Cyclooxygenase-2 expression in oral precancerous and cancerous conditions and its inhibition by caffeic acid phenyl ester-enriched propolis in human oral epidermal carcinoma KB cells

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Abstract: Oral cancer accounts for 3-5% of all cancers worldwide. The present study was undertaken to investigate the correlation between overexpression of cyclooxygenase-2 (COX-2) and various grades of oral cancer, and to ascertain the inhibitory effect of propolis in the human oral carcinoma cell line. For ex vivo studies, 45 patients with oral submucous fibrosis (OSF; n=15), oral leukoplakia (OLP; n=18) and oral squamous cell carcinoma (OSCC; n=18) were recruited, and a biopsy was done to determine COX-2 protein expression by Western blotting and immunohistochemistry (IHC). For the in vitro study, COX-2 levels were measured in human oral epidermal carcinoma cell line by immune blotting and IHC. The results of ex vivo studies by Western blotting revealed that COX-2 protein levels were highly upregulated in OSCC tissue, followed by OLP and OSF. The levels of COX-2 expression also showed a positive correlation with the grade (severity) of each oral precancerous and cancerous condition. Immunohistochemistry analysis revealed the presence of intense COX-2 staining in the cells of OSCC tissue, equivalent to the OLP and OSF specimens. In the in vitro study of oral carcinoma KB cells, Western blotting and IHC analysis showed that caffeic acid phenyl ester (CAPE)-rich propolis and celecoxib, a standard COX-2 inhibitor, markedly downregulated COX-2 expression. These results suggest that propolis exhibits a chemopreventive potential by lowering COX-2 expression in the oral carcinoma KB cell line. Hence, propolis might be used as an adjuvant therapy for treating oral cancer with standard chemotherapy drugs.

Key words: cyclooxygenase-2; oral submucous fibrosis; oral leukoplakia; oral squamous cell carcinoma; propolis

INTRODUCTION

Oral cancer is the most common type of cancer significantly linked to morbidity and mortality. The World Health Organization (WHO) has reported that the rate of oral cancer incidence will continue to escalate globally in the future [1]. In Taiwan, oral cancer is one of the top ten leading causes of cancer death [2]. Various oral mucosal lesions such as OSF and OLP are considered as a precancerous condition, which originates in epithelial surfaces chronically exposed to carcinogens such as tobacco and alcohol. This oral precancerous condition can be developed to a cancerous condition such as OSCC at the site of the lesion(s) as well as at other sites in the upper digestive tract, and its transformation rate is high in tobacco-using patients [3,4].

Cyclooxygenase (COX) is a key enzyme required for the conversion of arachidonic acid to prostaglandins (PGs) and other eicosanoids. At least two COX isofoms, the constitutive COX-1 and the inducible COX-2, have been identified. COX-1 is expressed in most mammalian tissue and is responsible for maintaining normal cellular physiological functions. COX-2 has been reported to play important roles in
certain inflammatory diseases, tumorigenesis, metastatic properties of cancer cells, and tumor-related angiogenesis [5,6]. Some experiments also suggested that hyperexpression of COX-2 is a core factor in various types of cancer, including colon, stomach, breast, lung, esophagus, pancreas, bladder, prostate and oral squamous cell carcinoma [7-9]. Moreover, it can serve as a diagnostic and prognostic index, which implicates the necessity for advance research related to COX-2 expression in different grades of oral precancerous (OSF, OLP) and cancerous conditions (OSCC).

Propolis is a resinous honeybee product and widely used in traditional or folk medicine for various ailments, owing to its diverse photo components, in particular polyphenols, terpenoids (artepillin C) and steroids. Propolis is widely used in food and nutraceutical industries (supplements) due to its various biological properties, such as immunomodulatory, antitumor, antimicrobial, hepatoprotective, anti-inflammatory and antioxidant [10-12]. The active components of propolis are caffeic acid phenyl ester (CAPE) and some flavonoids (pinocembrin, galangin, quercetin, chrysin, naringenin and pinobanksin). CAPE has been reported for its anticancer, immunomodulatory, antioxidant properties. It inhibits the development of azoxymethane-induced aberrant crypts in the colon of rats. The anti-inflammatory properties of CAPE have been assigned to the suppression of PG synthesis via downregulating LOX and COX enzymes [13], CAPE is a well-known and well-documented inhibitor of the transcription factor nuclear factor kappa B (NF-κB), as well as an inducer of apoptosis [14]. The antitumor activity of CAPE is favored as it inhibits angiogenesis, tumor metastasis, and invasiveness [15].

