Biomedicine & Pharmacotherapy 70 (2015) 181–189

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Original article

The cancer pain related factors affected by celecoxib together with cetuximab in head and neck squamous cell carcinoma

Yaqiong Yang [1], Jia Yan [1], Yan Huang, Hui Xu, Ying Zhang, Rong Hu, Jue Jiang, Zhifeng Chen *, Hong Jiang *

Department of Anesthesiology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Zhizaoju Road 639, Shanghai 200011, China

ARTICLE INFO

Article history:
Received 22 December 2014
Accepted 5 January 2015

Keywords:
Celecoxib
Cetuximab
COX-2
HNSCC
ET-1
NGF

ABSTRACT

Pain is the most disruptive influence on the quality of prognosis among head and neck squamous cell carcinoma (HNSCC) patients. The development of pain is closely associated with tumor growth and inflammation in the cancer patients. Notably, cyclooxygenase-2 (COX-2) is an important mediator during inflammation. Celecoxib, a selective inhibitor of COX-2, was hailed as a promising chemopreventive agent for HNSCC. Dose-dependent cardiac toxicity limits long-term use of celecoxib. However, the toxicity can be diminished by lowering the dosage. In this study, we hypothesized that a combinatory strategy to reduce cancer pain via two distinct pathways, tumor grown inhibition and inflammation blockade, which would enhance analgesia effect induced by HNSCC. We found that treatment of cetuximab (C225), a monoclonal anti-epidermal growth factor receptor (EGFR) antibody, with low-dose celecoxib results in a more pronounced anticancer effect in HNSCC than either agent alone. More noticeably, the combination could downregulate the phosphorylation of constitutively active extracellular signal regulated kinase (ERK) in CAL-27 and Fadu cells. Furthermore, combination therapy enhancing S phase arrest and downregulating cyclin D1 was observed in Fadu cells. The COX-2 expression was significantly blocked by celecoxib combined with C225, and other cancer pain related factors, such as ET-1 and NGF, was also downregulated by combination treatment. Taken together, these results strongly suggest that combination of celecoxib with C225 holds potential as a new therapy strategy in developing cancer pain treatment in HNSCC.

1. Introduction

Head and neck cancer is the sixth most common cancer with an annual incidence of over 560,000 cases worldwide, of which nearly 90% are squamous cell carcinomas (HNSCCs) [1]. Despite recent advances in HNSCC cancer treatment, pain control remains as an important clinical challenge, especially since cancer patients are living longer [2]. Pain is the most disruptive influence on the quality of prognostic life of cancer patients, especially in HNSCC, which is one of the most painful among all cancers [3]. Currently, a number of approaches have been aiming at reducing the levels of cancer-related pain. Therapies that aim to decrease tumor size are often effective and include radiation, chemotherapy and/or surgery – but these can be burdensome to administer and are accompanied by significant unwanted side effects. Moreover, medications that are targeted at decreasing inflammation-associated pain, such as non-steroidal anti-inflammatory drugs (NSAIDs) or opiates, also have many unwanted side effects [4]. About 45% of cancer patients have inadequate and under-managed pain control due to the treatment-associated side effects [5,6]. Cancer pain is a complex pathologic process; it has been a challenge to develop new approaches to relieve cancer-associated pain.

Cyclooxygenase-2 (COX-2), an isoform of the COX enzymes, inducible in response to pro-inflammatory cytokines and growth factors, catalyzes the biosynthesis of prostanoids including prostaglandin E2 (PGE_2), thereby, playing important roles in the regulation of various cellular functions under physiologic and pathologic conditions, including carcinogenesis [7]. The expression of COX-2 has increased in a variety of human malignancies, including HNSCC [8]. Celecoxib is a highly selective NSAID that helps to control inflammation by inhibiting COX-2 activity [9], it was hailed as a promising cancer prevention drug and has been

* Corresponding authors. Tel.: +86 021 150 021 739 83.
E-mail addresses: dzfc2013@163.com (Z. Chen), dr_jianghong@163.com (H. Jiang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.biopha.2015.01.018
0753-3322/

tested in several clinical studies [10,11]. However, this drug was found to be associated with a dose-dependent cardiovascular morbidity, which limited its long-term use as a cancer prevention agent [9].

Epidermal growth factor receptor (EGFR) belongs to the human epidermal growth factor receptor (HER) family, which includes three other closely related members: HER2, HER3, and HER4. EGFR mRNA [12] and protein [13] are upregulated in 80% to 90% and 40% of HNSCC cases, respectively, and are positively correlated with poor prognosis, advanced disease, and reduced survival [14]. Cetuximab (C225) is a monoclonal anti-EGFR antibody that was approved for the treatment of HNSCC in 2006. Cancer pain is a complex pathologic process, malignant cells are known to secrete prostaglandins, cytokines, endothelins (ET), transforming growth factor (TGF), and nerve growth factor (NGF), many of which have been shown to excite primary afferent nociceptors [15]. Utilizing the anti-tumor therapy C225 may reduce the cancer pain caused by these factors.

In recent years, combination treatment has received increasing attention due to its potential to enhance the therapeutic effect and reduce toxicity by lowering the dose required for each agent [16]. In this study, we tested the hypothesis that combination of C225 and low-dose celecoxib may result in a more pronounced effect on analgesia and inhibited tumor growth in HNSCC cells when compared with using either agent alone in vitro.

