Cyclooxygenase-2 Inhibition Suppresses αvβ6 Integrin–Dependent Oral Squamous Carcinoma Invasion

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Abstract

Worldwide oral squamous cell carcinoma (OSCC) represents about 5.5% of all malignancies, with ~30,000 new cases each year in the United States. The integrin αvβ6 and the enzyme cyclooxygenase-2 (COX-2) are implicated in OSCC progression and have been suggested as possible therapeutic targets. Each protein also is reported to identify dysplasias at high risk of malignant transformation, and current clinical trials are testing the efficacy of nonsteroidal anti-inflammatory drugs (NSAID) at preventing OSCC development. Given the probable role of NSAIDs in malignant transformation, and current clinical trials are expressing both αvβ6 and COX-2 in OSCC and the inhibition of several integrins by NSAIDs, we investigated whether NSAIDs affected αvβ6–dependent cell functions. We found that expression of both αvβ6 and COX-2 was significantly higher in OSCC compared with oral epithelial dysplasias. Neither protein preferentially identified those dysplastic lesions that became malignant. Using OSCC cell lines, modified to express varying levels of αvβ6, we assessed the effect of COX-2 inhibition on cell invasion. We found that the COX-2 inhibitor NS398 inhibited specifically αvβ6-dependent, but not αvβ3-independent, OSCC invasion in vitro and in vivo, and this effect was modulated through prostaglandin E2 (PGE2)–dependent activation of Rac-1. Transient expression of constitutively active Rac-1, or addition of the COX-2 metabolite PGE2, prevented the anti-invasive effect of NS398. Conversely, RNA interference down-regulation of Rac-1 inhibited αvβ6-dependent invasion. These findings suggest that COX-2 and αvβ6 interact in promoting OSCC invasion. This is a novel mechanism that, given the ubiquity of αvβ6 expression by head and neck cancers, raises the possibility that NSAIDs could protect against OSCC invasion. (Cancer Res 2006; 66(22): 10833–42)

Introduction

Cyclooxygenases (COX) catalyze the key step in prostanoid and thromboxane biosynthesis and are targets of nonsteroidal anti-inflammatory drugs (NSAID). Two human isoforms exist: COX-1, expressed constitutively in most mammalian cells, generates prostaglandins necessary for normal physiologic function, whereas COX-2, normally undetectable, is induced rapidly by stimuli, including cytokines, oncogenes, and tumor promoters (1, 2). Elevated COX-2 expression occurs in many carcinomas, including oral squamous carcinoma (OSCC), where it contributes to tumor progression (1–3). Transgenic female mice overexpressing COX-2, for example, have a high frequency of breast dysplasia and metastatic tumors (4), whereas genetic inactivation of COX-2 in a murine model of familial adenomatous polyposis reduced both the size and number of intestinal polyps (5).

The epithelial integrin αvβ6 is a receptor for fibronectin, vitronectin, tenasin, and the latency-associated peptide (LAP) of transforming growth factor–β (e.g., ref. 6). Generally found to have minimal expression in adult epithelium, αvβ6 is up-regulated during epithelial remodeling (e.g., wound healing) and tumorigenesis. Aberrant αvβ6 expression occurs in numerous carcinoma types, particularly OSCC (7–11). Moreover, increased expression of both αvβ6 and COX-2 have been reported in oral dysplastic epithelium, suggesting a role in tumor progression (12, 13).

NSAIDs have marked antitumor activity via COX-2 inhibition. Selective COX-2 inhibitors are chemopreventive in animal models of colon, bladder, and breast cancer (1, 2), whereas epidemiologic studies indicate that NSAIDs reduce breast, esophageal, and colon cancer mortality (1, 2). The effects of NSAIDs on tumor growth and progression are likely to be through multiple mechanisms, including indirect effects on angiogenesis and inflammation, as well as direct effects on tumor cell proliferation and apoptosis (1, 2). Particularly interesting are studies where NSAIDs inhibit tumor cell adhesion, migration, and invasion (1, 2, 14). These changes may be mediated via effects on various integrins (15, 16). For example, aspirin and indomethacin inhibit activation of the platelet integrin αIβ3 (17) whereas Dormond et al. inhibited endothelial cell migration and angiogenesis using the selective COX-2 inhibitor NS398, effects that were modulated through inhibition of the αvβ3-dependent activation of Rac-1 and Cdc42 (18). This may be a reciprocal effect because integrin-mediated adhesion can contribute to stabilization of COX-2 protein levels (19).

Given that both αvβ6 and COX-2 are expressed in OSCC and have been reported to promote invasion (20–22), and that NSAIDs may inhibit integrin function, we determined whether NSAIDs could inhibit αvβ6–mediated invasive activity.

