Interleukin-1 Regulates Interleukin-6 Secretion in Human Oral Squamous Cell Carcinoma in Vitro: Possible Influence of p53 but not Human Papillomavirus E6/E7

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ABSTRACT

We have previously shown that interleukin-1 (IL-1) and IL-6 are constitutively produced by human oral squamous cell carcinoma (SCC) and some derived cell lines but not by cultured normal oral keratinocytes. To elucidate possible cytokine regulatory pathways that may contribute to oral SCC growth and/or progression, we tested the hypotheses that exogenous and/or endogenous IL-1 regulates IL-6 production in vitro. We investigated the effects of exogenous IL-1 and IL-6 on secondary cytokine secretion. Our studies revealed that IL-1 strongly up-regulated IL-6 protein secretion in all three cell lines tested. This effect was completely abrogated by IL-1 receptor antagonist. IL-1 receptor antagonist also inhibited the secretion of IL-1α and IL-1β in two of three cell lines. These data show for the first time that IL-1 strongly up-regulates IL-6 and support the notion of autocrine regulation of IL-1 in certain oral SCC cell lines. Additionally, because human papillomavirus (HPV) infection and p53 mutation have been implicated in the malignant transformation of SCC, we explored a second hypothesis, that HPV and/or p53 mutation contribute to cytokine dis-regulation. We investigated HPV DNA presence, transcriptional activation of HPV E6/E7 (in HPV DNA-positive cell lines), and p53 gene status in our cell lines. No association between HPV DNA and cytokine expression was found. However, the oral SCC cell lines secreting the most IL-6 had mutant rather than wild-type p53.

INTRODUCTION

Oral cancer accounts for approximately 3% of the incidence of malignant tumors in the United States, with an estimated $400 deaths expected this year, usually due to local or regional failure to control metastatic disease (1). Oral SCC progresses through a series of clinically and histopathologically distinct stages from carcinoma in situ to invasive SCC, with tumor cells ultimately entering lymphatic vessels and metastasizing to regional lymph nodes. Although the molecular mechanisms underlying the development of malignant oral SCC are currently unknown, application of the clonal origin of metastases theory (2) suggests that subpopulations of advanced primary invasive squamous carcinoma cells acquire autonomous growth properties or, conversely, may actually become stimulated to grow by normal cells and/or their products (3). How these metastatically competent oral SCC tumor cells acquire their growth advantage and invasive ability has been the subject of extensive study. It has been proposed that the cells exhibit either acquisition of resistance to one or more inhibitory paracrine growth factors or endogenous production of growth factors with autocrine or intracrine regulatory capability (4).

Although tumor cell production of cytokines is rarely documented in vivo, some tumors are able to elaborate a multiplicity of cytokines that have been shown to act in solid tumor systems by altering the tumor microenvironment, by functioning as autocrine growth factors, by varying the expression of adhesion molecules, or by regulating antitumor immune responses (5). Cytokines secreted from the primary tumor site may initiate regulatory molecular cascades that ultimately down-regulate host defense, thus supporting tumor proliferation, fostering invasion, promoting progression, and/or subsequently enhancing metastasis along lymphatic channels. We have previously detected endogenous production of IL-1 and IL-6 in some head and neck SCC cell lines and universal intracellular production of these ILs in primary invasive head and neck squamous cell tumors from 12 patients (6). We have also reported that exogenous and/or endogenous IL-1 produced by melanoma cell lines regulates IL-6 production in malignant melanoma (7).

