RESEARCH ARTICLE

Antiproliferative Effects of Celecoxib in Hep-2 Cells through Telomerase Inhibition and Induction of Apoptosis

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Abstract

Background: To investigate the effect of celecoxib on telomerase activity and apoptosis in a human laryngeal squamous carcinoma cell line (Hep-2 cells). Materials and Methods: The growth inhibition rate of Hep-2 cells *in vitro* was measured by MTT assay, and apoptosis by TUNEL assay and flow cytometry (FCM). The TRAP-ELISA method was used to determine telomerase activity in Hep-2 cells. The mRNA expression of human telomerase RNA component(hTR), human telomerase reverse transcriptase (hTERT) and human telomerase-associated protein(hTEP1) was determined by RT-PCR assay. Expression of Bax and Bcl-2 proteins was assessed by Western blotting. Results: Celecoxib can inhibit proliferation and induce apoptosis in a dose- and time-dependent manner, repress telomerase activity, decrease hTERT mRNA and Bcl-2 protein expression and increase Bax protein expression, PGE2 had no effect on telomerase. Conclusions: Celecoxib had the antiproliferative and pro-apoptotic effect in Hep-2 cells. Apoptosis was accompanied by a decrease in telomerase activity which was directly correlated with hTERT mRNA and up-regulation of Bax/Bcl-2. Bcl-2 may thus play an important role in telomerase activity as well as apoptosis.

Keywords: Celecoxib - cyclooxygenase-2 - apoptosis - telomerase - Bcl-2 - hTERT

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Introduction

Larynx cancer is 3rd common familiar carcinomas of the head neck cancers and nearly 4, 000 larynx cancer patients die each year in America (Jemal et al., 2010). In China, the death toll was about 15, 000 each year for the past few years (Du et al., 2012). A study showed the five-year overall survivals for larynx cancer was 38.0% (Pruegsanusak et al., 2012). Chemotherapy or chemotherapy combined radiotherapy could improve the overall survival and organ retention for recurrence of advanced laryngeal cancer patients (Gino et al., 2006). Squamous cell carcinoma accounts for 90% of all malignancies of the larynx and chromosomal aberrations accumulate can result in a more aggressive cancer phenotype as disease stage advance (Guo et al., 2012).

Cyclooxygenase (COX) is a critical enzyme involved in prostaglandin production and has two isoforms. COX-2 is overexpressed in many human epithelial tumors, such as colorectal, lung, breast, prostate, and head/neck cancer (Klsmet et al., 2004; Perez-Ruiz et al., 2012; Ninomiya et al., 2012). However, COX-1 is expressed constitutively in normal tissues. Upregulated expression of COX-2 is an early event during carcinogenesis, and is mostly associated with poor prognosis as it promotes tumor cell proliferation, angiogenesis, invasion (Harizi et al., 2002).

Telomerase consists of telomerase reverse transcriptase (hTERT), telomerase RNA (hTR) and telomerase associated protein-1 (TEP-1). So far, telomerase activation is considered to associate with essentially all major types of human cancer, while most normal tissues and cells contain inactivated telomerase (Liu et al., 2002). Thus, cancer-specific activation of telomerase provides an attractive avenue for developing novel anticancer therapeutics.

Preclinical and clinical data indicate that the COX-2 inhibitors, such as celecoxib, can inhibit carcinogenesis in many cancers (Pang et al., 2007; Jeon & Suh, 2013). Celecoxib showed the anti-proliferative ability via inducing apoptosis in MGC803 cells has been proved (Wang et al., 2013). However, there is few particular reports on effects of celecoxib in Hep-2 cells. Here our research first show that inhibition of cell proliferation in Hep-2 cells by celecoxib, is associated with the induction of apoptosis and inhibition of telomerase activity via regulation of proteins involved Bax and Bcl-2 and hTERT mRNA.

