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ORIGINAL PAPER

Circulating cycloxygenase-2 in patients with tobacco-related intraoral squamous cell carcinoma and evaluation of its peptide inhibitors as potential antitumor agent

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Abstract
Purpose The aim of this study was to quantitate circulating COX-2 levels in patients with tobacco-related intraoral cancer and to evaluate antitumor activities of COX-2 peptide inhibitors in vitro on KB cell lines.
Patients and methods We used a novel biosensor-based surface plasmon resonance (SPR) technique for estimation of circulating COX-2 levels in 76 patients with oral cancer and 43 normal individuals. Antitumor activities of five COX-2 inhibitory peptides were evaluated using propidium iodide labeling and flow cytometry, alamar blue, MTS, and annexin-V binding assays.
Results Patients with oral cancer showed threefold increase in serum COX-2 level when compared to normal controls ($P < 0.0001$). Further, late-stage tumors and lymph node metastasis were associated with significant increase in serum COX-2 levels. Patients with higher circulating COX-2 also showed higher immunoreactivity to anti-COX-2 antibody in the lesions. The peptides significantly reduced viability and inhibited growth/proliferation, induced cytotoxicity and apoptosis in tumor cells. However, no such effect was observed either on normal human leukocytes or on MCF-7 cell line that did not over express COX-2.
Conclusion Our results indicate that SPR may be a useful proteomic technique for quantitative assessment of COX-2 and to identify patients with high-risk oral premalignant or occult cancer, as well as in monitoring response to novel COX-2 targeting strategies. Furthermore, COX-2 peptide inhibitors appear to be a new class of potent anticancer agent for human oral carcinoma.

Keywords COX-2 · Quantitative assessment · Spr · Peptide inhibitors · Anti cancer · Oral cancer

Introduction

Oral squamous cell carcinoma (OSCC), which falls into the head and neck cancer category, is a major health problem in the world, particularly in the developing countries. Out of 400,000 annually reported new cases world over, 100,000 OSCC cases occur in India alone (GLOBOCAN 2002). These tumors have male predominance with a male: female ratio of over 2:1. Although survival rate of patients depends on tobacco and alcohol consumption, age, sex, and ethnic background; the mortality rates have been increasing in recent years especially among young males in the United States (Jemal et al. 2007; Mackenzie et al. 2000). Despite advances in treatment modalities, the 5-year survival rate for oral cancer still remains 50–55 percent since past several decades (Silverman 2001). Cancer of the oral cavity presents certain special problems. These tumors are asymptomatic in the early stage hence ignored for longer time until they spread to regional lymph nodes. However,

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distant metastases are uncommon, and most patients present with T3/T4 lesions with multiple nodes that are beyond reach of surgery and radiotherapy (Khanna et al. 1983). In addition, higher rate of recurrence after surgery or radiotherapy possibly due to poor monitoring of the disease in absence of a reliable biomarker increases morbidity and mortality. Therefore, identification and defining suitable biomarker that could provide prognostic assessment of the disease at the time of presentation would help in designing strategies for treatment of oral cancer and monitoring the disease activity during follow-up period.

Oral cancer development is a multi-step process involving multifocal carcinogenesis and intraepithelial clonal spread. Aberrant expression of COX-2, the key enzyme in the conversion of arachidonic acid to prostaglandins, has been suggested to be involved in critical steps of onset and progression of oral cancer (El-Hakim and Langdon 1991). Two COX isoforms have been characterized. COX-1 is ubiquitous and constitutively expressed throughout the gastrointestinal system, kidneys, vascular smooth muscle, and platelets (Morita 2002). COX-2 is undetectable in most tissues, but its expression can be rapidly induced by a variety of stimuli like growth factors, inflammatory cytokines, tumor promoters, ionizing radiation, and carcinogens. Immuno-histochemical, reverse transcription (RT)-PCR and in situ hybridization studies have shown very low or no expression of COX-2 in normal human and hamster oral tissues, while it was up-regulated in oral precancerous lesions and overexpressed in dysplasia and OSCC (Li et al. 2005; Shibata et al. 2005; Sawhney et al. 2007; Pandey et al. 2008) besides other malignancies (Howe et al. 2001; Marnett and DuBois 2002; Rigas and Kashfi 2005; Wu 2005; Zhao et al. 2008). The increased COX-2 expression was reported to be associated with malignant transformation and advancing clinical stage and prognosis (Grau et al. 2004; Saba et al. 2009) in patients with head and neck cancer. COX-2 expression may be up-regulated by tobacco and areca nut constituents (Jeng et al. 2003) and also during 7,12-dimethylbenz[α]anthracene (DMBA)-induced oral carcinogenesis (Li et al. 2005). Although exact role of COX-2 in oral carcinogenesis is not well understood, it is able to cause multiple effects through enhanced production of several prostaglandins that regulate diverse physiological processes including Wnt and β-catenin signaling (Castellone et al. 2005; Lim et al. 2008). Over expression of COX-2 has been shown to promote tumorigenesis by enhanced tumor cell proliferation and differentiation, inhibition of apoptosis, increased cell adhesion to extracellular matrix, increased metastasis, neoangiogenesis, and host immune response to tumor cells (Wu 2005). Hence, COX-2 expression has been used as a potential tumor marker in most cancers.

