Expression of Proinflammatory and Proangiogenic Cytokines in Patients with Head and Neck Cancer


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ABSTRACT

Altered immune, inflammatory, and angiogenesis responses are observed in patients with head and neck squamous cell carcinoma (HNSCC), and many of these responses have been linked with aggressive malignant behavior and a decrease in prognosis. In this study, we examined the hypothesis that HNSCC cells produce cytokines that regulate immune, inflammatory, and angiogenesis responses. We identified important regulatory cytokines in supernatants of well-defined and freshly cultured HNSCC cell lines by ELISA and determined whether these cytokines are detected in tumor cell lines and tissue specimens by immunohistochemistry. The serum concentration of the cytokines and cytokine-dependent acute phase inflammatory responses (i.e., fibrinogen, C-reactive protein, and erythrocyte sedimentation rate) from patients with HNSCC was determined, and the potential relationship of serum cytokine levels to tumor volume was analyzed. Cytokines interleukin (IL)-1α, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor were detected in similar concentration ranges in the supernatants of a panel of established University of Michigan squamous cell carcinoma (UM-SCC) cell lines and supernatants of freshly isolated primary HNSCC cultures. Evidence for the expression of IL-1α, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, and VEGF in HNSCC cells within tumor specimens in situ was obtained by immunohistochemistry. In a prospective comparison of the cytokine level and cytokine-inducible acute-phase proteins in serum, we report that cytokines IL-6, IL-8, and VEGF were detected at higher concentrations in the serum of patients with HNSCC compared with patients with laryngeal papilloma or age-matched control subjects (at $P < 0.05$). The serum concentrations of IL-8 and VEGF were found to be weakly correlated with large primary tumor volume ($R^2 = 0.2$ and $0.4$, respectively). Elevated IL-1- and IL-6-inducible acute-phase responses were also detected in cancer patients but not in patients with papilloma or control subjects (at $P < 0.05$).

We therefore conclude that cytokines important in proinflammatory and proangiogenic responses are detectable in cell lines, tissue specimens, and serum from patients with HNSCC. These cytokines may increase the pathogenicity of HNSCC and prove useful as biomarkers or targets for therapy.

INTRODUCTION

Alterations in host immunity, inflammation, angiogenesis, and metabolism have been noted to be prominent clinical features in patients with HNSCC (1–3). These tumor-induced T-lymphocyte, granulocyte, and neoangiogenesis responses in the local tumor microenvironment (1–3) have been associated with increased growth and metastasis and decreased survival (4–6). Pathological changes in systemic responses have also been observed, including induction of antibody and other acute-phase inflammatory protein responses (7, 8); a decrease in delayed-type hypersensitivity (9, 10), T-cell and natural killer cell-mediated immunity (11, 12); and changes in catabolism that often lead to marked weight loss and hypercalcemia (13, 14). Although the origin of the signals and mechanisms underlying these responses are not well understood, the local and systemic nature of these responses suggests the hypothesis that cytokines with proinflammatory, proangiogenic, and immunoregulatory activity are produced by squamous cell carcinomas and could contribute to the pathogenesis of HNSCC.

We reported previously that murine SCC cell lines derived after tumor progression exhibit increased malignant behavior in association with expression of a repertoire of proinflammatory and proangiogenic cytokines (15–17). We found that human

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3 The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; IL, interleukin; VEGF, vascular endothelial growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; TNF, tumor necrosis factor; TGF, transforming growth factor; FGF, fibroblast growth factor.
HNSSC cell lines express a similar repertoire of cytokines, including IL-1α, IL-6, IL-8, and GM-CSF, and that IL-1α induces the expression of other members of this cytokine repertoire by HNSSC cells (18). One or more of these cytokines have also been detected in tumor homogenates, primary cultures, and established HNSSC lines from patients by others (19–22). The extent to which these and other cytokines important in the regulation of immune, inflammatory, and angiogenic responses can be detected in the local tumor environment and systemically in patients remains to be determined.