Materials and Methods

Cell culture

Human laryngeal squamous carcinoma cell lines (Hep-

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2 cells) were obtaind from Shandong Academy of Medical Sciences .Hep-2 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco, USA), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were maintained in a humidified atmosphere of 95% air and 5%CO_2 at 37°C. They were subcultivated every 2-4 d and given fresh medium every other day. Hep-2 cells at 80-90% subconfluent were employed in all experiments.

Cell proliferation assay

The effect of celecoxib on cell proliferation was determined with the colorimetric MTT assay. Briefly, the cell suspension at an initial density of 8×10^4 cells/ml was seeded into 96-well plates and allowed to incubate for 24 h. Then cells were treated with celecoxib (0, 30, 50, 100 μM) for 0, 12, 24, or 48 h. After the drug treatment, the MTT solution at a final concentration of 0.5 mg/ml was added to the cell culture. Following incubation 4 h, the supernatant was aspirated and 150 μl of DMSO was added to each well to dissolve the formed blue formazan crystals. The absorbance at wavelength of 570 nm was measured by an enzyme-linked immunoabsorbent assay reader. The negative control well contained medium only and was used as zeropoint of absorbance. The relative inhibition rate was calculated as a percentage, as follows: (Acontrol-Aexperiment/Acontrol-Anegtive control)×100%.

Apoptosis assay by flow cytometry

Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime, China) was used. Hep-2 cells (2×10^5) were cultured in a 6-well plate for 24 h prior to treatment. Samples were exposed to 0, 45, 50, 55, and 65 hr of celecoxib. Cells were trypsinized, washed twice with PBS. The cells were centrifuged at 1000 r/min for 6 min, and the cells were resuspended in 1×binding buffer. The sample solution (400 μl) was transferred to a 5-ml culture tube and stained with 5 μl of fluorescein isothiocyanate (FITC)-conjugated annexin V at room temperature in the dark for 15 min and 10 μl of PI on ice in the dark for 5 min. All samples were analyzed by flow cytometry (BD FACSCALIBUR, USA) using the Cell Quest Research Software.

Apoptosis analysis by TUNEL

Terminal deoxynucleotide transferase-mediated dUTP nick-end labelling (TUNEL) system was carried out using the kit (Roche, Germany) according to the manufacturer's protocol. In brief, cells grown on coverslips were fixed with a freshly prepared 4% paraformaldehyde for 30 min at 20°C, washed and incubate with 3% H_2O_2 for 10 min at 20°C, washed 2 times with ice-cold PBS and permeabilized with 0.2% Triton X-100 in PBS on ice for 3 min. Then samples were incubated with fluorescein-conjugated TUNEL reaction mixture in a humidified box at 37°C for 1 h. TUNEL-positive cells were analyzed under the fluorescence microscope (e600, NIKON). And samples were stained by Hoechst 33342 as the total control.

TRAP-ELISA analysis

Telomerase activity was determined by the telomeric repeat amplification protocol (TRAP assay) using the kit TeloTAGGG Telomerase PCR ELISA PLUS (Roche, Germany) according to manufacturer's instructions. Collected and counted Hep-2 cells which were treated with celecoxib by different hours (Hep-2 cells were cultivated in the 6-well plate.) Briefly, 1×10^4 Hep-2 cells of each sample were lysed, respectively in 30 μl of ice-cold lysis buffer for 30 min. Supernatants were collected and rapidly frozen and stored at -80˚C. 5μl of each extract was added to 25 μl of reaction mixture with the appropriate amount of sterile water to make a final volume of 50 μl. TRAP reactions were carried out on each sample. PCR was carried out as follows: primer elongation (30 min, 25°C), telomerase inactivation (5 min, 94°C), and 30 cycles of amplification (94°C for 30 s, 50°C for 30 s, 72°C for 90 s). the PCR product was denatured and then hybridized with the digoxigenin (DIG)-labeled telomeric repeat-specific probe. Finally, the PCR product was detected with the anti-DIG-POD antibody measured at 450 nm using an ELISA reader. And cells in another group added Prostaglandin E2 (PGE2; Kangnuo Co., China)to the cell culture medium 2 hours before adding celecoxib. After treatment with celecoxib (50 μM) for24 and48 hours, whole cells lysates were collected and measured for telomerase activity.

