Celecoxib Enhances Radiosensitivity via Induction of G\textsubscript{2}-M Phase Arrest and Apoptosis in Nasopharyngeal Carcinoma

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Key Words
Celecoxib • COX-2 • Nasopharyngeal carcinoma • Radiosensitivity

Abstract

Background: Previous work has proposed that celecoxib may be able to enhance the effects of radiotherapy. However, the underlying mechanism of this activity has not yet been determined.

Methods: The cell colony formation assay after the combination of celecoxib and radiation treatment was done on C666-1, CNE-1 and CNE-2 nasopharyngeal carcinoma cells, which expressed different COX-2 levels. Moreover, COX-2 knocked down or overexpressed cells were developed, and apoptosis and cell cycle analysis were performed.

Results: Celecoxib enhances radiation cytotoxicity in C666-1 and CNE-1 nasopharyngeal carcinoma cells that expressed high COX-2 but not in CNE-2 cells that expressed low COX-2. The radiosensitization of celecoxib in C666-1 cells disappeared after the COX-2 knocked down, while the CNE-2 cells were radiosensitized by celecoxib after the transfection of COX-2. Moreover, celecoxib enhanced radiation-induced G\textsubscript{2}-M phase arrest was observed in some of the tested cells. Furthermore, we found that the radiosensitivity of celecoxib in nasopharyngeal carcinoma was correlated with the apoptosis induction. Additionally, the combination of celecoxib (25 mg/kg) and radiation (6 Gy) treatment significantly reduced tumor volume in C666-1 and CNE-2 nasopharyngeal carcinoma xenograft models.

Conclusion: These results indicate that the combination of celecoxib and radiation treatment has potential application in radiotherapy, and these effects may be attributable to the G\textsubscript{2}-M cell phase arrest and enhancement of cell apoptosis.

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Introduction

Nasopharyngeal carcinoma is a kind of epithelial malignant lesions associated with genetic factors, which is characterized by the highly invasive and early lymph node metastasis. Recently, the preferred treatment of nasopharyngeal carcinoma is radiotherapy [1, 2], which is the most effective therapeutic method for this disease. However, the radiation sensitivity to nasopharyngeal carcinoma is not high and many patients fail with recurrence and metastasis [3]. Due to the high local and distant recurrence rate, more effective strategies and drugs to prevent and treat nasopharyngeal carcinoma are needed. Increasing evidences report that the chemotherapies have clear synergistic responses to inhibit cancer cell growth and metastasis when combined with appropriate radiotherapy [4, 5]. Hence, it is necessary to combine radiotherapy with other therapeutic methods for the nasopharyngeal carcinoma patients to improve the survival rates.

Cyclooxygenase (COX)-2 is an inducible enzyme, which is regulated by various inflammatory mediators and growth factors [6, 7]. Increased COX-2 expression is closely correlated with poor differentiation and angiogenesis in a wide variety of tumor types, and COX-2 activation is associated with cancer invasion and multidrug resistance [8, 9]. Given that COX-2 plays an important role in carcinogenesis and neoplastic progression, nonsteroidal anti-inflammatory drugs (NSAIDs) are agents of interest for cancer prevention [10]. NSAIDs could suppress COX-2 expression and prevent the effects of prostaglandins. Celecoxib, the first selective COX-2 inhibitor approved by the U.S. Food and Drug Administration, has been reported to prevent carcinogenesis in both animals and humans [11-13]. Moreover, it has been reported that the chemotherapy, including celecoxib when used at low dose, may be able to enhance the efficacy of radiation treatment on the cancer cells [14-16].

Recently, the efficacy of celecoxib has been investigated in the nasopharyngeal carcinoma cells, wherein it exerts the effects by inhibiting COX-2 expression and prostaglandin E2 synthesis [5, 17]. It is helpful to evaluate the effects of celecoxib combined with radiation therapy in the management of nasopharyngeal carcinoma which expresses high COX-2 protein. Although the previous study has showed the potential advantage of the combination of celecoxib and radiation treatment on nasopharyngeal carcinoma in clinical trial [5], the rational mechanism is still unclear. In the present study, we investigate the potential of celecoxib to enhance response of nasopharyngeal carcinoma to radiation therapy and determine the mechanism underlying the anticancer effects.