In the present study, we surveyed the expression of 14 cytokines important in the regulation of immune, inflammatory, and angiogenic responses in well-defined and freshly cultured HNSSC lines and sought to determine whether these cytokines are expressed and can be detected in the local tumor environment and systemically in vivo. Proinflammatory and proangiogenic cytokines were most commonly detected, including IL-1α, IL-6, IL-8, GM-CSF, VEGF, and basic FGF. Other important proinflammatory cytokines, such as IL-1β, TNF-α, and TGF-β, and immunoregulatory cytokines, such as IL-2, IL-12, IFN-γ, IL-4, and IL-10 were not detected in the HNSSC supernatants. We reported here that cytokines IL-1α, IL-6, IL-8, GM-CSF, and VEGF were detected in HNSSC in situ by immunohistochemistry. Furthermore, elevation in the levels of IL-6, IL-8, and VEGF, as well as cytokine-inducible acute phase responses, were found in the serum from patients with HNSSC. We therefore conclude that expression of cytokines important in proinflammatory and proangiogenic responses were detected in the tumor environment and systemically in patients with HNSSC.

**MATERIALS AND METHODS**

**Human Subjects.** All tissue for derivation of cell lines and immunohistochemical analysis and serum for cytokine and acute-phase reactant studies were obtained with informed consent under Institutional Review Board-approved protocols at the University of Michigan (UM-SCC cell lines) or the NIH (97-DC-0044; primary NIH-SCC cell cultures, tissue, and serum). Patients with HNSSC treated at the University of Michigan or NIH received standard treatment with surgery without or with radiation or investigational therapy with concurrent paclitaxel and radiation therapy under NIH Institutional Review Board-approved protocol 95-C-0162. Patients with papilloma underwent standard microlymangiocscopic excision with CO2 laser.

**Culture of Established UM-SCC Squamous Cell Carcinoma Lines for Analysis of Cytokine Secretion.** To identify cytokines produced by human HNSSC, we screened seven well-characterized cell lines from the UM-SCC series for production of a panel of 14 important regulatory cytokines with proinflammatory, proangiogenic, and immunoregulatory activity by ELISA. The panel of seven established SCC cell lines from the University of Michigan series (UM-SCC) were obtained from Dr. T. E. Carey at the University of Michigan (Ann Arbor, MI; Ref. 23). The UM-SCC cell lines were derived from five patients with SCC of the upper aerodigestive tract (Table 1). The seven UM-SCC cell lines selected were obtained from patients with stages I–IV tumors, distributed among oral, pharyngeal, and laryngeal sites, and who died within 2 years of therapy (Table 1). Cell lines established from single isolates of a patient specimen are designated by a numeric designation, and where isolates from two time points or anatomical sites were obtained, the designation includes an alphabetical suffix (i.e., A or B). Karyotype and epithelial marker analyses of the panel of UM-SCC cell lines used have been published previously (24, 25). The expression of the SCC tumor-associated A9 antigen by the cell lines was confirmed in our laboratory prior to use (26). Cell lines were tested and found to be free of Mycoplasma contamination by two independent assays, Gibco MycoTect kit (Life Technologies, Inc., Gaithersburg, MD) and Mycoplasma Detection kit (Boehringer Mannheim, Mannheim, Germany). Cell lines from relatively early (UM-SCC9, P 13; UM-SCC-11A, P 26 and B, P 48) or later passages (UM-SCC-38, P 49; UM-SCC-1, P 59; UM-SCC-22A and B, P 51, P 60) that were available were included. Studies were performed using frozen stocks within five passages of receipt. The cell lines were maintained in Eagle’s minimal essential media supplemented with 10% fetal bovine serum and penicillin/streptomycin. To prepare supernatants from UMSCC cell lines for ELISA, 12 ml of fresh medium were added to established UM-SCC tumor cell lines when 60–80% confluent in 75-cm² tissue culture flasks and collected after 48 h. Supernatants were centrifuged at