RT-PCR

Expression of the telomerase catalytic subunits, hTR, hTEP1, and hTERT mRNA were determined by Reverse transcription (RT)-PCR. RT-PCR was performed using the two-step RT-PCR kit (Takara Co., Dalian, China). Extraction of total RNA was performed using the Beyozol (Beyotime, China). Collected and counted Hep-2 cells which were treated with celecoxib by different hours (Hep-2 cells were cultivated in the 6-well plate.) Total RNA was collected from samples, using the Beyozol (Beyotime, China). The total volume of the RT-PCR reaction was 25 μl, containing 0.2 mM dNTP, 1 mM of each primer, 1 mM MgSO4, 0.1 U/ml AMV transcriptase, 0.1 U/ml Tf1 DNA polymerase, 1X reaction buffer, 3%DMSO and 1μl of cDNA. The amplification condition was as follows: hTR 30 cycles (94˚C, 45 sec; 54˚C, 45 sec; 72˚C, 1.5 min), hTEP1 28 cycles (94˚C, 45 sec; 60˚C, 45 sec; 72˚C, 1.5 min), hTERT 35 cycles (94˚C, 45 sec; 60˚C, 45 sec; 72˚C, 2 min). The amplified product was analyzed by gel electrophoresis in a 2% agarose gel and EB staining. The primer sequences and PCR product sizes are shown in Table 1.

Western blot analysis

After treatment with different hours (0, 4, 6, 12, 24

Table 1.RT-PCR Primer Sequence and Product size of Telomerase Subunits and GAPDH

Gene	Primer sequence	Product size(bp)
GAPDH	Up 5'AATGACCCCTTCATTGAC3	191
	Down 5'TCCACGACGTACTCAGCGC3	
hTERT	Up 5'CCGAAGAGTGTCTGGAGCAA3	145
	Down 5'GGATGAACCGCACTCTGCA3	
hTP1	Up 5'TCAAGCGAAACCTGAATCTGAC3	264
	Down 5'CCCGAGTGAATCTTTCTACGC3	
hTR	Up 5'TCTAACCCTAACTGAGAAGGGCGTAG3	126
	Down 5'CTTTGCTCTAGAATGAACGGTGCAAC3	

and 48 h) by 50 celecoxib, Hep-2 cells were harvested and then treated with cell lysis buffer. Protein extracts were separated by 10% SDS-PAGE and transferred to a PVDF membrane. 5% defat milk powder-PBS solution was used to block the blots for one hour. Primary antibodies of Bcl-2 and Bax (Chicago, USA)at the appropriate dilutions were hybridized to the membrane overnight at 4°C. The membranes were washed 3 times with TBS-T and were then incubated with a horseradish peroxidase-linked secondary antibody (Boster , China)for 1 h at room temperature. The protein bands were visualized using ECL Plus substrate and then exposed to X-ray films in a dark room. The BioRad Laboratories Quantity One software (BioRad, USA) was used to quantify the blots.

Statistical analysis

Statistical analysis was carried out using SPSS 16.0 for Windows .All data were analyzed by the Student's t-test. P values less than 0.05 were considered statistically significant.

Results

Celecoxib inhibited cell proliferation in Hep-2 cells

We examined the effect of celecoxib treatment on Hep-2 cells proliferation using the MTT assay which reflects total number of live cells. In Figure 1, showed differential killing with increasing concentrations of celecoxib or increasing hours of celecoxib, and revealed the inhibition rate was gradually enhanced as the concentrations or hours increased. The IC_{50} value was about 60. 2 μM and 43.7μM in Hep-2 cells incubated with celecoxib for 24 h and 48h, respectively.