Most of the patients are generally debilitated at the time of treatment, having low performance status and poor immunologic response (Khanna et al. 1983). The administration of chemotherapy leads to further decline in the general condition of these patients, even though there may be regression of tumor (Bertino et al. 1973). Recent evidence suggests that while over expression of COX-2 enhances prostaglandin E2 (PGE2) production and promotes tumor growth, depletion of COX-2 attenuates its growth (Han et al. 2004; Wu et al. 2004). Inhibitors of the COX-2 enzyme are associated with a reduction of up to 50% in the morbidity and mortality of colorectal cancer (Marnett and DuBois 2002). The use of non-steroidal anti-inflammatory drugs (NSAIDs; i.e. ibuprofen, indomethacin, and aspirin), as non-specific inhibitors of COX-2, has shown beneficial effects in prevention of oral and head and neck cancer (Perkins and Shklar 1982; Cornwall et al. 1983; Ondrey et al. 1996). Similarly, specific inhibitors of COX-2 such as celecoxib reduced the incidence of DMBA-induced OSCC with concomitant reduction in leukotriene B4 and PGE2 levels in hamster and rat models (Li et al. 2005; Shiotani et al. 2001). We, therefore, postulated that COX-2 peptide inhibitors aimed to selective disruption of COX-2 activity might be a potent therapeutic strategy for tumors associated with overexpression of COX-2 including OSCC.

In this study, we for the first time successfully performed quantitative assessment of circulating COX-2 levels in patients with oral cancer by a novel biosensor-based SPR technique and evaluated antitumor activities of COX-2 peptide inhibitors KB cell line in vitro.

Materials and methods

Patients and controls

This study was undertaken on 76 patients with biopsy proven primary OSCC of various sites (buccal mucosa, tongue, alveolus, floor of mouth, etc.) attending the Out Patient Department of Otorhinolaryngology, All India Institute of Medical Sciences, New Delhi. Tumor, node, and metastasis (TNM) criteria laid down by the American Joint Committee on Cancer (1988) was used for clinical staging of the tumor. All patients had history of tobacco use at least till 6 months before the onset of the disease, and none of them had received any anti-inflammatory or anticancer treatment prior to the study. The control group included 43 age-, sex-, and ethnicity-matched normal healthy subjects. Five milliliter of venous blood was aseptically drawn from each individual; serum was separated, aliquoted, and stored at −20°C until further use.

The study protocol was approved by 'Ethics Committee' of the All India Institute of Medical Sciences, and informed consent was obtained from all participants.

Estimation of serum COX-2 by surface plasmon resonance (SPR) technique

The serum levels of COX-2 were measured by the biosensor-based SPR technique using an automatic apparatus BIAcore 2000 (Pharmacia, Sweden) and BIA evaluation software 3.0. The technology involves immobilization of one interacting partner onto the surface of a sensor chip, while the other flows over the surface in solution. Interaction between the two generates a response which is proportional to the bound mass. The resonance angle expressed in resonance unit (RU) depends on the refractive index of the vicinity of the surface and changes with concentration of the molecules on the surface. The SPR technique provides accurate detection of the analyte at pico and nano-molar range.

Preparation of sensor chip

In the first step, preparation of CM5 sensor chip (Pharmacia Biosensor AB, Sweden) surface and immobilization of the anti-COX-2 antibody (Santa Cruz Biotechnology, CA) was performed using amine coupling kit (Pharmacia Biosensor AB, Sweden). Experiments were performed at 25°C in HBS-EP buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% P20 (surfactant). Briefly, equal volume (115 µl) of N-hydroxysuccinimide (NHS, 2.3 mg in 200 µl of water) and *N*-ethyl-*N*′-3 (diethylamino propyl) carbodiimide (EDC, 15 mg in 200 µl of water) was mixed first, and 75 µl of this solution was injected into the flow cell at the flow rate of 5 µl/min across the CM5 sensor chips to activate the carboxy methylated dextran surface for 15 min. Next, 210 µl of antibody (250 µl in 100 µl of 10 mM sodium acetate, pH 4.7) was injected at the flow rate of 5 µl/min across the activated surface for 25 min. The residual NHS esters were inactivated with ethanolamine (50 µl) for 10 min. Under these conditions, 19347 RU of anti-COX-2 antibody was immobilized on the sensor chip where 1.0 RU corresponds to protein concentration of $\sim$1.0 pg/mm^2 (Fig. 1a).

Measurement of serum COX-2 levels

Next, the recombinant COX-2 (rCOX-2) was prepared as described elsewhere (Somvanshi et al. 2007). Ten different concentrations of rCOX-2(4.4, 8.8, 13.2, 17.6, 22.0, 26.4,