Materials and Methods

Immunohistochemistry. Antibodies used were anti-cytokeratin, AE1/AE3 (1:50; DAKO, High Wycombe, United Kingdom); anti-αv, 6.2G2 (0.5 μg/mL; Biogen Idec, Cambridge, MA); or anti-COX-2 (1:500; Cayman Chemical Co., Ann Arbor, MI). Antigen retrieval varied according to primary antibody: 0.1% α-chymotrypsin, 0.1% calcium chloride (pH 7.8) for

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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20 minutes at 37°C (AE1/AE3); Digest-All 3 Pepsin Solution (Invitrogen Corporation, Carlsbad, CA) for 5 minutes at 37°C (6.262); microwaving for 30 minutes in 0.1 mol/L citrate buffer (pH 9; COX-2). Endogenous peroxidase was neutralized with 0.45% hydrogen peroxide in methanol for 15 minutes and primary antibodies applied in TBS (pH 7.6) for 1 hour. Anti-mouse IgG biotinylated secondary antibody (Vectastain Elite ABC Reagent; Vector Laboratories, Burlingame, CA) was applied for 30 minutes followed by peroxidase-labeled streptavidin (Vectastain Elite ABC Reagent; Vector Laboratories) for 30 minutes. Peroxidase was visualized using DAB+ (DAKO) for 7 minutes and counterstained in Mayer's hematoxylin (Sigma-Aldrich, Dorset, United Kingdom).

Twenty OSCC, 39 epithelial dysplasias (18 transforming to OSCC and 21 nontransforming; clinical follow-up range, 5-31 years), and 14 benign polyps showing fibroepithelial hyperplasia were chosen at random; stained for α5b6 and COX-2 and scored according to the Quickscor method (23). The staining intensity was scored out of three (1, weak; 2, moderate; 3, strong), and the proportion of epithelial cells staining positively was scored out of 4 (1, <25%; 2, 25-50%; 3, 51-75%; 4, 76-100%). The score for intensity was added to the score for proportion to give a score in the range of 0 to 7 and grouped as − (score = 0), + (score = 1-3), ++ (score = 4-5), or +++ (score = 6-7).

**Cell culture.** Using cDNA transfection techniques, we created a panel of cell lines expressing various levels of α5b6 (20, 21). H357 is an α5-negative OSCC cell line (24) from which the V3 cell line was generated by transfection of α5 cDNA (25); V3 cells express low levels of α5b6. This cell line was retrovirally infected with b5b6 cDNA, creating the VB6 cell line, which has high α5b6 expression. A null transfectant control cell line for the VB6 cells (C1) also was generated at this time (20, 21). BIRC6, CAI, and H157 OSCC cell lines were also used; provided by Professors K. Parkinson (BICR6) and I. Mackenzie (CA1) of the Queen Mary’s University, London and Professor S. Prime (H357 and H157) of the University of Bristol Dental School. OSCC cells were grown in keratinocyte growth medium (KGM; ref. 20). Human foreskin fibroblasts (supplied by Cell Services of Cancer Research UK, London Research Institute) were maintained in fibroblast growth medium (DMEM supplemented with 10% FCS) at 37°C in an humidified atmosphere.

**Flow cytometry.** Flow cytometry was done (20), using anti-α5b6 antibody (E7P6; Chemicon International, Harrow, United Kingdom) and FITC-conjugated secondary antibody (DAKO). Negative control used secondary antibody only and was subtracted from the results. Labeled cells were scanned on a FACSCalibur cytometer (BD BioSciences, Oxford, United Kingdom) and analyzed using CellQuest software, acquiring 1 × 10^5 events. Results show mean fluorescence (arbitrary units, log scale).

**RNA interference.** RNA interference (RNAi) SMART pool reagents targeting b5b6, Rac-1, COX-2, or control (random) sequences were obtained from Dharmacon (Chicago, IL). Cells were seeded into six-well plates and left for 24 hours until ~40% confluent, then transfected with 100 pmol/well of the relevant duplex pool using Oligofectamine transfection reagent (Invitrogen, Paisley, United Kingdom). Cells were used in assays after 24 to 48 hours. Cells were also lysed and used to verify protein knockdown by Western blotting analysis.

**Modulation of Rac-1 activity.** Cells were transfected with enhanced green fluorescent protein (EGFP)-tagged, constitutively active Rac-1 [V12Rac-1-GFP (construct made by J. Monypenny, GKT, London, United Kingdom) or vector control (pEGFP-C2; Invitrogen)]. Cells at 70% confluency in six-well dishes were transfected with 0.8 μg of DNA using Effectene transfection reagent (Qiagen, Reutlingen, Germany). Results are expressed relative to invasion of VB6 control treatment (= 100). For blocking experiments, anti-α5b6 antibody (1D05, 1:100 or 6.3G9, 1:100; Biogen Idec), NS398 (20 μmol/L), SC-560 (10 μmol/L), indomethacin (500 μg/mL), prostaglandin E2 (PGE2; 1-10 ng/mL; Cayman Chemical), PGE2 (1-10 ng/mL; Cayman Chemical), and the thromboxane A2 mimetic U46619 (1-10 μmol/L; CN Biosciences) were added to the cells for 30 minutes before plating and were present throughout the experiment.