Materials and Methods

Reagents and antibodies

Celecoxib was obtained from LKT Laboratories (Minneapolis, MN, USA). Antibodies against COX-2, β-actin, caspase-3, cyclin B1, small interfering RNA (siRNA) specific for human COX-2 mRNA and control siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies specific for Poly (ADP-ribose) polymerase (PARP), phosphorylated H2AX (γ-H2AX), cell division cycle 25C (Cdc25C) and cyclin-dependent kinase-1 (CDK1) were obtained from Cell Signaling Technology (Beverly, MA, USA). Unless otherwise indicated, all other reagents used in the study were obtained from Sigma Chemicals (St. Louis, MO, USA).

Cell culture

The human nasopharyngeal carcinoma cell lines CNE-1 (well-differentiated nasopharyngeal carcinoma cells), CNE-2 (poorly differentiated nasopharyngeal carcinoma cells), C666-1 (undifferentiated nasopharyngeal carcinoma cells) and human breast cancer cell MCF-7 were cultured in RPMI 1640 (Gibco, USA) supplemented with 10 % fetal bovine serum at 37°C, 5 % CO2.
**Cell treatments**

The cells were exposed to various concentrations of celecoxib for 24 h, and then the cells were cultured in drug-free medium. In the radiation study, radiation was conducted before celecoxib treatment using the Gammacell 3000 Elan system (MDS Nordion, Inc., Ontario, Canada) at a dose rate of 1 Gy/min.

**Western blot analysis**

The cells were harvested after the treatment, and equal amounts of proteins were subjected to SDS-PAGE analysis and subsequent immunoblotting was carried out with specific antibodies against COX-2 (1:400), β-actin (1:400), caspase-3 (1:400), cyclin B1 (1:400), PARP (1:1000), γ-H2AX (1:1000), Cdc25C (1:1000), CDK1 (1:1000) or phosphorylated CDK1 (1:1000) at 4°C overnight, and then incubated with the horseradish peroxidase-conjugated secondary antibody (1:8000) for 2 h. The bands were detected using the enhanced chemiluminescent detection reagents (Amersham Pharmacia Biotech). The membranes were also probed with β-actin antibody to normalize the differences between the samples. All the bands were evaluated by densitometry. Bands of interest were normalized against β-actin and data were presented as relative density ratios.

**Colony Formation Assay**

The soft agar colony formation assay was used to assess the growth inhibitory effects of celecoxib and radiation on cells in six-well tissue culture plates. After the treatments, the cells were cultured in drug-free medium and maintained for 7 days to allow for the formation of colonies and then stained with 0.5% crystal violet (Sigma Chemical Co.). The number of colonies counted under low magnification (×100) at four points on each well. Experiments were repeated at least three times, and the data were expressed as the mean±SEM.

**Transfection of cells**

The stably transfected cells were conducted using COX-2 expressed plasmid (full-length human COX-2 cDNA cloned in pcDNA3 vector) and G418 (Invitrogen) as described previously [18]. Briefly, the transfected cells were trypsinized and cultured in medium containing a final concentration of 0.8 mg/ml G418 to select stably transfected cells. The discrete colonies formed by 14 days after selection. Stable clones were continuously cultured in the presence of 0.8 mg/ml G418. The drug-resistant individual colonies were isolated and cultured for the further experiments.

**RNA interference**

The cells were seeded onto six-well tissue culture plates at 60-70% confluence in the absence of antibiotic for 24 h before transfection. The siRNA specific for COX-2 (COX-2 siRNA) and control siRNA were transfected into cells using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer’s instructions. After 48 h of transfection with siRNA, cell culture media were changed and the cells were used for the subsequent experiments. Depletion of endogenous COX-2 by the siRNA was confirmed by western blot analysis.

