ORIGINAL ARTICLES

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Anticancer effects of celecoxib through inhibiton of STAT3 phosphorylation and AKT phosphorylation in nasopharyngeal carcinoma cell lines

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Previously, we showed that treatment with celecoxib obviously inhibited proliferation of nasopharyngeal carcinoma (NPC) cell lines in a dose-dependent manner. However, the underlying molecular mechanisms of its anticancer effect on NPC have not been fully clarified. The present *in vitro* study was performed to investigate the mechanisms involved in the anticancer effect of celecoxib in NPC. NPC cell line HONE1 was treated with celecoxib at varying concentrations. The antiproliferation effect of celecoxib on the HONE1 cell line was assessed with methyl thiazolyl tetrazolium (MTT) assay. Western blot analysis of signal transducer and activator of transcription 3 (STAT3), phosphorylated $STAT3^{Y705}$ ($pSTAT3^{Y705}$), Survivin, Mcl-1, Bcl-2 and Cyclin D1 was carried out at various concentration of celecoxib for 48 h in HONE1 cell line. Western blot analysis of Protein Kinase B (AKT), phosphorylated AKT (pAKT) was performed at increasing doses of celecoxib for 48 h in HNE1, CNE1-LMP1 and HONE1 cells. The results showed that celecoxib inhibited proliferation of HONE1 cell line in a dose-dependent manner. Celecoxib inhibited the activation of STAT3 phosphorylation in HONE1 cells and the downstream genes of STAT3 (Survivin, Mcl-1, Bcl-2 and Cyclin D1) were downregulated after treatment with celecoxib. Furthermore, celecoxib could inhibit AKT phosphorylation in HNE1, CNE1-LMP1 and HONE1 cell lines. These data suggested that celecoxib was a promising agent for the chemoprevention and treatment of NPC.

1. Introduction

Traditional non-steroidal anti-inflammatory drugs are non-selective inhibitors of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 is a housekeeping enzyme constitutively expressed in almost all tissues, which is capable to produce prostaglandins regulating normal physiological function (Wang et al. 2007). COX-2 is induced by growth factors and cytokines and aberrantly expressed in various types of cancers. It has been established that overexpression of COX-2 participated in carcinogenesis, tumor cell proliferation, apoptosis inhibition, angiogenesis, invasion and metastasis (Khan et al. 2011). Celecoxib is a COX-2 selective inhibitor which has shown anticancer effects in multiple cancer cell lines including those arising from the lung, breast, liver and so on (Yin et al. 2011; Zhang et al. 2011; Dai et al. 2012; Jendrossek 2013). Furthermore, the US Food and Drug Administration has approved the use of celecoxib for the adjuvant treatment of familial adenomatous polyposis (FAP) because of its chemopreventive properties (Steinbach et al. 2000). Although the precise molecular mechanisms of the anticancer effect of celecoxib is not fully clear, celecoxib has become an effective agent for cancer prevention and therapy (Amir and Agarwal 2005).

Nasopharyngeal carcinoma (NPC) is endemic in southern China where the incidence rate of NPC is approximately 20 to 50 per 100,000 individuals (Yu and Yuan 2002). The study of celecoxib on NPC chemoprevention and treatment are scarce and the underlying molecular mechanisms of its anticancer effect on NPC need to be further elucidated (Chen and Long 2004; Chan et al. 2005; Chen et al. 2010). Previously, we have demonstrated that celecoxib could induce apoptosis and cell cycle arrest in NPC HNE1 and CNE1-LMP1 cell lines *via* inhibition of STAT3 phosphorylation, which indicated that the inhibitory effect of celecoxib on NPC cells may be mediated by a COX-2-independent pathway (Liu et al. 2012). Phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) signaling pathway can regulate tumor cell survival, apoptosis, and a series of cell physiological and biochemical processes (Bartholomeusz et al. 2012). Phosphorylation of AKT regulates a range of important functions of functional proteins in tumor cells, and thus indirectly regulates cell proliferation, cell death, cell viability, adhesion, cell transformation, angiogenesis and apoptosis (Pal and Mandal 2012; Xue and Hemmings 2013). Many studies showed that PI3K/AKT pathway played an important role in cancer development (Kobayashi et al. 2006; Sourbier et al. 2006; Mei et al. 2007; Ye et al. 2012). Recent studies found that celecoxib could exert anticancer effects in a variety of tumors through COX-2-independent molecular mechanisms including apoptosis induction, the inhibition of cell cycle progression, angiogenesis, and metastasis, while the PI3K/AKT signaling pathway inhibition appears to play a central role in these molec-