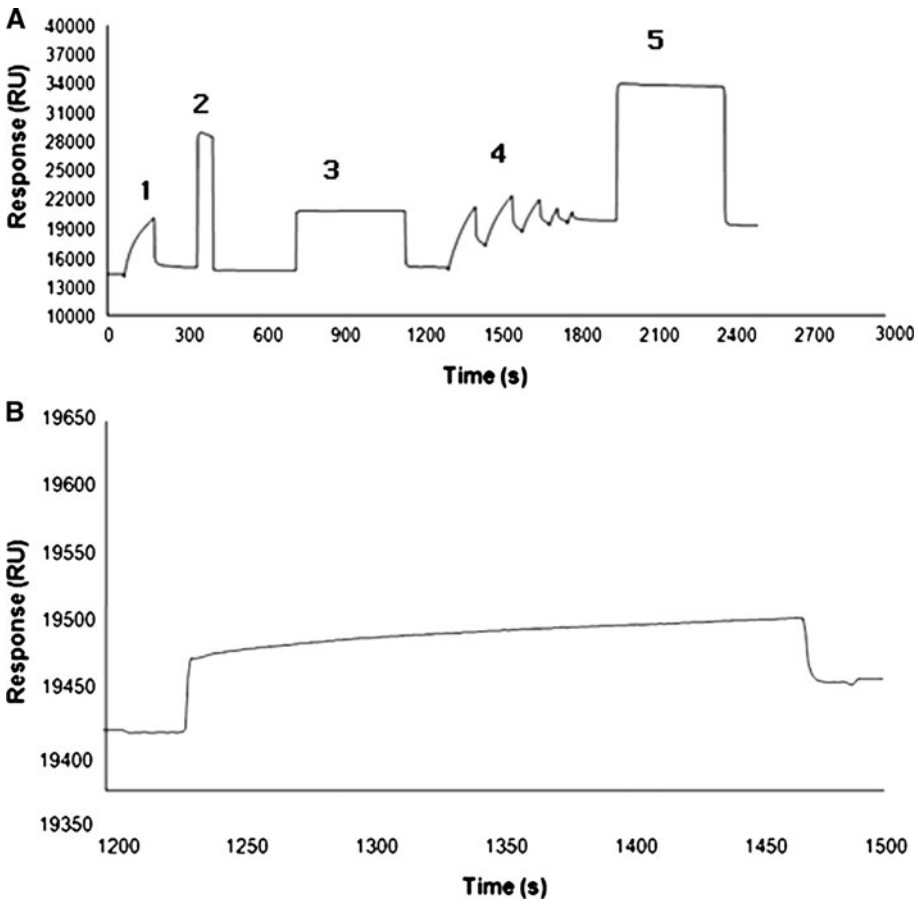

Fig. 1 Typical sensogram of immobilized antibody on the sensor chip and resonance response of the serum COX-2. The COX-2 antibody was immobilized on the sensor chip by amine coupling method as described in the "Materials and methods" section. **a** Response curve for immobilization sequence of anti-COX-2 antibody on the sensor chip (*1* Pre-concentration, *2* ethanolamine wash, *3* activation of the sensor surface by NHS/EDC, *4* antibody injection, and *5* deactivation); **b** Response curve for binding of serum COX-2 to the anti-COX-2 antibody on the sensor chip

30.8, 35.2, 39.6, and 44.0 μg) in HBS-EP buffer (pH 7.4) were passed over the immobilized COX-2 antibody, and the corresponding RUs (19,438.1, 19,501.2, 19,568.6, 19,685.5, 19,775.5, 19,875.5, 19,985.5, 20,105.5, 20,225.5, and 20,378.6, respectively) were obtained. A standard curve was plotted with RU against the concentrations of rCOX-2.

Serum samples diluted with HBS-EP buffer (1:99) were passed (40 μl) over the immobilized COX-2 antibody on the activated sensor chip at the flow rate of 10 μl/min, and RU for each sample was recorded (Fig. 1b). The RU values (RU range 19,451.7–20,090.8) of serum samples at this dilution were within linear range of the standard curve. After each binding event, the sensor surface was regenerated with 1 mM NaOH and 10 mM glycine–HCl. HBS-EP buffer and recombinant COX-2 were run along with the serum as negative and positive controls, respectively. The concentration of the serum COX-2 was derived from the standard curve.

Immunohistochemical staining

Immunohistochemical detection of COX-2 was carried out on 5-micron paraffin sections using streptavidin–biotin Universal Detection Kit (Immunotech, France). Briefly, after sequential rehydration through acetone, ethanol, and distilled water, the endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol at room temperature for 5 min. The sections were washed with water, and antigen was retrieved by heating sections in microwave (700 W) in 10 mM citrate buffer (pH 6.0) for 20 min. Before incubation in antibody solution, the sections were covered with protein blocking agent (PBA) for 5 min at room temperature, excess PBA was taped off, and sections were covered with mouse monoclonal anti-COX-2 antibody (1:75 dilution; Alexis Biochemicals, Switzerland) for 2 h in a wet chamber. The sections were washed and treated with biotinylated secondary antibody (anti-mouse 1gG) for 30 min at room temperature followed by treatment with streptavidin-peroxidase for 30 min at room temperature. The color was developed using freshly prepared chromogen (DAB). The sections were counter stained with Mayer's hematoxylin and mounted with D.P.X. mountant. The negative control was prepared similarly by omitting primary antibody. Slides were examined under light microscopy and scored positive in case with positive staining of more than 5% tumor cells in three randomly selected areas of dense staining by two independent observers on multiple experiments.

Synthesis of peptide inhibitors

The peptides were synthesized by solid phase Peptide Synthesizer PS3 (Protein technology, USA) using Fmoc and Wang resin chemistry (Merrifield 1963). To synthesize WCS peptide, the resin used was Fmoc-Ser-Wang resin, and solvent used for synthesis was dimethylformamide (DMF). In the first step, Fmoc-Ser-Wang resin was deprotected by 20% piperidine in DMF to form H_2N-Ser-Wang resin. The uronium salt 2-(1Hbenzotrizole-1yl)-1, 1, 3, 3-tetramethyl uronium hexafluoro-phosphate (HBTU) in the presence of base N-methylmorpholine (NMM) activated the amino acid to form an active ester of Fmoc-Cys-OH. This was coupled with H_2N-Ser-Wang resin to get Fmoc-Cys-Ser Wang resin. The above procedure was repeated for Fmoc-Trp-OH to get the final sequence Fmoc-Trp-Cys-Ser-Wang resin. Piperidine (20%) was then used to remove Fmoc protecting group, and the resin was cleaved from the peptide Trp-Cys-Ser (WCS) with trifluoroacetic acid. All other peptides with different amino acid sequence were synthesized in similar way and purified by reverse phase chromatography on C18 Pep-RPC column (1.6 × 10 cm; Amersham Bioscience, Sweden).

Six peptide inhibitors (P1-P6) with tripeptide sequences (WCS, ASW, WCA, WCY, GYW, and WYS, respectively) were synthesized that contained a hydrophobic amino acid with aromatic ring, a cysteine residue containing sulfur atom and a charged residue at the C-terminal end, whose free carboxylate end can form charge–charge interaction with Arg 120 in the active site of COX-2 with the aim to provide selective COX-2 inhibition with minimum side effects.

