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Heterogeneous nuclear ribonuclear protein C is increased in the celecoxib-induced growth inhibition of human oral squamous cell carcinoma

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Abbreviations: COX-2, cyclooxygenase-2; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; hnRNP C, heterogeneous nuclear ribonuclear protein C; NSAIDs, nonsteroidal anti-inflammatory drugs; OSCC, oral squamous cell carcinoma; YD, Yonsei University College of Denstry

Abstract

Celecoxib is a selective inhibitor of cyclooxygenase-2 (COX-2) that is a critical factor in carcinogenesis, but precise mechanism of its action remains to be elucidated. Here we evaluated the inhibitory effect of celecoxib on cell growth of human oral squamous cell carcinoma (OSCC) YD-10B, which was established to be used as *in vitro* OSCC model, and identified celecoxib-regulated protein by proteomics techniques. Celecoxib (IC_{50} = 37 μM) inhibited the growth of YD-10B cells with the decrease of COX-2 protein expression. Its inhibition could be linked in the arrest of G_1 phase with increased levels of p27 protein, a specific CDK inhibitor. Using proteomics, the 10- to 20-fold increase of heterogeneous nuclear ribonuclear protein C (hnRNP C), which has been suggested to be related with the translation of p27 mRNA, was observed in celecoxib-treated YD-10B cells. In summary, celecoxib has a potential to induce the protein expression of hnRNP C and its increase subsequently induce the translation of p27 mRNA, which trigger the inhibition of cell growth *via* p27-regulated cell cycle arrest in YD-10B cells. In addition, YD-10B cells could be useful to study the pathological mechanism of OSCC.

Keywords: carcinoma, squamous cell; celecoxib; cell cycle; cyclin-dependent kinase inhibitor p27; cyclooxygenase 2 inhibitors; heterogeneous-nuclear ribonucleoprotein C

Introduction

Oral malignancy, the commonest form of which is squamous cell carcinoma (SCC), constitutes a major worldwide health problem (Jemal *et al.*, 2003). Moreover, despite curative therapy for oral squamous cell carcinoma (OSCC), patients may experience debilitating changes in appearance, speech, swallowing, and breathing.

Chemoprevention appears to be a promising strategy for the inhibition of carcinoma before the development of invasive tumors, but current strategies are far from satisfactory, primarily due to the significant toxicity associated with these approaches. In the search for agents with potentials to prevent or delay the onset of cancer, it has become apparent that cyclooxygenase-2 (COX-2) is a promising therapeutic target (Fosslien *et al.*, 2000; Hayashi *et al.*, 2001; Sirica *et al.*, 2001; Nzeako *et al.*, 2002; Mohan *et al.*, 2003; Subbaramaiah *et al.*, 2003). Epidemiological studies and clinical observation suggest that the regular consumption of nonsteroidal anti-inflammatory drugs (NSAIDs) may reduce the risk of developing several types of human cancer by regulating the expression/activity of COX-2 (Fosslien *et al.*, 2000; Hayashi *et al.*, 2001; Sirica *et al.*, 2001; Subbaramaiah *et al.*, 2003). Recently many groups have reported that NSAIDs can suppress growth of human cancer cells, including those associated with head and neck carcinomas by inducing the cells to arrest at the G_0/G_1 or G_2/M phase; often they induce apoptosis as well (Fosslien *et al.*, 2000; Hayashi *et al.*, 2001; Sirica *et al.*, 2001; Nzeako *et al.*, 2002; Wu *et al.*, 2002). However, considering the report showing that the anti-proliferative effects of NSAIDs on cancer cells are both dependent and independent on

the inhibition of COX-2 activity and prostaglandin synthesis (Gupta *et al.*, 2001; Choi *et al.*, 2004; Maier *et al.*, 2004), the precise mechanism and involvement of NSAIDs on the process of carcinogenesis still remain to be elucidated.

Celecoxib is a newly developed COX-2 inhibitor with significantly less toxicity. Recently, chemopreventive activity of celecoxib in oral carcinoma cell has been reported showing that celecoxib significantly delayed cell growth and reduced tumor volume in mice to which oral carcinoma cells were intradermal inoculated (Wang *et al.*, 2002). In this report, no toxic effect was found by means of measurement of body weight loss and microscopic dissection of organs. Based on this report, here the inhibitory mechanism of celecoxib on the cell growth was evaluated to understand its functional mechanism in OSCC. Especially, in this study, COX-2-overexpressed OSCC (named YD-10B) was established and used. This was for evaluating whether YD-10B cells could be used as *in vitro* model of OSCC. In addition, proteins regulated by the treatment of celecoxib were identified by proteomics technologies.

