Abstract

**Purpose:** Inflammatory cytokines have been implicated in the progression of head and neck squamous cell carcinoma (HNSCC). Herein we investigate the mechanisms by which interleukin-1β (IL-1β) might contribute to Epithelial-Mesenchymal Transition (EMT) in HNSCC.

**Experimental Design:** We evaluated the effect of IL-1β on the molecular events of EMT in surgical specimens and HNSCC cell lines. We examined the correlation with tumor histologic features, and a SCID xenograft model was used to assess the effects of Snail overexpression.

**Results:** Cyclooxygenase-2 (COX-2)-dependent pathways contribute to the modulation of E-cadherin expression in HNSCC. An inverse relationship between COX-2 and E-cadherin was shown *in situ* by double immunohistochemical staining of human HNSCC tissue sections. Treatment of HNSCC cells with IL-1β caused the downregulation of E-cadherin expression and upregulation of COX-2 expression. This effect was blocked in the presence of COX-2 small hairpin RNA. IL-1β–treated HNSCC cell lines showed a significant decrease in E-cadherin mRNA and an increase in the mRNA expression of the transcriptional repressor Snail. IL-1β exposure led to enhanced Snail binding at the chromatin level. Small hairpin RNA–mediated knockdown of Snail interrupted the capacity of IL-1β to downregulate E-cadherin. In a SCID xenograft model, HNSCC Snail-overexpressing cells showed significantly increased primary and metastatic tumor burdens.

**Conclusions:** IL-1β modulates Snail and thereby regulates COX-2–dependent E-cadherin expression in HNSCC. This is the first report indicating the role of Snail in the inflammation-induced promotion of EMT in HNSCC. This newly defined pathway for transcriptional regulation of E-cadherin in HNSCC has important implications for targeted chemoprevention and therapy. (Clin Cancer Res 2009;15(19):6018–27)
IL-18 Regulates Snail and E-cadherin in HNSCC

Translational Relevance

The presence of regional metastases in head and neck squamous cell carcinoma (HNSCC) patients is a common and adverse event associated with poor prognosis. Understanding the molecular mechanisms that mediate HNSCC metastasis may enable identification of novel therapeutic targets. Here, we provide the first report indicating the role of E-cadherin transcriptional repressors in the inflammation-induced promotion of EMT in HNSCC. We also document cyclooxygenase-2 (COX-2)-dependent transcriptional regulation of E-cadherin in HNSCC. These findings suggest that therapies targeting the cyclooxygenase pathway may diminish the propensity for tumor metastasis in HNSCC by blocking the prostaglandin E2–mediated induction of E-cadherin transcriptional repressors. This newly defined pathway for transcriptional regulation of E-cadherin in HNSCC has important implications for chemoprevention as well as therapies utilizing COX-2 inhibitors in combination with other agents. COX-2 inhibitors may enhance HNSCC E-cadherin expression and may therefore augment sensitivity to epidermal growth factor receptor Tyrosine-Kinase Inhibitor (TKI) therapy. The tailoring of individual treatment strategies to aggressively treat HNSCC will improve long-term survival.

Materials and Methods

Reagents and cell lines. Recombinant human IL-1β was purchased from BD Bioscience. 16,16-dimethyl-PGE2 was purchased from Cayman Chemicals. Recombinant human IL-1α was purchased from ProSpec Protein Specialists. IL-1β and IL-1α were dissolved in the diluent 0.1% bovine serum albumin in 1× PBS. Other reagents were purchased from Sigma Chemicals unless otherwise specified. HNSCC cells utilized in this study included Tu686, Tu212 (generously provided by Dr. D. Shin; ref. 21), OSC, HOC, and TSU (generously provided by Dr. M. Nagayama; ref. 22). SNAIL sense (SNAIL-S) and pLHCX (vector alone) clones were generated for the Tu686 and OSC cell lines using retroviral transfection as previously described (13, 14). Briefly, for each cell line, an approximately 10-fold higher level of SNAIL was noted in SNAIL-S compared with parental or vector controls (13). These cells were then expanded for further studies. The following cell line terminology is used in the text: (a) Tu-686 SNAIL-S and OSC-SNAIL-S are the cell lines transfected with SNAIL in the sense orientation, and (b) Tu686-V and OSC-V are the cells transfected with the expression vector pLHCX alone. E-cadherin–overexpressing cells were generated as follows: wild-type E-cadherin cDNA pcDNA3.1 (a generous gift from A.S.T. Wong and B.M. Gumbiner, University of Virginia, Charlottesville, VA) was excised from the plasmid with HindIII and XbaI and subcloned into PC3.1 vector (Invitrogen). A 2.7-kb E-cadherin cDNA was further excised from PC3.1 construct with Pmel and HindIII and subcloned into the retrovirus vector pLHCX (Clontech), which contains the cytomegalovirus promoter for controlling expression of the cDNA insert and hygromycin (Mediatech) resistance gene for selection (13). Snail-overexpressing cells were generated as follows: wild-type Snail cDNA pcDNA3 (a generous gift from Dr. E. Fearon, University of Michigan) was excised from the plasmid with HindIII and EcoRV and subcloned into the retrovirus vector pLHCX. All constructs were verified by restriction endonuclease digestion. For virus production, 70%–confluent 293T cells were cotransfected with pLHCX-Snail or pLHCX-E-cadherin and pLHCX (vector alone). Tumor cells were then transduced with high-titer supernatants producing either Snail, E-cadherin, or pLHCX virus. Following transduction, the tumor cells were characterized by Western blot for expression of Snail or E-cadherin.