Tumor cell culture and treatment

KB cell line was obtained from National Centre for Cell Sciences (Pune, India) and maintained in DMEM (Sigma–Aldrich, USA) supplemented with 10% fetal bovine serum and antibiotics (streptomycin, penicillin, and fungizone). These tumor cells were used in subsequent experiments. Antitumor activity of peptide inhibitors was estimated by cell viability, proliferation, growth inhibition, and apoptosis assay. In all the experiments, a known COX-2 specific inhibitor, celecoxib (Zydus-Cadila), was used for comparison of the data, and the known anticancer drug, cisplatin (Sigma, USA), was used as positive control. Pilot experiments were done to determine optimum dose of peptides, celecoxib, and cisplatin in 48 and 72 h culture using cytotoxicity assays. Optimum response was obtained at 48-h culture period. Therefore, subsequent experiments were done using optimum doses of peptides, celecoxib, and cisplatin in 48-h tissue culture. All experiments were repeated at least three times.

Cell viability/cytotoxicity assay by PI labeling and trypan blue dye exclusion test

Briefly, KB cells were plated in 24-well plates (0.5×10^5 cells/well) in duplicates and cultured for 24 h at 37°C in

CO_2 incubator to synchronize the culture. After 24 h, the tumor cells were pulsed with 200 µl of optimum doses of peptides, celecoxib, and cisplatin. The untreated control wells received equal volume of phosphate-buffered saline (PBS, pH 7.4). The cells were harvested after 48 h, and viability was checked in 50-µl aliquots from each well using trypan blue dye exclusion test. The tumor cells were further labeled with 10 µl of 50 µg/ml of propidium iodide (PI), and about 10,000 events were acquired in flow cytometer (BD LSR II, Becton–Dickinson, San Diego). The percentage of dead cells (PI-labeled) was determined using BD FACSDiva™ software.

Cell proliferation assay

The proliferation assay was performed using alamarBlue™ technique (Fields and Lancaster 1993). Briefly, tumor cells were plated in 96-well plates (10^4 cells/well) in duplicate and cultured for 24 h at 37°C in CO_2 incubator. The cells were then treated with 20 µl of optimum doses of different peptides, celecoxib, and cisplatin as positive control and equal volume of medium as negative control and cultured for further 48 h. Subsequently, each well received 20 µl of alamarBlue™ (Biosource International, CA) and immediately read at 570 and 600 nm. The plate was incubated at 37°C in CO_2 incubator and monitored every 15 min for change of color from blue to red. The plate was read again at the above two wavelengths as soon as the color change was observed. Reduction of alamarBlue™ is proportional to proliferation of the cells and expressed as a percentage (% reduced) as below:

$$\%\,\text{Reduced} = \frac{(\varepsilon_{ox}\lambda_2)(\text{A}\lambda_1) - (\varepsilon_{ox}\lambda_1)(\text{A}\lambda_2)}{(\varepsilon_{\text{red}}\lambda_1)(\text{A}'\lambda_2) - (\varepsilon_{\text{red}}\lambda_2)(\text{A}'\lambda_1)} \times 100$$

where A = absorbance of test wells (containing cells with and without treatment); A' = Absorbance of control wells with only medium but no cells; $\lambda_1 = 570$ nm; $\lambda_2 = 600$ nm; ($\varepsilon_{\text{red}}\ \lambda_1$ = molar extinction coefficient of reduced alamarBlue™ at 570 nm (155,677); ($\varepsilon_{\text{red}}\ \lambda_2$ = molar extinction coefficient of reduced alamarBlue™ at 600 nm (14,652); ($\varepsilon_{\text{ox}}\ \lambda_1$ = molar extinction coefficient of oxidized alamarBlue™ at 570 nm (80,586); ($\varepsilon_{\text{ox}}\ \lambda_2$ = molar extinction coefficient of oxidized alamarBlue™ at 600 nm (117,216).

Assessment of cell growth inhibition by MTS assay

Effect of peptide treatment on tumor cell growth inhibition was studied by MTS assay using CellTiter 96® AQueous Assay kit (Promega, USA) as per the manufacturer's instructions. Briefly, KB cells were seeded in duplicates in 96-well culture plates at a density of 10,000 cells per well and cultured for 24 h at 37°C in CO_2 incubator. They were then treated with optimum doses of different peptides, celecoxib, and cisplatin and incubated for 48 h. To each well, 20 µl of MTS working solution was added, and the plate was incubated for 1 h at 37°C in CO_2 incubator. The tumor cell growth inhibition was assessed on the basis of mitochondrial conversion of MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] into aqueous, soluble formazan. The quantity of formazan product that is directly proportional to the number of living cells in culture was measured by determining the $A_{490\text{nm}}$ of the cell culture medium. The frequency of tumor cell growth inhibition was calculated using the formula: % inhibition = $(1 - \text{OD}_{\text{test}}/\text{OD}_{\text{control}}) \times 100$.

Assessment of apoptosis by annexin-V binding assay and flow cytometry

The frequency of apoptosis in KB cells following treatment with peptides was assayed by annexin-V binding assay. Briefly, KB cells were pulsed with peptides, celecoxib, and cisplatin for 48 h as described above. After termination of the culture, cells were washed twice with PBS, and 1×10^6 cells were resuspended in 100 µl annexin-V binding buffer. To the cell suspension, 5 µl of FITC-conjugated annexin-V (BD Biosciences, USA) and 10 µl of PI (50 µg/ml) solution were added and further incubated for 15 min at room temperature. After staining, 400 µl of annexin binding buffer was added, and samples were stored on ice until acquisition. About 10,000 events were acquired in LSR II (Becton–Dickinson, USA) flow cytometer. The frequency of annexin-positive (apoptotic) cells was determined using BD FACSDiva™ software.