IL-1 is known for its extensive ability to mediate host defenses. It exerts its pleiotropic effects on nearly every tissue and organ system in the body. The IL-1 family includes two distinct IL-1 proinflammatory gene products, IL-1α and IL-1β, which recognize the same receptors, and one naturally occurring inhibitor, the IL-1Ra (8). IL-1 is potent in the pico- and femtomolar range and is capable of inducing a response by binding to only one or two IL-1 type I receptors per cell. IL-1 may affect the host-tumor relationship through its effect on the endothelium with its ability to up-regulate adhesion molecules specific for leukocytes and through stimulation of endothelial cell morphological changes that allow for extravasation of infiltrating immune cells (7, 9, 10). IL-1 is also capable of stimulating the hematopoietic system and fibroblasts, leading to induction of a selective cytokine cascade that includes the production of IL-6 through NF-κB (11).

IL-6 is a multifunctional cytokine produced by both lymphoid and nonlymphoid cells. Some of its many effects are regulation of immune responses, acute-phase reactions, and hematopoiesis (12). IL-6 is also a regulator that gives immunity cells the potential to control or influence immune responses by mediating communication between stromal and hematopoietic cells. The plethora of IL-6 actions contribute to indirect suppressor and/or antitumor effect, and some of its activities overlap with IL-1.

Diminished antitumor immunity is a determinant of head and neck tumor progression (13). The relationship between immune impairment and oral SCC has been described (14, 15) and involves local and systemic deficiencies in both the humoral and cellular immune systems (reviewed in Ref. 13). We have previously documented an association between HPV infection and oral SCC (16) that is consistent with current understanding of the importance of tumor suppressor factors in malignant transformation, the multifactorial causation of cancer, and the immunosuppression seen in many oral cancer patients. Inactivation of the tumor suppressor gene products p53 and p105, either directly, by point mutation, or indirectly, by the products of the oncogenic HPV E6 and E7 genes, respectively, has been proposed as...
a method of malignant transformation in SCC. We are unaware of any studies that have attempted to address the functional contribution of cytokines from primary oral SCC cell lines in the context of HPV infection or p53 status. Hence, to approach the mechanisms of cytokine regulatory pathways active in oral SCC tumors that may contribute to their growth and/or progression, we tested the following hypotheses: (a) that the secretion IL-1 and IL-6 by oral SCC cell lines can be regulated by the addition of extracellular ILs and/or IL-1Ra; and (b) cytokine secretion correlates with oncogenic HPV E6/E7 expression and/or the status of the p53 gene.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. MDA 686L.N, derived from a poorly differentiated lymph node metastasis from the base of the tongue, and MDA Tu 1483, derived from a well-differentiated tumor of the retromolar trigone, were kindly provided by Dr. Peter Sacks (Ref. 17; Memorial Sloan-Kettering Hospital, New York, NY); MDA Tu 138, derived from a well-differentiated tumor from the mucosal surface of the lower lip, was provided by Dr. Gary Clayman (Ref. 18; University of Texas M. D. Anderson Cancer Center); UMSCC-1 cells, derived from a moderately well-differentiated tumor from the floor of the mouth, were provided by Dr. Thomas Carey (Ref. 19; University of Michigan, Ann Arbor, MI); and HOK16B, a HPV-immortalized human oral keratinocyte cell line, was provided by Dr. N-H. Park (Ref. 20; University of California at Los Angeles, Los Angeles, CA). HeLa and SiHa, cervical cell lines known to harbor HPV 18 and 16, respectively, as well as SK Mel 1, a melanoma-derived cell line, were acquired from American Type Culture Collection (Manassas, VA) and cultured according to standard protocols. All SCC lines used in the studies on cytokine expression and regulation were grown in 1:1 DMEM-F12 culture medium supplemented with 10% FCS and penicillin and streptomycin. Cells were incubated in 5% CO₂ in humidified air at 37°C. Stock cultures were grown to 85% confluency and were subcultured after disruption of adherence with a 0.05% trypsin, 0.5% EDTA solution.