In the past, a few studies concentrated on COX-2 expression in different oral cancer conditions, but the different grade of oral precancerous and cancerous conditions is yet to be explored. Hence, the levels of COX-2 and the severity (grade) of precancerous (OSF/leukoplakia) and cancerous conditions (OSCC) were determined, which might be helpful in treating various oral cancerous conditions. Moreover, suppressing the levels of COX-2 in a precancerous patient may have a greater impact on lowering the incidence of oral cancer by reducing the transformation rate. Therefore, we selected a propolis, which is a well-known anti-inflammatory/COX-2 inhibitor, to evaluate the influence of propolis on COX-2 levels in oral carcinoma conditions (KB cells). The present study was designed to elucidate the effect of propolis (an anti-inflammatory agent) on the regulation of 12-o-tetradecanoylphorbol-13-acetate (TPA)-induced inflammatory responses in KB cells, with a focus on COX-2 levels.

MATERIALS AND METHODS

Chemicals

Brazilian Brown propolis was provided by Golden Chia-Fong Biotech Co., Taiwan. Sodium dodecyl sulfate (SDS), 12-o-tetradecanoylphorbol-13-acetate (TPA), trypsin, triton X and Tween 20 were purchased from Sigma (USA); fetal bovine serum (FBS), Folin-Ciocalteu reagent, Trypsin-EDTA and antibodies for blotting were purchased from Gibco (Germany). Secondary antibody-horseradish peroxidase (HRP)-IgG was obtained from Santa Cruz Biotechnology (CA, USA). All other chemicals were of analytical grade.

Ethanolic propolis extract (EPE)

Propolis (10 g) was shredded into small pieces and extracted with 40 ml of 95% ethanol at room temperature, and the resulting extract was filtered. Residue from the filtration was extracted again by repeating the same procedure twice. The filtrates were combined and then freeze-dried by a vacuum pump to remove excessive solvent, filled with nitrogen and then stored at -20°C until used.

Ex vivo study

Subjects

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human patients were approved by the Institutional Review Board of the Chung Shan Medical University Hospital (CCH-94-02-01). A questionnaire was given at the beginning of the study providing information on any concurrent diseases and the patient's medical history (including cancer location and tumor staging). OSF and OLP
are precancerous conditions, but OSCC is a cancerous condition.

Experimental design

Forty-five patients with OSF (n=15; 3 mild, 6 moderate, 6 severe stage), OLP (n=15; 5 mild, 5 moderate, 5 severe stage doesn’t add up…) and OSCC (n=15; 5 mild, 5 moderate, 5 severe stage), patients of the Department of Oral and Maxillofacial Surgery (Changhua Christian Hospital and Chung Shan Medical University Hospital, Taiwan) were enrolled. Patients with concurrent malignancy, chronic diseases (diabetes mellitus, renal disease, hepatitis) and those who refused to cooperate in the study were excluded. The study protocol was elaborated in detail, and then a written informed consent was obtained. Biopsy specimens were obtained from histologically normal and affected areas from 45 patients (with or without areca quid-chewing habits) under mild local anesthesia. The clinical diagnosis was confirmed by histopathological examination (using hematoxylin and eosin stain) of the biopsy specimens of the respective grades (mild – I, moderate – II and severe – III) based on the criteria described in the report from the International Union Against Cancer (UICC) in the year 2000 [16,17].

In vitro study

Human oral epidermal carcinoma cells (KB cells) were obtained from the American Type Culture Collection (ATCC). KB cells were cultured in Dulbecco’s modified Eagle medium (DMEM) (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin. Cells were maintained at 37°C in a humidified incubator containing 5% CO₂.