**Table 1** Tumor treatment and response characteristics of patients from which UM-SCC cell lines were developed

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>Stage</th>
<th>TNM</th>
<th>Primary site</th>
<th>Specimen site</th>
<th>Prior therapy</th>
<th>Status</th>
<th>Survival</th>
<th>Cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72</td>
<td>M</td>
<td>I</td>
<td>T2N0M0</td>
<td>FOM</td>
<td>Local recur</td>
<td>R</td>
<td>DWOD</td>
<td>15</td>
<td>UMSCC-1</td>
</tr>
<tr>
<td>2</td>
<td>72</td>
<td>F</td>
<td>II</td>
<td>T2N0M0</td>
<td>Tonsil/BOT</td>
<td>Local recur</td>
<td>R</td>
<td>DOD</td>
<td>15</td>
<td>UMSCC-9</td>
</tr>
<tr>
<td>3</td>
<td>65</td>
<td>M</td>
<td>IV</td>
<td>T2N2aM0</td>
<td>Hypopharynx</td>
<td>Pri bx</td>
<td>N</td>
<td>DOD</td>
<td>14</td>
<td>UMSCC-11A</td>
</tr>
<tr>
<td>4</td>
<td>59</td>
<td>F</td>
<td>III</td>
<td>T2N1M0</td>
<td>Hypopharynx</td>
<td>Pri bx</td>
<td>N</td>
<td>DOD</td>
<td>10</td>
<td>UMSCC-22A</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>M</td>
<td>IV</td>
<td>T2N2aM0</td>
<td>Tonsil/BOT</td>
<td>LN met</td>
<td>N</td>
<td>DOD</td>
<td>11</td>
<td>UMSCC-38</td>
</tr>
</tbody>
</table>

*Patient age in years at diagnosis.

*Tumor Node Metastasis system of staging.

*FOM, floor of mouth; BOT, base of tongue.

*Origin of tissue used to establish cultures; recur, recurrence, Pri, primary tumor site; bx, biopsy; resect, surgical resection specimen; LN, lymph node; met, metastasis.

*Therapy given before the specimen used for culture was obtained: N, none; R, radiation; C, chemotherapy.

*DWOD, died without disease; DOD, died with disease.

*Survival in months from diagnosis to last follow up.
Tumor, treatment, and response characteristics of patients with head and neck squamous cell carcinoma from which primary SCC cell lines, serum, and tissue were obtained

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>Stage</th>
<th>TNM</th>
<th>Primary site</th>
<th>Specimen site*</th>
<th>Prior therapy</th>
<th>Status*</th>
<th>Months*</th>
<th>Cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>F</td>
<td>IV</td>
<td>T&lt;sub&gt;4&lt;/sub&gt;N&lt;sub&gt;1&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Lateral tongue</td>
<td>Pri bx</td>
<td>N</td>
<td>NED</td>
<td>19</td>
<td>NIH-SCC-1</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>M</td>
<td>IV</td>
<td>T&lt;sub&gt;4&lt;/sub&gt;N&lt;sub&gt;1&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Nasopharynx</td>
<td>Pri bx</td>
<td>N</td>
<td>NED</td>
<td>18</td>
<td>NIH-SCC-2</td>
</tr>
<tr>
<td>3</td>
<td>77</td>
<td>M</td>
<td>IV</td>
<td>T&lt;sub&gt;4&lt;/sub&gt;N&lt;sub&gt;1&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Lateral tongue</td>
<td>Pri bx</td>
<td>N</td>
<td>AWD</td>
<td>17</td>
<td>NIH-SCC-3</td>
</tr>
<tr>
<td>4</td>
<td>63</td>
<td>M</td>
<td>IV</td>
<td>T&lt;sub&gt;4&lt;/sub&gt;N&lt;sub&gt;1&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Retromolar</td>
<td>Pri bx</td>
<td>N</td>
<td>AWD</td>
<td>16</td>
<td>NIH-SCC-4A</td>
</tr>
<tr>
<td>5</td>
<td>53</td>
<td>F</td>
<td>IV</td>
<td>T&lt;sub&gt;4&lt;/sub&gt;N&lt;sub&gt;1&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Supraglottis</td>
<td>Pri bx</td>
<td>N</td>
<td>NED</td>
<td>16</td>
<td>NIH-SCC-4B</td>
</tr>
<tr>
<td>6</td>
<td>55</td>
<td>M</td>
<td>IV</td>
<td>T&lt;sub&gt;4&lt;/sub&gt;N&lt;sub&gt;1&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Tongue base</td>
<td>Pri bx</td>
<td>N</td>
<td>DOD</td>
<td>3</td>
<td>(–)</td>
</tr>
<tr>
<td>7</td>
<td>29</td>
<td>M</td>
<td>IV</td>
<td>T&lt;sub&gt;4&lt;/sub&gt;N&lt;sub&gt;1&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Nasopharynx</td>
<td>Pri bx</td>
<td>N</td>
<td>AWD</td>
<td>13</td>
<td>NIH-SCC-7</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
<td>M</td>
<td>IV</td>
<td>T&lt;sub&gt;4&lt;/sub&gt;N&lt;sub&gt;1&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Maxillary sinus</td>
<td>Pri bx</td>
<td>N</td>
<td>NED</td>
<td>12</td>
<td>NIH-SCC-8A</td>
</tr>
<tr>
<td>9</td>
<td>72</td>
<td>F</td>
<td>IV</td>
<td>T&lt;sub&gt;4&lt;/sub&gt;N&lt;sub&gt;1&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Tonsil</td>
<td>Pri bx</td>
<td>N</td>
<td>DOD</td>
<td>9</td>
<td>NIH-SCC-8B</td>
</tr>
<tr>
<td>10</td>
<td>46</td>
<td>M</td>
<td>III</td>
<td>T&lt;sub&gt;4&lt;/sub&gt;N&lt;sub&gt;1&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Tonsil</td>
<td>Pri bx</td>
<td>N</td>
<td>NED</td>
<td>13</td>
<td>NIH-SCC-9</td>
</tr>
<tr>
<td>11</td>
<td>46</td>
<td>M</td>
<td>IV</td>
<td>T&lt;sub&gt;4&lt;/sub&gt;N&lt;sub&gt;1&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Pyriform sinus</td>
<td>Pri bx</td>
<td>N</td>
<td>NED</td>
<td>6</td>
<td>(–)</td>
</tr>
</tbody>
</table>