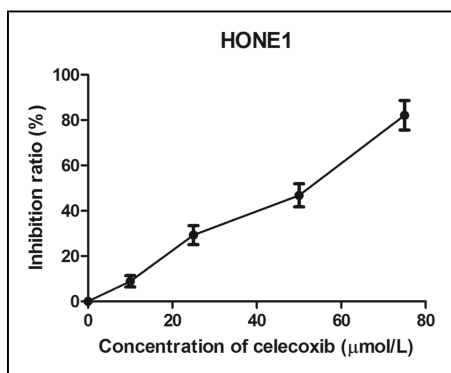

Fig. 1: HONE1 cells were incubated with increasing doses of celecoxib (0, 10, 25, 50 and 75 μmol/L) for 48 h. Celecoxib induced dose-dependent growth inhibition in HONE1 cells. Values are the mean ± SD (n = 3).

ular mechanisms (Grösch et al. 2006). Whether the anticancer effect of celecoxib on NPC cells is associated with AKT signaling pathway has not been investigated.

The present *in vitro* study was carried out to investigate whether the underlying mechanism of celecoxib-associated anticancer effects is related to AKT signaling. Moreover, NPC HONE1 cell line was used to confirm our previously reported results that celecoxib can exert anticancer effect through phosphorylation inhibition of STAT3 and down-regulation of its downstream protein expression.

2. Investigations and results

2.1. Celecoxib inhibited HONE1 cells proliferation

To investigate whether celecoxib was capable of inhibiting NPC cell growth, HONE1 cells were treated with increasing doses of celecoxib (0, 10, 25, 50, 75 μmol /L) for 48 h, followed by an MTT assay. As shown in Fig. 1, we observed that celecoxib inhibited HONE1 cells proliferation in a concentration dependent manner at 48 h.

2.2. Celecoxib inhibited STAT3 phosphorylation

To investigate whether celecoxib could inhibit STAT3 phosphorylation, western blot was used to analyze the levels of STAT3 phosphorylation change after celecoxib treatment. HONE1 cells were treated with increasing doses of celecoxib (0, 25, 50, 75 μmol /L) for 48 h. As shown in Figs. 2A and 2B, we observed that the amount of STAT3 phosphorylation level was significantly decreased ($p < 0.01$, $p < 0.01$) after celecoxib treatment at concentrations of 50 and 75 μmol /L.

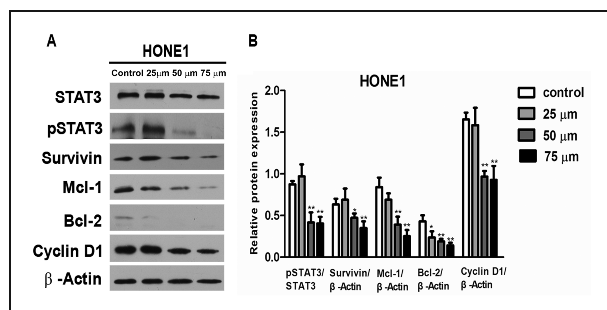

Fig. 2: Celecoxib inhibited STAT3 phosphorylation and downregulated downstream proteins expression of STAT3. HONE1 cells were treated with DMSO (control) and celecoxib (25, 50 and 75 μmol/L concentrations) for 48 h. Celecoxib significantly inhibited STAT3 phosphorylation in HONE1 cell line. The downstream proteins of STAT3 (Survivin, Mcl-1, Bcl-2 and Cyclin D1) were also downregulated after celecoxib treatment. The blots shown are from a representative experiment repeated three times with similar results. *$p < 0.05$ vs control, ** $p < 0.01$ vs control.

To provide further evidence of the inhibition of STAT3 phosphorylation, we analyzed the important downstream proteins of STAT3 by western bolt. Survivin ($p < 0.05$, $p < 0.01$ at 50 and 75 μmol /L), Mcl-1 ($p < 0.01$, $p < 0.01$ at 50 and 75 μmol /L), Bcl-2 ($p < 0.05$, $p < 0.01$, $p < 0.01$ at 25, 50 and 75 μmol /L) and cyclin D1 ($p < 0.01$, $p < 0.01$ at 50 and 75 μmol /L) proteins showed decreased expression with escalating doses of celecoxib in HONE1 cell (Figs. 2A and 2B).