**Cell apoptosis assay**

Cells were stained with annexin V and propidium iodide (PI) for 15 min at room temperature using the FITC-annexin V apoptosis detection kit (BD Biosciences, San Diego, CA) according to the manufacturer’s instructions. Early apoptotic (annexin V-positive and PI-negative) cells and late apoptotic (annexin V and PI double-positive) cells were analyzed on a flow cytometry (Becton Dickinson, San Jose, CA, USA) using the Cellquest software (Becton Dickinson). The bitmap gate was placed around the cell population on the basis of forward and orthogonal light scatter to discriminate cell aggregates and debris.

**Cell cycle assay**

The cells were fixed with 75% ethanol for 30 min and washed in PBS containing 25 μg/ml RNase and 0.5% Triton X-100. Cells were then stained with PI (50 μg/ml) at 37°C for 30 min. The percentage distribution of cells in the different phases of the cell cycle was determined with flow cytometry. Three independent experiments were performed for each assay.
Xenograft model

All animal experiments used the BALB/c nude mice and were conducted in accordance with the governmental committee for animal research. Female mice (4-6 weeks old) were injected s.c. with C666-1 or CNE-2 cells (10^7) suspended in Matrigel (BD Biosciences) per mice at left flank and randomly assigned to different groups (five mice per group). After 8 days, when a small tumor (80-100 mm^3) had developed, control group was treated with vehicle (1 % methylcellulose and 0.05 % Tween 20 in water) daily by orally gavage. The celecoxib group (25 mg/kg/day) was treated daily by oral administration for 29 days as described previously [19]. Radiation group was treated in single doses of 6 Gy to the tumor site using a radiator at a rate of 1 Gy/min. When radiation treatment was combined with celecoxib, radiation was administered after celecoxib treatments within 2 h. Mice were weighed at least twice a week as an assessment of health. The tumor size in two dimensions (width and length) was measured between the skin surface layers at least three times per week using a digital caliper. The length was measured along the imaginary longitude of the leg and the width was measured in the direction of the latitude [20]. Tumor volumes were calculated according to a standard formula: width^2 × length/2. Mice were killed after 29 days of treatment and the tumor specimens were removed for western blot analysis.

Statistical analysis

Data were presented mean±SEM. Statistical analyses of the differences between two groups were determined with the unpaired Student’s t-test, and among three or more groups were determined by the analysis of variance test (ANOVA) using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). P < 0.05 was considered to be significant.

Results

Cytotoxicity of celecoxib in nasopharyngeal carcinoma cells

As shown in Fig. 1A, C666-1 cells exhibited a high constitutive COX-2 protein expression, the CNE-1 cells expressed modest COX-2 protein, and the CNE-2 cells manifested relative low COX-2 expression level. MCF-7 cells, which showed no COX-2 expression, were used as negative control. The nasopharyngeal carcinoma cells were exposed to various celecoxib dosages (0, 20, 40, 60 and 80 μM) for 24 h and cell survival was assessed using the colony formation assay. The results showed that celecoxib treatment remarkably decreased colony
formation in a dose-dependent manner and there was no significant difference according to COX-2 expression in these cells (Fig. 1B).

**Radiosensitivity of celecoxib in nasopharyngeal carcinoma cells**

To investigate the effect of celecoxib on radiosensitization, the nasopharyngeal carcinoma cells were exposed to radiation (4 Gy) either with or without celecoxib and cell survival was determined by the colony formation assay. Both the COX-2-high-expressing C666-1 cells and the COX-2-expressing CNE-1 cells were sensitive to the combination treatment (Fig. 2A and 2B). However, celecoxib exhibited no radiosensitization in the COX-2-low-expressing CNE-2 cells (Fig. 2C).