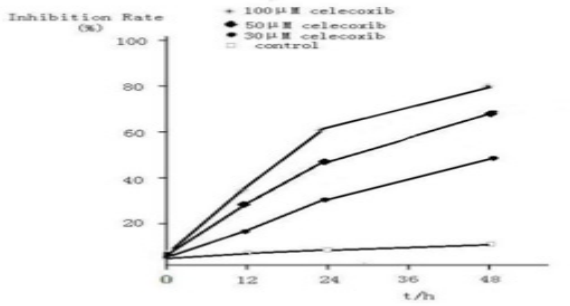

Figure 1. Inhibition of the Cell Growth by Celecoxib in Hep-2 cells. Hep-2 cells were treated with celecoxib at final (0, 30, 50, 100 μM) for 0,12, 24, or 48 h, respectively

Celecoxib induced apoptosis in Hep-2 cells

In order to ascertain the induction of Hep-2 cells apoptosis by celecoxib, the TUNEL assay and FCM assay were performed. The results showed that the rate of apoptosis increased in response to celecoxib administration. Hep-2 cells were treated with 50μM celecoxib for 3, 6, 12, 24 and 48 h, and subjected to flow cytometry analysis for apoptotic activity. The percentage of apoptotic cells was 2.1, 3.3, 8.0, 15.7 and 20.8%, respectively (Figure 2A). On the other hand, Hep-2 cells were treated with 50μM celecoxib for 24h. Result showed the TUNEL-positive cells appeared to be towards more as celecoxib treated hours increased. And all cells were stained with Hoechst 33342 as the total control (Figure 2B). Overall, at the tested levels, celecoxib induced a time- and dose-dependent apoptosis in Hep-2 cells.

Celecoxib repressed of telomerase activity in Hep-2 cells

To evaluate the role of celecoxib in the regulation of telomerase activity in Hep-2 cells, cells were cultured with 50 μM celecoxib for different period of time. Addition of celecoxib in Hep-2 cells substantially reduced the telomerase activity gradually .The percentage of relative activity of telomerase decreased from 100% in the control group to 96.43, 73.87, 51.28, 28.03 and 17.11% in groups treated for 3, 6, 12, 24, and 48 h respectively

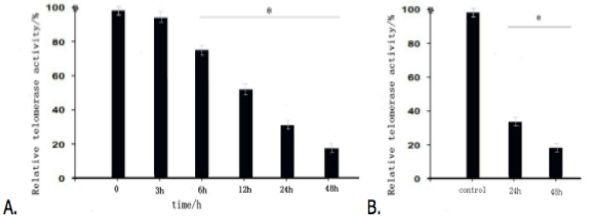

Figure 3. The Effect of Celecoxib on Telomerase Activity in Hep-2 Cells by TRAP-ELISA. Activity of control cells was regarded as100%. **A)** Cells were treated with celecoxib at 50μM for 0,3, 6, 12, 24 and 48 h, respectivel. **B)** Exogenous PGE2 a was added to the celecoxib group for 24 and 48h (*p<0.05, compared with control cells)

