The Cyclooxygenase 2-selective Inhibitor NS398 Inhibits Proliferation of Oral Carcinoma Cell Lines by Mechanisms Dependent and Independent of Reduced Prostaglandin E\(_2\) Synthesis\(^1\)

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ABSTRACT

Purpose: We investigated the potential of cyclooxygenase (COX)-2 as an appropriate chemopreventive and/or therapeutic target for oral cancer.

Experimental Design: Immunohistochemical analysis of COX-2 expression was carried out on 37 oral squamous cell carcinomas (OSCCs) and 23 normal oral epithelium samples. We investigated whether the COX-2-selective inhibitor NS398 induced growth inhibition in four human OSCC cell lines and whether this was COX-2 dependent.

Results: COX-2 staining was more intense in the carcinomas compared with normal epithelium (P < 0.001). Early-stage tumors (stages I and II) had significantly higher epithelial COX-2 staining than late-stage tumors (stages III and IV; P = 0.034), and overexpression of COX-2 was detected in hyperplastic and dysplastic epithelium. Treatment of OSCC cells with NS398 for 72 h at concentrations of 50 \(\mu\)M and above resulted in growth inhibition accompanied by a reversible G\(_0\)-G\(_1\) arrest, but no apoptosis or terminal differentiation. However, a concentration of 10 \(\mu\)M was sufficient to abolish secreted prostaglandin E\(_2\) (PGE\(_2\)) production. Over a longer treatment time, lower concentrations of NS398 were growth inhibitory. Growth inhibition of the OSCC cell line H357 was detected after treatment with 5 \(\mu\)M NS398 as well as 100 \(\mu\)M NS398 for 6–12 days. In cultures treated with 5 \(\mu\)M NS398, but not in those treated with 100 \(\mu\)M NS398, restoration of PGE\(_2\) to control levels abrogated growth inhibition.

Conclusions: NS398 inhibits the growth of OSCC cells by mechanisms that are dependent and independent of suppression of PGE\(_2\) synthesis. Molecular targeting of COX-2, PGE\(_2\) synthase, or PGE\(_2\) receptors may be useful as a chemopreventive or therapeutic strategy for oral cancer.

INTRODUCTION

Oral malignancy, of which the commonest form is OSCC,\(^3\) is a major health problem worldwide (400,000 cases/year). Treatments often fail due to local recurrence, lymph node metastases, and second primary tumors. Yet oral cancer is an excellent candidate for a chemopreventive strategy because of the potential for treatment with locally active agents. There is evidence from epidemiological and animal studies that long-term use of NSAIDs can prevent cancer development. This is particularly striking in the case of colorectal cancer, where the incidence is reduced by 40–50%, but there is also evidence that NSAIDs reduce the risk of many other tumor types, such as esophageal, gastric, breast, and lung cancers (1).

NSAIDs inhibit the activity of the COX enzymes. COX is responsible for the conversion of arachidonic acid to the endoperoxide intermediate prostaglandin H\(_2\), which is subsequently converted to prostaglandins by specific synthases. There are two forms of COX enzyme, COX-1 and COX-2, which are encoded by different genes. Traditional NSAIDs such as aspirin inhibit COX-1 and COX-2 activities. Cox-1 is constitutively expressed in most tissues, whereas the Cox-2 gene is induced rapidly in response to growth factors, oncoproteins, tumor promoters, and carcinogens, as well as physiological stress stimuli (2).

For example, in oral keratinocytes, Cox-2 is induced by benzo-(a)pyrene, a mutagenic carcinogen of tobacco smoke (3).

In addition to the chemopreventive effect of NSAIDs, there is considerable direct evidence for the involvement of COX-2 in intestinal carcinogenesis. Tsujii and DuBois (4) demonstrated that overexpression of COX-2 in rat intestinal epithelial cells resulted in an increased tumorigenic potential of the epithelial cells. Furthermore, in the APC\(^{2\rightarrow 16}\) mouse model of FAP, null mutation for Cox-2 markedly reduced the number and size of intestinal adenomas (5). Overexpression of COX-2 in human colon cancer cells increases their metastatic potential (6) and promotes angiogenesis (7). Consistent with these properties of COX-2, COX-2-selective inhibitors are effective as antitumor

\(^1\)The abbreviations used are: OSCC, oral squamous cell carcinoma; COX, cyclooxygenase; PGE\(_2\), prostaglandin E\(_2\); NSAID, nonsteroidal anti-inflammatory drug; FAP, familial adenomatous polyposis; EGF, epidermal growth factor; PARP, poly(ADP-ribose) polymerase; Rb, retinoblastoma protein.

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agents in animal models of colon cancer or FAP (7, 8). COX-2-selective inhibitors include SC-58125 (9), celecoxib (10), and NS398 (11). These inhibitors do not induce the gastric ulcers associated with the use of traditional NSAIDs, and in clinical trial, celecoxib reduced duodenal polyposis in FAP patients (12).

In the 1980s, work using a number of animal models of oral cancer chemoprevention suggested that nonselective NSAIDs could inhibit tumor development (13, 14). Furthermore, indomethacin, a nonselective COX inhibitor, inhibited the growth of squamous carcinomas of the head and neck in a clinical study (15). Recently, this idea has been revisited exploring the potential of COX-2-selective inhibitors for oral cancer prevention (16, 17). Shiotani et al. (17) showed inhibition of postinitiation tumor development by the NS398 analogue nimesulide in a rat oral carcinogenesis model in which COX-2 was overexpressed during carcinogenesis (17). These observations underscore the importance of understanding the action of COX-2-selective inhibition in oral carcinoma cells.