**Western blotting.** Cells were lysed in NP40 (COX-2) or radioimmuno precipitation assay (Rac-1) buffer. Samples containing equal protein were electrophoresed under reducing conditions in 10% (COX-2) or 15% (Rac-1) SDS-PAGE gels. Protein was electroblotted to nitrocellulose (COX-2) or polyvinylidene difluoride (Rac-1) membranes (GE Healthcare, Buckinghamshire, United Kingdom). Blots were probed with antibodies against COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA) or Rac-1 (Upstate, Lake Placid, NY). Horseradish peroxidase-conjugated anti-goat or anti-mouse (DAKO) and Pierce, Rockford, IL) were used as secondary antibodies. Bound antibodies were detected with the enhanced chemiluminescence Western blotting detection kit system (GE Healthcare or Pierce). Blots were probed for HSC70 (Santa Cruz Biotechnology) as a loading control.

**Determination of Rac-1 activity.** The Rac-1 interactive binding (CRIB) domain of the downstream Rac-1 effector, p21-activated kinase (PAK), was used to pull down active Rac-1 from cell lysates. A construct encoding glutathione S-transferase (GST) linked to the amino acid residues 57 to 141 of the PAK-CRIB domain (GST-PAK-CRIB; provided by J. Collard, King’s College, London, United Kingdom) was transformed into BL21 bacteria, and production of the fusion protein induced using 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside (Sigma). Bacteria were resuspended [50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L MgCl2, 100 mmol/L NaCl], sonicated, and centrifuged. The supernatant was incubated with glutathione-coupled Sepharose 4B beads (Amersham Biosciences) for 30 minutes at 4°C. Beads were washed in resuspension buffer and stored in glycerol at ~80°C. Cells were plated onto LAP-coated 10-cm cell culture dishes (0.5 μmol/L) for 24 hours following targeting of b5b6 using RNAi, or treated with 50 μmol/L NS398 ± PGE2 (2-4 ng/mL) and compared with DMSO control. Cells were lysed with 50 mmol/L Tris-HCl (pH 7.5), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 10 mmol/L MgCl2, 5 μg/mL leupeptin, 5 μg/mL Pefabloc, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)]. Cleared cell lysates were incubated with the GST-PAK-CRIB beads for 40 minutes at 4°C and washed thrice [50 mmol/L Tris-HCl (pH 7.5), 1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L MgCl2, 5 μg/mL leupeptin, 5 μg/mL Pefabloc, 1 mmol/L PMSF]. Samples of lysate preincubation and postincubation with the GST-PAK-CRIB beads were probed by Western blot under reducing conditions. The proportion of active Rac-1 relative to the total amount in the cells was determined by densitometry (Scion software).

**Organotypic culture.** Organotypic cultures with an air-interface tissue were prepared as described (26). Gels comprised a 50:50 mixture of Matrigel (Becton Dickinson, Oxford, United Kingdom) and type I collagen (Upstate) containing 5 × 10^3/mL human foreskin fibroblasts, to which were added 5 × 10^3 OSCC cells. For inhibition studies, NS398 (20 μmol/L) with or without PGE2 (1-4 μmol/L) was added to the KGM. The medium was changed every 2 days. After 6 or 14 days, the gels were bisected, fixed in formal-saline, and processed to paraffin. Four-micrometer sections were immunostained with the pan-cytokeratin antibody AE1/AE3 (DAKO).
Quantitative analysis. Invasion in organotypic culture was quantified as described (26). Immunostained sections were viewed at ×100 magnification and digitally photographed. The digital image was converted to greyscale (OptiLab 2.6.1, Grafeik Imaging, Inc., Austin, TX), and an “invasion index” was calculated from this image. This gave a quantitative value for tumor invasion (the product of the average depth of invasion, the number of invading particles, and the area of the invading particles; ref. 26). Three 4-μm sections spaced at 150 μm were analyzed for each in vitro gel.

Transplantation of organotypic cultures onto nude mice. All animal experiments were done according to the guidelines of the local ethics committee and were as specified in the project license. Organotypic cultures were grown in vitro for 7 days before being transplanted into 10-week-old athymic nude (nu/nu) mice as described previously (26). Seven days after transplant, test animals were given 5 mg/kg NS398 i.p. every 72 hours, whereas control animals received vehicle (1:100 DMSO in PBS) only. At 6 weeks, animals were killed, and the transplants were disected en bloc and further processed for histology. Five 4-μm sections spaced at 150 μm were analyzed for each in vivo gel. Sections where the tumor epithelium deviated significantly from the horizontal were excluded from analysis.

Statistical analysis. Data are expressed as the mean ± SD of a given number of observations. Where appropriate, one-way ANOVA was used to compare multiple groups. Comparisons between groups were by Fisher’s PLSD,  unknowable MS was considered significant. Figures show representative examples of independent repeats with error bars representing SD unless stated otherwise.

Results

αvβ6 and COX-2 expression in hyperplastic and dysplastic epithelium and in OSCC. We examined the expression and distribution of αvβ6 and COX-2 expression in hyperplastic and dysplastic epithelium and OSCC by immunohistochemistry. Results are summarized in Table 1. Of 39 dysplastic lesions examined, moderate (+++) to strong (+++++) expression of αvβ6 and COX-2 was present in 21% and 31%, respectively. Neither protein preferentially identified lesions, which subsequently transformed to OSCC, and similar staining sometimes was found in hyperplastic epithelium. In contrast, αvβ6 and COX-2 were both up-regulated in established OSCC, with moderate to strong expression in 85% and 80% of tumors, respectively. Staining for both proteins was often more intense at the periphery of invading tumor islands (Fig. 1).

