. 2007). As a housekeeping enzyme expressed constitutively in almost all tissues, COX-1 is responsible for the production of prostaglandins that mediate normal physiological function. COX-2, undetectable in most normal tissues, is an inducible protein by various factors including mitogens, growth factors, and cytokines. It is well established that aberrant expression of COX-2 is frequent in various types of cancers and induction of COX-2 participates in carcinogenesis, inflammation, immune response suppression, apoptosis inhibition, angiogenesis, and tumor cell invasion and metastasis (Cao and Prescott 2002; Kim et al. 2009a; Toomey et al. 2009).

The potential for application of non-steroidal anti-inflammatory drugs as well as the recently developed COX-2 specific inhibitors in cancer clinical practice has drawn substantive attention in the recent years (de Souza 2009; Lanan 2009; Fujimura et al. 2007). For the reason that NSAIDs also inhibit COX-1 function which has a protective effect on

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gastrointestinal and renal tissue, selective COX-2 inhibitors such as celecoxib were developed to avoid the side effects of NSAIDs. Abundant evidence for functional involvement of celecoxib in tumor suppression has been reported in multiple studies including breast cancer, esophageal cancer, prostate cancer and cervical cancer (Singh and Mittal 2008; Tfayli et al. 2008; Dawson et al. 2007; Antonarakis et al. 2009; Gaffney et al. 2007). Although the precise mechanism of anti-tumor functions of celecoxib remains unclear, COX-2 blockade by celecoxib, a promising anticancer agent is effective for cancer prevention and therapy.

Although some studies have demonstrated that celecoxib treatment suppressed tumorigenesis and angiogenesis of NPC, the mechanisms underlying these phenotypic changes are still unknown (Chen and Long 2004; Yee et al. 2005; Chan et al. 2005). In the present study, we emphasized on the functional role of celecoxib in NPC cells. To elucidate the possible tumor-suppressing activity of celecoxib, HNE-1 cells were treated with celecoxib at various concentrations and *in vitro* cell growth and invasion analyses were conducted. In addition, we further investigated the possible mechanism of VEGF-A downregulation and radiosensitivity enhancement by celecoxib. Our results provide potent evidence to suggest important characteristics for celecoxib in suppressing NPC progression.

Materials and methods

Reagents

Celecoxib was obtained from Pharmacia Corp. (Skokie, IL). Stock celecoxib solutions were constructed via the dissolution of the compound in DMSO (Sigma–Aldrich, St. Louis, MO) and then stored at -20°C . Immediately before the commencement of the experiments, we diluted the stock solutions in DMSO to the appropriate concentrations and final DMSO concentrations in all cultures were 0.1%.

Cell lines and culture conditions

Human NPC cell line HNE-1 and MCF-7 were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco), 10 mM

glutamine (Invitrogen, Carlsbad, CA), 100 units/mL of penicillin (Invitrogen), and 100 $\mu\text{g}/\text{mL}$ of streptomycin (Promega, San Luis Obispo, CA) in 25-cm² tissue flasks at 37°C in 5% CO₂. Various concentrations of celecoxib dissolved in culture medium at a final concentration of 0.1% DMSO were added to the cells for subsequent analyses. The controls were treated with a DMSO vehicle at a concentration equal to that of the drug-treated cells.

RNA extraction and reverse transcription PCR analysis

Total cellular RNA was extracted from HNE-1 cells with TRIzol reagent (Invitrogen) and then cDNA was prepared from 3 μg of RNA with the random hexamers and avian myeloblastosis virus reverse transcriptase (Invitrogen) according to the manufacturers' instructions. Semi-quantitative PCR for COX-2 expression was performed as detailed previously (You et al. 2008). Briefly, cDNA was subjected to PCR for 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s. The resulting products were separated on 2% agarose gel and stained with ethidium bromide. We conducted PCR analyses using primers for COX-2 (5'-TGGCTACAAAAGCTGGGAAG-3' and 5'-GGGGATCAGGGATGAACTTT-3') and VEGF-A (5'-CCTGGTGGACATCTTCCAGGAGTACC-3' and 5'-AGCTCATCTCTCCTATGTGCTGGC-3'). β -actin transcripts in each sample were also amplified as an invariant housekeeping gene internal control.

Western blot analysis

Whole cell lysates were prepared in RIPA lysis buffer (50 mM Tris–Cl pH 7.4, 150 mM NaCl, 0.05% SDS, 0.1% Tween 20, 0.1% NP40). Approximately 50 μg of total proteins for each sample was separated on an 12% polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA), and incubated overnight in PBS containing 5% bovine serum albumin (BSA, Sigma) at 4°C . After briefly washing with 0.1% Tween 20 (Sigma) in PBS, the membrane was incubated with polyclonal rabbit COX-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:5000. The membrane was washed again and incubated in goat anti-rabbit horseradish peroxidase-conjugated IgG (Amersham,

Arlington Heights, IL) at a dilution of 1:5000. The expression of COX-2 protein was detected using the enhanced ECL kit (Amersham). To verify equal loading of the samples, the same membrane was stripped using 0.2 M NaOH for 5 min and incubated with the mouse monoclonal antibody against β -actin (Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham).