Materials and Methods

Establishment of cell lines and cell culture

The surgical specimens utilized in this study were obtained from a patient (67 yr-old male with moderately differentiated squamous cell carcinoma in tongue; no pathological nodal status; no clinical history for anti-cancer therapy). Tissues were transported in high glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1×10^{-10} M cholera toxin, 0.4 mg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 2×10^{-11} M triiodothyronine. Tissues were treated with trypsin and then the isolated cells were grown with a feeder layer composed of mitomycin C-treated NIH 3T3 fibroblasts. The cells were fed with a mixture of DMEM and Ham's nutrient mixture F12 at a 3:1 ratio, supplemented with 10% FBS, 1×10^{-10} M cholera toxin, 0.4 mg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 2×10^{-11} M triiodothyronine. All materials used in cell culture experiment were purchased from Life Technologies, Inc. (Grand island, NY). The cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. Cells were subcultured twice a week by trypsinization for 1 yr after initial cultivation, and the cell line was considered to be "established" after 40 such passages. In this experiment, we used the cell line between 150 and 160 such passage. This carcinoma cell line, which was acquired from human tongues, was designated as the YD-10B cell line. The YD- 10B cell line exhibits frameshift p53 mutation. (Lee *et al.*, 2005)

Cell viability assay

Celecoxib (Pfizer, Groton, CN) was dissolved in DMSO (Sigma, Indianapolis, IN). Each treatment condition contained the same amount of DMSO. As DMSO modulates keratinocyte differentiation and proliferation, blank controls and solvent controls were also included to confirm that the DMSO had extended no such effects at the concentrations used in this study. The percentage of growth inhibition was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. Oral carcinoma YD-10B cells was seeded, cultured onto a 96-well plate (2.0×10^3 cells/well) for 24 h, treated with various concentrations (1-100 μM) of celecoxib, and incubated for an additional 3 days. Subsequently, 100 μl of MTT at a concentration of 5 mg/ml was added to each well, and cells were incubated for an additional 4-6 h. The supernatant was aspirated, and 100 μl of DMSO was added to the wells in order to dissolve any precipitate present. The absorbance was then measured at a wavelength of 570 nm using an ELX800 reader (Bio-Tek Instruments, Inc., Winooski, VT).

Cell cycle analysis

A total of 2.0×10^5 cells/well was plated onto 60 cm^2 dish, and incubated for 24 h. Celecoxib (37 μM) was added to the wells, and then incubated for an additional 3 days. Cells were washed, pelleted, fixed with cold 100% ethanol for at least 30 min, and incubated at 37°C with 100 μg/ml RNase A and 50 μg/ml propidium iodide in PBS for 30 min at room temperature. Samples were immediately analyzed by flow cytometry (Becton Dickinson, San Jose, CA). Cell cycle phase distribution was determined using Modfit software (Verity Software House, Topsham, ME).

Two-dimensional (2D) gel electrophoresis

Cells were washed well with PBS three times and homogenized in 0.5 ml sample buffer consisting of 40 mM Tris, 7 M urea (Merck, Darmstadt, Germany), 2 M thiourea (Sigma, St. Louis, MO), 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate, Sigma), 60 mM 1,4-dithioerythritol (Merck), 1 mM EDTA (Merck), 0.5% (v/v) IPG buffer with pI 4-7, 1 mM PMSF (phenylmethylsulfonyl fluoride, Sigma), and protease inhibitor cocktail tablet (Roche, Mannheim, Germany). The suspension was sonicated for approximately 30 s and

centrifuged at 12,000 × *g* for 1 h. The protein concentration of the supernatant was determined by the Coomassie blue method (Bradford *et al.*, 1976). Samples (100 μg) were applied on Immobiline Drystrip (pH 4-7, 18 cm, Amersham, Seoul, Korea). After the rehydration for 12 h, the proteins were focused at total 57,000 Vhrs. The second-dimensional separation was performed on 12% polyacrylamide gels. After protein fixation with 40% methanol containing 5% phosphoric acid for 12 h, the gels were stained with silver staining kit (Amersham) according to manufacturer's protocol. The molecular mass was determined by running standard protein markers at the right side of selected gels. The size markers (Gibco, Basel, Switzerland) covered the range 10-200 kDa. Isoelectric point (pI) values were used, as given by the supplier of the IPG strips. The gels were destained with water and scanned by GS-800 imaging densitomer (Bio-Rad). The images were analyzed by PDQuest software (Bio-Rad). Among ~ 800 proteins spots which were increased more than 10-fold after the treatment of celecoxib were decided to be identified.