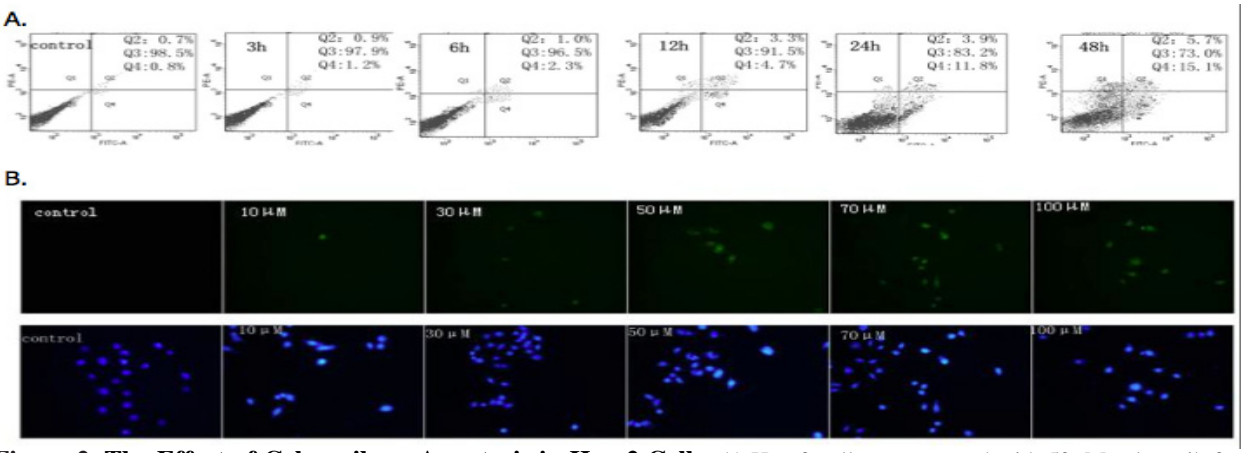

Figure 2. The Effect of Celecoxib on Apoptosis in Hep-2 Cells. A) Hep-2 cells were treated with 50μM celecoxib for 0,3, 6, 12, 24 and 48 h by flow cytometry analysis.B) Hep-2 cells were treated with 0, 10, 30, 50, 70, 100 μM celecoxib for24h byTUNEL (×200)

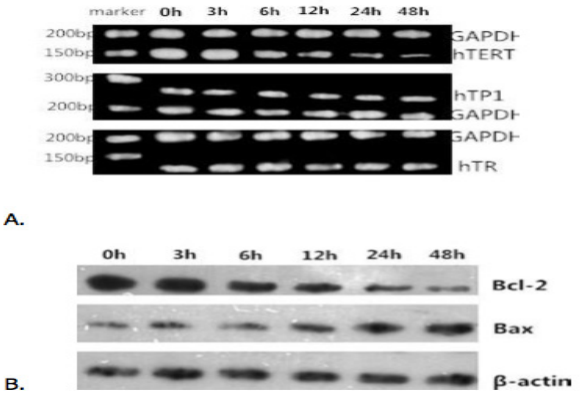

Figure 4. mRNA Expression of hTR, hTEP1, hTERT and Protein Expression of Bcl-2, Bax. Hep-2 cells were treated with celecoxib at 50μM for 0, 3, 6, 12, 24 and 48 h, respectivel. A) hTR, hTEP1 and hTERT mRNA expression was decreased by RT-PCR. B)Bcl-2 and Bax protein expression was decreased by western blot

(Figure 3A). PGE2, one of the primary cyclooxygenase products of arachidonic acid through the COX pathway. It was found that exogenous PGE2 did not restore the telomerase activity inhibited by celecoxib, suggesting that the inhibitory effect of celecoxib on telomerase activity was independent of the COX-2-prostaglandin pathway (Figure 3B).

Celecoxib decreased the mRNA expression of hTERT in Hep-G2 cells

To determine whether hTR, hTEP1, and hTERT are involved in the mechanism of the inhibition of telomerase activity by celecoxib, those mRNA expressions were were measured by RT-PCR. We found that celecoxib did not inhibit expression of both hTR and hTEP-1 mRNA compared with control. Conversely, hTERT mRNA detected by RT-PCR found which expression was gradually inhibited as the hours treaed by celecoxib increased. The levels of hTERT were in same relationship with the activity of telomerase presented in the treated samples (Figure 4A).

Detection of bcl-2 expression using western blot

Western blot results showed that Hep-2 cells treated with 50 μM celecoxib increased the Bax (pro-apoptotic protein) expression at protein levels, meanwhile, decreased Bcl-2 (anti-apoptotic protein)expression. Bax/ Bcl- 2 ratio enhanced as the hours treated by celecoxib increased (Figure 4B).