Extraction of DNA. DNA was extracted from cells cultured in four 75-mm² tissue culture flasks. The extraction procedure was slightly modified from the DNA preparation procedure by Hermann and Frischauf (21). In brief, 2.8 ml of lysis buffer (2.5 ml of TEN9 (50 mM Tris-HCl, pH 9, 100 mM EDTA, 200 mM NaCl), 37.5 µl of proteasome K at 300 µg/ml, and 250 µl of 1% SDS) was added to each flask, and the flasks were incubated overnight at 37°C. The lysed cell suspensions were pooled into one 50-ml sterile centrifuge tube and extracted with a 1:1 mixture of phenol-chloroform for 1 h at room temperature on a rocking platform. The aqueous layer was extracted twice more with phenol-chloroform for 30 min each time, followed by extraction with chloroform for 30 min. After the chloroform extraction, RNase was added to the cellular DNA mixture at a final concentration of 100 µg/ml, and the mixture was incubated for 15 min at 37°C. Following two additional phenol-chloroform extractions and one last chloroform extraction, water-saturated ether was added to the DNA to remove any excess chloroform, and the DNA was dialyzed against Tris-EDTA (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) overnight at 4°C. The DNA was then concentrated by the addition of an equal volume of 2-butanol, mixed for 10 min, and centrifuged at 1500 rpm for 5 min; hence, the aqueous phase was removed. Butanol concentration was repeated until the concentration of DNA was between 200 and 240 µg/ml as determined spectrophotometrically. Excess butanol was removed by ether extraction.

Cell lines containing HPV DNA and plasmids containing cloned HPV DNA were used as positive controls to test the validity of PCR with HPV consensus primers. DNAs of HPV types 6, 11, 16, and 18, which were cloned into the plasmid pBR322, were recovered from bacterial lysates using a Qiagen plasmid preparation (Qiagen, Studio City, CA). DNA from two HPV-containing cell lines, SiHa (containing 1-2 copies of HPV 16 per cell) and HeLa (containing 10-50 copies of HPV 18 per cell; Refs. 22-25), was extracted and used as an additional control for PCR amplification. HeLa and SiHa served as positive controls and showed a band of 450 bp when visualized on agarose gel after electrophoresis; this is the size expected for amplification of HPV DNA from these primers.

Extraction of RNA. Cultured cells were trypsinized and washed once in PBS, after which 3-6 × 10⁶ cells were pelleted by centrifugation at 7000 rpm in a microcentrifuge. The pellets were frozen at −70°C for no longer than 72 h until RNA could be extracted. TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH) was used to extract total RNA from the cell pellets according to the manufacturer’s instructions. Briefly, the cells were homogenized by phenol-chloroform extraction, followed by precipitation of RNA with isopropanol. The RNA was washed with 75% ethanol and was solubilized in DEPC-treated water. Finally, the RNA was resuspended in 24 µl of DEPC-treated water and frozen at −70°C.

Primers and Probes. Oligonucleotide primers for amplification of HPV gene sequences were synthesized in the laboratory of Dr. Edwin Murphy (University of Texas M. D. Anderson Cancer Center) on an Applied Biosystems Model 380 DNA synthesizer (Applied Biosystems, Foster City, CA). Oligonucleotide primers for amplification of the ILs were purchased from Clontech Laboratories (Palo Alto, CA). Each of these cytokine oligonucleotide primer pairs was constructed to span an intron to distinguish cDNA transcribed from viral DNA and mRNA. Although these primers bind to both DNA and RNA sites, the sizes of the bands differ. Our bands correspond to the expected sizes of products amplified from transcribed mRNA not the larger DNA (see Table 3). G3PDH primers, also spanning an intron, were derived from the published gene sequences and synthesized in the laboratory of Dr. Jack Roth (University of Texas M. D. Anderson Cancer Center) on similar equipment. The primers were purified on NACS columns (Bethesda Research Laboratories, Gaithersburg, MD) according to the manufacturer’s instructions. Oligonucleotide primers for the amplification of HPV E6/E7 open reading frames were synthesized, based on published sequences, by Genesys (The Woodlands, TX). All oligonucleotide primers were tested, aliquoted, and stored frozen at −20°C according to the manufacturer’s instructions. The primers and probes are shown in Table 1. The development of consensus HPV amplification oligonucleotide primers by Manos et al. (26) used regions of DNA sequence homology among the five mucosotropic types of HPVs. The choice of the 3’ end of the HPV L1 open reading frame for amplification
reflects the fact that this region of viral DNA is likely to be retained if viral DNA has become integrated within an infected cell (27).