Western blot analysis. HNSCC cells were washed with PBS and whole cell lysate was prepared with modified radioimmunoprecipitation assay buffer at 4°C for 15 min. The cell lysates were centrifuged at 13,000 rpm for 10 min and the supernatant was collected. Protein concentration was measured with a protein assay reagent (Bio-Rad). Protein for E-cadherin (20 μg), COX-2 (20 μg), and Snail (50 μg) was resolved by SDS-PAGE and analyzed by Western blot using polyvinylidene difluoride membranes (Millipore) according to the manufacturer’s instructions. Membranes were blocked with 5% nonfat dry milk in TBS plus 0.1% Tween 20. The membranes were probed with anti–E-cadherin antibody (BD Biosciences Pharmingen/Transduction Laboratories) at 1:2,500 dilution and anti–COX-2 antibody (Santa Cruz Biotechnology) at 1:1,000 dilution in TBS plus 0.1% Tween 20 containing 1.0% nonfat dry milk. The membranes were developed by the ECL chemiluminescence system (Amersham Pharmacia Biotech) and exposed to X-ray film (Optimum Brand X-Ray Film). Equal loading of samples was confirmed by probing the membranes with β-actin or GAPDH antibody.

Total RNA preparation, cDNA synthesis, and real-time PCR. To analyze the COX-2/PGE2-dependent regulation of E-cadherin, Snail mRNA expression, total RNA from 1 × 106 control and IL-1β (200 U)-treated HNSCC cells were extracted using Trizol reagent according to the manufacturer’s instructions (Invitrogen). The cDNA was prepared with a kit (Invitrogen) according to the manufacturer’s instructions. E-cadherin and Snail mRNA levels were quantified by real time reverse transcription-PCR using the several early transcription factors involved in the transcription of proinflammatory cytokine genes. IL-1α is known to induce the activation of immediate-early transcription factors and genes that promote the survival and proliferation of HNSCC (5–7). This suggests that IL-1α may serve as an important autocrine and/or exocrine factor in coordinating expression of this repertoire of cytokines in HNSCC. IL-1β has also been implicated in the progression of HNSCC. Increased secretion of IL-1β has been shown to be the profile of resistant or progressing oral tumors (8, 9). IL-1β is one of several cytokines known to potently upregulate cyclooxygenase-2 (COX-2) expression in a variety of cells (5, 6, 10, 11). Tumor COX-2 and its metabolites play important roles in regulating diverse cellular functions under physiologic and pathologic conditions (12–14). Loss of E-cadherin is frequently observed at sites of EMT during cancer development and progression, and is closely correlated with poor prognosis (15–18). Several E-cadherin transcriptional repressors have been characterized (ZEB1, Snail, E12/E47, Slug, Twist, and SIP-1). In head and neck tissues, both malignancy and local recurrence following treatment have been associated with a gene expression signature that includes the zinc-finger E-box-binding transcriptional inhibitor Snail (19). Recently, Lyons et al. reported that Snail upregulates proinflammatory mediators in oral keratinocytes, which have been shown to correlate with malignancy (20). Herein, we show that proinflammatory mediators upregulate Snail, thus further defining the cycle by which inflammation promotes tumor progression. We report that IL-1β upregulates Snail and suppresses E-cadherin in a Cox-2–dependent manner. Immunohistochemical staining of HNSCC tissue sections confirm that these relationships exist in situ. This is the first report implicating inflammation-dependent regulation of E-cadherin transcriptional repressors in head and neck cancer.
Tu686 cells were treated with the indicated concentrations of IL-1β for 18 h. Protein from whole cell lysates was analyzed for COX-2 and E-cadherin expression by Western blot as described in Materials and Methods. A, IL-1β upregulates COX-2 expression in a concentration-dependent manner. Lane A, no treatment; lane B, IL-1β (100 U/mL); lane C, IL-1β (200 U/mL). B, IL-1β causes downregulation of E-cadherin expression in a concentration-dependent manner. Lane A, no treatment; lane B, IL-1β (100 U/mL); lane C, IL-1β (200 U/mL). C, upon addition of celecoxib, E-cadherin is no longer downregulated in Tu686 HNSCC cells, indicating that functional COX-2/PGE2 is required for its downregulation. Lane A, IL-1β (100 U/mL) plus celecoxib (1 μmol/L); lane B, diluent (0.1% bovine serum albumin in 1× PBS) alone; lane C, IL-1β (100 U/mL). D, in the presence of COX-2 shRNA, E-cadherin is no longer downregulated in Tu686 HNSCC cells, indicating that functional COX-2/PGE2 is required for its downregulation. Lane A, diluent (0.1% bovine serum albumin in 1× PBS) alone; lane B, IL-1β (100 U/mL); lane C, IL-1β (100 U/mL) plus COX-2 shRNA. E, PGE2 causes the downregulation of E-cadherin expression in Tu686 cell lines. Lane A, PGE2 (10 μg/mL); lane B, diluent (0.1% bovine serum albumin in 1× PBS) alone.