NSAIDs are known to exert their effects by mechanisms dependent and independent of the inhibition of prostaglandin synthesis (18). Our objective was to explore the possibility that NS398 might induce growth inhibition in oral cancer cells by either of these mechanisms. High concentrations of NS398 (greater than those required to inhibit prostaglandin synthesis) have been shown to induce COX-2-independent apoptosis in colon carcinoma cell lines (19). Because topical treatment is a viable option for oral cancer chemoprevention, effects of relatively high concentrations of COX-2-selective inhibitors would be clinically relevant. However, COX-2-dependent effects of NS398 occur at low concentrations and would rely on the expression of COX-2 in oral tumors. In this study we show that COX-2 is overexpressed in OSCC, particularly in early-stage tumors. The COX-2 inhibitor NS398 was found to inhibit cell proliferation by mechanisms that, at low and high concentrations, are dependent on or independent of reduced PGE2 synthesis, respectively. Furthermore, suppression of the production of secreted PGE2 is a major mechanism of COX-2-dependent growth inhibition.

MATERIALS AND METHODS

Immunohistochemistry. The antibody used was rabbit COX-2 polyclonal antibody (Cayman Chemical Co., Ann Arbor, MI). This antibody was raised against murine COX-2 protein but also recognizes human COX-2. Two-μm sections were mounted on polylysine-coated slides. Sections were dewaxed in xylene and then rehydrated in descending alcohol to running tap water. Endogenous peroxidase activity was quenched using 3% (v/v) hydrogen peroxide (H2O2) for 15 min. For antigen retrieval, sections were microwaved in a 960 W oven for 30 min in 0.1 M citrate buffer (pH 6.0). Slides were incubated with COX-2 antibody diluted 1:700 in PBS containing 1% (v/v) normal goat serum (DAKO) for 18 h at 4°C. Antibody binding was detected using a “link and label” kit (Biomen Diagnostics). Sections were exposed to a diaminobenzidine peroxidase substrate (DAKO) for 5 min, washed with distilled water, and counterstained with Mayers’ hematoxylin. As a positive control, colon carcinoma tissue known to express high levels of COX-2 was used. Negative controls were included with omission of the primary antibody. In addition, antibody specificity was confirmed by preincubation with blocking peptide (Cayman Chemical Co.) at a 1:70 dilution in PBS containing 1% (v/v) normal goat serum. Overall COX-2 staining intensity in normal and tumor epithelial cells was graded by two independent scorers (J. W. E. and S. H.) as follows: 0, negative; +, low; ++, medium; and ++++, high.

Cell Lines and Culture. Established oral carcinoma-derived cell lines (20, 21) were cultured in DMEM/F12 (1:1) medium (Life Technologies, Inc.) containing 10% (v/v) fetal bovine serum, 0.6 μg/ml l-glutamine (Sigma), and 0.5 μg/ml hydrocortisone (Sigma). H314 was derived from a poorly differentiated tumor of the floor of the mouth, H376 was derived from a moderately differentiated tumor of the floor of the mouth, and H357 and H413 were derived from well-differentiated tumors of the tongue and buccal mucosa, respectively (20). These four oral carcinoma cell lines all have mutant p53 (22), although in H376 this is not expressed as protein (23). Only the H357 cell line has mutant Ha-ras (22).

Primary cultures of normal human oral keratinocytes were established from normal human palate. These were isolated and cultured using the method of Rheinwald and Green (24) with growth medium (as above) with additional supplements of 0.05 μg/ml cholera toxin (Sigma), 0.05 μg/ml transferrin (Sigma), 0.04 IU/ml insulin (Novo Nordisk Pharmaceuticals Ltd.), 200 IU/ml penicillin (Sigma), and 200 μg/ml streptomycin (Sigma). Swiss 3T3 cells were added at a density of 2 × 105 cells/6-cm dish. Ten ng/ml EGF (Sigma) was added to cultures subsequent to cell attachment. For preparation of cells for Western blot analysis, 3T3 feeder cells were first removed using 0.02% (v/v) EDTA.

The HT29 colon carcinoma cell line was used as a positive control for NS398-induced apoptosis and was cultured in DMEM (Life Technologies, Inc.) containing 10% (v/v) fetal bovine serum, 0.6 μg/ml glutamine, and 0.5 μg/ml hydrocortisone.

Treatment with NS398. Oral carcinoma cells were treated with 10, 20, 50, 75, and 100 μM NS398 (Sigma) 24 h after seeding. For treatment of the HT29 colon carcinoma cell line, cells were treated after 3 days of culture. This ensured that the cells were in exponential growth at the time of treatment. NS398 (Sigma) was dissolved in DMSO at 0.1 M. Each treatment condition contained the same amount of DMSO. Because DMSO can modulate keratinocyte differentiation and proliferation (25), blank controls and solvent controls were included to check that the DMSO had no such effects at the concentration used [0.1% (v/v) for experiments with doses up to 100 μM, and 0.005–0.01% (v/v) for low-dose experiments (1–10 μM)].

Measurement of Adherent Cell Yield and Apoptosis. After 72 h of treatment, attached cells (those remaining adherent to the flask) and shed cells (those that had become detached from the adherent monolayer and shed into the medium) were counted separately. For longer treatments, the shed cells were collected and counted at each medium change. Unfixed samples of each of these cell populations were examined for apoptotic morphology by staining with acridine orange and ethidium bromide (5 μg/ml of each stain in PBS), which allows apoptotic cells to be distinguished by their characteristically condensed chromatin. We previously used these methods to demonstrate
the induction of apoptosis in colonic tumor cell lines (26). Therefore, HT29 colon cancer cells were used as a positive control for NS398-induced apoptosis. In addition, because Li et al. (27, 28) have shown that NS398-induced apoptosis is dependent on the mitochondrial pathway of apoptosis and that caspase-3 activation and PARP cleavage are characteristic molecular markers of apoptosis in both colonic (27) and esophageal (28) cells, the cleavage of caspase-3 and PARP was measured in control and treated cells. Adherent and floating cells were pooled to detect cleaved PARP by Western blotting. In addition, adherent cultured cells were examined by immunocytochemistry for cleaved caspase-3 (presence of active enzyme).