Oligonucleotide probes were purified on NACS columns (Life Technologies, Inc., Gaithersburg, MD). Fifty pmol of each detection sequence was 5'-end labeled with [γ-32P]ATP by exchange of 5'-terminal phosphate groups against [γ-32P]ATP (Amersham Pharmacia Biotech) by T4 polynucleotide kinase using a commercially available kit (Boehringer Mannheim, Indianapolis, IN). The radiolabeled probes were purified on G-25 Sephadex Quick Spin columns (Life Technologies, Inc., Gaithersburg, MD). Fifty pmol of each detection sequence was 5' end labeled with [γ-32P]ATP by exchange of 5'-terminal phosphate groups against [γ-32P]ATP (Amersham Pharmacia Biotech) by T4 polynucleotide kinase using a commercially available kit (Boehringer Mannheim, Indianapolis, IN). The radiolabeled probes were purified on G-25 Sephadex Quick Spin columns (Boehringer Mannheim). The specificity of each probe was 1–2 × 10^6 cpm per ml.

PCR. PCR amplifications of DNA were carried out as described by Woods et al. (16). The high sensitivity of PCR demanded rigorous preparation of reagents and material to eliminate all possible sources of contamination. Positive-displacement pipettors with disposable pistons (Rainin, Woburn, MA) were used for all pipetting procedures. Two physically separated laboratories and separate equipment were used for all experiments because the amplification products of one experiment can be a source of contamination for the next experiment (carry-over). Prior to the addition of template DNA, each PCR mixture was placed on a transilluminator and irradiated with UV light for 10 min to denature anycontaminating double-stranded DNA. Preparation and amplification were performed in separate rooms. All experiments were performed in duplicate using both positive and negative controls. Manipulation, analysis, and storage of amplified products was isolated in a separate laboratory using different equipment than that used in reagent preparation and amplification. Several glove changes were made.

RT-PCR. RT-PCR was used according to a published protocol by Brenner et al. (28). Amplification was performed in a Perkin-Elmer 9600 thermocycler. Amplification of the template with G3PDH primers via the same method was carried out to verify that the mRNA was intact. Positive-control cDNAs corresponding to each IL (Clontech) were amplified to confirm primer specificity. Amplification of mRNA with primers to pAWT09 (IL-1α) RNA (supplied in the RNA PCR kit by Perkin-Elmer Corp., Foster City, CA) also served as positive controls for each experiment. Negative controls and amplification of HPV E6/E7 cDNA were the same as previously indicated above in the section on PCR.

After reverse transcription of RNA extracted from MDA 686LN and MDA Tu 1483, the resulting strands were PCR amplified using oligonucleotide primers synthesized according to the E6 and E7 sequences of HPV 16 and HPV 18. The 5' oligonucleotide primer was complementary to E6, whereas the 3' oligonucleotide was complementary to E7. The amplicon obtained represented an E6/E7 mRNA splice product known to be produced by other cellular models (23).

Southern Analysis of Amplified DNA. Twelve μl of each PCR-amplified product were separated by electrophoresis through 4% agarose gels (3% NuSieve agarose, 1% SeaKem agarose, FMC Corp., Rockland, ME). The PCR products were made visible by staining with ethidium bromide and exposure to UV light (254 nm) on a transilluminator. The molecular weights of the amplification products were determined by comparison with either HincII or HaeIII restriction fragments derived from bacteriophage φX 174 DNA (United States Biochemical Corp., Cleveland, OH).