Statistical analysis

Statistical analysis of the data was done using Epiinfo version 6.04d software (CDC, Atlanta). One-way analysis of variance (ANOVA) and Student's t-test were applied for evaluation of statistical significance of the data. Test statistics with 95% confidence limit ($P < 0.05$) was considered to be significant.

Results

Patient characteristics

Of 76 randomly recruited patients, 63 (83%) were men and 13 (17%) were women. The age of the patients was ranging from 22 to 75 with a mean ± SD = 51.7 ± 13.3. Majority of patients presented with tumor at base of the tongue (30.3%) followed by buccal mucosa (23.7%), tongue, and retromolar trigon (11.8% each) besides lip and alveolus being the less common sites. The TNM stage of tumors was

T1 6 (8%), T2 22 (29%), T3 25 (33%), and T4 23 (30%); N_0 25 (33%), N_1 23 (30%), N_2 24 (32%), and N_3 4 (5%). For the convenience of analysis, the patients were divided into two groups each for tumor (T1 + T2, T3 + T4) and lymph node (N_0 and N_+) stages. Among them, 2 (3%), 10 (13%), 21 (28%), and 43 (56%) patients had tumors of clinical stages I, II, III, and IV, respectively. Thus, 12 (15.7%) of 76 patients had early-stage (stages I + II) tumors, while 64 (84.2%) patients presented with late-stage (stages III +IV) tumors. Majority of patients (>90%) presented with well differentiated tumors rest with moderately differentiated tumors and none with poorly differentiated tumors.

Quantitative SPR analysis of COX-2 expression in patients with oral cancer

The circulating COX-2 level was quantified in 76 patients with oral cancer and 43 normal individuals. Patients with oral cancer expressed almost three-times more COX-2 when compared to the normal controls (9.4 ± 4.4 vs. 3.5 ± 1.3 μg/ml; $P < 0.0001$). Circulating COX-2 levels further correlated with clinical stage of the disease and lymph node involvement, when compared independently (Table 1). Patients with metastatic cervical lymph node and with late-stage tumor expressed significantly higher levels of COX-2 when compared to those without lymph node involvement ($P < 0.002$) and with the early-stage tumor ($P < 0.0004$). However, tumor size as an independent variable did not show any correlation.

The circulating COX-2 levels also correlated with tissue expression as observed by immunohistochemistry in representative samples. The patients with higher serum COX-2 levels also showed higher COX-2 immunoreactivity in their tissues. However, site of the tumor was not related to COX-2 immunoreactivity. The normal buccal mucosa did not show immunoreactivity (Fig. 2).

Table 1 Circulating COX-2 levels in relation to clinical features of oral cancer patients

Group	*n*	Serum COX-2 levels (μg/ml)		*P*-value
		Range	Mean ± SD	
Normal	43	2.4–5.2	3.5 ± 1.3	<0.0001
Patients	76	3.1–27.4	9.4 ± 4.4	
Tumor size				
T1 + T2	28	3.1–25.0	8.7 ± 4.8	<0.3
T3 + T4	48	7.0–27.4	9.8 ± 4.1	
Lymph node				
N0	25	3.4–10.1	7.2 ± 1.8	<0.002
N+	51	3.1–27.4	10.4 ± 4.9	
Clinical stage				
Early (I + II)	12	3.1–7.6	5.8 ± 1.6	<0.0004
Late (III + IV)	64	4.3–27.4	10.5 ± 4.4	

Antitumor activity of COX-2 peptide inhibitors on KB cell line

We next examined the cytotoxic activity of the peptide inhibitors on KB cell line by viability assay using PI labeling and flow cytometry, trypan blue dye exclusion test, and antiproliferative activity by alamarBlue™ assay. In the pilot experiment, all peptides, celecoxib, and cisplatin induced cytotoxicity in KB cells in a dose-dependent

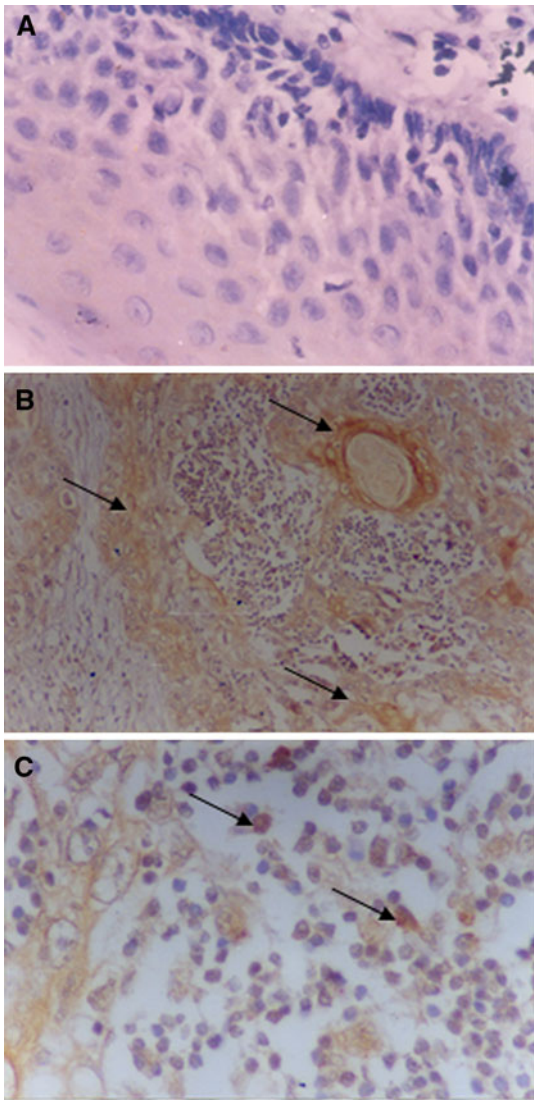

Fig. 2 Immunohistochemical staining of COX-2 in tobacco-related intraoral squamous cell carcinoma using mouse monoclonal antibody as described in "Materials and methods" [**a** Normal buccal mucosa showing negative staining for COX-2; **b** tumor cells showing intense staining (*arrows*; original magnification × 10×), **c** tumor cells showing intense staining (*arrows*; original magnification × 40×)]

manner from 75 to 200 μM concentration in 48 h culture. The optimum cytotoxic dose of P4 and cisplatin was 100 μM while that of celecoxib and peptides P1, P2, P3, and P6 was 150 μM. The subsequent experiments were done with the optimum doses of peptides, celecoxib, and cisplatin in 48-h culture.