**Western Blot Analysis.** Cell pellets (10⁶) were lysed in ice-cold radioimmunoprecipitation assay buffer [0.15 m NaCl, 1% (v/v) NP40, 0.5% (v/v) sodium deoxycholate, 0.1% (w/v) SDS, and 50 mM Tris-HCl (pH 8.0)] with protease inhibitors (cocktail set III; Calbiochem). The lysates were vortexed and incubated on ice for 15 min, followed by centrifugation at 7000 × g for 20 min at 4°C. The supernatant was collected, and protein estimation was performed using the Bio-Rad DC protein estimation assay according to the manufacturer’s instructions. An equal volume of 2× gel sample buffer [100 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 200 mM DTT, and 4% (w/v) bromphenol blue] was added to the remaining sample volume, and the samples were placed in a boiling water bath for 5 min. Proteins were separated on a 10% acrylamide gel for detection of COX-2, p21WAF1, p27KIP1, and involucrin or on a 7.5% gel for detection of PARP and Rb. Western blotting was carried out as described previously (26).

COX-2 protein was detected using a rabbit polyclonal COX-2 antibody kindly provided by Stephen Prescott (Huntsman Institute, University of Utah) or a goat polyclonal COX-2 antibody (Santa Cruz Biotechnology), each at 1:500. Involucrin was detected using the SYS mouse monoclonal antibody, a kind gift of Fiona Watt (Cancer Research United Kingdom, London, United Kingdom), at 1:10,000. PARP was detected using a mouse monoclonal antibody (Oncogene Research Products) at 1:100. Rb was detected using a mouse monoclonal antibody (Oncogene Research Products) at 1:100, and p27KIP1 was detected using a rabbit polyclonal antibody (Oncogene Research Products) at 1:100, and p27KIP1 was detected using a rabbit polyclonal antibody (Santa Cruz Biotechnology) at 1:500. As a loading control, α-tubulin was detected using a mouse monoclonal antibody (Sigma) at 1:10,000. The horseradish peroxidase-conjugated secondary antibodies were goat antirabbit antibody (Sigma) at 1:4000, donkey antigoat IgG antibody (Santa Cruz) at 1:3333 (COX-2), or goat antimouse antibody (Sigma) at 1:1000 (involucrin, PARP, and α-tubulin). Peroxidase activity was detected using an enhanced chemiluminescence detection kit (Amersham) following manufacturer’s instructions and developed on Kodak X-Omat film.

**Immunocytochemistry.** Because the levels of apoptosis in the oral tumor cell cultures were low, apoptosis was quantified by immunocytochemical detection of the active form of caspase-3 using a mouse monoclonal antibody (New England Biolabs), which detects the large fragment of activated caspase-3 (Mr, 17,000–19,000) formed upon cleavage of the proenzyme, at a 1:80 dilution. This antibody does not recognize full-length caspase-3 or other known caspases. Cycling cells were detected using a rabbit polyclonal Ki67 antibody (DAKO) at 1:800. Cells were cultured on three well slides for 24 h prior to 24 h of treatment with either solvent control [0.1% (v/v) DMSO] or 100 μM NS398. Cells were fixed in acetone for 10 min. The primary antibody was applied in PBS containing 1% (v/v) normal goat serum (DAKO) for 60 min at room temperature and detected using a link and label kit (Biomax Diagnostics). Cells were exposed to a diamobenzidine peroxidase substrate (DAKO) for 5 min, washed with distilled water, and counterstained with Mayer’s hematoxylin. As negative controls, primary antibody was omitted, and cells were incubated in PBS containing 1% (v/v) normal goat serum.

**Cell Cycle Analysis after Treatment with NS398.** Detached cell suspensions were centrifuged at 150 × g for 10 min at room temperature and washed in PBS. Cell pellets were resuspended in ice-cold 70% ethanol at 1 × 10⁶ cells/vial and stored −20°C. Cells fixed in ethanol were stored at −20°C for at least 7 days, but not longer than 4 weeks. Twenty-four h before analysis, fixed cells were centrifuged at 150 × g for 10 min at room temperature. Cell pellets were resuspended in PBS containing 20 μg/ml propidium iodide (Sigma) and 15 μg/ml RNase A (Sigma). Samples were incubated in the dark at 37°C for 30 min and then at 4°C overnight. Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). For each sample, 10,000 fluorescent cells were counted. The data were analyzed using ModFit software.

**Measurement of PGE2 Production.** PGE2 is the major end product of the metabolism of arachidonic acid by COX in oral carcinoma cells (29). PGE2 production was measured by a competitive enzyme immunoassay using a PGE2 detection kit (Cayman Chemical Co.). Oral carcinoma-derived cell lines were cultured and treated with NS398 for 72 h in duplicate. For H314 cells, 1 h before harvesting, 50 μM arachidonic acid (Sigma) was applied to one of each of the duplicates. One-ml aliquots of culture medium were snap frozen in liquid nitrogen. Adherent cell yields were determined. Immediately before assay, samples were centrifuged (7000 × g at 4°C) for 5 min to remove any cellular debris. The supernatant was then transferred to fresh tubes and kept on ice. The PGE2 assay was performed according to the manufacturer’s instructions (Cayman Chemical Co.) after dilutions were made to ensure that readings were within the limits of accurate detection by the assay.