Electrophoretically separated DNA fragments were prepared for Southern transfer and hybridized according to our previously described protocol (16). The HPV E6/E7 detection sequence was hybridized in the same manner.

RESULTS

Oral SCC Tumor Cell Lines Constitutively Express IL-1α, IL-1β, and IL-6 in Contrast to Cultured Normal Oral Keratinocytes. UMSCC-1, MDA Tu 138, MDA 686LN, and MDA Tu 1483 express mRNA and produce intracellular and secreted IL-1 and IL-6 (Ref. 6 and MDA 1483 data not shown). Additionally, each cell line produced significant amounts of each cytokine when compared with...
HPV-16-immortalized human oral keratinocytes after 72 h of incubation in serum-free medium (6). To answer the question of whether exogenous IL-1 or IL-6 alter the proliferation rate of these cell lines, MTT proliferation assays were performed on days 1–3 by adding varying concentrations (0, 15.4, 62.5, 250, and 1,000 units/ml) of IL-1α, IL-1β, IL-6, and IL-1Ra in multiple experiments. There was no consistent proliferative effect of these recombinant cytokines (data not shown).

IL-1Ra Differentially Affects the Secretion of IL-1α and IL-1β in Oral Cell Lines. Because our experiments showed that all three cell lines consistently produce IL-1, we investigated the possibility of an autocrine system by testing whether administration of extracellular IL-1Ra inhibits the secretion of IL-1α or IL-1β by these cell lines. ELISA was used to measure the secretion of IL-1α or IL-1β from cell lines cultured for 52 h in the presence of IL-1Ra versus medium alone. The addition of exogenous IL-1Ra at 1000 ng/ml significantly inhibited the secretion of IL-1α by MDA Tu 138 and MDA 686LN (Fig. 1). Unexpectedly, IL-1Ra appeared to stimulate the secretion of IL-1β in MDA Tu 138. Fig. 2 shows that exogenous IL-1Ra significantly inhibited the secretion of IL-1β in both MDA Tu 138 and MDA 686LN but appeared to exert no effect on UMSCC-1. These data suggest that IL-1 may regulate its own secretion in an autocrine fashion.

Exogenous IL-1 Induces IL-6 Secretion. To evaluate whether exogenous IL-1 induces IL-6 secretion in a sequential pathway in oral SCC cell lines as we had reported previously in melanoma (7), we performed ELISAs to quantitate the IL-6 secreted from each oral SCC cell line incubated for 52 h with 100 units/ml IL-1α or IL-1β versus medium alone. Fig. 3 demonstrates that the addition of IL-1α significantly increased the secretion of IL-6 by all three lines. Curiously, administration of exogenous IL-1β had no consistent effect on IL-6 secretion (MDA Tu 138 and MDA 686LN secreted less IL-6, whereas UMSCC-1 secreted more). Therefore, IL-1α, but not IL-1β, appears to regulate IL-6 secretion.

Specific Regulation of IL-6 Secretion by IL-1. To assess whether the induction of secretion of IL-6 by IL-1α seen in the last experiment was specific, we tested IL-6 secretion of oral SCC cell lines grown with and without IL-1Ra (Fig. 4). IL-1Ra clearly and significantly (P < 0.05 by two methods) reduced the secretion of IL-6 in all 3 cell lines at both doses tested, confirming the specificity of IL-1 in regulating IL-6.
can bind to and subsequently inactivate the p53 tumor suppressor gene product and the retinoblastoma tumor suppressor gene product, p105\(^\text{r}\), respectively (29).