2. Materials and methods

2.1. Cell lines and reagents

Celecoxib (Pfizer) was extracted from commercially available 200 mg Celebrex capsules with dimethyl sulfoxide (Sigma, Saint-Louis, MO). C225 was purchased from Merck Serono, Inc. HNSCC cell lines (CAL-27 and Fadu) were obtained from FDCC (The Institutes of Biomedical Sciences, Fudan University). The HNSCC cells were grown in DMEM. The cultures were supplemented with 10% fetal bovine serum and an antibiotic–antimycotic mixture (100 U/mL penicillin and 100 μg/mL streptomycin; Cellgro). All cells were grown in 5% CO_2 at 37 °C and subcultured at an initial density of 1×10^5/mL every 3 to 4 days. All experiments were done with cells in the logarithmic phase of growth.

2.2. Cell survival analysis

The viability of the CAL-27 and Fadu cells was evaluated by CCK-8 assay. In brief, the cells (5×10^3) were plated in 96-well cell culture plates in DMEM with10% FBS in a final volume of 0.1 mL. At 60% confluence, cells were treated with celecoxib (1–10 μg/mL) and C225 (0.1–100 μg/mL) for 24 h. Cell survival was assessed by 10 μL CCk-8 solution to the 100 μL medium for another 3 h in 37° C, before reading the absorbance at 450 nm using a Bio-Rad Technologies Microplate Reader. Three independent experiments were performed.

2.3. Quantitative real-time PCR

Total RNA from treated cells was isolated using Trizol reagent (Invitrogen Life Technologies) and reverse-transcribed into cDNA using random hexamer primer and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The expression level of COX-2 was measured by real-time reverse transcription-PCR using Taqman Universal PCR Master Mix (Applied Biosystems) with COX-2 primer (forward 5′-AA ATCCTTGCTGTTCCCACC-3′, revere 5′-TTTCTCCATAGAATCCTGT CCG-3′). A β-actin (forward 5′-CACCCAGCACAATGAAGATCAAGAT -3′, reverse 5′-CCAGTTTTTAAATCCTGAGTCAAGC-3′) primer was used as an endogenous control. The real-time PCR was performed in triplicate for each gene. AmpliTaq Gold Enzyme was first activated at 95 °C for 10 min for 40 cycles, including denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The data were analyzed using MxPro-Mx3000p software and $2^{-\Delta\Delta CT}$ method.

2.4. ELISA

PGE_2 level was evaluated to indicate COX-2 activity. CAL-27 and Fadu cells were treated with celecoxib (10 μg/mL), C225 (10 μg/mL), and combination (5 μg/mL celecoxib plus 5 μg/mL C225) in DMEM with 10% fetal bovine serum. The culture medium was collected, and the level of PGE_2 was determined by a Correlate-EIA Prostaglandin E_2 Enzyme Immunoassay kit (Assay Designs) at absorbance (Bio-Rad) of 450 nm. The PGE_2 level was calculated at pg/mL according to the manufacturer provided protocol for the assay kit.

2.5. Flow cytometry

To analyze cell cycle, Fadu cell was cultured without serum for 24 h and then treated with celecoxib (10 μg/mL), C225 (10 μg/mL), and a half dose of combination for 24 and 48 h. The treated cells were collected, washed, and fixed in chilled 70% ethanol. The fixed cells were washed twice in PBS and then incubated with a solution containing 100 μg/mL RNase at 37 °C water bath for 30 to 45 min. Then, 25–50 μL of propidium iodide (final concentration, 50 μg/mL) were added to the cells, which were incubated at 37 °C water bath for 15 min. The cell cycle was analyzed by flow cytometry. Ten thousand cells per sample were analyzed for both assays.

2.6. Immunoblot analysis

The CAL-27 and Fadu cells were seeded and incubated in six-well plates in DMEM with 10% FBS for 24 h and exposed to 10 μg/mL concentrations of celecoxib, C225, or a half dose of combination in 10% FBS-supplemented DMEM for 48 h. The protein concentrations were measured using the BCA Kit (Pierce, Rockford, IL). The proteins (20 μg) were separated with 10% SDS–PAGE gels and transferred to polyvinylidene fluoride membranes (Millipore Billerica, MA). The membranes were blocked with 5% skim milk and incubated overnight at 4 °C with primary antibodies. The following antibodies were used: anti-GAPDH, anti-phospho-ERK1/2, anti-ERK1/2, anti-COX-2, anti-ET-1, anti-NGF, anti-cyclin D1 antibodies purchased from Abcam (Cambridge, United Kingdom). The anti-phospho-Akt (Ser473) and anti-Akt antibodies were obtained from Cell Signaling (Cell Signaling Technology, Danvers, MA). The immune complexes were detected through incubation of the membrane with horseradish peroxidase-conjugated goat anti-rabbit antibody (YeaenBio Technology Co., Ltd. Shanghai) for 1 h at room temperature and subsequent exposure of the membrane to enhanced chemiluminescence reagents (Millipore Billerica, MA).

2.7. Statistical analysis

All data are presented as the mean ± SE. Data were examined using an analysis of variance (ANOVA) and the least significant differences method for multi-sample comparisons. $P < 0.05$ was considered statistically significant.

3. Result

3.1. Celecoxib and C225 inhibited cell survival in HNSCC cells

We first examined the cytotoxic effect of celecoxib and C225 in human HNSCC cells, CAL-27 and Fadu cell lines, by CCK-8 assay. As