If the lack of secreted PGE2 were the cause of the growth-inhibitory effect of 5 μM NS398, adding exogenous PGE2 to the medium would be predicted to restore the growth rate of the cells. For these experiments, it was necessary to replace the medium containing fresh vehicle control, 5 μM NS398, PGE2, and 5 μM NS398 + PGE2 every 48 h. To take into account the possibility that PGE2 may degrade or be internalized into cells, we wished to determine the concentration of PGE2 to add to the medium to restore concentrations such that 48 h after addition of PGE2, the concentration in the medium of the NS398- and PGE2-treated cultures would be approximately equal to that in the control cultures. Cells were treated first for 24 h with NS398; the medium was then replaced with fresh NS398 with the addition of 100-5000 pg/ml PGE2 (0.28–14.1 nM) and incubated for 48 h before medium collection for the PGE2 assay as described above.
Statistical Analysis. For analysis of COX-2 expression against other parameters in the immunohistochemical study Mann-Whitney, Kruskal-Wallis, and Wilcoxon signed ranks tests were used. Dunnett’s t test was used to assess the significance of growth inhibition and cell shedding in response to NS398 treatment. Growth curve data were analyzed by Tukey’s test at each of the time points. Paired t tests were used to analyze G0-G1 arrest with fluorescence-activated cell-sorting analysis, cleaved caspase-3, and Ki67 expression in response to 100 μM NS398 treatment.

RESULTS

Cox-2 Protein Is Overexpressed in Human OSCCs. In this study, COX-2 protein expression was compared between 37 OSCCs and 23 samples of normal oral mucosa from patients without neoplastic disease. The COX-2 antibody was that used previously by Wolff et al. (30) to demonstrate COX overexpression in human lung carcinomas. Cancer patient and tumor characteristics are summarized in Table 1. All tumors used in this study were specifically oral. The normal oral tissue was collected from different areas of the oral cavity (tongue, inner lip, palate, and floor of mouth) from patients undergoing routine treatment for benign disease (Table 2), and the epithelium was confirmed to be histologically normal. Although the normal tissue samples came from a younger set of patients, COX-2 expression did not differ significantly with patient age in either the normal tissue series or the tumor series.

COX-2 was weakly expressed in the basal epithelial cells of the majority of normal oral tissue samples (Fig. 1A), but COX-2 expression was increased in areas of inflammation, hyperplasia (Fig. 1B), or dysplasia (data not shown). In 6 of 23 normal samples, COX-2 could not be detected in the epithelial cells, yet granular cytoplasmic COX-2 staining was detected in endothelial cells and in lymphocytes in these tissue sections as well as in those that exhibited positive epithelial cell staining. Others have previously found that endothelial cells, smooth muscle, and lymphocytes stain positively for COX-2, and these served as internal positive controls (31). Of the six negative normal samples, five were palate samples from the <16-year-old group, and one was a tongue sample from a 60-year-old individual. In the tumors, the epithelial cells of all 37 tumors stained positively for COX-2 with a granular cytoplasmic pattern (Fig. 1C). COX-2 was overexpressed in tumors compared with separate samples of normal oral epithelium (Table 3; P < 0.001, Mann-Whitney test). In addition, 27 of the tumor sections incorporated adjacent histologically normal epithelium, and in these sections, 20 tumors (74%) overexpressed COX-2 relative to their adjacent epithelium (P < 0.001, Mann-Whitney test; Fig. 1D). There was no significant difference in staining intensity between the independent series of normal oral epithelial tissues and the histologically normal epithelium adjacent to the tumors. Therefore, the overexpression of COX-2 protein in OSCCs is highly statistically significant. Furthermore, in some tumors, COX-2 expression was highest at the leading edge of the tumor, with strong perinuclear expression (Fig. 1, E and F). To confirm specificity of the antibody for COX-2, one of the tumors was stained with COX-2 antibody with and without preincubation with blocking peptide. Blocking peptide completely abrogated staining (Fig. 1, G and H).

The intensity of COX-2 staining in the tumors varied significantly with site of the primary tumor (P = 0.021, Kruskal-Wallis test). In general, buccal mucosa tumors had the highest intensity of COX-2 staining. No correlation was found between the intensity of staining and degree of differentiation, the presence of neck lymph node metastasis, or age at presentation. However, there was a significant difference in COX-2 staining intensity in epithelial cells between early-stage and late-stage tumors comparing STNMP (32) stages I and II with stages III and IV (P = 0.034; Mann-Whitney test). Interestingly, the early-stage tumors tended to have higher COX-2 expression. In addition, within the tumor series, greater intensity of COX-2 protein expression correlated with patients who had never smoked tobacco (P = 0.039, Mann-Whitney test).

Cox-2 Expression in Oral Cancer Cell Lines and Cultured Normal Oral Epithelial Cells. Having established that COX-2 is overexpressed in OSCC, we wished to address whether a COX-2-selective inhibitor could induce growth inhibition and/or apoptosis in oral cancer cells. We first examined nine human oral cancer cell lines for COX-2 expression by Western blotting. These cell lines were H103, H1157, H314, H357, H376, H400, H413, BICR31, and BICR56. The deriv-
tion and tumor characteristics of these cell lines have been described by Prime et al. (20) and Edington et al. (21). All of these cell lines expressed COX-2, although H314 had very low levels compared with the other cell lines. Fig. 2 shows Western blot analysis for COX-2 protein in the cell lines selected for treatment with NS398. COX-2 expression was also examined in cultured normal human oral epithelial cells by Western blotting, and the levels of COX-2 in these cells were found to be consistently high (Fig. 2, Lane 1, NK). In addition, there was a pronounced band of approximately M, 65,000, corresponding with the size of uncleaved COX-2 (33), possibly reflecting the synthesis of new COX-2 protein. We consistently found that COX-2 expression was induced by the disaggregation and culture conditions required for the growth of normal keratinocytes (i.e., in the presence of Swiss 3T3 feeder cells, insulin, cholera toxin, transferrin, and EGF), whereas parallel histology of the same tissue samples gave results similar to that shown in Fig. 1A. Because the differential expression of COX-2 that was seen in vivo could not be maintained in vitro, comparing the response of normal and tumor cells could not explore the possible tumor specificity of COX-2-selective growth inhibition. Interestingly, although COX-2 protein production was stimulated, a 72-h treatment with 100 μM NS398 did not induce growth inhibition in normal oral epithelial cells under these culture conditions (data not shown).