**Immunochemistry.** With Institutional Review Board approval, immunohistochemistry was done on formalin-fixed, paraffin-embedded HNSCC tissues from the UCLA Pathology Department archives. Twenty-four HNSCC specimens were obtained anonymously and randomly. They comprised eight well differentiated, eight moderately differentiated, and eight poorly differentiated tumors. Tissue sections (4 μm thick) were cut, deparaffinized in xylene, rehydrated in alcohols, and washed twice with water. Samples were then incubated in 0.01 mol/L citrate buffer (pH 6.0) for 25 min in a steamer to unmask antigens as previously described (24, 25). Following cooling to room temperature and rinsing with dH2O, samples were treated for 15 min with 3% H2O2 diluted in methanol. Tissue sections were washed in dH2O, then PBS, then blocked with 10% normal horse serum for 30 min at room temperature. For COX-2 and E-cadherin staining, the sections were first stained for COX-2, followed by E-cadherin. The sections were incubated with goat anti-human COX-2 polyclonal IgG 1 μg/mL (Santa Cruz Biotechnology) Transfection Kit (Invitrogen). The supernatant was harvested 36 h after transfection and used to infect HNSCC cells (Tu212 and Tu686) for 6 h. The cells were then selected in 1 μg/mL puromycin containing medium until all the control cells died.

**Table 1.** IL-1β–dependent regulation of COX-2/PGE2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PGE2 levels (pg/mL)</th>
<th>Control</th>
<th>IL-1β 100 U/mL</th>
<th>IL-1β 200 U/mL</th>
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<tbody>
<tr>
<td>HOC</td>
<td></td>
<td>0</td>
<td>366 ± 13*</td>
<td>440 ± 11*†</td>
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<tr>
<td>TSU</td>
<td></td>
<td>70 ± 3.5</td>
<td>303 ± 10*</td>
<td>413 ± 9.6*†</td>
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<tr>
<td>Tu686</td>
<td></td>
<td>8 ± 0.6</td>
<td>15 ± 1*</td>
<td>35 ± 1.9*†</td>
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NOTE: HOC, TSU, and Tu686 HNSCC cells were stimulated with IL-1β (100 and 200 U/mL) for 18 h. PGE2 concentration was then measured by ELISA. Upon treatment with IL-1β, significant increases in PGE2 levels were noted. Mean values are reported. All measurements were made in triplicate and repeated in at least three separate experiments. *P < 0.001 vs. control. †P < 0.01 vs. 100 U/mL.