Cell growth assay

The anti-proliferative activity of celecoxib was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay. Briefly, HNE-1 cells were cultured in 96-well plates at a density of 5×10^3 cells per well in triplicate. After 24 h culture to allow reattachment, the cells were then treated with celecoxib at various concentrations (0, 50, 75, 100, 125, 150, 175, 200 $\mu\text{mol/L}$) and cell growth was measured at 24, 48 or 72 h. Controls were treated with a DMSO vehicle at a concentration equal to that in drug-treated cells. At designed time points, cells were replaced in DMEM without phenol red containing 20 μL of 5 mg/mL MTT (Sigma) at 37 °C for 4 h. The supernatants were then removed, and the formazan crystals in each well were solubilized by the addition of 150 μL of DMSO by vigorous shaking. The colored formazan product was measured at 490 nm using a microplate reader. Measurements of cell growth by MTT assay were expressed as percent inhibition according to the following formula: % inhibition = [(absorbance of untreated control group – absorbance of celecoxib treated group)/absorbance of vehicle group] $\times$ 100%. The experiment was repeated three times.

Cell invasion assay

The invasive capacity of HNE-1 cells after celecoxib treatment was determined by an invasion chamber assay. Briefly, after 24 h treatment with celecoxib at various concentrations (0, 50, 100, 200 $\mu\text{mol/L}$), 2×10^5 cells was resuspended in 100 μL serum-free DMEM and seeded into the insert chamber of a 24-well-size Matrigel-coated micropore membrane filter with 8 μm pores (Millipore), and the bottom chamber was filled with 750 μL DMEM medium containing 10% fetal bovine serum. After 24 h of incubation at

37 °C, the inserts were removed and the non-invasive cancer cells that remained on the upper side of the filter were scraped off. The membranes were fixed and stained crystal violet reagent (Sigma) before counting in five 100 $\times$ fields under phase-contrast microscopy. Each experiment was performed in triplicate wells and repeated 3 times.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was conducted to determine the secreted levels of VEGF-A in cell supernatants using Quantikine kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Briefly, after 24 h treatment with celecoxib at various concentrations (0, 50, 100, 200 $\mu\text{mol/L}$), HNE-1 cells were seeded into 6-well plate at 2×10^5 cells per well in triplicate and cultured for 24 h. Then the cells were rinsed with phosphate-buffered saline (PBS) and incubated in serum-free medium for 48 h. The culture supernatants were then collected and assayed in 96-well microplates. Optical density of each well was determined using an automatic plate reader at 405 nm wave length. Serial dilutions of recombinant human VEGF-A was used to prepare the standard curve.

Clonogenic assay for radiation survival experiment

3×10^4 mL HNE-1 cells were resuspended in 200 μL DMEM containing 10% fetal bovine serum and plated out in triplicate into 25-cm² cell culture dishes. The cells were then permitted to attach for 24 h at 37 °C and exposed to a DMSO vehicle or to 150 $\mu\text{mol/L}$ celecoxib for 48 h. The cells were then irradiated with graded doses of γ -rays (0, 1, 2, 4, 6 Gy) using the Gammacell 3000 Elan system (MDS Nordion Inc., Ontario, Canada), at a dose rate of 600 cGy/min, as determined by thermoluminescence dosimetry for the specific system employed. The cells were rinsed with PBS and then maintained at 37 °C for 9 days to allow for the formation of colonies and then stained with Giemsa reagent (Sigma). The colonies were counted visually with a cutoff value of 50 viable cells. The surviving fraction was then calculated as follows: [mean number of colonies/(number of cells inoculated $\times$ plating efficiency)] $\times$ 100%, where plating efficiency is defined as the mean

number of colonies/number of cells inoculated for untreated controls. Surviving fractions in the cells exposed to radiation plus celecoxib were normalized by dividing by the surviving fraction obtained for celecoxib alone. The experiment was repeated three times.

Statistical analysis

Data were analyzed by Student's test. Results were expressed as the means $\pm$ standard deviations (SD). Differences were considered significant when the *P* value was less than 0.05.

Results

COX-2 expression in HNE-1 cells

To evaluate COX-2 expression in HNE-1 cells, we performed RT-PCR and western blot analyses. As shown in Fig. 1, we found that HNE-1 cells expressed significant amounts of COX-2, whereas the control MCF-7 cells exhibited no constitutive COX-2 expression.