Western blot analysis

COX-2 protein expression was measured by Western blotting assay. KB cells were plated in 10-cm plates at 2.5×10⁶ cells, allowed to adhere to the plate overnight, and the medium was replaced. Cells underwent 24-h treatment with different concentrations of propolis extracts (10, 15 and 20 μg/mL), 50 ng/mL of TPA, and 100 ng/mL of celecoxib (NSAID). Similarly, biopsy tissues were minced using sterile techniques and washed twice with phosphate buffer saline (PBS). Cell lysates (20 μg proteins) were resolved on 10% SDS-polyacrylamide gels and blotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 h at room temperature in a 5% non-fat dry milk dissolved in TBS buffer (25 mM TRIS, pH 7.4, 150 mM NaCl, 0.3% Tween-20). The membrane was incubated with the anti-COX-2 specific monoclonal antibody (1:1000; Upstate Biotechnology Inc, Lake Placid, NY, USA) overnight at 4°C. Incubation with the HRP-conjugated secondary antibody (1:5000; Santa Cruz, CA, USA) was carried out for 1 h at room temperature. The reacted bands were revealed by enhanced chemiluminescence (ECL) using a commercial kit (Amersham Pharmacia Biotech, NJ, USA) according to manufacturer’s instructions. β-actin was used as an internal control. The band intensity was determined using a densitometer.

Histopathology and immunohistochemistry

The biopsy specimens were taken from the lesion (tumor) of different grades of OSF, OLP and OSCC patients under local anesthesia, and fixed in 10% formalin solution. KB cells were also fixed in formalin solution. Paraffin-embedded formalin-fixed tissues were processed, and 4-μm-thick sections (from the edge of the tumor) were prepared by the routine method and stained with hematoxylin and eosin (H&E). For IHC, the stained sections were treated with the primary polyclonal anti-COX-2 rabbit anti-human antibody (1:500; Santa Cruz Biotechnology, CA, USA). Sections were incubated overnight at 4°C with primary antibody in a humid chamber. The following day, sections were stained using labeled HRP secondary antibody solution (1:1000; Santa Cruz Biotechnology, CA, USA). Bound peroxidase was revealed using 0-5% 3-diaminobenzidine tetrahydrochloride (DAB) in TBS. Sections were dehydrated, mounted and counterstained with hematoxylin. Negative controls were included with the omission of the primary antibody. COX-2 (IHC) positive cells (cytoplasmatic stained epithelial cells) were counted (1000 neoplastic cells in 5 different fields) using a light microscope (Nikon Microscope ECLIPSE E400, model 115, Tokyo, Japan) at ×400 magnification. The sections were blindly counted by two independent pathologists and the mean value of each section were noted.
Statistical analysis

The experiments were performed in triplicate. The quantitative data are expressed as mean values ± standard deviation (SD). The difference in the results of this study was assessed using one-way ANOVA for comparison between each group, and the Student t-test for comparison within the group, using SPSS software (version 17.0; SPSS Inc.). A p-value of less than 0.05 was considered statistically significant.

RESULTS

Ex vivo study

The COX-2 protein expression by Western blot is shown in Fig. 1A. Mild OSF tissue did not exhibit COX-2 protein expression (0/3), but two patients with mild OSF and OLP displayed a prominent COX-2 protein expression (2/5 and 2/6, respectively). Similarly, three patients with severe OSF, moderate and severe OLP showed strong COX-2 protein expression (3/6, 3/5 and 3/5, respectively). In the case of patients with mild OSCC, 4 out of 5 displayed COX-2 protein expression, whereas all five moderate and severe OSCC patients displayed strong COX-2 protein expression (5/5).

The intensity of COX-2 protein expression was significantly upregulated in a severe grade of all cancer conditions (OSF, OLP and OSCC). Intercomparison between each condition based on different grades exposed a substantial difference between the grades. On comparing the moderate and severe OSF grades, the COX-2 protein expression showed a 2.67-fold increase. The COX-2 protein expression was upregulated significantly (p<0.05) by 5.05-fold in severe OLP tissue when compared to mild OLP tissue. Similarly, a 1.54-fold increase in the levels of COX-2 expression was noted in severe OLP tissue over moderate OLP tissue. When comparing severe and mild OSCC, the COX-2 expression was elevated to 1.54-fold. However, comparison with severe and moderate OSCC showed only a 1.33-fold increase in the COX-2 levels by Western blot. The order of COX-2 protein expression in the different conditions of different grade is OSCC>OLP>OSF.