The COX-2-selective Inhibitor NS398 Inhibits Growth of OSCC Lines. Four oral carcinoma cell lines (H314, H357, H376, and H413; Fig. 2) were used to determine whether the COX-2-selective inhibitor NS398 is able to inhibit growth of OSCCs. Concentrations of 10, 20, 50, 75, and 100 μM NS398 were used to treat the four oral carcinoma cell lines for 72 h. Growth inhibition was consistently obtained in response to 75 and 100 μM NS398 in all four human oral carcinoma-derived cell lines (Fig. 3). The lowest dose of NS398 to produce statistically significant growth inhibition was 50 μM in H314, H357, and H376 cells and 75 μM in H413 cells.

NS398 Inhibits PGE2 Production at Concentrations Below Those Required to Induce Growth Inhibition at 72 h. To determine the effect of NS398 on PGE2 production, a PGE2 immunoassay was performed on medium collected from the four oral carcinoma-derived cell lines after treatment with NS398 (10–100 μM) for 72 h. PGE2 is the major prostaglandin produced by oral carcinoma cells (29), and the production of secreted PGE2 is therefore an appropriate measure of COX activity. Because very little PGE2 is produced by untreated H314 cells, which have only low levels of COX-2 protein, exogenous arachidonic acid was added to these cultures for 1 h before harvest of the medium. NS398 dramatically reduced PGE2 secretion by all of the four cell lines (Fig. 4). Concentrations of 10 and 20 μM NS398 were effective at inhibiting PGE2 secretion; however, under these conditions, growth inhibition was not observed over 72 h.

Cell Death Is Not the Major Mechanism by Which 50–100 μM NS398 Inhibits Growth of Oral Carcinoma Cells. NS398 has previously been shown to induce COX-2-independent apoptosis in colorectal carcinoma cells (19, 26). To determine whether oral carcinoma cells responded similarly, the extent of apoptosis was measured in parallel with the assessment of the effect of NS398 on cell growth. Cells shed into the medium were counted and examined for apoptotic morphology by acridine orange/ethidium bromide staining. The proportion of cells floating in the medium can be used as a measure of the extent of apoptosis in a culture if the apoptotic nature of the floating cells is confirmed by morphological analysis (4, 19, 34). For these studies, the colon carcinoma cell line HT29, used previously to demonstrate the ability of NS398 to induce apoptosis in colorectal tumor cell lines (19), was used as a positive control. In the HT29 colon carcinoma cell line, a 2.7-fold increase in floating cells was detected in response to 100 μM NS398, and a greater proportion of the floating cells were morphologically apoptotic in the treated cultures compared with the controls (data not shown). This shows that, as in previous studies (19, 26), 100 μM NS398 induces apoptosis to a highly significant extent (P = 0.0008, Student’s t test). By contrast, treatment with NS398 for 72 h did not induce extensive numbers of floating cells in oral carcinoma cell cultures, and a lower proportion of the floating cells were apoptotic than in HT29 (40–55% compared with >90%) in both control and treated cultures. The percentage of floating cells in three of the cell lines remained unchanged by treatment (0.9% in H314 and H376 and 1.5% in H413). The proportion of cells floating in the medium was significantly increased only in H376 cells after treatment with 75 and 100 μM (2.3- and 2.5-fold control, respectively; P < 0.01 for both concentrations). The percentage of cells floating increased from 0.9% in the control cultures to 1.9% in the H376 cultures treated with 100 μM NS398. The percentage of floating cells that were apoptotic was similar in treated and control cultures (48–52%). As a result of relatively limited induction of cell shedding in H376 cells, we examined this further for each cell line. The floating cells shed into the medium were pooled with the adherent cells for Western blot analysis of PARP cleavage, but as expected, no induction of PARP cleavage was detected after treatment with 100 μM NS398 (data not shown). Because levels of apoptosis may be too low to detect by Western blotting for PARP cleavage, cleaved caspase-3 was measured by immunocytochemistry in adherent cells in situ. In these experiments, cells were cultured on glass slides and treated with 100 μM NS398. No increase in cleaved caspase-3-positive cells was detected after NS398 treatment (Fig. 5A), whereas a very significant 2.2-fold increase was detected in HT29 (P = 0.006). Therefore, by contrast to previous findings in colorectal tumor cells, where NS398 induces growth inhibition primarily by apoptosis (19), we did not find apoptosis to be the major mechanism by which NS398 induces growth inhibition in oral carcinoma cells.

Involutin is a cornified envelope protein used as a marker of terminal differentiation (35). To determine whether NS398 induced differentiation of oral carcinoma cells, levels of involucrin were measured by Western blot analysis. Involutin levels were not altered by a 72-h treatment with NS398 at concentrations of 10–100 μM (Fig. 5B).