SYBR Green quantitative PCR kit from Bio Rad in a MyiQ Cycler (Bio Rad) following the manufacturer’s protocol. Amplification was carried out in a total volume of 20 μL for 40 cycles of 15 s at 95°C, 20 s at 60°C, and 30 s at 72°C. Samples were run in triplicate and their relative expression was determined by normalizing expression of each target either to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) or β-actin. The results were then compared with the normalized expression in a reference sample to calculate a fold-change value. Primers were designed as previously described (23). Primer sequences were as follows: human G3PDH 5′- TGACACCCAACTGCTTAGC-3′, and 5′-GACATG-GAICTGTGCTACATGAGC-3′; β-actin 5′-GATGAGATTTGCCATGGCTTT-3′, and 5′-CACCCTCAAGCCGCTTCACTTT-3′; human E-cadherin 5′-CGGAAGATCGAGTGGAGATC-3′ and 5′-AGGATGTGTTAAGCGGATGGC-3′; and human Snail 5′-CGGAGCCTTCCCTCCTGTCAG-3′ and 5′-GACCAGGATGAGATTCG-3′.

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation analysis was done using the ChIP-IT Enzymatic kit (Active Motif) following the manufacturer’s protocol. Briefly, control Tu686, IL-1β–treated Tu686 cells, OSC control cells, and IL-1β–treated OSC were first fixed with 1.0% formaldehyde and were used for chromatin preparation as described in the manufacturer’s protocol. Chromatin was then precleared with protein G beads for 2 h at 4°C. Antibodies to Snail and control IgG (normal goat IgG) were then added to the precleared chromatin and incubated overnight at 4°C. Subsequently, protein G beads were added to the immunoprecipitated chromatin DNA and incubated for 1 h at 4°C. The beads were then collected by centrifugation and washed extensively. The cross-linked DNA then eluted from protein G beads. The eluted clued-linked protein-DNA complexes were treated with RNase A at 65°C overnight for the removal of RNA and then treated with proteinase K at 42°C for 2 h to reverse protein-DNA complex. The resulting DNA was purified by columns and then subjected to PCR analysis. PCR reactions were done with the following human E-cadherin promoter primers: forward 5′-GCGCGCTCTTTCCTCGTCAG-3′ and reverse 5′-GGCCGGCAGGTCTGAACTGA-3′.

**RNA interference with shRNA.** The Hush 29mer small hairpin RNA (shRNA) constructs against Human Snail1 were purchased from ORI-GENE. The shRNA constructs against Human Cox-2 were purchased from GeneCopoeia. The plasmids were cotransfected with the Amphotropic (package plasmid) into 293T cells using the Calcium Phosphate Transfection Kit (Invitrogen). The supernatant was harvested 36 h after transfection and used to infect HNSCC cells (Tu212 and Tu686) for 6 h. The cells were then selected in 1 μg/mL puromycin containing medium until all the control cells died.
overnight at 4°C, rinsed, and incubated for 40 min at room temperature with horse anti-goat IgG-biotin 7.5 μg/mL (Vector Laboratories; ref. 26). Samples were then incubated for 30 min at room temperature, with avidin-horseradish peroxidase diluted 1:1,000 in PBS (Vector Laboratories), washed, and treated with Nickel DAB (DAB kit, Vector Laboratories) for black color development to augment contrasting color in double-stained slides. Samples were extensively washed in PBS (three times, 5 min each) in preparation for E-cadherin staining. Samples were incubated overnight at 4°C with 250 μg/mL mouse anti-human E-cadherin diluted in normal horse serum (BD Transduction Biosciences). After extensive rinsing with PBS, samples were incubated for 40 min with 7.5 μg/mL horse anti-mouse IgG-biotin (Vector Laboratories) and rinsed with PBS. Samples were then incubated for 30 min at room temperature with the Vectastain ABC- kit (Vector Laboratories), followed by PBS washing, and then incubated with an alkaline phosphatase substrate kit (Vector Laboratories). Color development was followed under the microscope for 20 min. The color reaction was stopped by rinsing with dH 2O. Samples were counterstained with hematoxylin. Normal human kidney was used as a positive control for both COX-2 and E-cadherin staining. Negative controls included incubation with nonimmune pooled rabbit or goat IgG (rabbit IgG was from Vector Laboratories and goat IgG was from Zymed, Invitrogen) at the same concentration as the primary antibody.

Single staining for Snail and COX-2 and double staining for Snail and E-cadherin were done (n = 24) essentially as described above with the following modifications. Goat anti-human Snail polyclonal IgG (1:50 dilution; Abcam) was used for Snail immunohistochemistry. All slides were reviewed by two of the investigators (MCF and CL). The following findings were recorded for each slide: (a) % cells positive for each stain, and (b) intensity of stain (0 to +3). We first examined the relationship between E-cadherin and Snail using the E-cadherin histochemistry results (0+, 1+, etc.) and found that they were significantly negatively correlated (p = -0.52, P = 0.02). Next, the staining for E-cadherin and Snail were dichotomized as positive (2+ or 3+) or negative (0+1+). Using Fisher's exact test we found that E-cadherin and Snail had a significant association (P = 0.0006) after adding tumor differentiation status.