Matrix-assisted laser adsorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis

Two protein spots, which were increased more than 10-fold after the treatment of celecoxib, were excised from the gels and destained with buffer containing 30 mM potassium ferrycyanide and 100 mM sodium thiosulfate and washed with distilled water. The protein spots were then dehydrated in a SpeedVac for 10 min. Gel pieces were rehydrated in 10 μl (10 ng/ml) of sequencing grade trypsin solution (Promega, Madison, WI) in 25 mM ammonium bicarbonate buffer, pH 7.8, at 37°C overnight. After removing the trypsin solution, the resulting peptides were extracted with 5 ml of 0.5% TFA containing 50% ACN by sonication. Peptide extraction was done at room temperature for 40 min. The tryptic digested peptide samples (1 ml) were mixed with equal volumes of CHCA (5 mg/ml in 50% v/v ACN/0.1% TFA) and loaded onto a target plate. MALDI-TOF mass spectra were obtained on a Voyager-DE STR mass spectrometer (Per-Spective Biosystems, Framingham, MA). Peptide matching and protein searches against the Swiss-Prot and NCBI databases were performed using the ProFound program (http://prowl.rockefeller.edu/).

Immunoblotting analysis

Proteins were extracted from the cells using a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40, and 1 × protease inhibitors (Roche Applied Science, Indianapolis, IN). Proteins (15 μg) were separated by electrophoresis through a 10% SDS polyacrylamide gel and then transferred onto a nitrocellulose membrane. The membrane was blocked in a blocking buffer (5% dry milk, 20 mM Tris-HCl (pH7.5), 100 mM NaCl, and 0.1% Tween 20) for 2 h. The membrane was incubated for 2 h at room temperature with 1:1,000 diluted primary antibody. After washing three times for 15 min with blocking buffer, membranes were probed with 1:2,000 diluted secondary antibodies for 1 h. The antibodies used in this study were purchased from the Santa Cruz Biotechnology (Delaware Avenue, CA) with the exception of the anti-human β-actin monoclonal antibody (Sigma). The membrane was washed three times for 15 min and developed with Western Blotting Luminol Reagents (Santa Cruz Biotechnology). The densities of bands were measured by Multi Gauge version 3.0 software (Fuji Photo Film Co., Ltd, Tokyo, Japan).

Results

Having established COX-2-overexpressing human OSCC YD-10B cells, we attempted to ascertain whether celecoxib, a COX-2-selective inhibitor, would inhibit the growth of oral cancer cells. When YD-10B cells were exposed to the continuous treatment of celecoxib at a concentration of 0-100 μM, in the presence of serum, the cell growth was inhibited in a dose-dependent manner and IC_{50} of celecoxib on cell growth of YD-10B was 37 μM (Figure 1). In the treatment of celecoxib (37 μM), the protein expression level of COX-2 was dramatically decreased to 72% and 48% after 48 h and 72 h, respectively (Figure 2).

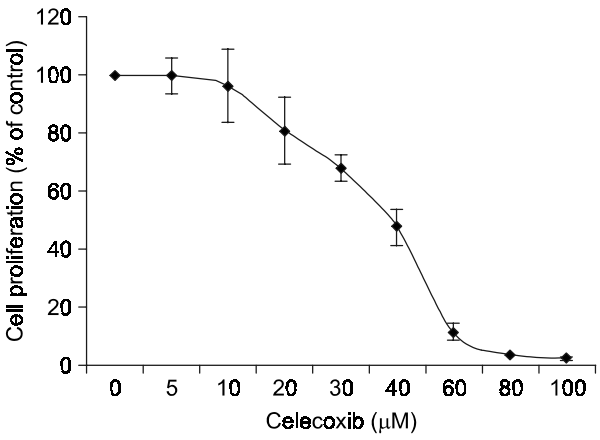

Figure 1. Celecoxib-inhibited cell growth of YD-10B cells. Cells were seeded onto 96-well plates at 2.5×10^3 cells/well, and were treated with different concentrations of celecoxib. The percentages of growth inhibition were determined by MTT assay after 72 h of treatment.