2.3. Celecoxib inhibited AKT phosphorylation

To investigate whether celecoxib may inhibit AKT phosphorylation, western blot was performed to detect the phosphorylated AKT expression in HNE1, CNE1-LMP1 and HONE1 cell lines after treatment with increasing doses of celecoxib (0, 10, 25, 50 or 75 μmol /L) for 48 h. As shown in Figs. 3A and 3B, phosphorylated AKT expression was downregulated after treatment with celecoxib in HNE1 cells ($p < 0.05$, $p < 0.01$, $p < 0.05$ at 10, 25 and 50. μmol /L), CNE1-LMP1 cells ($p < 0.01$, $p < 0.01$, $p < 0.01$ at 25, 50, 75 μmol /L), and HONE1 cells ($p < 0.05$, $p < 0.05$, $p < 0.01$ at 25, 50, 75 μmol /L).

3. Discussion

NPC is the most common head and neck cancer in China. Many studies showed that celecoxib was capable of inhibiting cancer cell growth in various tumors. Previously, we demonstrated that celecoxib strongly inhibited STAT3 phosphorylation in CNE1-LMP1 and HNE1 cell lines, a critical step to activate downstream signaling. Subsequently, the critical downstream proteins survivin, Mcl-1, Bcl-2, and cyclin D1 were down-regulated, ultimately leading to cell growth inhibition, apoptosis induction and cell cycle arrest at G0/G1 phase (Liu et al. 2012). To further confirm our previously reported results, HONE1 was chosen as experimental model. MTT showed that celecoxib could inhibit HONE1 cell growth in a concentration dependent manner. In consistence with previous results, STAT3 phosphorylation was obviously inhibited in HONE1 cells after celecoxib treatment. And the downstream proteins survivin, Mcl-1, Bcl-2, and cyclin D1 were down-regulated accordingly. These results indicated that inhibition of STAT3 phosphorylation may be one of the underlying molecular mechanisms of celecoxib-associated anticancer effects in NPC cell lines. As celecoxib could exert anticancer effects in many different COX-2-independent molecular mechanisms in various tumor cells, we investigated the effect of celecoxib on AKT signaling pathway. After treatment with various doses of celecoxib for 48 h, we observed that the phosphorylated AKT expression was significantly down-regulated in HNE1, CNE1-LMP1 and HONE1 cells. In line with our results, many studies showed that celecoxib could inhibit AKT phosphorylation leading to apoptosis and cell cycle arrest and proliferation inhibition in gastric cancer, breast cancer, osteosarcoma and other tumors cell lines (Arico et al. 2002; Pang et al. 2007; Liu et al. 2008; Kim et al. 2009). The AKT signaling pathway may play a crucial role in the COX-2-independent anticancer molecular mechanisms of celecoxib.

There are some limitations of the present study. Firstly, further studies are needed to differentiate the anticancer effect of celecoxib in NPC from different molecular pathways. Celecoxib could act through COX-2 dependent and/or COX-2 independent mechanisms including IL-6/STAT3 pathway (Li et al. 2011), NF-κB pathway (Sareddy et al. 2012), ERK pathway