Histopathological examination by H&E staining of normal oral epithelium revealed the cells organized as shown in Fig. 2A. Mild OSF tissue showed increased degeneration of collagen fibers (F), with juxta-epithelial hyalinization (H). C – Leukoplakia tissue showing hyperorthokeratosis (I) with excessive epithelial cell layer acanthosis (A) and hyalinization (H). D – OSCC tissue exhibiting hyperplastic changes (neutrophil infiltration) accompanied with scaly-shaped squamous cells with condensed nucleus and intraepithelial keratinization. Magnification x400.
into a stratified epithelium, with large polyhedral, eosinophilic cytoplasm and large vesicular nuclei (Fig. 2A). OSF tissue showed increased degeneration of collagen fiber (F) with juxta-epithelial hyalinization (H) (Fig. 2B). Leukoplakia tissue showed hyperorthokeratosis (O) with an excessive epithelial cell layer acanthosis (A) and hyalinization (H) (Fig. 2C). The OSCC tissue exhibited hyperplastic changes (neutrophil infiltration) accompanied with scaly-shaped squamous cells with a condensed nucleus and intraepithelial keratinization (Fig. 2D).

The immunoreactive product observed by IHC indicated the presence of COX-2 in the cytoplasm and was detected at relatively high levels in stromal cells, including macrophages and some neutrophils, fibroblasts and vascular endothelial cells (Fig. 3). The intensity of COX-2 expression was stronger at the edge of the lesion or tumor (perinuclear), as shown by an arrow mark in severe OSF (3C), OLP (3F) and OSCC (3I). The mean value of COX-2 positive cells in severe OSCC was 61%, with OSF, OLP and control tissue showing 16, 21 and 2%, respectively.

**Cell line in-vitro study**

Western blot analysis was used to check COX-2 protein expression in KB cells treated with TPA and propolis (Fig. 1B). TPA is a well-known inflammatory activator triggering the protein kinase C (PKC) pathway, and in our study TPA (50 ng/mL) influenced a significant elevation (5.05-fold) in the levels of COX-2 protein expression in KB cells. Whereas, incubation with different concentrations of propolis (10, 15 and 20 mg/mL) and celecoxib (100 ng/mL) markedly downregulated (31, 60.4, 76.8 and 79.3%, respectively) the levels of COX-2 protein expression when compared with the TPA-induced group. However, 20 mg/mL of propolis exhibited the best COX-2-suppressive activity. No substantial difference was noted between the propolis 20 mg, celecoxib and control groups.

Based on the results of immunoblot, the 20 mg/mL of propolis was used for further examination by IHC.

IHC was employed to verify the expression of COX-2 at the cellular level of KB cells treated with TPA and propolis (Fig. 4). Immunostained KB cells induced with TPA exhibit hyperexpression of COX-2 protein, which is indicated by an arrow (B); the addition of propolis (20 mg/mL) (C) and celecoxib (100 ng/mL) (D) significantly lowered COX-2 expression (magnification x400).
DISCUSSION

The present study was designed to demonstrate the association between overexpression of COX-2 and various grades of oral precancerous (OSF, OLP) and cancerous conditions (OSCC) as well as to check the inhibitory potential of propolis in human oral carcinoma cell line KB. OSF is a chronic, debilitating disease and a premalignant condition of the oral cavity that is characterized by a juxta-epithelial inflammatory reaction followed by a fibroblastic change in the lamina propria and associated epithelial atrophy [18]. OLP is strictly a clinical term used to characterize a white lesion that carries an increased risk for malignant potential [19]. OSCC is the most common malignant neoplasm of the oral cavity and is thought to originate from a progressive dysplasia of the oral mucous membrane. Both OLP and OSF are clinically distinct precancerous lesions that precede the development of oral cancer, especially OSCC [4].