RT-PCR of MDA 686LN and MDA Tu 1483 was performed to detect HPV E6/E7 RNA transcription (Fig. 6). A band of approximately 700 bp was obtained in the lanes labeled \(\text{HeLa, SiHa, 1483, and MDA 686LN}\). This band was of the size expected for an E6/E7 product and was detected in HeLa and SiHa as well as MDA Tu 1483 and MDA 686LN. However, the band was not detected in UMSCC-1 (Fig. 6), as expected since this cell line was shown not to contain HPV DNA. Southern analysis was used to confirm the identity of the PCR products (Fig. 7). When the membrane was probed with two oligonucleotide detection sequences, internal to the flanking primers, the expected band was detected in HeLa, SiHa, MDA 686LN, and MDA Tu 1483 suggesting that the E6/E7 mRNA of HPV 16 was expressed in these cell lines but not in Mel-1, the negative control.

**Secretion of IL-1 or IL-6 Does Not Correlate with Transcriptional Activation of HPV E6/E7.** Table 2 summarizes the HPV DNA status of and the cytokine production by these oral SCC lines. There was no apparent association between HPV DNA status and cytokine expression. All cell lines secreted IL-1\(\alpha\), IL-1\(\beta\), and IL-6 regardless of whether they contained or expressed HPV DNA.

**Association of IL-6 Secretion with p53 Status.** The secretion of IL-6 by the oral SCC cell lines is shown in Fig. 8. We graded the maximal IL-6 secretion from these cell lines on a scale of 0 (lowest) to 4+ (highest) and compared the scores with p53 gene status [as reported in Refs. 29 and 30 or established by single-strand conformation polymorphism and DNA sequencing of exons 4–9 in independent laboratories by two of our authors (K. A-S. and G. L. C.); data not shown]. As shown in Table 3, the two cell lines with mutated p53 usually secreted high levels of IL-6; however, IL-6 production from

**Fig. 5. Analysis of PCR products from HPV L1 amplification by Southern hybridization.** Thirty \(\mu\)l of PCR product were loaded onto a 4% agarose gel and resolved by electrophoresis. The DNA was then transferred to a Hybond N\(^+\) membrane. The membrane was probed with HPV L1 consensus detection sequences. Negative control (—control), water instead of DNA template amplified with glyceraldehyde-3-phosphate dehydrogenase primers.

**Fig. 6. Analysis of HPV E6/E7 mRNA by RT-PCR.** Thirty \(\mu\)l of PCR product were loaded onto a 4% agarose gel and resolved by electrophoresis. The DNA was visualized by staining with ethidium bromide. Controls: + control, Perkin-Elmer RNA PCR kit control amplified with kit primers (DM151, DM 152) after reverse transcription; —control, water instead of RNA template amplified with glyceraldehyde-3-phosphate dehydrogenase primers.

**Fig. 7. Analysis of RT-PCR products from HPV E6/E7 amplification by Southern hybridization.** Thirty \(\mu\)l of PCR product were loaded onto a 4% agarose gel and resolved by electrophoresis. The DNA was then transferred to a Hybond N\(^+\) membrane. The membrane was probed with a mixture of two internal detection sequences, one from E6 and one from E7.

**Table 2. Summary of cytokine expression and HPV DNA status in oral SCC cell lines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IL-1(\alpha)</th>
<th>IL-1(\beta)</th>
<th>IL-6</th>
<th>HPV DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMSCC-1</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MDA Tu 138</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MDA 686LN</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MDA Tu 1483</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>HPV 16</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>HPV 18</td>
</tr>
</tbody>
</table>
UMSCC-1, a wt p53-positive cell line, strongly indicates other regulatory influences, particularly that of endogenous IL-1. Therefore, in conclusion, our results reveal that the only consistent regulation of IL-6 in these cell lines was via IL-1. IL-1 receptor studies are in progress.