NS398 Induces a G0-G1 Arrest in Oral Carcinoma Cells. To determine whether growth inhibition by 50–100 μM NS398 was due to changes in cell cycle progression, flow cytometric analysis was performed on cells treated for 24 h with 100 μM NS398. In each of the four cell lines, NS398 induced an increase in the proportion of cells in the G0-G1 phase of the cell cycle and a decrease in S-phase cells. This is illustrated in Fig.
Fig. 1 Expression of COX-2 protein in normal oral epithelium and OSCCs. The COX-2 antibody used was the rabbit COX-2 polyclonal antibody at 1:700. A, normal labial mucosa (inner lip) staining weakly (+) for COX-2 in basal cells. B, overexpression of COX-2 in hyperplasia (H) compared with lower levels of COX-2 staining in adjacent histologically normal epithelium (N). C, OSCC demonstrating cytoplasmic COX-2 expression (+++). D, a comparison of COX-2 staining in a squamous cell carcinoma (T; ++++) compared with weaker staining in adjacent histologically normal epithelium (N; +). E shows the leading edge of a tumor with pronounced cytoplasmic staining. F shows pronounced perinuclear COX-2 staining in the leading edge of a different tumor to E. G and H show the results of COX-2 peptide blocking, demonstrating the specificity of COX-2 staining. G, oral carcinoma stained with 1:700 rabbit anti-COX-2 polyclonal antibody. H, serial section of carcinoma showing the results of incubation of the primary COX-2 antibody at 1:700 in the presence of 1:70 blocking peptide.
6A as an increase in the G₁-G₂-S ratio, which for each of the four cell lines was significantly greater in cells treated with 100 μM NS398 compared with control. The 24 h time point was selected such that the controls had the maximum proportion of cells in S phase. Control cells treated for 72 h had a low percentage of cells in S phase, even if the medium was replenished every day, suggesting that increasing confluence led to a reduction in the proportion of cells that are actively dividing. However, in treated cultures, a G₀-G₁ arrest persisted at 72 h (data not shown). Even after 72 h of NS398 treatment, the S phase was restored 24 h after NS398 removal, indicating that the G₁ arrest was reversible (data not shown). To determine whether the cells were arrested in G₀-early G₁ or whether they were at the G₁-S checkpoint, control cells and those treated with 100 μM NS398 for 72 h were examined by immunocytochemistry for Ki67 expression. Ki67 staining demonstrates a nuclear antigen expressed in all phases of the cell cycle, except G₀ and early G₁ (36). Significantly more cells were negative for Ki67 in the NS398-treated cultures (Fig. 6B), indicating an increase in the proportion of cells in G₀-G₁. Because others have reported that the cyclin-dependent kinase inhibitors p21⁰⁰⁰¹⁰⁰⁰¹ and p27⁰⁰⁰¹⁰⁰⁰¹ may be involved in NS398-induced growth arrest (37, 38), we examined the levels of these proteins at 24, 48, and 72 h of treatment. In two of the cell lines, H357 and H376, p21⁰⁰⁰¹⁰⁰⁰¹ was elevated by NS398 at 24 h. However, only in H357 did this elevation persist throughout the time course (Fig. 6C). In H376 cells, p21⁰⁰⁰¹⁰⁰⁰¹ was reduced by 72 h to levels below those seen in the controls. In H314 and H413 cells, p21⁰⁰⁰¹⁰⁰⁰¹ expression remained low and unchanged throughout the time course. We did not find any marked induction of p27⁰⁰⁰¹⁰⁰⁰¹ after NS398 treatment in any of the cell lines (data not shown). In addition, Western blotting for Rb did not show any changes in Rb phosphorylation (data not shown).

Growth Inhibition of H357 Cells Induced by 5 μM NS398 Is Abrogated by PGE₂ Addition and Is Therefore COX-2 Dependent. To confirm that lower concentrations of NS398 could suppress COX-2 activity, a PGE₂ assay was conducted on culture medium from H357 cells treated with 1, 5, and 10 μM NS398 for 72 h. At these concentrations, NS398 is highly selective for COX-2 rather than COX-1 (11). Concentrations of 5 and 10 μM NS398 reduced the secreted PGE₂ levels in undiluted medium to levels below the limits of accurate detection of the assay (Fig. 7A), whereas the control medium had to be diluted to obtain values within the standard curve. To determine whether NS398 could induce COX-2-dependent growth inhibition if cells were treated for a longer time period, H357 cells were treated for 12 days, and medium was changed with fresh reagents every 48 h. Five μM NS398 was consistently growth-inhibitory (Fig. 7, B–D). We hypothesized that this growth inhibition was COX-2 dependent and resulted from depletion of secreted prostaglandins. If this were the case, addition of the most commonly secreted prostaglandin, PGE₂, might be expected to abrogate the growth inhibition. To determine the appropriate amount of PGE₂ to add to the culture medium, an experiment was first conducted in which varied concentrations of PGE₂ were added into cultures in the presence of 5 μM NS398. This was done to take into account the possibility that PGE₂ may degrade or be internalized into cells and served to determine the concentration of PGE₂ to add to the medium such that 48 h after addition, the concentration of PGE₂ in the medium of the cultures receiving the combination treatment of NS398 and PGE₂ would be approximately equal to that in the control cultures. Fig. 8A shows that addition of 2000 pg/ml PGE₂ results in similar concentrations of PGE₂ in the medium to the controls at the time of harvest. This concentration of PGE₂ was then used to evaluate the importance of reduced PGE₂ secretion in the growth-inhibitory response of the cells to 5 μM NS398. Addition of 2000 pg/ml PGE₂ completely abrogated the growth inhibition induced by 5 μM NS398 (Fig. 8, B and C). It should be noted that under these experimental conditions, addition of PGE₂ alone did not stimulate the growth of H357 cells, suggesting that the amount of endogenously produced PGE₂ in control cultures is not limiting the growth of the cells (Fig. 8B). We then addressed whether the growth inhibition induced by 100 μM NS398 over 6 days of treatment could be restored by addition of 2000 pg/ml PGE₂. The cell yields from cultures treated with the combination of 100 μM NS398 and 2000 pg/ml PGE₂ were not significantly different from those treated with 100 μM NS398 alone (Fig. 8C). In these experiments, the proportion of cells shed into the medium was evaluated to determine whether longer treatments with NS398 induced cell death by either COX-2-dependent or -independent pathways. No induction of cell shedding was obtained after treatment with either 5 or 100 μM NS398 after 6 days of treatment (data not shown). This indicates that COX-2 inhibition does not induce apoptosis or other forms of cell death in this cell line and that the extensive growth inhibition that is induced by a 6-day treatment with 100 μM NS398 is not accompanied by cell death. Using the same experimental approach, we confirmed that inhibition of COX-2 by a 6-day treatment with 5 μM NS398 does not induce apoptosis in OSCCs using a different cell line (H376; data not shown).