Results of research have documented the link between cancer and inflammation, as chronic inflammation might constitute the risk factor for initial cell transformation and cancer progression [20,21]. During various oral precancerous and cancerous conditions, inflammation plays a substantial role in the progression based on severity and grade [21], and thus the protein expression of COX-2 was evaluated. COX-2 protein expression in oral cancer and precancerous conditions by Western blot increased with the severity of OSF, OLP and OSCC accordingly. Also, OSCC (malignant) showed greater COX-2 protein expression. In several malignant conditions, a strong correlation was observed between COX-2 and p53 expression, which are known to be involved in the early stages of carcinogenesis [22]. As the severity or grade of the cancerous condition increases with the alteration in the levels of p53, a tumor suppressor gene, as seen in increased COX-2 expression, this might be the reason for increased COX-2 expression in the malignant condition (OSCC) when compared with the premalignant condition. Hence, a strong association was maintained between severities of cancer and COX-2 expression levels. COX-2 expression is highly upregulated in the advanced stage compared with an early stage of squamous dysplasia and cell squamous carcinoma of the esophagus [23]. Moreover, COX-2 protein and mRNA expression were significantly enhanced in cancerous conditions as compared to normal cells [24,25]. In addition to COX-2, we also evaluated the protein expression of other pro-inflammatory cytokines, IL-1β and IL-8; however, no significant changes were noted (data not shown).

The histopathological results after H&E staining of severe OSF, OLP and OSCC tissue showed marked cellular changes. OSF and OLP showed fewer undifferentiated epithelial cells with increased degeneration of collagen fiber and hyperorthokeratosis, whereas OSCC tissue showed more undifferentiated malignant epithelial cells with hyperplastic changes and intraepithelial keratinization. The above histological changes indicated that COX-2 could be involved in these cellular changes owing to increased invasive and metastasis processes by disrupting the cytoskeleton structure of epithelial cells. Thus, IHC was used to check the expression of COX-2 in cellular levels of OSF, OLP and OSCC biopsy tissue.

The COX-2 specific antibody staining of various oral tissue (severe OSF, OLP and OSCC) biopsies was found to be positive for COX-2 expression in both premalignant and malignant epithelial cells. The COX-2 stained cell intensity was found to vary according to the severity of the disease. In OSF and OLP, the premalignant epithelial cells are poorly undifferentiated (dysplasia) in the cytoplasm and hence the COX-2 stain intensity is lower. However, OSCC malignant epithelial cells are highly undifferentiated (hyperplasia), displaying considerable COX-2 staining. It has been demonstrated the normal epithelial cells show very low levels of COX-2 expression (IHC) as compared to OSF, OLP or OSCC [26,27]. COX-2-containing epithelial cells were involved in invasion and metastasis processes and thus enhanced the severity or stage of cancer. Our results are in concordance with Shibata et al. [17] and Itoh et al. [28] who indicated that COX-2 expression was predominant in the cytoplasm of grade 3 neoplastic cells. The hyperexpression of COX-2 in OSF, OLP and OSCC is related to increasing metastasis, tumor size and histological changes [29,30]. Similarly, the overexpression of COX-2 in the present study was correlated with the increased histological (IHC) changes during various stages of premalignant and malignant tissue.

The ex vivo study showed that a very strong association exists between the intensity of COX-2 ex-
expression and severity or grade of the oral cancerous condition, i.e. between the early stages (I & II) and late stage (III), but the exact mechanism is still unknown. Therefore, we designed an in vitro model (KB cells) to determine whether a COX-2-selective inhibitor could inhibit the development or proliferation of oral cancer cells by downregulating COX-2 expression. Propolis was used to investigate chemopreventive potential because of its various phytoconstituents. Miguel and Antunes [31] indicated that propolis works better in a holistic way (traditional medicine) rather than specific active components and we therefore used crude propolis. KB cells treated with TPA are a well-known experimental model of tumor promotion (in which the inflammatory cascade is triggered), would be an excellent tool (cell line model) to investigate the chemoprotective effect of propolis, which would be a new rationale for anticancer therapy [32]. A few studies also proved that NSAIDs can significantly reduce the risk of many other tumor types, such as esophageal, gastric, breast, lung and oral cancers in both ex vivo and in vitro models [7,33,34]. Therefore, chemotheraphy agents such as NSAIDs that block COX-2 activity could aid in managing oral carcinoma. Celecoxib, a well-known COX-2 inhibitor, was used in the present study as a standard for comparison.