Table 1. αvβ6 and COX-2 expression in epithelial hyperplasia, dysplasia, and OSCC

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<th>αvβ6 expression</th>
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<td>Hyperplastic epithelium</td>
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NOTE: Twenty OSCCs, 39 epithelial dysplasias with a minimum of 5 years of clinical follow-up (21 nontransforming and 18 transforming to OSCC), and 14 benign polyps showing fibroepithelial hyperplasia were chosen at random and stained for αvβ6 and COX-2 before being scored according to the Quickscore method (23). Briefly, the staining intensity was scored out of three (1, weak; 2, moderate; 3, strong), and the proportion of the tumor cells staining positively was scored out of four (1, <25%; 2, 25-50%; 3, 51-75%; 4, 76-100%). The score for intensity was added to the score for proportion to give a score in the range of 0 to 7 and grouped as − (score = 0), + (score = 1-3), ++ (score = 4-5), or +++ (score = 6-7). There was significantly less expression of both αvβ6 and COX-2 in hyperplastic or dysplastic epithelium compared with OSCC. Neither protein preferentially identified the subgroup of dysplasias that subsequently transformed.

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NSAIDs Suppress αvβ6-Dependent Invasion

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COX-2 inhibition suppresses αvβ6-dependent cell invasion in Transwell assays. From the H357 αv-negative OSCC line, a high αvβ6 expressing line (VB6) was generated using cDNA transfection and retroviral infection (20, 21). A null transfectant line (C1), expressing low levels of αvβ6, also was created (Fig. 2B; ref. 20). As shown previously, VB6 cells are significantly more invasive than C1 control cells, and the increased invasion is αvβ6 dependent (Fig. 2A; ref. 20).

The cell lines were examined for COX-2 expression by Western blotting. All three lines expressed the 72-kDa COX-2 protein at similar levels (Supplementary Fig. S1A). An additional band (~ 65 kDa), corresponding in size with unglycosylated COX-2 (3), was apparent, possibly reflecting synthesis of new COX-2 protein. Inhibition of αvβ6 with blocking antibodies did not effect COX-2 expression in VB6 cells (data not shown).

We assessed the effects of NSAIDs on αvβ6-dependent OSCC Transwell invasion using SC-560 (COX-1 inhibitor), NS398 (COX-2 inhibitor), and indomethacin (nonselective COX inhibitor). Over 16 experiments, invasion of the high αvβ6-expressing VB6 cells was inhibited significantly using NS398 (Fig. 2A; P < 0.00001). Indomethacin produced similar results (Fig. 2A; P < 0.0001). COX-2 inhibition in VB6 cells reduced invasion to control C1 levels, comparable with inhibiting αvβ6 with blocking antibodies. NS398 combined with αvβ6 blocking antibodies did not further reduce VB6 invasion. Because VB6 invasion of Matrigel is αvβ6 dependent (Fig. 2A; ref. 20), COX-2 seems to promote αvβ6-dependent invasive activity. Inhibition of COX-1 did not affect VB6 invasion (P < 0.19). C1 control cells invaded poorly and were unaffected by these treatments (Fig. 2A). H357 cells lack αv integrins and invade Matrigel using α3β1- and α6-dependent mechanisms (data not shown). Inhibition of COX-2 (NS398 or indomethacin) had no significant effect on H357 invasion (P < 0.424 and P < 0.331, respectively; Fig. 2A). Inhibition of VB6 but not H357 cells by NS398 indicates the effects are αvβ6 specific.

The invasion assay was repeated using higher concentrations of NS398 (titrated at 0.5, 5, and 50 μmol/L). VB6 invasion inhibition was evident at 0.5 μmol/L (P = 0.014) with maximum inhibition at 5 μmol/L (P < 0.002; Fig. 2A). No effect on H357 or C1 invasion was seen even at 50 μmol/L NS398.

To confirm that suppression of VB6 invasion was not due to growth inhibition. Cells were grown on Matrigel gels (diluted 1:2 in α-MEM) for 72 hours ± NS398, extracted using cell dispersal
solution (BD Biosciences), and counted (Casy 1 counter; Sharpe System). No inhibition of growth occurred in H357, VB6, or C1 cells (data not shown; \( P < 0.448, P < 0.306, \) and \( P < 0.581 \), respectively). Similarly, NS398 had no effect on proliferation of cells on uncoated plastic (data not shown).

Because VB6 cells have been genetically manipulated to express high levels of \( \alpha_v\beta_6 \), invasion assays were carried out using three OSCC cell lines (BICR6, CA1, and H157), which express high levels of endogenous \( \alpha_v\beta_6 \) and show \( \alpha_v\beta_6 \)-dependent invasion in Transwell assays (Fig. 2B). Invasion of all three cell lines was inhibited by NS398 (Fig. 2B; BICR6, \( P < 0.0001; \) CA1, \( P < 0.0001; \) H157, \( P < 0.011 \)).