In order to determine whether the celecoxib-induced suppression of YD-10B cell growth was associated with cell cycle arrest, cell cycle distribution was evaluated by flow cytometric analysis. Interestingly, as shown in Table 1, celecoxib significantly induced a G_0/G_1 phase arrest, coupled with a concomitant decrease in the S phase. Next, the involvement of p21 and p27 in celecoxib-induced cell cycle arrest in YD-10B cells was evaluated by measuring the protein expression levels. As shown in Figure 3, celecoxib treatment induced protein levels of p27 (4-11 fold compared to control). Whereas the YD-10B cells express little or no p21 at baseline culture conditions, a minor increase in p21 levels was observed after treatment with celecoxib (data not shown).

To identify celecoxib-regulated proteins in YD-10B cells, proteomics techniques were performed. Pro-

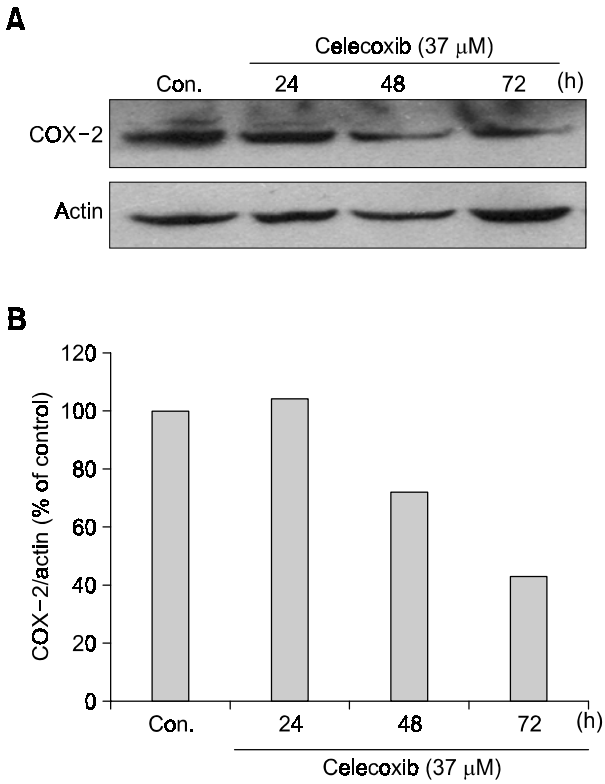

Figure 2. Celecoxib-inhibited protein expression of COX-2 in YD-10B cells. (A) Cells were incubated in the presence or absence of celecoxib (IC_{50} = 37 μM). At different times, YD-10B cells were lysed and using these proteins, Western blot analysis was performed as described in Materials and Methods. β-actin was used as an internal control. (B) The densities were normalized with that of β-actin and normalized values were represented to % of control.

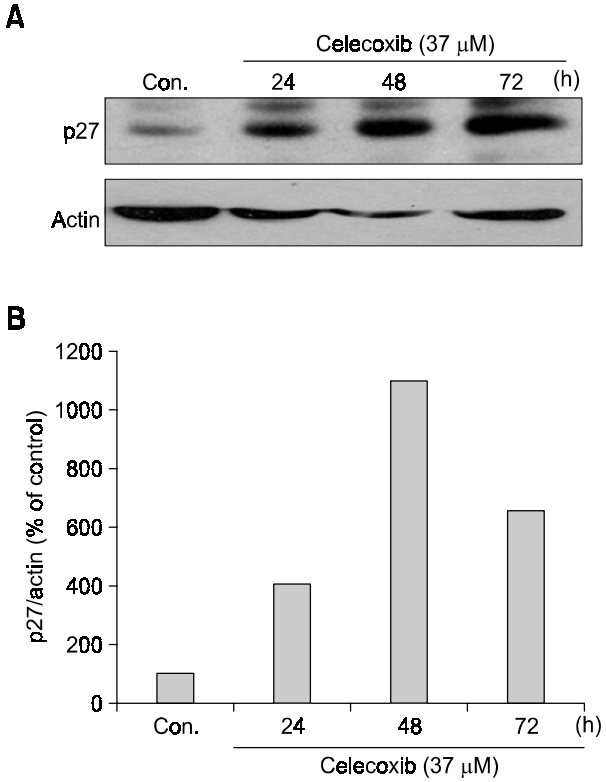

Figure 3. Celecoxib-induced protein expression of p27 in YD-10B cells. (A) Cells were incubated in the presence or absence of celecoxib (IC_{50} = 37 μM). At different times, YD-10B cells were lysed and using these proteins, Western blot analysis was performed as described in Materials and Methods. β-actin was used as an internal control. (B) The densities were normalized with that of β-actin and normalized values were represented to % of control.