DISCUSSION

Because the basis behind the concept of using COX-2-selective inhibitors for cancer prevention or treatment is the observation that COX-2 protein is overexpressed in tumor relative to normal tissue, if COX-2 selective inhibitors are to be considered for prevention or treatment of human oral cancer, it is important to know the extent to which COX-2 is overexp
pressed and whether this is a feature of specific subsets of oral carcinomas. We investigated COX-2 expression in a series of 37 OSCCs and 23 samples of normal oral epithelium. COX-2 was only weakly expressed in normal epithelium, and a gradient of expression was observed with COX-2 confined to the basal and suprabasal proliferative layers. COX-2 protein has been reported to be absent from oral stratified squamous epithelium in some studies (39–41). However, Shiotani et al. (17) detected weak basal staining in rat normal tongue epithelium, and, more recently, Renkonen et al. (42) found only 25% of 38 normal human tongue samples were totally negative for COX-2 staining. In our study, 6 of the 23 normal samples were totally negative for COX-2 expression (26%). Our findings are therefore in close agreement with those of Renkonen et al. (42), who used the same COX-2 antibody, and this may reflect antibody sensitivity because we found that COX-2-blocking peptide completely blocked staining.

In our tumor series, the epithelial cells of the tumors had stronger COX-2 staining than either independent samples of normal epithelium or adjacent histologically normal epithelium. In tumors, we noted that COX-2 expression was often highest, with pronounced perinuclear localization, at the leading edge of the tumor. Well-differentiated tumors often retained the gradient of expression seen in normal oral epithelium with high levels in the basal cells. This is in contrast to the observations made by Jaeckel et al. (41) that COX-2 is highly expressed in keratin whorls of well-differentiated tumors. However, our observations of high COX-2 expression in hyperplastic and dysplastic oral epithelium agree with observations by Nathan et al. (40) and Renkonen et al. (42). Our observations that COX-2 protein is expressed in the proliferative compartment of normal oral epithelium and that this pattern is retained in well-differentiated tumors suggest that there may be a relationship between COX-2 expression and cell proliferation. This is substantiated by our functional studies of COX-2 in oral carcinoma cells, to be discussed below, which demonstrate that COX-2 contributes to cell proliferation.

Increased Cox-2 transcriptional activity has been shown to result from genetic changes such as p53 mutation (43), Ha-ras mutation (9), or EGF receptor overexpression (44) that are common in oral carcinogenesis. In their study of tongue tumors, Renkonen et al. (42) found that stage III tumors had higher COX-2 expression than stage I or II tumors. By contrast, in our series of diverse oral tumors, we found that stage I or II tumors tended to have higher COX-2 expression than stage III and IV tumors. Interestingly, further novel findings shown by our immunohistochemical study were that COX-2 expression in OSCC depended on the site of origin of the tumor within the oral cavity (tumors of the buccal mucosa had the highest levels of expression) and also on whether or not the patient smoked tobacco. There was significantly higher COX-2 expression in the tumors of those patients who had never smoked. It would be interesting to confirm this in a larger study. The increased expression of COX-2 during carcinogenesis may therefore depend on tumor etiology, different regions of the oral epithelium being subjected to slightly different environmental insults, and may reflect different oral carcinogenesis pathways.

In other tumor types in which COX-2 is overexpressed, COX-2-selective inhibitors are showing antitumor activity in vitro and in vivo. However, the dose required to inhibit tumorigenesis in vivo by nonselective NSAIDs or COX-2-selective inhibitors is frequently far greater than that required to inhibit COX-2 activity, suggesting the existence of additional mechanisms of action of these drugs (45). For oral cancer chemoprevention, topical treatment may be a viable option, and hence the effects of relatively high concentrations of these inhibitors are of clinical relevance. We initially set out to determine whether NS398 would induce growth inhibition over a concentration range that has previously been shown to induce apoptosis in several other cell lines, for example in colon carcinoma (19), prostate carcinoma (46), esophageal carcinoma (28, 47), and hepatocellular carcinoma cell lines (48). In these cell types, apoptosis is a major mechanism of NS398-induced growth inhibition.

The COX-2 inhibitor NS398 induced growth inhibition in four oral cancer cell lines. Although H314 cells had very low
levels of COX-2, they were significantly growth inhibited by 50 \mu M NS398. However, by contrast to previous findings of the induction of apoptosis in colorectal tumor cells (19), no marked apoptosis was detected in the oral carcinoma cells in response to NS398. Instead, a G0 -G1 arrest was induced, which proved to be reversible. The proportion of cells staining positively for Ki67 was reduced similarly in all four oral carcinoma cell lines after NS398 treatment, suggesting that the arrested cells were not at the G1-S boundary. In H357 and H376 cells, NS398 induced a greater G0-G1 arrest and a greater reduction in cell yield at 72 h than in the other two cell lines. This correlates with the induction of p21WAF-1 in these two cell lines and suggests that p21WAF-1 may contribute to the growth arrest in some carcinoma cells but that the arrest cannot always be attributed to its induction. Although we cannot rule out the accumulation of cells in early G1, it is likely that at least some of the cells were stimulated to exit the cell cycle. Despite this, there was no increase in the terminal differentiation marker, involucrin. Nor did we detect any changes in cellular morphology that would indicate induction of replicative senescence, as previously observed in response to some NSAIDs in head and neck squamous cell carcinoma cell lines (49). Indeed, the S phase recovered 24 h after removal of the NS398 from the culture. The reversibility was further confirmed by experiments showing that NS398 treatment for 72 h did not decrease the colony-forming efficiency of H376 cells (data not shown).