**DISCUSSION**

To elucidate possible cytokine regulatory pathways active in oral SCC tumors that may contribute to their growth and/or progression, we investigated the effects of exogenous IL-1 and IL-6 on cell line proliferation and secondary cytokine secretion. Our data suggest that the exogenous administration of rIL-1 or rIL-6 does not provide these cell lines with a growth advantage. We hypothesize that as the original primary tumor progressed, the cells underwent a transition to produce the endogenous ILs that we detected. This process may have at one time provided a growth advantage that contributed to the aggressive and invasive phenotype now seen in these derived cell lines. The acquisition of endogenous IL-1 production and its possible contribution to autocrine cell growth have been implicated in other tumor systems and should be investigated fully in oral SCC. The participation of endogenous IL-1 in constitutive IL-6 secretion has been established in acute myeloblastic leukemia (32), in CSF-producing carcinoma cell lines (33), and in melanoma cell lines (7).

Although IL-1 is produced by both normal and abnormal epithelium, the enhanced production of IL-6 is seen in epithelium only under pathological conditions. To assess what role IL-1 plays in regulating IL-6 secretion in these tumors, exogenous IL-1 was administered to cell lines, and IL-6 secretion was measured. Our results indicate that exogenous IL-1a strongly up-regulated IL-6 secretion in all three cell lines. This augmentation was specifically abolished by exogenous administration of IL-1Ra. The finding that exogenous IL-1 regulates IL-6 in oral SCC is novel. Moreover, the ability of exogenous IL-1 to activate IL-6 at the gene level to induce transcription, translation, and secretion in oral SCC is an important observation. IL-1 is known to regulate IL-6 transcription in other cells via at least two transcription factors, NF-IL-6 and NF-kB. To clarify the mechanism involved in oral SCC, these cell lines could be studied for their expression (either constitutive or IL-1-induced) of these transcription factors.

Furthermore, association of the oncogenic HPV types with SCC is well documented; hence, our detection of HPV DNA in some of these oral SCC cell lines was not unexpected (Fig. 5). To specifically address the hypothesis that HPV DNA-containing tumors of the oral cavity would express different cytokines than tumors without HPV, we compared HPV DNA presence with cytokine expression. No association between HPV DNA presence and cytokine expression was found in the small number of cell lines we tested (Table 2).

Bryan et al. (34) found that a cultured human oral keratinocyte cell line transfected with the entire HPV 16 early gene sequence secreted high levels of IL-6, and Malejczyk et al. (35) suggested that constitutive release of IL-6 may result from persistence and expression of an integrated HPV 16 genome. However, we found that although MDA 686LN contains and transcribes HPV 16 E6 and E7 and also secretes IL-6 at a relatively high concentration (3+, shown in Table 3) when compared with the other oral SCC cell lines, HOK 16B contains integrated HPV 16 that expresses HPV E6 and E7 (36) but, as shown in Fig 1, does not constitutively secrete IL-6. Nevertheless, it is important to consider the immunology of HPV infection. Inflammatory cytokines affect HPV-infected cells and regulate HPV early gene expression (reviewed in Ref. 37). Tumor necrosis factor α and IL-1 have been shown to repress the transcription of HPV 16 early gene expression, whereas IL-6 and IFN-γ have not (38). Cell-mediated antiviral effects are mediated through down-regulation of viral early gene products. However, if HPV has subverted host defense, HPV DNA integrates stably into the genome, expression and production of the putative transforming proteins occurs, and then differential qualitative and quantitative cytokine profiles may emerge. This hypothesis is supported by the decreased cytokine production observed in cervical malignancy (39, 40) and by the constitutive production of IL-1 and IL-6 detected *in vitro* and *in vivo* in our investigations of oral SCC.