In vivo mouse model of HNSCC metastasis. Pathogen-free SCID Beige CB17 (8-12 weeks of age) mice were obtained from Charles River Laboratories, and maintained in the West Los Angeles VA Animal Research vivarium. All studies were approved by the institution's animal studies review board. Five million HNSCC cells (Tu686-S or Tu686-V) were implanted via s.c. injection on the right supra scapular area of the SCID mice. Tumor growth was assessed three times each week following implantation with nonimmune pooled rabbit or goat IgG (rabbit IgG was from Vector Laboratories and goat IgG was from Zymed, Invitrogen) at the same concentration as the primary antibody.

To assess the metastatic potential of the HNSCC cells in SCID mice, a single cell suspension was prepared from lungs and livers in collagenase digestion buffer [type IV collagenase, 1 mg/mL (Sigma), DNase 50 U/mL, in RPMI media] for 45 min at 37°C. A single cell suspension of lungs or livers from tumor-bearing or naive non-tumor controls was stained by CXC4R (R&D) to detect HNSCC by histochemistry results (0+, 1+, etc.) and found that they were significantly negatively correlated (p = -0.52, P = 0.02). Next, the staining for E-cadherin and Snail were dichotomized as positive (2+ or 3+) or negative (0+1+). Using Fisher's exact test we found that E-cadherin and Snail had a significant association (P = 0.0006) after adding tumor differentiation status.

Results

IL-1β upregulates Snail and downregulates E-cadherin expression in HNSCC. IL-1β has been implicated in the progression of tobacco-related malignancies and is one of several cytokines known to potently upregulate COX-2 expression in a variety of cells (10, 11). We examined the effects of adding IL-1β on COX-2 expression in Tu686, Tu212, and OSC HNSCC cell lines. These HNSCC cell lines were used for all of the subsequent experiments unless otherwise specified. IL-1β caused the upregulation of COX-2 expression in these cell lines in a concentration-dependent manner (Fig. 1A). In addition to assessing COX-2 expression using Western blot analysis, we carried out PGE 2 assays. Upon treatment with IL-1β, significant increases in PGE 2 levels were noted (Table 1). As IL-1α has been shown to induce the activation of immediate-early transcription factors and genes that promote the survival and proliferation of cytokines that mediate inflammatory responses (7–9), we also examined the effect of IL-1α on E-cadherin, COX-2, and Snail levels in the Tu686, Tu212, and OSC HNSCC cell lines. We repeated the exact protocols we used for IL-1β, however, no change in the expression levels of E-cadherin, COX-2, or Snail was noted (data not shown).

We examined E-cadherin levels in HNSCC cells treated with IL-1β and discovered that its expression was downregulated in a concentration-dependent manner (Fig. 1B). To establish that the downregulation of E-cadherin was due to a COX-2–dependent mechanism, we added the COX-2 inhibitor celecoxib (1 μmol/L) to cells prior to treatment with IL-1β. Upon addition of celecoxib, E-cadherin was no longer downregulated, indicating that functional COX-2 is required for its downregulation (Fig. 1C).

In order to determine more definitively the import of COX-2 in the IL-1β–induced reduction of E-cadherin, we used shRNA to knockdown COX-2 expression in Tu686 and Tu212 cells. When the HNSCC cells were transfected with COX-2 shRNA, E-cadherin was no longer down-regulated (Fig. 1D). This supports the celecoxib data and more definitively indicates that functional COX-2 is required for IL-1β–mediated E-cadherin down-regulation.

We also treated HNSCC cells directly with PGE 2 (16,16-dimethyl-PGE2 at 2 μg/mL, 5 μg/mL, and 10 μg/mL) and consistent with the celecoxib results, noted a significant decrease in E-cadherin levels (Fig. 1E). This decrease in E-cadherin levels was associated with an increase in N-cadherin levels (data not shown). Immunohistochemical staining of oral tongue squamous cell carcinoma tissue sections confirmed that these relationships exist in situ. There is reciprocal expression of E-cadherin and COX-2 in HNSCC in these specimens (Fig. 2).