NSAIDs have been reported to have antitumor effects through both COX-2-dependent and COX-2-independent mechanisms (1, 2). To test whether suppression of \( \alpha_v\beta_6 \)-dependent invasion by NS398 was modulated specifically through inhibition of COX-2, the invasion assays were repeated following RNAi knockdown of COX-2 protein. Forty-eight hours after RNAi treatment, levels of COX-2 protein were reduced in H357, VB6, and C1 cells by 50%, 50%, and 69%, respectively; the (presumed) 65-kDa unglycoylated form had disappeared completely (Supplementary Fig. S1B). Suppressing COX-2 protein reduced invasiveness of VB6 cells to control C1 levels but had no effect on H357 or C1 invasion (Supplementary Fig. S1B; \( P < 0.003, P < 0.86, \) and \( P < 0.89 \), respectively).

**PGE\(_2\) restores \( \alpha_v\beta_6 \)-dependent invasion.** To determine whether COX-2 modulated \( \alpha_v\beta_6 \)-dependent invasion through a metabolic product of COX, invasion assays were repeated, but adding NS398 with PGE\(_2\), PGF\(_{2\alpha}\), or U46619 (a thromboxane A\(_2\) mimetic). PGE\(_2\) at 2 to 4 ng/mL completely abrogated the anti-invasive activity of NS398 (Fig. 2C; \( P < 0.0017 \)), whereas PGF\(_{2\alpha}\) and U46610 had no effect (Fig. 2C; \( P < 0.444 \) and \( P < 0.276 \), respectively). PGE\(_2\) also seemed to produce some restoration of invasion in the presence of \( \alpha_v\beta_6 \) blocking antibodies, although this did not reach significance (\( P = 0.09 \); data not shown).

Interestingly, the dose response of VB6 cells to PGE\(_2\) produced a bell-shaped curve, with restoration of invasion between a narrow spectrum of concentrations (2-4 ng/mL) but not at higher concentrations (Fig. 2C). Indeed, in the absence of NS398, PGE\(_2\) at concentrations of \( \geq 10 \) ng/mL inhibited invasion of all three cell lines (data not shown). Similar biphasic response curves have been described previously, and it is suggested that this may result from prostaglandins interacting nonspecifically with other classes of prostanoid receptors (27).

PGE\(_2\) levels were measured by ELISA (Cayman Chemicals); the cell lines produced similar levels of PGE\(_2\). NS398 treatment or RNAi knockdown of COX-2 inhibited prostaglandin production by >95%, suggesting that synthesis of the prostaglandin was mediated largely through COX-2 rather than COX-1 (Fig. 2C).

**COX-2 inhibition suppresses \( \alpha_v\beta_6 \)-dependent invasion in organotypic culture.** NS398 consistently inhibited the \( \alpha_v\beta_6 \)-dependent invasion of VB6 cells in Transwell assays. Such assays, however, oversimplify the invasive process because stromal cells interact dynamically with tumor cells *in vivo* to promote invasion (28). We, therefore, used organotypic cultures containing fibroblasts to measure invasion (26).

We transfected VB6 cells with \( \beta_6 \) RNAi and measured invasion. Because RNAi effects are transient, organotypic cultures were harvested after 6 days (7 days after transfection). Sections were analyzed to generate an invasion index, as described (26). Seven days after transfection, RNAi knockdown of \( \beta_6 \) was \( \sim 50\% \) (3 days, 91%; 5 days, 54%; data not shown), and VB6 invasion was inhibited by >95% (Fig. 3A and D). As in Transwell assays, the high \( \alpha_v\beta_6 \)-expressing VB6 cells were significantly more invasive than C1 control cells (\( P < 0.0002 \)) but invaded at similar levels to the invasive, \( \alpha_v\)-negative H357 cells (Fig. 3B-D; \( P < 0.07 \)). NS398

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**Figure 1.** Immunohistochemistry showing representative \( \alpha_v\beta_6 \) and COX-2 expression in hyperplastic oral epithelium (A), dysplastic oral epithelium (B), and OSCC (C). Although expression of both proteins was present in some dysplasias (see Table 1), expression did not correlate with malignant transformation and was significantly lower than in established OSCC. Expression in OSCC often was most prominent at the periphery of invading tumor islands (C).
treatment reduced VB6 invasion significantly by ~90% over three experiments (Fig. 3B and D; \( P < 0.0001 \)). Similar to Transwell assays, addition of 2 ng/mL PGE2 completely abolished NS398-induced inhibition of invasion (Fig. 3B and D). C1 cells, expressing low levels of \( \alpha_v^b_6 \), invade organotypic cultures poorly (Fig. 3B and D), but this limited invasion was still inhibited by NS398 (Fig. 3D). NS398 had no effect on the invasion of \( \alpha_v \)-negative H357 cells (Fig. 3C and D; \( P < 0.572 \)).