After reviewing the p53 status of our cell lines in the context of HPV, we concur with other authors (41–43) that, unlike lung and cervical carcinoma models, there is no "inverse correlation" between high-risk HPV infection and p53 expression in oral SCC. In fact, our data could suggest different mechanistic scenarios of tumorigenesis for each of the four oral SCC cell lines tested (Table 3). MDA 686LN contains both a mutant p53 gene and transcriptionally active HPV 16, leading to speculation that two mechanisms of transformation may have occurred. MDA Tu 1483 contains transcriptionally active HPV 18 DNA and wild-type p53, suggesting that E6 may have bound normal p53, resulting in tumor formation. Tu 138 does not contain HPV DNA; however, its p53 gene is mutated, suggesting one explanation for transformation, although it would be necessary to establish whether the p53 mutation is a functional one. UMSCC-1 has neither HPV DNA nor mutated p53, so the mechanism of transformation for this cell line is unclear. In the HPV-positive lines it is also possible that HPV infection occurred subsequent to tumorigenesis in some cases and is unrelated to the process. It is important then to investigate the status of the Rb gene and the newly reported putative tumor suppressor on chromosome 13 (44) to speculate upon alternative mechanisms of transformation.

To interpret the secretion of IL-6 by these cell lines in the context of the p53 gene according to the current paradigm, our findings support the evolution of a complex interrelationship among transcriptional induction and repression of cytokine elaboration, as well as HPV early gene regulation mediated by p53 and NF-IL-6.

Examination of our findings in the light of these paradigms (Table 3) reveals that when the p53 status of our cell lines is compared with

| Table 3 Summary of IL-6 expression, HPV DNA status, and p53 status in oral SCC cell lines |
|---------------------------------|-------------------------------------------------|-------------------------------------------------|
| IL-6   | HPV DNA status | p53 status |
| HOK 16B | HPV 16 | *a* |
| UMSSCC-1 | HPV 16 | *a* |
| MDA Tu 1483 | HPV 16 | *a* |
| MDA 686LN | HPV 16 | *a* |
| MDA Tu 1483 | HPV 16 | *a* |

*a* = wild type; *mut* = mutant.

*Tested independently in the laboratories of our authors K. A-S. and G. L. C.
IL-1 REGULATES IL-6 SECRETION IN HUMAN ORAL SCC IN VITRO

towards their cytokine secretion, the cell lines secreting the most IL-6 also have mutant p53 and/or HPV 16 E6/E7 expression. The production of high concentrations of IL-1 by UMSCC-1, MDA Tu 138, MDA 686LN, and MDA 1483 may also contribute to high levels of NF-IL-6 as well as NF-κB, both of which induce IL-6 expression and secretion. It is also likely that p53 interacts with and inhibits the function of other cellular transcription factors.

The aberrant production of IL-6 by neoplastic cells has been strongly implicated in the growth of multiple myeloma and other B-cell dyscrasias, T cell lymphoma, renal and ovarian carcinomas, and Kaposi sarcoma and the inhibition of tumor cell apoptosis (12, 45–50). In some of these models, autocrine regulation of IL-6 has been hypothesized. Inducible expression of IL-6 by serum components, cytokines, second messengers, and viruses may all be involved, leaving unanswered questions about constitutive IL-6 production by neoplastic cells. As in vivo gene therapy using adenoviral transfer of wild-type p53 emerges as a treatment modality to prevent microscopic residual head and neck SCC (51), monitoring IL-6 might serve as a useful marker of p53 regulation.

Our cell line data may or may not accurately reflect in vivo cytokine regulation due to artifacts introduced by culture over time; however, the results described here are corroborated by our detection of universal elaboration of IL-1 and IL-6 in surgical oral SCC specimens (6). Consequently, future studies should focus on differences in the expression and regulation of ILs in advanced carcinomas versus premalignant lesions and in situ carcinomas to access the role that cytokines play in tumor progression, as well as to establish whether altered cytokine regulation correlates with tumor progression. Additional studies should also address the influence of cytokine elaboration on invasion and angiogenesis.

Therefore, understanding the complex elaboration and regulation of cytokines in the oral SCC model may be of major importance to immunological tumor control. Although numerous studies have addressed the relationship between specific immunological impairments and poor prognosis, few have addressed the complex network of factors that affect the primary tumor microenvironment. This study should be viewed as a step toward defining the biological factors present within the intricate tumor microenvironment that may predispose an oral cancer patient toward disease progression conferring poor prognosis.

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