All peptide inhibitors significantly ($P < 0.001$) induced cytotoxicity in KB cells. While frequency of cytotoxicity in P1, P4, and P6 treated cultures was 87 ± 1.6, 70 ± 2.6, and 82 ± 1.3%, respectively, and celecoxib and cisplatin induced cytotoxicity in 80 ± 3.4 and 90.2 ± 1.1% cells, respectively (Fig. 3a), suggesting a higher cytotoxic activity of P1 and P6 when compared to celecoxib and almost similar to that of the cisplatin. Similarly, all peptides significantly ($P < 0.001$) reduced viability of KB cells, the reduction in tumor cell viability by P1 (11.1 ± 2.3%) and P6 (11.7 ± 2.6%) was similar to that of the cisplatin (11.8 ± 1.3%), but it was higher than celecoxib (20.2 ± 1.3%), while untreated cells showed highest (93.8 ± 1.4%) viability. However, none of these peptides or celecoxib showed cytotoxic effect on either normal human leukocytes or on MCF-7 cells that did not show COX-2 expression as against ubiquitous expression of COX-2 in KB cells (Suppl. Fig. 1a–d).

In proliferation assay, while untreated cells showed 85.7% proliferation, peptides P1, P4, and P6 reduced proliferation to 5.7, 3.5, and 4.1%, respectively ($P < 0.0001$). A significant reduction in KB cell proliferation was also observed with peptides P2 (58%, $P < 0.05$), P3 (45.6%, $P < 0.009$), celecoxib (11.6%, $P < 0.0005$), and cisplatin (14.4%, $P < 0.0003$). These results show higher antiproliferative activity of peptides P1, P4, and P6 on KB cells when compared to celecoxib and cisplatin (Fig. 3b).

The results of MTS assay have been shown in Fig. 3c. While peptides P1 and P6 inhibited KB cell growth by >95%, the inhibition with peptide P4 was almost 90 percent. The tumor cell growth inhibition by peptides P1 and P6 was similar to that of the celecoxib and cisplatin.

Apoptotic activity of COX-2 peptide inhibitors on KB cells

We next examined the apoptotic activity of the three best peptides (P1, P4, and P6) as well as celecoxib and cisplatin using annexin-V binding assay (Fig. 4). In this experiment, while peptides P1, P4, and P6 induced apoptosis in 32.0, 33.0, and 49.6% KB cells, respectively, while only 2.0% of untreated cells showed apoptosis. The frequency of apoptotic cells was significantly higher in peptide-treated cells when compared to the untreated controls ($P < 0.0001$). The frequency of apoptosis in celecoxib- and cisplatin-treated cultures was 83 and 68.1%, respectively.

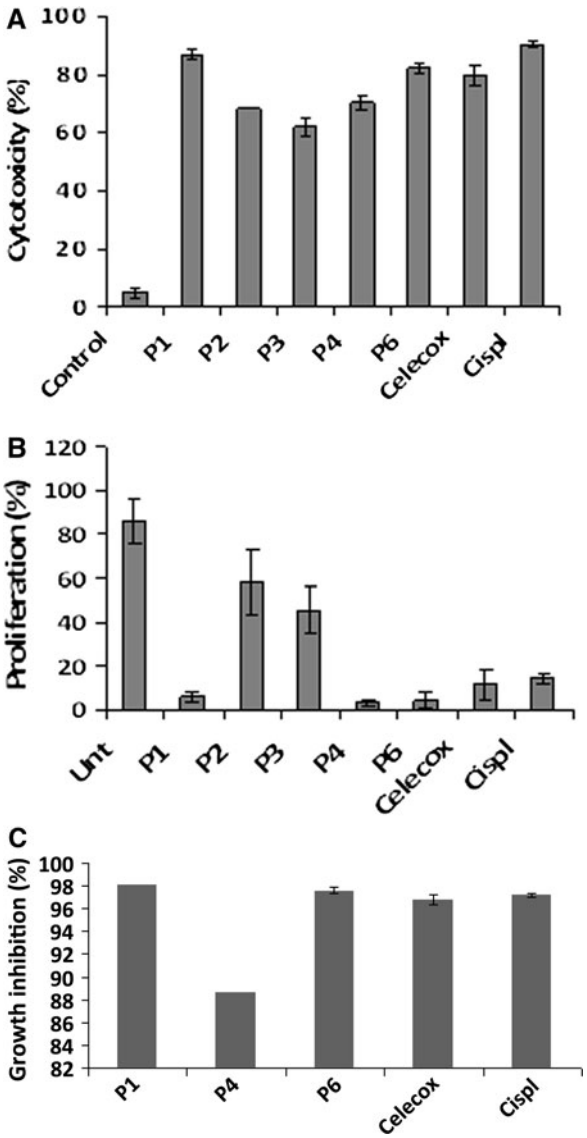

Fig. 3 Antitumor activity of COX-2 peptide inhibitors on KB cells (**a** Tumor cell cytotoxicity. KB cells were treated with optimum concentrations of peptides, celecoxib, and cisplatin for 48 h, labeled with PI, and acquired in flow cytometer; **b** tumor cell proliferation as determined by almarBlue™ assay; **c** tumor cell growth inhibition as determined by MTS assay)