We next determined the Snail and E-cadherin mRNA expression by real-time reverse transcriptase-PCR in control and IL-1β–treated HNSCC cell lines. When these lines were exposed to IL-1β, Snail mRNA expression levels were elevated. Consistent with these findings, E-cadherin mRNA expression was decreased under these conditions (Fig. 3A). As determined by quantitative real-time PCR, IL-1β down-regulated E-cadherin and up-regulated Snail in a dose-dependent manner in all three cell lines.
<table>
<thead>
<tr>
<th>Differentiation</th>
<th>Ecad</th>
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<tr>
<td>Poor</td>
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In order to further define the role of Snail in HNSCC, we generated genetically modified Snail overexpressing (Snail-S) HNSCC cells (as well as vector controls). We determined that in these HNSCC cell lines that constitutively express high levels of E-cadherin, the introduction of Snail down-regulates the expression of E-cadherin (Fig. 3B).

To determine the importance of Snail in the IL-1β–mediated down-regulation of E-cadherin, we used shRNA to knockdown Snail expression in Tu686 and Tu212 cells (Fig. 4A). Transfection with Snail shRNA resulted in a 4-fold reduction of Snail mRNA levels compared with control transfected cells. Concomitantly, shRNA-mediated knockdown of Snail led to an 18-fold increase in E-cadherin mRNA (Fig. 4A). Treatment of HNSCC cells with IL-1β increased Snail mRNA 6-fold and decreased E-cadherin mRNA expression (0.45-fold). Knockdown of Snail expression prevented the IL-1β–mediated downregulation of E-cadherin (Fig. 4A). These findings implicate IL-1β as an autocrine or paracrine modulator of Snail and define a pathway by which COX-2 decreases E-cadherin expression in HNSCC.

**Snail binds to E-box elements of the E-cadherin promoter.** Snail is known to bind the E-boxes present in the E-cadherin promoter and thus repress E-cadherin transcription. In order to test whether the IL-1β–mediated elevation in Snail is associated with an increase in binding to the E-cadherin promoter, we examined the binding of Snail to E-boxes in ChIP studies in HNSCC cells. Anti-SNAIL antibody directed against the NH2-terminal domain efficiently pulled down Snail protein complexes with the chromatin fragment comprising the -84 to +64 E-cadherin promoter region (Fig. 4B). Control goat IgG did not precipitate the E-cadherin promoter region. Importantly, an enhanced expression of Snail in IL-1β–treated cells resulted in an increase in chromatin binding as shown by the increase in intensity of the band in Fig. 4B. Our ChIP assays show that Snail specifically binds to the E-cadherin promoter region.

**Snail overexpression increases metastasis and upregulates proinflammatory mediators in murine models of HNSCC.** In order to further understand the role of Snail in metastasis and angiogenesis in HNSCC, we made use of xenograft murine models. Tu686 and OSC HNSCC cell lines were genetically altered to produce stable Snail-overexpressing lines.

Fig. 3. IL-1β–dependent regulation of E-cadherin and Snail in HNSCC. A, IL-1β regulates expression of E-cadherin and its transcriptional repressor Snail. Tu686 cells were treated with the indicated concentrations of IL-1β for 18 h and levels of mRNA for E-cadherin and Snail were evaluated by quantitative real time PCR analysis as described in Materials and Methods. Upon addition of IL-1β, Snail mRNA expression levels were elevated and E-cadherin mRNA expression was decreased significantly in HNSCC cell lines. Data shown are the mean of three experiments. Bars, SE; *P < 0.05. B, transfection of Snail into the Tu686 HNSCC cell line (that constitutively expresses high levels of E-cadherin) completely diminishes E-cadherin expression. Lane A, parental cell line; lane B, vector control; lane C, Snail.

Five million HNSCC cells (Tu686 Snail-overexpressing or Tu686-vector controls; OSC-Snail overexpressing or OSC-vector controls) were implanted via s.c. injection on the right supracapsular area of the SCID mice. Primary tumor burden increased significantly in Snail-overexpressing tumors as compared with vector controls (P < 0.005; Fig. 5A-C). Pulmonary and hepatic metastases were also significantly greater in mice bearing Snail-overexpressing tumors (P < 0.001). ELISA studies revealed increased levels of the angiogenic and proinflammatory mediators: VEGF, IL-8, and...
In Chromatin Immunoprecipitation (ChIP) assay, Snail associates with the binding of Snail to the E-box sequences of the E-cadherin promoter.

Snail; N, Negative Control.

antibodies to Snail and control IgG as described in Materials and Methods. Separate experiments. Bars, SE; P < 0.01. A, IL-1β-mediated downregulation of E-cadherin. The data represent the mean ± SD of triplicate determinations in one representative experiment out of three.

downregulation of E-cadherin. The data represent the mean ± SD of triplicate determinations in one representative experiment out of three.