Table 1. Effect of celecoxib on the cell cycle distribution in YD-10B cells. YD-10B cells were incubated in the presence or absence of celecoxib (IC_{50} = 37 μM) for 24, 48, and 72 h. Cells then were harvested, and the cell cycle profile was analyzed using flow cytometry as described in Materials and Methods. Results were presented as mean (%) ± SD (*n* = 3).

Phase of cell cycle	Vehicle	+ Celecoxib (37 μM)		
		24 h	48 h	72 h
G1	57.93 ± 4.91	73.74 ± 2.02	76.47 ± 1.88	82.07 ± 0.59
S	32.90 ± 2.23	18.73 ± 0.10	19.17 ± 0.43	13.01 ± 0.32
G2	10.18 ± 4.10	7.47 ± 2.202	4.37 ± 1.44	4.50 ± 1.63

teins from cells treated with 37 μM for 48 h were compared with those from control cells. Interestingly, of the spots investigated, two protein spots were increased more than 10-fold after the treatment of celecoxib (Figure 4). These spots were subjected to MALDI-TOF/MS and identified to heterogeneous nuclear ribonucleoprotein C (hnRNP C) isoform b (Table 2). The protein expression levels of hnRNP C were confirmed using specific antibody against hnRNP C. As shown in Figure 5, hnRNP C1/C2 expression was initially 3-fold induced by the treatment of celecoxib (37 μM) and it was 20-fold induced when cells were cultured with celecoxib for 72 h.

Discussion

It has been shown that inhibition of COX-2 may cause growth arrest and reduced tumor formation in some human cancer, but the mechanism is not fully known. Recent report suggested that COX-2 could be a major target molecule showing a moderate to high COX-2 expression in 35 out of the 45 OSCC specimens (77.8%) (Pannone *et al.*, 2004). Here, we used human oral squamous YD-10B carcinoma cells to study the growth inhibition and changes in cell cycle by the treatment of the selective COX-2 inhibitor celecoxib.

Celecoxib inhibited the growth of YD-10B cells in a concentration-dependent manner with the decreased protein expression of COX-2. This is consistent with the report showing that celecoxib inhibits proliferation *via* prostaglandin E2 pathway (Wu *et al.*,

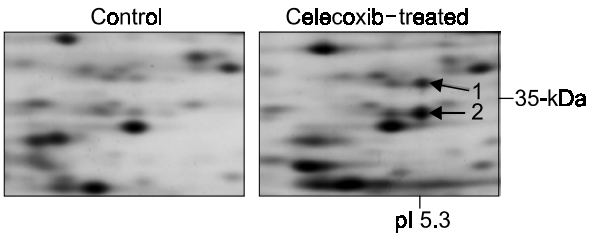

Figure 4. Partial image of 2D gel electrophoresis showing celecoxib-regulated proteins in YD-10B cells. Cells were incubated in the presence or absence of celecoxib (IC_{50} = 37 μM). After 48 h, 2D gel electrophoresis was performed using cell homogenates as described in Materials and Methods. Arrows (1 and 2) indicate proteins whose expressions were evaluated to be 10 and 20-fold higher in celecoxib-treated YD-10B cells compared to control, respectively. Numbers refer to protein identities in Table 2.