Discussion

Earlier studies have shown higher tissue expression of COX-2 during oral carcinogenesis through pre-malignant, dysplasia, and OSCC (Li et al. 2005; Sawhney et al. 2007; Pandey et al. 2008). However, most of these studies represent either subjective or semi-quantitative evaluation of COX-2 expression in tissues. Since circulating COX-2 levels may be a useful marker for study of disease progression as well as monitoring the response to novel COX-2 targeting strategies, we for the first time designed a novel

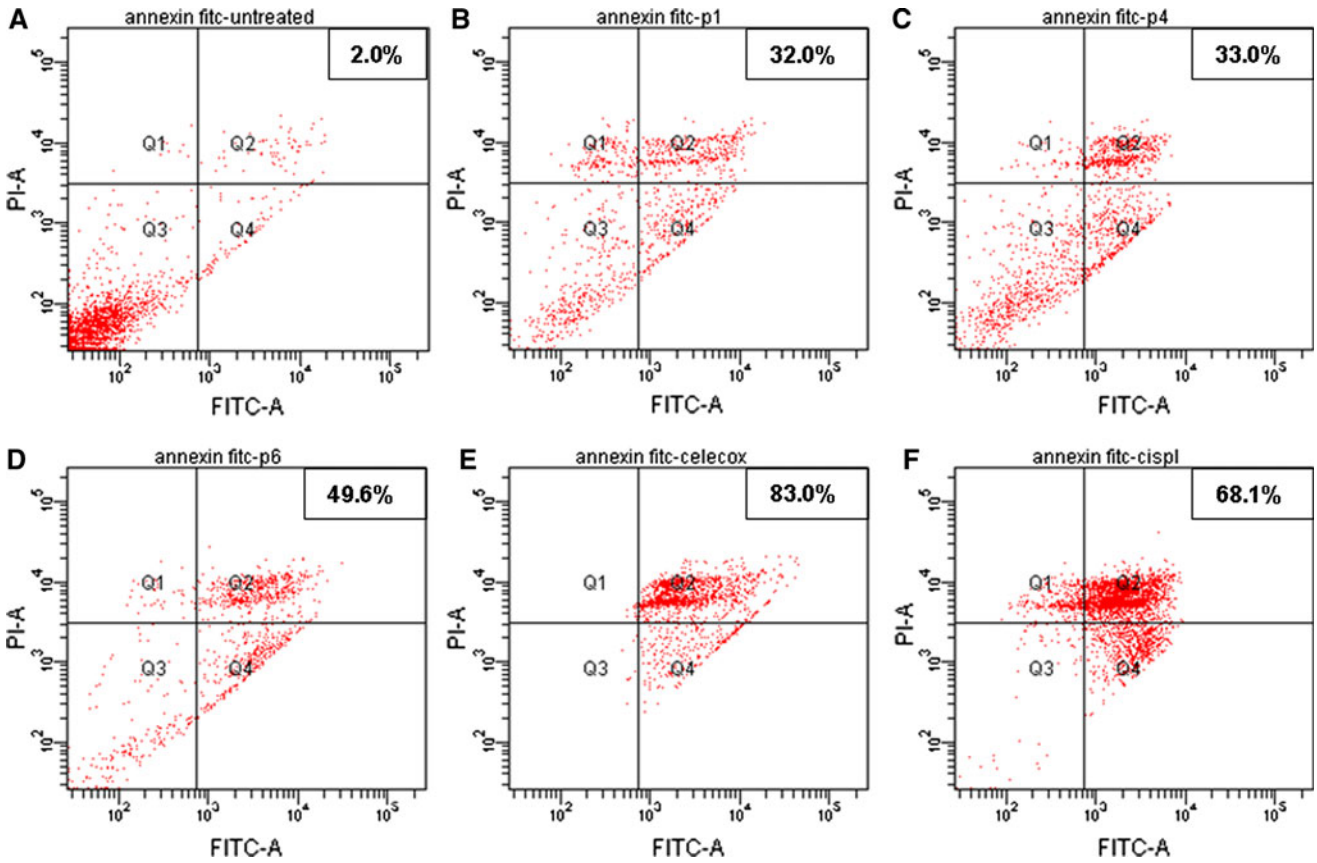

Fig. 4 Representative dot plots showing apoptotic activity of peptides on KB cells (**a** Untreated control; **b–d** treated with peptides P1, P4, and P6, respectively; **e** treated with celecoxib and **f** treated with cisplatin)

strategy to quantify the circulating level of COX-2 using biosensor-based SPR technique by measuring interaction phenomenon of two biological molecules (COX-2 and anti-COX-2 antibody in this case) in real time. We found almost threefold increase in circulating COX-2 levels in patients with oral cancer when compared to the normal controls. Patients with metastatic cervical lymph node and with late-stage tumors expressed higher COX-2 than those with early-stage disease and without lymph node involvement, suggesting that expression of COX-2 is related to invasive potential of OSCC rather than the local spread (tumor size). Similarly, COX-2 has been reported to be over expressed through normal to premalignant and malignant tissues in patients with oral and head and neck carcinoma as well as in oral tumor cell lines (Li et al. 2005; Shibata et al. 2005; Sawhney et al. 2007; Pandey et al. 2008). While correlation between tissue expression of COX-2 and grade and stage of the disease has been controversial and inconclusive (Sawhney et al. 2007; Pandey et al. 2008), the circulating COX-2 in our study showed a clear correlation with higher stage and invasive potential of the tumor. The immunohistochemical observations also clearly support our quantitative SPR data. It appears that the circulating COX-2 levels may be discriminatory between similarly staged oral cancer patients with higher invasive potential, and it may be used as potential tumor marker for OSCC. Moreover, the circulating COX-2 measurement by SPR technique seems to be appropriate than the measurement of serum prostaglandins which have a shorter half-life. Therefore, SPR appears to be a useful proteomic technique for monitoring circulating COX-2 levels in patients with cancer.