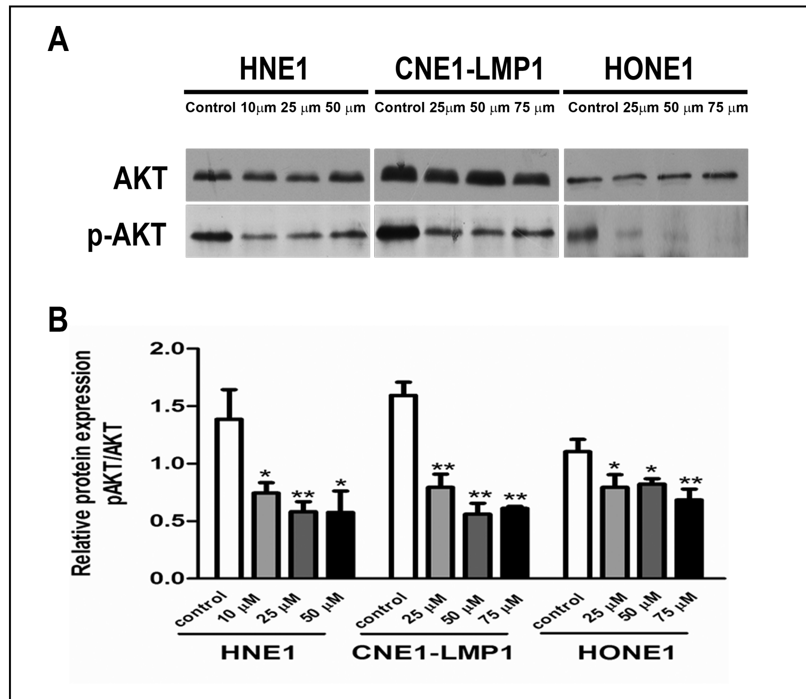

Fig. 3: Western blot analysis of phosphorylation AKT expression after increasing concentration of celecoxib for 48 h. Celecoxib significantly inhibited AKT phosphorylation in HNE1, CNE1-LMP1 and HONE1 cell lines. The blots shown are from a representative experiment repeated three times with similar results. *$p < 0.05$ vs control, ** $p < 0.01$ vs control.

(Wang et al. 2013) and so on. Additionally, *in vivo* studies are also needed.

In summary, in this study we demonstrated that celecoxib could inhibit HONE1 cell proliferation in a concentration dependent manner. This is associated with down-regulation of STAT3 phosphorylation and AKT phosphorylation. These data suggest that celecoxib is a promising agent for chemoprevention and treatment of NPC.

4. Experimental

4.1. Cell lines

HNE1, HONE1 and CNE1 are all low differentiated NPC cell lines. CNE1-LMP1 is a stably transfected cell line, established by introducing Latent membrane protein 1 (LMP1) cDNA into the CNE1 cell. HNE1, HONE1 and CNE1-LMP1 cell lines were all provided by Cancer Research Institute of Central South University (Changsha, China). All cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, USA) supplemented with 10% newborn calf serum (Gibco, Grand Island, USA) in a 5% CO_2 atmosphere.

4.2. MTT assay

The growth inhibition activity effect of celecoxib on HONE1 cells was analyzed by MTT assay. Cells were seeded into 96-well plates and allowed to attach for 24 h. The cells were then treated with escalating doses of celecoxib (Sigma Chemical Co., MO, USA) (0, 10, 25, 50 and 75 μmol/L) dissolved in DMSO (final concentration 0.1%) and incubated for up to 48 h. Afterwards, 20 μl MTT (5 mg/ml) were added to each well and cells were incubated for 4 h at 37 °C. After removal of the supernatants, the crystals were dissolved in DMSO and the absorbance was measured at 490 nm. The percentage growth inhibition was calculated as (ODcontrol − ODdrug)/OD control × 100%. The experiment was performed in triplicate and repeated for at least three times.

4.3. Westetn Blotting analysis

Cells were treated with DMSO (control) or celecoxib with increasing concentrations (10, 25, 50 and 75 μmol/L) for 48 h. Cells were then washed with phosphate balanced solution (PBS) and lysed in Radio-Immunoprecipitation Assay (RIPA) buffer with phosphatase and protease inhibitors (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined using the Bradford protein assay kit (Bio-Rad Laboratories Inc., Hercules, California, USA) according to the manufacturer's instructions. Protein samples were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, Massachusetts, USA). After blocking non-specific binding by non-fat milk, the membranes were separately incubated with anti-phsopho-STAT3-Tyr705, anti-Survivin, anti-Mcl-1, anti-Cyclin D1 (Epitomics, Burlingame, USA), anti-Bcl-2, anti-STAT3, anti-AKT, anti-phsopho-AKT (Cell Signaling Technology, Danvers, USA) primary antibodies overnight and then probed with the appropriate secondary antibodies. Specific protein was then visualized by an enhanced chemiluminescence (ECL) detection system. Each blot was stripped and reprobed with a ß-actin antibody as an internal control. Densitometric quantification of the bands was performed using AlphaEaseFC software tool (Alpha Innotech, San Leandro, CA).The experiment was performed in triplicate and repeated for at least three times.

4.4. Statistical analysis

Statistical analysis was carried out using SPSS 15.0 software (SPSS inc., Chicago, IL, USA). All data were expressed as mean ± standard deviations (SD). Statistical analysis was performed by Student's t test. p value < 0.05 was considered statistically significant.

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