**Celecoxib enhances radiation cytotoxicity through COX-2-dependent manner**

To determine whether the radiosensitivity of celecoxib in C666-1 cells occur in a COX-2-dependent route, COX-2 knocked-down cells (C666-1-KD), which transfected COX-2 siRNA, and the mock-transfected cells (C666-1-mock) were established from C666-1 cells. The COX-2 protein expression was notably reduced in C666-1-KD compared with C666-1 cells, whereas we observed no changes in COX-2 expression in C666-1-mock cells (Fig. 3A). As shown in Fig. 3B, both C666-1-KD and C666-1-mock cells were sensitive to celecoxib. However, the increase of radiosensitivity induced by celecoxib was observed in C666-1-mock cells (Fig. 3C), but this effect was completely disappeared in C666-1-KD cells (Fig. 3D).

In addition, we constructed the stably COX-2 transfected CNE-2 cells (CNE-2-COX-2) and the mock-transfected cells (CNE-2-mock). The CNE-2-COX-2 cells expressed high expression of COX-2 protein, whereas the CNE-2-mock cells expressed low COX-2 (Fig. 4A). As shown in Fig. 4B, the cytotoxicity of celecoxib was similar in CNE-2-COX-2 and CNE-2-mock cells. However, the radiation-enhancing effect of celecoxib was showed in COX-2-overexpressing CNE-2-COX-2 cells (Fig. 4C) but was not observed in the COX-2-low-expressing CNE-2-mock cells (Fig. 4D).

**The radiosensitizing effect of celecoxib is not a result of impaired cell cycle control**

To determine whether the radiosensitization of celecoxib in the C666-1-mock and CNE-2-COX-2 cells was related to cell cycle regulation, the cells were exposed to celecoxib either with or without radiation, and then the cell cycle phase was determined by flow cytometry.
analysis. Celecoxib combined with radiation treatment in C666-1-mock cells caused an increase in the proportion of cells in G\textsubscript{2}-M phase compared with what was observed with radiation alone (Fig. 5A). However, the combination treatment in CNE-2-mock and CNE-2-COX-2 cells did not affect the cells in the G\textsubscript{2}-M phase (Fig. 5B). These data clearly showed that the observed COX-2-dependent effect of celecoxib and radiation on survival fraction cannot be caused by impairment of cell cycle control. Considering the role of the CDK1/cyclin B complex and Cdc25C in regulating G\textsubscript{2}-M phase transition, we also examined the expression of these proteins. It showed that the combination treatment in C666-1-mock cells decreased cyclin B1, CDK1 and Cdc25C expression, while phosphorylated CDK1 were significantly upregulated (Fig. 5C). γ-H2AX is a regulator of DNA repair and is required for cell cycle arrest [21]. The expression of γ-H2AX was also clearly elevated after the combination treatment. Similar results were also observed in the C666-1-KD cells (Fig. 5D).

**Celecoxib enhances radiation cytotoxicity by apoptosis induction**

To determine whether the radiosensitivity of celecoxib in the C666-1-mock and CNE-2-COX-2 cells but not in the C666-1-KD and CNE-2-mock cells was correlated with the differences of apoptosis induction, cells were exposed to radiation (4 Gy) with or without celecoxib (60 μM), and then cell apoptosis was detected by flow cytometry analysis. The apoptosis induction effects of celecoxib or radiation alone were moderately upregulated in C666-1-mock (Fig. 6A), CNE-2-COX-2 and CNE-2-mock (Fig. 6B) cells, and the apoptosis induction rates seemed to have undergone a synergistic increase in C666-1-mock and CNE-
2-COX-2 cells after the combination treatment. Moreover, the results were further confirmed by the detection of cleavage caspase-3 and PARP in C666-1-mock cells (Fig. 6C). Similar results were also observed in the CNE-2-COX-2 cells (Fig. 6D). These data showed that the combination-induced apoptosis in nasopharyngeal carcinoma cells occurred through COX-2 dependent mechanism.