Since overexpression of COX-2 has been shown to promote various aspects of tumorigenesis such as tumor cell proliferation and differentiation, inhibition of apoptosis, increased cell adhesion to extracellular matrix, increased metastasis, neoangiogenesis, and host immune response to tumor cells (Wu 2005) and based on the recent reports on antitumor effects of COX-2 inhibitors on human oral cancer cells (Yang et al. 2003; Li et al. 2005), we examined the antitumor activity of peptide inhibitors (P1–P4 and P6) with higher enzyme inhibitory activity, on KB cells. All peptide inhibitors significantly induced cytotoxicity in KB cells; the frequency of cytotoxicity by P1, P4, and P6 was higher than P2 and P3. Similarly, P1 and P6 showed a higher cytotoxicity when compared to celecoxib and almost similar to that of the cisplatin. Although all

peptides significantly reduced viability of KB cells, the reduction in tumor cell viability by P1 and P6 was similar to that of the cisplatin, but it was higher than the celecoxib. Similarly, KB cell growth inhibition by peptides P1 and P6 was comparable to that of the celecoxib and cisplatin. However, neither peptides nor celecoxib induced cytotoxicity in MCF-7 cells. Unlike KB cells, MCF-7 cells do not express COX-2 (Zhao et al. 2008). Although all peptides significantly reduced tumor cell proliferation, peptides P1, P4, and P6 showed maximum inhibition. These peptides also induced apoptosis in almost 50% of KB cells that was similar to cisplatin but higher than the celecoxib.

We earlier reported the binding kinetics of these peptide inhibitors with recombinant COX-2 and their enzyme inhibitory activity by arachidonic acid and *N*,*N*,*N*′,*N*′-tetramethyl p-phenylenediamine oxidation method (Somvanshi et al. 2007). Interestingly, peptides P1, P4, and P6 showed a very low dissociation constant (2×10^{-8} to 4×10^{-10}) and very high (80–90%) COX-2 enzyme inhibitory activity, while peptides P2 and P3 showed relatively higher dissociation constant (1×10^{-6}) and lower enzyme inhibitory activity (50%). In addition, P1, P4 and P6 also showed higher anti-inflammatory activity that was comparable to selective COX-2 inhibitor celecoxib. Thus, it appears that the antitumor activities of these peptides are related to their COX-2 enzyme inhibitory and anti-inflammatory activities. Furthermore, the antitumor activity of the peptides seems to be directly related to their affinity to COX-2 in human OSCC.

The exact mechanisms of action of COX-2 inhibitors on oral cancer cells are not well understood. COX-2 inhibitors retarded growth of human oral cancer cells in both PGE2-dependent and independent manner (Yang et al. 2003). In a recent study, out of four selective COX-2 inhibitors, celecoxib and NS-398 strongly suppressed proliferation of KB cells at 10–100 μM doses, whereas nimesulide and meloxicam were less potent proliferation inhibitors (Ko et al. 2008). Moreover, celecoxib at 100 and 200 mg twice daily was ineffective in controlling oral premalignant lesions, besides its cardiovascular toxicity has discouraged the continued investigation into these agents in oral cancer chemoprevention (Papadimitrakopolou et al. 2008). In case with cholangiocarcinoma treatment with exogenous PGE2 increased tumor cell growth and prevented apoptosis (Han et al. 2004; Wu et al. 2004) and ω3 polyunsaturated fatty acid was shown to block tumor cell growth through inhibition of Wnt/β-catenin and COX-2 signaling pathway (Wu 2005). COX-2 over expression was shown to be parallel with NF-κB activation in tobacco-induced oral carcinogenesis (Sawhney et al. 2007). Although studies using COX-2 inhibitors known to be not particularly specific for COX-2 inhibition show reduced cell proliferation and tumorigenic behavior of cells (Howe et al. 2001; Rigas and Kashfi 2005), the knockdown of COX-2 with small interfering RNA (siRNA) showed no effect on cell cycle distribution (Denkert et al. 2003). However, in the earlier study, peptides have shown higher affinity (K_D 1×10^{-8} to 1×10^{-10}) to COX-2 as well as almost 80–90% inhibition of PGE2 synthesis in vitro (Somvanshi et al. 2007). The results of our present study clearly indicate that COX-2 peptide inhibitors may be potent antiproliferative, cytotoxic, and apoptotic agent for human OSCC. One of the prominent mechanisms for the antitumor and chemopreventive action of peptide inhibitors appears to be their selective high affinity binding with COX-2 enzyme leading to suppressive effects on arachidonic acid metabolism, particularly PGE2 synthesis. Hence COX-2 peptide inhibitors appear as a new class of anticancer agent for the treatment of cancer associated with overexpression of COX-2 including OSCC.

In conclusion, we have successfully quantified the circulating levels of COX-2 in patients with OSCC using surface plasmon resonance technique. Patients with oral cancer have significantly higher levels of COX-2 when compared to that of the normal controls and that the higher circulating COX-2 levels are related to disease activity such as advanced clinical stage and metastatic potential of the tumor. Furthermore, the COX-2 inhibitory peptides showed a potent antitumor activity on human OSCC cells in vitro. Thus, circulating COX-2 appears as a plausible tumor marker and its peptide inhibitors as a new class of potent anticancer agent for human OSCC.

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Conflict of interest statement We declare that we have no conflict of interest.

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