Discussion

Larynx cancer is one of the most common head and neck malignant tumor and seriously affects the quality of people's lives. Most patients with laryngeal carcinoma in advanced stage chose surgery, hence they lost the function of throat (Forastiere et al., 2013). Celecoxib is a non-steroidal anti-inflammatory drug (NSAID) that specifically inhibits COX-2 and has little effect on COX-1. It has significant anti-inflammatory and analgesic properties but lesser toxicity , which also has anti-tumor effect on some cancers (Bocca et al., 2012; Ramer et al., 2013). And most studies *in vitro* focused on the apoptosis induced by celecoxib. However, there is little research about the effect of celecoxib on telomerase activaty. Our study found celecoxib had the antiproliferative and pro-apoptotic effect in concentration- and duration-dependent manners. Apoptosis is accompanied by a decrease in telomerase activity which has directly correlated with hTERT mRNA and down-regulation of the pro-apoptotic gene Bcl-2.

Apoptosis is a tightly regulated process under the control of several signaling pathways and one of the major gene groups that regulate apoptosis is the Bcl-2 family. There has been evidence that Bcl-2 members (e.g., Bcl-2, Bcl-xL) protect against multiple signals that lead to cell apoptosis (Armstrong et al., 1996); whereas Bax members (e.g., Bax, Bad) induce apoptosis (Scorrano et al., 2003), indicating that the Bcl-2 family regulates a common cell apoptosis pathway and functions at a point where various signals converge (Jeong et al., 2008). Our study found the protein expression of Bax/Bcl- 2 ratio increased after treatetment by celecoxib also proved that Bcl-2 members can be an important contributing factor to the apoptosis of cancer cells.

In human normal body cells, which show little or no telomerase activity in synthesizing new telomeres at the ends of replicating chromosomes, the telomeric DNA gradually shortens with each cell division (Sato et al., 2012). However, telomerase are active in most cancer tumors (Murnane, 2010). As we know, Human telomerase is composed of hTP1, hTR and hTERT, which together promote the ability of telomerase to elongate telomeres *in vivo*. And RT-PCR results showed celecoxib had no effect on hTP1and hTR, but repressed hTERT. There is a good correlation between the expression of hTERT and the presence of telomerase activity in extracts from tissue culture cells and normal and cancer tissues (Takakura et al., 2005). Therefore, hTERT plays a vital role in telomerase activation (Rahmati et al., 2013). We observed the inhibition of hTERT mRNA expression keep in step with the repression of telomerase activity by celecoxib. Besides, telomerase activity was prostaglandin-independent.

The view that apoptosis plays a key part in the development of cancer is widely accepted (Johnstone et al., 2002). However, the concept that telomerase may have relationship with inhibiting apoptotic signaling cascades was not known widely. And telomerase activity may be involved in the regulated process of apoptosis. In the this study, concurrent suppression of hTERT mRNA, bcl-2 protein expression and telomerase activity was observed 6 h after celecoxib treatment, while the apoptotic cells did not detectable 6 h after celecoxib treatment, suggesting that the down-regulation of bcl-2 and telomerase may play a causative role in celecoxib-induced apoptosis. More over, it has been reported that the overexpression of Bcl-2 in human cancer cells resulted in increased telomerase activity and a resistance to apoptosis, indicating a link between Bcl-2 expression and telomerase activity in human cancer cells (Fu et al., 1999). Therefore, we deduceed that celecoxib suppressed telomerase activity by down-regulation hTERT mRNA via deactivation of the anti-apoptotic gene bcl-2.

In summary, our results in the present study demonstrate that celecoxib can inhibit proliferation of human laryngeal squamous carcinoma cell cells, which may involve its inducing apoptosis and inhibiting telomerase. The apoptotic events were associated with concurrent down-regulation of Bcl-2 and the telomerase activity and up-regulation of Bax.The inhibition of hTERT mRNA expression resulted in decreased telomerase activity.Bcl-2 may play an important role in apoptosis and telomerase activity.

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