**Evaluation of the combination of celecoxib and radiation treatment in nasopharyngeal carcinoma xenograft models**

To evaluate the therapeutic efficacy of the combination of celecoxib and radiation treatment in vivo, C666-1 and CNE-2 nasopharyngeal carcinoma xenograft models were used. Nude mice bearing tumors were treated with celecoxib (25 mg/kg) and radiation (6 Gy) individually, or in combination. As shown in Fig. 7A, the group treated with celecoxib alone had no change in tumor size compared with that of mice receiving vehicle. Radiation treatment alone showed moderate tumor size reduction in C666-1 xenograft model, whereas the combination treatment significantly reduced tumor volume after 29 days. The animals
in the experimental groups decreased body weight as the appetite loss. Similar results were also observed in the CNE-2 xenograft models (Fig. 7B).
Moreover, the extent of apoptosis in the C666-1 xenograft tumor specimens was assessed by western blot analysis. As shown in Fig. 7C, the combination treatment resulted in marked increased cleaved caspase-3 and PARP compared with what was observed in the celecoxib or radiation treatment alone.
Discussion

Abundant studies indicate that celecoxib suppress cell proliferation and carcinogenesis through different mechanism [22, 23]. Celecoxib is shown to suppress breast cancer cells growth through COX-dependent mechanism and the inhibitory effects are disappeared when COX-2 is knocked down [22]. The evidence from in vitro and in vivo experiments indicates that the underlying mechanism responsible for the anticancer activities of celecoxib involves the induction of cell apoptosis [24, 25]. Although celecoxib is a COX-2 inhibitor, it has been found...
to have potent proapoptotic activity in tumor cells through a mechanism that is independent of its COX-2 inhibitory activity [26, 27]. Celecoxib could affect cell survival and induce apoptosis in colon carcinoma cells irrespective of their COX-2 status [26]. Moreover, several experimental studies have showed that celecoxib-induced apoptosis is associated with the protein kinase Akt [28, 29] and the extrinsic death receptor pathway [30]. In addition, some studies suggest that celecoxib might suppress cell proliferation and carcinogenesis by reducing CDKs/cyclins activity or inducing of p21 and p27 expression, leading to cell cycle arrest [26, 31]. In the current study, we investigate the effects of the combination of celecoxib and radiation treatment in a variety of nasopharyngeal carcinoma cells. Moreover, we evaluate the therapeutic efficacy of the combination treatment on nasopharyngeal carcinoma xenograft models.

Recent evidence shows that the constitutive expression of COX-2 in cells and animal models promotes tumor cell growth, survival and angiogenesis [32, 33]. Moreover, the COX-2 levels of the cancer cells are associated with the drug resistance and the efficacy of therapeutic modalities [8, 9, 25]. Here, we found that the radiosensitivity of celecoxib was correlated with cellular COX-2 levels. To further clarify this issue, we constructed COX-2 knocked down cells from C666-1 cells as well as COX-2-overexpressing cells from CNE-2 cells to confirm the COX-2 dependency of the radiosensitization of celecoxib. It showed that the radiosensitization of celecoxib decreased when the COX-2 was knocked down in the C666-1 cells by siRNA, whereas the weak radiosensitization of celecoxib in the COX-2-lowexpressing CNE-2-mock cells was reversed after the stable transfection of COX-2. According to these results, it could be inferred that celecoxib affected the sensitization of high COX-2-expressing cells to radiation and hardly tended to confer this sensitivity to COX-2-lowexpressing cells. Furthermore, we found that the radiosensitivity of celecoxib was correlated with the apoptosis induction in C666-1-mock and CNE-2-COX-2 cells, and the results were further confirmed by the detection of cleaved caspase-3 and PARP. Therefore, it suggested that the radiosensitivity of celecoxib was dependent on the expression of COX-2 in the nasopharyngeal carcinoma cells.