A preliminary 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed the cytotoxicity and antiproliferative effect of propolis (data not shown). TPA is a well-known inflammatory activator (PKC activator) and triggers angiogenesis, invasion and thus, worsening the cancer condition [35]. During our in vitro study, KB cells treated with TPA exhibited a significant upregulation at the levels of COX-2 protein expression. Similarly, COX-2 expression was significantly upregulated in TPA-treated mammary epithelial cells (MCF-7), pancreas cells (BxPC-3 and Panc-1) and lung cancer (NCI-H292) cells [36,37]. COX-2 protein expression in KB cells treated with different concentrations of propolis displayed a positive impact by substantially downregulating the COX-2 level in a dose-dependent fashion, attributed to its phytoconstituents, especially polyphenols, terpenoids and steroids. CAPE in propolis might effectively suppress lipoxygenase (LOX) and cyclooxygenase (COX) enzymes via the inhibition of prostaglandin endoperoxide synthase during inflammation. Galangin, a flavonoid in propolis, is shown to inhibit COX-2 expression and its subsequent inflammatory cascade [38]. Chrysin (a component of propolis) administration significantly reduced the number and size of preneoplastic nodules and the expression of COX-2, NF-κB and p65 in a rat model [39]. Celecoxib, a standard antiinflammatory drug, also suppresses COX-2 protein expression [40].

The immunoreactivity of COX-2 expression using primary antibodies on KB cells treated with TPA revealed the presence of increased positively stained cells, especially at the edge of the slide, with a disrupted nuclear membrane with scaly-shaped epithelial cells, indicating its higher undifferentiated pattern due to the activation of inflammatory cytokines. Immunostained KB cells induced by TPA and propolis exhibited hypoexpression of COX-2 protein with less intensely stained epithelial cells and few cellular modifications. CAPE is a well-known NF-κB inhibitor and actively suppresses the proliferation of several human cancer cell lines, including breast, prostate, lung, cervical, and oral cancer cells [41,42]. The anti-inflammatory activity of CAPE is derived from the downregulation of COX-2 expression through activation of the expression of the tissue inhibitor of metalloproteinase-2 (TIMP-2) and inhibition of focal adhesion kinase (FAK) phosphorylation. Propolis is more likely to induce COX-2-independent apoptosis in KB cell lines, which is probably the reason for its chemopreventive potential. Several reports showed that propolis can act as a chemotherapeutic agent by stimulating multicellular immunity, preventing metastasis, speeding-up apoptosis of cancer cells, a mitosis-suppressing, anti-angiogenesis, immunomodulatory and antioxidant effects [43]. The present results confirmed that the chemoprotective effect of propolis was mainly due to significant downregulation of COX-2 expression. We plan to evaluate the effect of propolis on matrix metalloproteinases (MMP)-2 and 9 as well as its correlation with tissue inhibitors of metalloproteinase (TIMP), to validate the chemoprotective effect of propolis.

CONCLUSION

We observed a strong correlation between COX-2 protein expression and the severity of oral precancerous and cancerous conditions. COX-2, besides being an
inflammatory biomarker, can be used as a good indicator (marker) for oral cancer and as an indirect marker of the grade of cancer, but further evaluation is needed to strengthen our outcome. Additionally, the COX-2 inhibitory activity of propolis was also confirmed in TPA-induced human oral carcinoma KB cells. Hence, we recommend the usage of propolis (holistic effect) as an adjuvant therapy for treating oral carcinoma combined with other standard chemopreventive agents to reduce the incidence of oral cancer as well as to lower the adverse event caused by chemotherapeutic agents. Further studies are warranted to reveal the precise mechanism of how overexpression of COX-2 promotes oral cancer and how its inhibition might slow down or prevent the progression of oral cancer after propolis treatment in large-scale preclinical and clinical studies.

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Authors’ contribution: HFC, YCS, KV and CKW conceptualized and designed the study protocol. HFC, YCH, CSY, and HIC helped to carry out the experiments. CKW and KV collected data and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest disclosure: The authors declare that there is no conflict of interest.

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