The dramatic reduction of PGE2 production induced by 10 and 20 \mu M NS398 in oral carcinoma cells without the induction of growth inhibition suggests that the G0-G1 arrest induced by higher concentrations (50-100 \mu M) of NS398 is not simply due to reduced PGE2 secretion. Therefore, we questioned whether treatment with NS398 over a longer period of time would result in inhibition of cell proliferation that was dependent on inhibition of COX-2 activity. In H357 cells, we found growth inhibition with 5 \mu M NS398 after 6-12 days of treatment. We then investigated whether the growth inhibition was due to depletion of the major prostaglandin in oral cancer cells, PGE2. The answer to this question was not necessarily predictable because although PGE2 is the

Fig. 4 Inhibition of PGE2 secretion by NS398 treatment. The four oral carcinoma cell lines were treated with solvent control and 10, 20, 50, 75, and 100 \mu M NS398 for 72 h. Measuring production of secreted PGE2 assessed COX-2 enzyme activity. A, H314; B, H357; C, H376; D, H413. H314 produced low basal levels of PGE2; therefore, for this cell line, a 1-h arachidonic acid pretreatment (black bars) was used to enhance PGE2 production before harvest. Samples were diluted such that upper values were within the limits of accurate detection (i.e., within the standard curve). The lower levels of accurate detection of the assay were (A) 16 pg/ml, (B) 23 pg/ml, (C) 18 pg/ml, and (D) 25 pg/ml; \( \Psi \) indicates concentrations that were lower than these. The results shown are results of duplicate measurements. Similar results were obtained in repeat experiments.

Fig. 5 NS398 does not induce apoptosis or terminal differentiation in oral carcinoma cells. A, immunocytochemical staining for cleaved caspase-3. The four oral carcinoma cell lines were cultured on glass slides and treated in exponential growth with 100 \mu M NS398 or with DMSO (solvent controls) for 72 h. Cells were fixed and labeled with cleaved caspase-3 antibody, and the proportion of positively labeled cells was scored out of a total of over 1000 cells counted. Results shown are the means \pm SE of three experiments. **, \( P < 0.01 \). B, Western blot analysis of the effect of 100 \mu M NS398 treatment for 72 h on the expression involucrin in the four oral carcinoma cell lines. The cell lines were treated with DMSO only (Con) or with 100 \mu M NS398. Equal loading was confirmed by reprobing for \( \alpha \)-tubulin.
In our experiments, there was no evidence for NS398-induced apoptosis. Thus, we can conclude that NS398 inhibits growth of oral carcinoma cells through mechanisms other than apoptosis.

The most abundant prostaglandin produced by oral cancer cells is PGE2. PGE2 has been reported to be growth inhibitory to oral cancer cells rather than growth stimulatory. Although PGE2 did not stimulate growth by itself, it was able to abrogate the growth-inhibitory effects of 5 μM NS398. These results demonstrate that in oral carcinoma cells, the growth inhibition induced by 5 μM NS398 is COX-2 dependent because at this concentration, NS398 is highly selective for COX-2. In addition, the results are consistent with depletion of prostaglandin product resulting in growth inhibition rather than the build up of COX substrate. This is important because one of the mechanisms by which NSAIDs have been proposed to induce growth inhibition is through apoptosis induced by accumulation of arachidonic acid, which is then converted to the apoptosis signaling intermediate, ceramide. The growth-inhibitory response to prostaglandin depletion is not rapid but is sustained in the presence of the COX-2 inhibitor.

In our experiments, there was no evidence for NS398-
induced apoptosis, either at the higher concentrations that induce PGE₂-independent growth inhibition or at low concentrations that induce growth inhibition that is dependent on reduced PGE₂ secretion. However, COX-2 overexpression may promote cell survival in unfavorable environments such as situations of limited survival factor availability or where cell-cell or cell-matrix interactions are compromised. The recent observations that lung carcinoma cells undergo apoptosis in response to 100 μM NS398 only in reduced serum conditions and not in 10% serum conditions (52) and that glioma cells treated with 25 μM NS398 only underwent apoptosis in spheroid cultures and not in monolayer culture (53) would appear to lend support to this hypothesis, but it has yet to be shown if the apoptosis is due to COX-2 inhibition, or if this is a COX-2-independent response to NS398.

The biological significance of COX-2 overexpression in oral cancers may not only be related to increased cell proliferation. It is also possible that COX-2 may promote cellular motility, increase propensity for invasion and metastasis, promote angiogenesis, or inhibit immune surveillance mechanisms, as has been shown in other cell types. Clearly, additional studies of the mechanisms of action of COX-2-selective inhibitors are needed, in particular to determine which cellular responses are COX-2 dependent and which are COX-2 independent, but the overexpression of COX-2 in oral carcinogenesis and the involvement of PGE₂ in oral carcinoma cell proliferation strongly suggest that such studies are warranted. Our in vitro system allows the COX-2-dependent cellular responses to COX-2-selective inhibitors to be distinguished and can therefore be used in preclinical models to evaluate the functional importance of COX-2 in oral carcinogenesis and as a molecular target for oral cancer chemoprevention or therapy.

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