Growth suppression of HNE-1 cells by celecoxib

To investigate whether celecoxib treatment affects the growth of HNE-1 cells, a cell growth assay was conducted. After treatment with celecoxib at various concentrations, the inhibitory effect on cell viabilities

was determined using the MTT assay, a colorimetric method. As shown in Fig. 2, we observed that treatment with celecoxib significantly reduced the viabilities of HNE-1 cells. Moreover, this inhibitory effect was presented in a dose- and time-dependent manner.

Suppression of invasiveness of HNE-1 cells by celecoxib

To examine whether the invasive capacity of HNE-1 cells is inhibited by celecoxib, the drug-treated cells were placed on a Matrigel ECM layer in a Transwell chamber and their rate of migration through the Matrigel was assayed. The result revealed that the invasive capacity of HNE-1 cells was significantly suppressed by treatment of celecoxib in a dose-dependent manner (Fig. 3). Compared with that in DMSO vehicle treatment group, cell invasiveness was reduced by 34.3%, 47.2 and 67.4% in HNE cells receiving 50, 100 or 200 $\mu\text{mol/L}$ celecoxib, respectively (*P* < 0.05).

Suppression of VEGF-A expression of HNE-1 cells by celecoxib

Downregulation of VEGF-A expression may contribute to the anti-angiogenic effect by celecoxib administration. To investigate this possibility, HNE-1 cells after treatment with celecoxib were examined

Fig. 1 Expression status of COX-2 in HNE-1 cells. **a** Total RNA isolated from HNE-1 or MCF-7 cells was subjected to RT-PCR analysis with COX-2 specific primers, and β -actin was used as an internal loading control. **b** Total cell lysates from HNE-1 or MCF-7 cells was collected and the expression of COX-2 protein was determined by Western blot analysis

Fig. 2 Growth suppression of HNE-1 cells by celecoxib. HNE-1 cells were plated onto 96-well plates and treated with celecoxib at various concentrations. The cell growth was measured by MTT assay at designed time points. A time- and dose-dependent growth inhibition was induced by celecoxib treatment

Fig. 3 Inhibitory effect of celecoxib on the invasive activity of HNE-1 cells. Cells invaded through the matrigel-coated micropore membrane filter were counted in five random 100 × microscope fields. *Filled asterisk* $P < 0.05$ versus DMSO vehicle treatment group, *filled diamond* $P < 0.05$ versus 50 μmol/L celecoxib treatment group, *hat* $P < 0.05$ versus 100 μmol/L celecoxib treatment group

for VEGF-A expression using RT-PCR and ELISA. Figure 4 shows that mRNA levels of VEGF-A were gradually reduced in HNE-1 cells, corresponding to the increasing doses of celecoxib treatment. On the other hand, the treatment of various concentrations of celecoxib also induced a dose-dependent inhibition on VEGF-A secretion. After 24 h incubation with celecoxib, VEGF-A secretion was significantly suppressed by 18.3, 30.9 and 69.8% in HNE cells receiving 50, 100 or 200 μmol/L celecoxib compared with that in DMSO vehicle treatment group, respectively ($P < 0.05$).

Radiosensitivity enhancement of HNE-1 cells by celecoxib

Another biological function of celecoxib, i.e. radiosensitivity enhancement, was tested by clonogenic assay. HNE-1 cells were exposed to graded doses of γ -radiation either with or without celecoxib at a concentration of 125 μmol/L for 48 h. These cells were all then permitted to form colonies in drug-free medium and the surviving fractions were calculated. As shown in Fig. 5, celecoxib was determined to augment the effects of radiation in HNE-1 cells, and showed a significantly synergistic anti-tumor effect with radiation treatment. The surviving fractions in HNE-1 cells treated with celecoxib were inhibited by 12.9, 33.0 and 61.0% when receiving 1, 2 or 4 Gy of

Fig. 4 Suppression of VEGF-A expression of HNE-1 cells by celecoxib. **a** Total RNA isolated from HNE-1 cells treated with celecoxib at designated concentrations was analyzed by RT-PCR using VEGF-A specific primers. β -actin was used as an internal loading control. **b** HNE-1 cells were treated with celecoxib at designated concentrations; the expression of secreted VEGF-A protein was detected by Elisa method. *Filled asterisk* $P < 0.05$ versus DMSO vehicle treatment group, *filled diamond* $P < 0.05$ versus 50 μmol/L celecoxib treatment group, *hat* $P < 0.05$ vs 100 μmol/L celecoxib treatment group. Values are means $\pm$ SE

γ -radiation, compared with those in celecoxib-untreated cells ($P < 0.05$).