Snail expression levels were evaluated by quantitative real time PCR analysis as described in Materials and Methods. Transfection with Snail shRNA resulted in a 4-fold reduction of Snail mRNA levels compared with control transfected cells and an 18-fold increase in E-cadherin mRNA. Treatment with IL-1β increased Snail mRNA 6-fold and decreased E-cadherin mRNA expression. Knockdown of Snail expression prevented the IL-1β-mediated downregulation of E-cadherin. The data represent the mean ± SD of triplicate determinations in one representative experiment out of three separate experiments. Bars, SE; P < 0.05. B, IL-1β-dependent enhancement in binding of Snail to the E-box sequences of the E-cadherin promoter. In Chromatin Immunoprecipitation (ChIP) assay, Snail associates with the E-cadherin promoter at the chromatin level. TUN86 and Tu212 control and IL-1β (100 U/mL)-treated cells were subjected to ChIP analysis using antibodies to Snail and control IgG as described in Materials and Methods. IL-1β treatment resulted in an amplified E-cadherin promoter fragment. S, Snail; N, Negative Control.

CXCL5 in the Snail-overexpressing tumors (Fig. 5D). These in vivo studies further support our hypothesis that Snail may be an important factor in the growth and metastasis of HNSCC, and therefore is a potential target for therapy.

Expression of COX-2, Snail and E-cadherin in human HNSCC tissue sections. In Fig. 2 we show that reciprocal expression of E-cadherin and COX-2 is evident in HNSCC cells in situ. The COX-2-dependent reciprocal expression of Snail and E-cadherin observed in vitro prompted us to determine if this relationship is also present in human neoplasm sections obtained from surgical specimens. Reciprocal expression of Snail and E-cadherin in histologic sections of human HNSCC was evident in a manner analogous to that seen with E-cadherin and COX-2 staining (Fig. 2). Low-grade carcinomas showed decreased expression of SNAIL and COX-2 compared with high-grade neoplasms. Immunohistochemical staining of high-grade carcinomas with a solid pattern showed stronger staining for Snail and COX-2 (Fig. 2). Consistent with our in vitro findings, the examination of serial sections indicated that tumor cells that were positive for COX-2, were predominantly also Snail positive. We examined the relationship between E-cadherin and Snail using the ordinal immunohistochemistry results (0+, 1+, etc.) and found that they were significantly negatively correlated (ρ = -0.52, P = 0.02). Next, the staining for E-cadherin and Snail was dichotomized as positive (2+ or 3+) or negative (0+, 1+). Using Fisher’s exact test we found that E-cadherin and Snail had a significant association (P = 0.0006) after adding tumor differentiation status (Fig. 2M).

We found that the COX-2 staining paralleled Snail staining and was inversely proportional to E-cadherin staining. Thus, to summarize the findings of the immunohistochemical studies in human oral squamous cell carcinomas there is: (a) reciprocal expression of E-cadherin and COX-2; (b) reciprocal expression of E-cadherin and Snail; and (c) coexpression of COX-2 and Snail.

Discussion

Several studies of E-cadherin in thyroid carcinomas have shown that its reduction or loss is generally related to features that correlate with tumor aggressiveness, such as poorly differentiated or anaplastic histology and widely invasive growth (27–30). Distant metastasis–free survival was significantly worse in tumors showing reduced E-cadherin expression (31).

In HNSCC patient samples, expression of Snail in primary tumors correlates with a higher probability of metastasis and a poor prognosis (23, 32). Snail is expressed at the invasive front of epidermoid carcinomas (33), and has been associated with the lymph node status and/or invasiveness of various carcinomas, as well as local recurrences (26, 34–36).

Herein, we investigated the mechanisms by which Snail might contribute to the pathogenesis of HNSCC using molecular analyses and in vivo modeling. Inflammation is commonly associated with cancer, and the upregulation of proinflammatory mediators has been observed in HNSCCs (37). We showed that IL-1β and PGE2 lead to a COX-2–dependent upregulation of Snail. This then leads to Snail binding to the E-cadherin promoter, with resultant downregulation of E-cadherin expression. These results support our previous findings in non–small cell lung carcinoma (12). When HNSCC cell lines that overexpress Snail are injected into SCID mice, the primary tumor and metastatic burdens are significantly greater than HNSCC vector controls. It is quite important to highlight that the metastatic model used in this study is not an orthotopic model. Based on Paget’s “seed and soil” theory, this model may not be an ideal one to study the metastasis of HNSCC, but it is still informative in terms of the metastatic capacity of each of the cell lines (38).