**COX-2 inhibition interferes with \( \alpha_v^b_6 \) post-receptor events.** COX-2 inhibition might modulate \( \alpha_v^b_6 \) function by reducing cell surface expression, decreasing ligand binding affinity, and/or interfering with \( \alpha_v^b_6 \) signaling. Flow cytometry revealed that

Figure 2. A, Transwell invasion of VB6 cells was inhibited by blockade of \( \alpha_v^b_6 \) integrin (10D5, 1:100) and by 20 \( \mu \)mol/L NS398 (\( P < 0.001 \)). Indomethacin (500 \( \mu \)g/mL) produced a similar level of inhibition, whereas SC-560 (10 \( \mu \)mol/L) had no effect. H357 and C1 cell invasion were not affected by anti-\( \alpha_v^b_6 \) antibody or any of the NSAIDs. Although VB6 invasion was maximally inhibited by 5 \( \mu \)mol/L NS398, no inhibition of H357 or C1 invasion was seen up to 50 \( \mu \)mol/L NS398.

B, fluorescence-activated cell sorting analysis confirmed high and low \( \alpha_v^b_6 \) expression by VB6 and C1 cells, respectively, and high endogenous \( \alpha_v^b_6 \) expression in OSCC cell lines BICR6, CA1, and H157. H357 cells were \( \alpha_v^b_6 \) negative. Transwell invasion of BICR6, CA1, and H157 was inhibited using the anti-\( \alpha_v^b_6 \) antibody 6.3G9 (Biogen Idec). Similar levels of inhibition were achieved using NS398. C, inhibition of VB6 Transwell invasion by NS398 was abrogated completely by PGE2 at 2 ng/mL (\( P < 0.0017 \)), whereas PGF2\(_a\) and U46619 had no effect. Titration of PGE2 produced a bell-shaped curve. All cell lines produced similar levels of PGE2. Treatment with COX-2 RNAi or 20 and 50 \( \mu \)mol/L NS398 inhibited PGE2 production by >95%.
there was no effect on surface levels of \(\alpha_v\beta_6\) in VB6 cells following a 72-hour incubation with NS398 (Supplementary Fig. S2A). We next examined the effect of COX-2 inhibition on \(\alpha_v\beta_6\) ligand binding. We have shown previously that adhesion to the LAP in the cell lines is modulated solely through \(\alpha_v\beta_6\): VB6 cells adhere well; C1 cells adhere poorly; and \(\alpha_v\beta_6\)-negative H357 cells do not adhere to this substrate (29). Inhibition of \(\alpha_v\beta_6\) with 10D5 antibody inhibited adhesion completely (Supplementary Fig. S2B), whereas SC-560, NS398, indomethacin, or inhibitory antibodies against \(\beta_1\) integrin had no effect (\(P < 1.00, P < 0.60, P < 0.65, \) and \(P < 1.00\), respectively). These data suggest that COX-2 does not modulate \(\alpha_v\beta_6\) expression or activity.

\(\alpha_v\beta_6\) ligand binding activates Rac-1. The role of small GTPase Rac-1 in regulating cell migration and cell spreading following integrin-binding is well described (30–32). In particular, several lines of evidence indicate that Rac-1 may play a critical role in a number of aspects of tumor development (31–34), and Rac-1 has also been shown to modulate tumor cell invasion (32–34). Therefore, we examined the effect of \(\alpha_v\beta_6\) ligand binding on Rac-1 activation in VB6 cells using a pulldown assay. Compared with cells in suspension, binding of VB6 cells to LAP resulted in Rac-1 activation (Fig. 4A, i, lanes 3 and 4). Rac-1 activation increased over time and was proportionally greater after 24 hours than 4 hours (Fig. 4A, i, lane 6). This effect was \(\alpha_v\beta_6\)-dependent: C1 control cells on LAP showed reduced Rac-1 activation compared with VB6 cells (Fig. 4A, ii, quantified in Fig. 4A, v; 61% reduction; \(P < 0.0001\)). Rac-1 activation in VB6 cells was inhibited by 69% following \(\beta_6\) knockdown with RNAi (Fig. 4A, iii, quantified in Fig. 4A, v; \(P < 0.0001\)).

COX-2 inhibition prevents \(\alpha_v\beta_6\)-dependent Rac-1 activation. We next examined whether COX-2 activity was required for \(\alpha_v\beta_6\)-dependent Rac-1 activation. Rac-1 activation in VB6 cells was reduced in the presence of NS398 by 72% (Fig. 4A, iv, quantified in Fig. 5A, v; \(P = 0.0057\)). This effect was eliminated if NS398 was added combined with PGE \(_2\) (2-4 ng/mL; Fig. 5A, iv, quantified in Fig. 4A, v). Rac-1 activation in VB6 cells treated with NS398 and PGE \(_2\) (4 ng/mL) did not differ significantly from control cells (\(P = 0.146\)). This confirmed that PGE \(_2\) was required for \(\alpha_v\beta_6\)-dependent Rac-1 activation.
Constitutively active Rac-1 restores \( \alpha_v\beta_6 \)-dependent invasion in the presence of NS398. To confirm that the inhibition of Rac-1 activation by NS398 was responsible for the inhibition of VB6 invasion, we expressed constitutively active Rac-1 in VB6 cells using transient transfection experiments. Over four experiments, cells transfected with V12-Rac-1 in the presence of NS398 had their invasive activity restored to control levels (Fig. 4B; no inhibition of invasion; \( P < 0.88 \)) but not when transfected with empty vector controls (Fig. 4B; invasion inhibited; \( P < 0.0001 \)). Additionally, in the presence of NS398, those VB6 cells that had invaded were found to be enriched for V12 Rac-1-GFP (by 42%; data not shown).