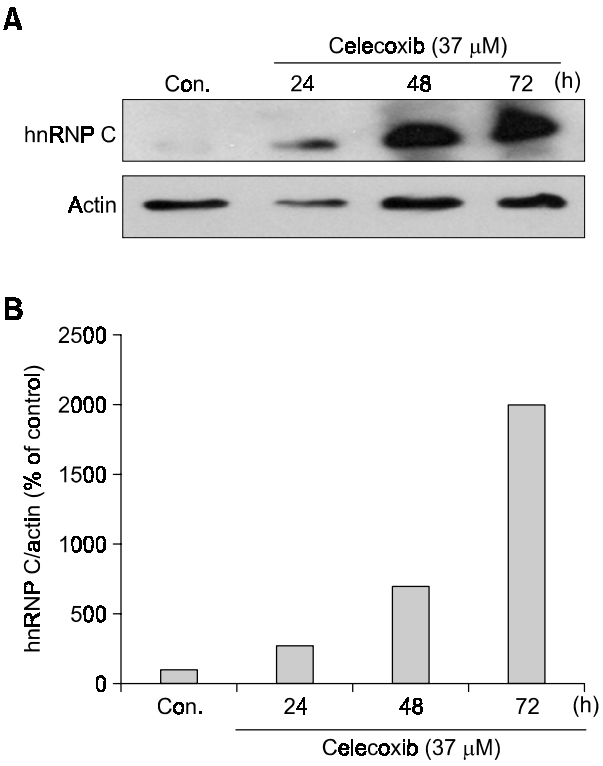

Figure 5. Celecoxib-induced protein expression of hnRNP C in YD-10B cells. (A) Cells were incubated in the presence or absence of celecoxib (IC_{50} = 37 μM). At different times, YD-10B cells were lysed and using these proteins, Western blot analysis was performed as described in Materials and Methods. β-actin was used as an internal control. (B) The densities were normalized with that of β-actin and normalized values were represented to % of control.

Table 2. Identification and expression levels of celecoxib-induced spot 1 and 2 in YD-10B cells.

Spot No.	Protein name	Species	Accession number	No. of matched peptides	Sequence coverage (%)	Theoretical Mr (kDa)/pI	Measured Mr (kDa)/pI	MOWSE score	Expression (vs. control)
1	heterogeneous nuclear ribonucleoprotein C isoform b (hnRNP C)	Human	4758544	5	20	31.967/5.1	35/5.3	153	10-fold increased
2	heterogeneous nuclear ribonucleoprotein C isoform b (hnRNP C)	Human	4758544	6	25	31.967/5.1	35/5.3	2942	20-fold increased

2003). In addition, the results of flow cytometric analysis indicated that celecoxib induced G_1-S arrest, with no significant effect on G_2-M transition. Makitie *et al*. reported that inhibition of COX-2 by celecoxib markedly induces the expression of critical cell cycle-regulating proteins including p27 in OSCC (Makitie *et al.*, 2003). In YD-10B cells, celecoxib dramatically induced the protein expression of p27. These results suggested that the celecoxib could induce the expression of p27, which consequently mediate the cell cycle arrest in G_1-S. In addition, considering that YD-10B cells exhibit frameshift p53 mutation, the inhibition of cell growth and induction of p27 by the treatment of a selective inhibitor of COX-2 celecoxib could be done in a p53-independent manner. However, the further study should be carried out to elucidate the relationship between the mutation of p53 and the inhibition of cell growth and induction of p27 by the treatment of celecoxib in YD-10B cells.

What proteins are involved in the inhibition of YD-10B cell growth by celecoxib? Using proteomics technologies, the up-regulation of hnRNP C protein was observed in this study. hnRNP C has been implicated in a variety of processes including splicing, polyadenylation, and RNA turnover, and its intrinsic ability to wrap or fold relatively long lengths of pre-mRNA in a peripherally accessible manner is consistent with an RNA chaperonin-like activity (Herschlag *et al.*, 1994; Herschlag *et al.*, 1995). Furthermore, the protein is preferentially phosphorylated during mitosis, suggesting that aspects of hnRNP C function are regulated during the cell cycle (Mayrand *et al.*, 1993; Pinol Roma *et al.*, 1993). Recent report showed that hnRNP C is not required for cell viability, but this protein may influence the rate and/or fidelity of one or more steps in mRNA biogenesis (Williamson *et al.*, 2000). Interestingly, it has been suggested that hnRNP C is one of factors that bind to a U-rich sequence in the 5' untranslated region (5'UTR) of p27 mRNA, which is necessary for the translation of p27 mRNA, and consequently regulate the translation of p27 mRNA, suggesting that this interaction may be a target of antimitogenic signals (Millard *et al.*, 2000). These data were also consistent with our data showing the induction of both p27 and hnRNP C in cell cycle-arrested YD-10B by the treatment of celecoxib.

In summary, celecoxib has a potential to induce the protein expression of hnRNP C and its increase subsequently induce the translation of p27 mRNA, which trigger the inhibition of cell growth via p27-regulated cell cycle arrest in YD-10B cells. Additionally, selective inhibition of COX-2 is a possible target for the therapeutic strategies to treat OSCC and established YD-10B cells could be useful to study the pathological mechanism of OSCC.

Acknowledgement

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