In HNSCC, IL-1 can induce activation of signal transduction pathways that regulate several early transcription factors involved in the transcription of proinflammatory cytokine genes. IL-1α and IL-1β have been reported to play a prominent role in enhancing the transcription and expression of cytokines IL-6 and IL-8 during the activation of the cytokine cascade. It has been shown that human HNSCCs constitutively express IL-1α and a repertoire of proinflammatory and proangiogenic cytokines that are potentially IL-1–inducible, but IL-1β was not detected in any of the HNSCC cell lines tested (39). IL-1α contributes to the transcriptional activation of NF-κB, to the expression of IL-8, and to cell survival and the growth of HNSCC in vitro (5–7, 39, 40). Herein, we have defined a new role for IL-1β in EMT. The role of IL-1 in enhancing activation of a cascade of proinflammatory cytokine mediators and responses suggests that IL-1 may serve as an important
autocrine and/or exocrine factor in coordinating expression of this repertoire of cytokines in HNSCC.

Proinflammatory mediators are upregulated in Snail-overexpressing tumors, including IL-8. IL-8 plays an important role in the stimulation of angiogenesis, proliferation, and chemotaxis of granulocytes and macrophages, which are prominent constituents in the stroma of HNSCCs. The upregulation of IL-8 has been observed in HNSCC patients and is linked to recurrence and metastasis (41). Lyons et al. have recently shown that Snail can upregulate proinflammatory cytokines in oral keratinocytes (20). Our work shows that proinflammatory mediators upregulate Snail, thus further defining the cycle by which inflammation promotes tumor progression.

Loss of E-cadherin and gain of the expression of Snail are associated with resistance to epidermal growth factor receptor TK inhibitors (42, 43). This evidence supports a key role for Snail as an inducer of tumor invasion as well as a potential contributor to tumor growth and/or chemoresistance mechanisms. Thus, there is a dual purpose in studying pathways that regulate E-cadherin expression in HNSCC: maintenance of E-cadherin expression may promote sensitivity to targeted therapy and prevent invasion and metastases (42, 43).

**Fig. 5.** In the SCID xenograft model, HNSCC Snail-overexpressing cells showed significantly increased primary and metastatic tumor burdens. A, B, and C, Tu686 and OSC HNSCC cell lines were genetically altered to produce stable Snail-overexpressing lines. Five million HNSCC cells (Tu686-Snail overexpressing or Tu686-vector controls; OSC-Snail overexpressing or OSC-vector controls) were implanted via s.c. injection on the right suprascapular area of the SCID mice as detailed in Materials and Methods. Primary tumor burden increased significantly in Snail-overexpressing tumors as compared with vector controls (P < 0.005). D, in the SCID xenograft model, HNSCC Snail-overexpressing cells showed increased levels of VEGF, IL-8, and CXCL5. ELISA studies on tumors removed from the animals revealed increased levels of the angiogenic and proinflammatory mediators: VEGF, IL-8, and CXCL5 in the Snail-overexpressing tumors.
The presence of regional metastases in HNSCC patients is a common and adverse event associated with poor prognosis. Understanding the molecular mechanisms that mediate HNSCC invasion and metastasis may enable identification of novel therapeutic targets for the prevention and management of metastasis. Here, we provide the first report indicating the role of E-cadherin transcriptional repressors in the inflammation-induced promotion of EMT in HNSCC. We also document COX-2–dependent transcriptional regulation of E-cadherin in HNSCC. Furthermore, in human oral squamous cell carcinoma we confirm a reciprocal relationship between COX-2 and E-cadherin as well as Snail and E-cadherin. The results presented here also indicate a positive correlation between COX-2 and Snail in human oral squamous cell carcinoma. These findings suggest that therapies targeting the cyclooxygenase pathway may diminish the propensity for tumor metastasis in HNSCC by blocking the PGF₂α-mediated induction of E-cadherin transcriptional repressors. This newly defined pathway for transcriptional regulation of E-cadherin in HNSCC has important implications for chemoprevention as well as therapies utilizing COX-2 inhibitors in combination with other agents. COX-2 inhibitors may enhance HNSCC E-cadherin expression and may therefore augment sensitivity to EGFR TKI therapy. The tailoring of individual treatment strategies to aggressively treat HNSCC will improve long-term survival.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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