Discussion

In contrast to earlier notion that NSAIDs are the most widely used therapeutic agents in the treatment of pain, inflammation and fever, increasing evidence implicates they also have a crucial impact on the management of cancer prevention (Agrawal and Fentiman 2008). As a COX-2 inhibitor being developed initially for the treatment of rheumatoid arthritis and osteoarthritis, celecoxib avoids the side effects of NSAIDs such as gastric and renal toxicity and inhibition of platelet function, and exhibits strong potential for the chemoprevention for different cancer types (Harris 2009). The effects of NSAIDs on tumor growth remain unclear, but are most likely to be multifocal.

Fig. 5 Radiation-enhancing effect of celecoxib on the surviving of HNE-1 cells. Cells attached to the dishes were treated with celecoxib for 48 h, irradiated and allowed to form colonies in drug-free medium. Surviving fractions for radiation plus celecoxib were normalized by dividing by the surviving fraction for celecoxib treatment only. Filled asterisks $P < 0.05$ versus radiation alone group at 1 Gy, filled diamond $P < 0.05$ versus radiation alone group at 2 Gy, hat $P < 0.05$ versus radiation alone group at 4 Gy

NPC is a tumor with a striking geographical and ethnic distribution (Lung et al. 2004). In spite of improved results with concurrent chemoradiation, significant numbers of patients with locally advanced disease still relapse after treatment and patients with metastatic disease are not cured with conventional treatment. Given that overexpression of COX-2 is identified in 71–79% of NPC and associated with nodal metastases and poorer prognosis, treatment with celecoxib may be a promising candidate for NPC therapy (Tan and Putti 2005; Peng et al. 2005; Chen et al. 2005). To date, evidence for anti-tumor involvement of celecoxib has been widely reported in multiple kinds of cancers, whereas the studies for the functional role of celecoxib in NPC are rare. For this purpose, we MCF-7se NPC cell line HNE-1 to obtain functional evidence of celecoxib, based on its high expression of COX-2. In this study, in vitro growth suppression on HNE-1 cells by celecoxib was demonstrated, as previously observed for other kinds of cancers (Kim et al. 2009b; Huang and Sinicrope 2010; Cui et al. 2005). Moreover, the result of in vitro cell invasion assay was consistent with that of growth assay. These data suggest a significantly suppression on multiple malignant phenotypes in HNE-1 cells by celecoxib treatment.

Angiogenesis is essential for the progression of solid tumors (Ferrara et al. 2003). The progressive

growth and metastasis of malignant neoplasms depend on new blood vessel formation from host vessels. The contributions of COX-2 in tumor angiogenesis include the increased expression of the proangiogenic growth factor VEGF. It has been well demonstrated that COX-2 generated prostaglandins can up-regulate VEGF expression through the activation of PKA and EP2 receptor-mediated cAMP pathways (Cheng et al. 1998; Wang and Klein 2007). As Soo et al., reported recently, reduced microvessel density was observed in clinical NPC samples after treatment with celecoxib (Soo et al. 2006). Because VEGF is a highly potent angiogenic agent that enhances vessel permeability, endothelial cell growth, proliferation, migration, and differentiation, we further investigated whether the VEGF-A expression could be suppressed by treatment with celecoxib. The expression of both mRNA and secreted protein of VEGF-A was dramatically downregulated in HNE-1 cells after celecoxib treatment. Thus, this result suggests the function of anti-angiogenesis by celecoxib would be caused, at least in part, by its significant inhibitory effect on VEGF-A expression.

In addition, COX-2 selective inhibitors are known to sensitize the tumor cells to both chemotherapeutic agents and ionizing radiation. As mentioned in previous studies, celecoxib treatments were shown to synergistically enhance the effect of radiation on cancer cells of lung, breast and colon (Kuipers et al. 2007; Park et al. 2006; Nakata et al. 2004). In the current study, we also studied celecoxib-associated radiosensitization in HNE-1 cells and demonstrated that celecoxib significantly reduced clonogenic survival. This finding implicates that selective COX-2 inhibitors have the potential to improve radiotherapy or radiochemotherapy for NPC, implicating a potential strategy for clinical therapy.

In conclusion, the current study demonstrated explicitly that celecoxib treatment suppressed growth and invasion of HNE-1 cells in vitro. Furthermore, we also showed that celecoxib has a significant impact on VEGF-A expression and radiosensitivity in HNE-1 cells. Although the molecular mechanisms underlying these celecoxib-associated effects requires further elucidation, these results may bear some clinical importance with regard to potential applications of celecoxib as a good candidate agent for NPC patients especially undergoing radiotherapy.

Acknowledgments We thank Dr. Caiwen Du for the gift of HNE-1 cells.

Conflict of interest There is no potential conflict of interest or financial dependence regarding this publication for any of the authors.

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