Rac-1 knockdown suppresses \( \alpha_v\beta_6 \)-dependent invasion. Additional experiments using RNAi to inhibit Rac-1 confirmed that \( \alpha_v\beta_6 \)-dependent invasion was mediated through Rac-1. RNAi treatment of VB6 cells reduced Rac-1 protein expression after 48 hours by 96% (Fig. 4C; 3 days, 93%; 5 days, 82%; 7 days, 78%; data not shown). Over five experiments, Rac-1 knockdown inhibited VB6 Transwell invasion by \( \sim 50\% \) (Fig. 4C; \( P < 0.0001 \)). Rac-1 knockdown also inhibited significantly invasion of VB6 cells in organotypic culture (Fig. 4D; \( P < 0.01 \)), an effect similar in magnitude to direct inhibition of \( \alpha_v\beta_6 \) or treatment with NS398 (Fig. 4A and B, respectively).

Figure 4. A, i, binding of VB6 cells to LAP over 4 hours induced Rac-1 activation compared with cells in suspension (S: lanes 3 and 4). Rac-1 activation increased over time and was proportionally greater at 24 hours (lanes 5 and 6). ii, C1 control cells compared with VB6 cells showed significantly less Rac-1 activation on LAP (61% reduction; \( P = 0 < 0.0001 \)). iii, RNAi \( \alpha_v \) knockdown in VB6 cells significantly reduced Rac-1 activation on LAP (69% reduction; \( P = 0.0001 \)). iv, NS398 suppressed \( \alpha_v\beta_6 \)-mediated Rac-1 activation in VB6 cells on LAP (72% reduction; \( P = 0.0057 \)). Addition of PGE_2 restored Rac-1 activation in the presence of NS398. v, densitometric analysis of three combined individual experiments confirmed that Rac-1 activation in VB6 cells on LAP was \( \alpha_v\beta_6 \)-dependent, and that this was inhibited by NS398 and restored by PGE_2. B, VB6 Transwell invasion in the presence of NS398 was restored by V12-Rac-1-GFP to \( \sim 85\% \) of that of empty vector controls. C, Rac-1 RNAi significantly inhibited VB6 Transwell invasion (\( P < 0.001 \)). Western blot verified protein knockdown. D, RNAi knockdown of Rac-1 inhibited invasion of VB6 cells in 6-day organotypic culture (\( P < 0.001 \)). Degree of invasion is quantified in the histogram.
COX-2 inhibition suppresses \( \alpha_v\beta_6 \)-dependent invasion in vivo.

Organotypic cultures can be transplanted onto the back muscle fascia of nude mice where they grow and invade into the host tissue (26). Using this method, VB6 cells produced significantly more invasion than C1 control cells (Fig. 5A and C). Transplants were established for 7 days before commencing treatment, when animals received NS398 (5 mg/kg i.p.) or DMSO every 72 hours for 5 weeks. Tumor take was 100%.

NS398 inhibited the invasion of VB6 cells in vivo by \( \sim 70\% \) (\( P < 0.009 \); Fig. 5A and C), suppression similar to that seen in Transwell assays and in vitro organotypic culture (Figs. 2 and 3B). There was no significant effect of NS398 treatment on the \( \alpha_v \)-negative H357 cells (\( P < 0.933 \); Fig. 5B and C). Because C1 cells invade poorly in vivo, the effect of NS398 on this line was not tested.

Discussion

Expression of \( \alpha_v\beta_6 \) occurs in many carcinoma types (6–10, 35–37) but is found most consistently and at its highest levels in OSCC (7, 37), where experimental studies indicate that it promotes invasion (20, 21, 38, 39). Similarly, high COX-2 expression is also found in OSCC, where it seems to be invasion promoting (22, 40, 41). Our results suggest a novel link among \( \alpha_v\beta_6 \), COX-2, and Rac-1 by showing that the selective COX-2 inhibitor NS398 suppresses \( \alpha_v\beta_6 \)-dependent, Rac-1-mediated invasion.

Elevated levels of both \( \alpha_v\beta_6 \) and COX-2 have (separately) been reported in oral dysplasia (12, 13, 40), where it is suggested that expression may identify lesions at risk of malignant transformation. We examined 39 dysplasias from patients with a minimum of 5 years of clinical follow-up. We found that neither \( \alpha_v\beta_6 \) nor COX-2 expression identified high-risk epithelial dysplasias. Thus, 20% to 30% of dysplasias were positive for one or both proteins. There were positive nontransforming lesions and, conversely, negative lesions that subsequently transformed. Additionally, focal basal expression of \( \alpha_v\beta_6 \) and COX-2 was present in several hyperplastic lesions. Although, generally, there was increased expression of \( \alpha_v\beta_6 \) and COX-2 in dysplasias compared with hyperplastic epithelium, this was much lower than expression in OSCC; most OSCC showed strong expression of both proteins with expression particularly prominent at the periphery of invading tumor islands. These data suggest that the invasion-promoting effects of \( \alpha_v\beta_6 \) and COX-2 primarily may occur in established malignancy. It is possible, however, that negative dysplasias subsequently may express the proteins at a later disease stage, before transformation.