The defects of tumor-related cell cycle are often mediated by the change of CDK activity. The cell growth suppression often occurs as a consequence of CDK inhibition and cell cycle block [34], so we speculate the radiosensitization of celecoxib may be attributable to the cell cycle regulation. After the combination of celecoxib and radiation treatment, the changes of cell cycle phase in C666-1-mock, C666-1-KD, CNE-2-mock and CNE-2-COX-2 cells were measured to determine the role of COX-2 in the radiosensitivity of celecoxib. Radiation treatment alone was shown to induce significant G2-M arrest, and the radiation-induced G2-M arrest was enhanced to a greater degree by celecoxib in C666-1-mock and C666-1-KD cells which expressed different COX-2 levels. These data showed that the COX-2-dependent cytotoxicity of the combination treatment on survival fraction can not be caused by the impairment of cell cycle control. It indicated that the combination-induced cell cycle arrest might be mediated by other factors besides COX-2 in the nasopharyngeal carcinoma cells. An early study showed that celecoxib-induced cell cycle inhibition in head and neck squamous cell carcinomas was acting through transcriptional induction of p21 and E2F-1 inhibition [35]. Moreover, it was demonstrated that the COX-2 inhibitor increased the accumulation of mouse mammary tumor virus HER-2/neu cell line NMF11.2 in the radiosensitive G2-M phase of the cell cycle by both dependent and independent of prostaglandin synthesis [36]. Further experiments are required to identify the direct molecular targets of the combination treatment. In addition, we noticed that a relationship was observed between the capacity of the combination treatment to induce G2-M phase arrest and apoptosis in the C666-1-mock cells, while the combination of celecoxib and radiation treatment induced mitotic arrest was not resulted in apoptosis in C666-1-KD cells. The mitotic arrest could allow the cell to extend the time to repair DNA damage. If the damage could not be repaired, the cell was eliminated via apoptosis. Therefore, the C666-1-mock and C666-1-KD cells might have different ability to repair DNA damage because of the different COX-2 levels.
To extend the observations made in cultured cells, we determined the combination of celecoxib plus radiation treatment on the growth of nasopharyngeal carcinoma xenograft in nude mice. Our results showed that the combination treatment caused a significant inhibition of tumor growth in xenograft models. It is speculated that the tumor size reduction by the combination treatment may be due to the effects of celecoxib on the tumor microenvironment resulting in better oxygenation of the tumor and the radiosensitizing effect of oxygen. In addition, it was noticed that a reduction in size of the CNE-2 derived tumor was observed in vivo while an enhanced radiosensitization was not observed in CNE-2 cells in vitro. The radiosensitivity of celecoxib in vivo involved the absorption by the intestine and metabolism by the liver, and the in vitro study could not completely provide physiological conditions and represent in vivo response. Moreover, the combination treatment significantly decreased the tumor size in vivo while the radiosensitivity of celecoxib on survival rate was relative low in vitro experiments. So we speculated that it could be related to the fact that celecoxib required a biotransformation to release the active compound that was not reached in the in vitro setting. Therefore, further study is necessary to investigate the related molecular mechanisms responsible.

In conclusion, we conclude that the increase of radiosensitivity induced by celecoxib in nasopharyngeal carcinoma is associated with G2-M phase arrest and apoptosis induction. Given the impressive data, these findings may be useful with regard to potential applications of celecoxib in nasopharyngeal carcinoma patients undergoing radiotherapy.

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Erratum

In the article by Zhang et al., entitled “Celecoxib Enhances Radiosensitivity via Induction of G2-M Phase Arrest and Apoptosis in Nasopharyngeal Carcinoma” [Cell Physiol Biochem 2014;33:1484-1497 (DOI: 10.1159/000358713)], is a printing error in the affiliations.

All the authors of this paper are from “Department of Radiology, Guangdong General Hospital/Guangdong Academy of Medical Sciences, Guangzhou, Guangdong, PR China”, not from “Department of Radiology, Guangdong General Hospital, Guangzhou, Guangdong, PR China”