VB6 cells, expressing high levels of \( \alpha_v\beta_6 \), are significantly more invasive than C1 control cells (this work and refs. 20, 21). Interestingly, \( \alpha_v \)-negative H357 cells invade as well as VB6 by using \( \alpha_v\beta_1 \) integrin– and \( \alpha_v \) integrin–dependent mechanisms.\(^5\) Thus, not all OSCC tumor cell invasion is due to \( \alpha_v\beta_6 \). NS398 inhibition of invasion was specific to VB6 cells, and therefore \( \alpha_v\beta_6 \), with no effect on H357 or C1 cells. Additionally, \( \alpha_v\beta_6 \)-dependent invasion of other OSCC lines through Matrigel was inhibited significantly by NS398 (Fig. 2B).

As well as having direct effects on invasion (4, 41–43), NSAIDs may inhibit tumor proliferation and promote apoptosis (2). However, NS398 did not affect growth of our cells at the concentrations used in this study, suggesting that effects of NS398 seem to be via a direct inhibition of \( \alpha_v\beta_6 \) activity.

Although NSAIDs have both COX-2-dependent and COX-2-independent antitumor effects (1), we show that inhibition of VB6 invasion was COX-2 activity dependent; \( \alpha_v\beta_6 \)-dependent invasion was suppressed using COX-2 RNAi and restored, in the presence of NS398, by exogenous PGE\(_2\). Recently, we developed a quantitative invasion assay using organotypic cultures (26). High \( \alpha_v\beta_6 \)-expressing VB6 cells are significantly more invasive than C1 control cells in such cultures, and invasion is \( \alpha_v\beta_6 \) dependent. NS398 markedly inhibited VB6 cell invasion by \( \sim 90\% \). As with Transwell assays, addition of exogenous PGE\(_2\) (at 2 ng/mL) restored VB6 invasion in the presence of the COX-2 inhibitor. However, NS398 had no effect on H357 invasion.

Although COX-2 and \( \alpha_v\beta_6 \) expression correlate in OSCC in vivo, we found no direct evidence that either regulates expression of the other. Antibody blockade of \( \alpha_v\beta_6 \) did not affect levels of COX-2 protein in VB6 cells, nor did COX-2 inhibition affect \( \alpha_v\beta_6 \) levels or ligand-binding affinity. Rather, NS398 affected \( \alpha_v\beta_6 \) post-receptor events.

\(^5\) Unpublished data.
Based on previous observations (18), we examined the role of Rac-1 in the COX-2/αvβ6-dependent invasion. The small GTPase Rac-1 regulates cell migration and spreading following integrin binding (30) and may also modulate tumor cell invasion (31, 32). Unlike Ras, however, mutation of Rac-1 has not been found in human cancer: activation probably occurring through aberrant, or overactive, signaling pathways (33, 34). Rac-1 was activated by αvβ6 ligand binding in VB6 cells, and this activation was not transient but was maintained, indeed increased, over a 24-hour time period. It is possible that pathologic up-regulation of αvβ6 results in overactive Rac-1 signaling, leading to Rac-1 activation. αvβ6-dependent Rac-1 activation was COX-2 dependent because it was inhibited by NS398 and restored on addition of PGE2. Functional studies confirmed that αvβ6-dependent invasion was mediated through Rac-1. Thus, transfection with constitutively active Rac-1 restored VB6 invasion in the presence of NS398. Additionally, RNAi-mediated Rac-1 knockdown inhibited VB6 invasion to levels comparable with NS398 treatment. Thus, transfection with constitutively active Rac-1 restored VB6 invasion in the presence of NS398. Additionally, RNAi-mediated Rac-1 knockdown inhibited VB6 invasion to levels comparable with NS398 treatment or αvβ6 blockade, both in Transwells and in organotypic cultures.

In summary, our data suggest that neither αvβ6 nor COX-2 expression predicts malignant transformation of oral dysplasia. However, there is strong coexpression of both proteins in established OSCC, in accord with our proposal that these molecules might interact in promoting invasion. We show, for the first time, that αvβ6-dependent invasion in vitro and in vivo by OSCC cells requires COX-2-dependent activation of Rac-1, via up-regulation of PGE2, abrogating COX-2, thus, blocks αvβ6-dependent invasion.

Worldwide, OSCC represents about 5.5% of all malignancies (44). Most patients are treated by surgery, and >50% of patients die within 5 years (44). New types of therapy obviously are required, and both COX-2 and αvβ6 have been suggested as possible therapeutic targets. Our data suggest that NSAIDs may inhibit the pro-inflammatory effect of both proteins. The significant morbidity and mortality rates of OSCC are such that the therapeutic benefit of these drugs should be considered, particularly in αvβ6-positive tumors.

Acknowledgments
Received 5/4/2006; revised 9/1/2006; accepted 9/8/2006.

Grant support: Cancer Research UK, The Health Foundation, U.K. Medical Research Council, DeBKA, ACRB, and University of London core research fund.

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