* Pri, primary tumor site; bx, biopsy; resect, surgical resection specimen; LN, lymph node; met, metastasis.

Cultured UM-SCC-11B cells (1–2 × 10<sup>4</sup>) were plated on 8-well chamber slides (Lab-Tek, Naperville, IL) for 2–3 days and were stained as positive controls (18). Cultured cells and frozen tissue sections (9–10 μm) from SCC specimens preserved in OCT were fixed and permeabilized in freshly made cold 4% paraformaldehyde (15 min) and 0.1% saponin (Sigma Chemical Co., St. Louis, MO) in HBSS (Mediatech, Herndon, VA) containing 0.2% sodium azide (Sigma) and 1% H<sub>2</sub>O<sub>2</sub> (Fisher Scientific, Fair Lawn, NJ) for blockade of endogenous peroxidase. The remaining procedures were carried out at room temperature. Ten % serum (Vector Lab, Inc., Burlingame, CA) was used to block the nonspecific binding sites for 20 min and removed without washing. The samples were incubated with the following primary antibodies: 10 μg/ml of mouse anti-human IL-1α (IgG1; Pharmingen, San Diego, CA), 5 μg/ml of rat anti-human IL-6 (IgG2a; Pharmingen), 10 μg/ml of mouse anti-human IL-8 antibody (IgG1; Genzyme, Cambridge, MA), 5 μg/ml of mouse anti-human GM-CSF (IgG2a; Pharmingen), 0.2 μg/ml of mouse anti-human VEGF (IgG1; Santa Cruz Biotechnology, Santa Cruz, CA), 5 μg/ml of mouse anti-human pan cytokeratin (IgG1; Novoceastra Lab, Newcastle upon Tyne, United Kingdom), or isotype controls, mouse IgG (Vector Lab) or rat IgG2a (Pharmingen), diluted in 0.1% saponin in balanced salt solution with 10% serum overnight at 4°C. The samples were blocked with 5% serum for 20 min and incubated with the secondary biotinylated antibody for 30 min (1:200 in 0.1% saponin in HBSS containing 5% serum; Vector Lab), followed by 30-min incubation with biotin/avidin horseradish peroxidase conjugates (Vectastain Elite ABC kit; Vector Lab) and 5–8 min with chromogen diaminobenzidine tetrahydrochloride (Vector Lab) according to the manufacturer’s specifications.

Serum for Cytokine and Acute-Phase Reactant Studies.
Serum from 11 patients with HNSCC, 12 patients with squamous papilloma of the upper aerodigestive tract, and 12 unaffected age- and gender-matched control subjects were obtained for a prospective comparison of cytokine concentration and cytokine-dependent acute-phase responses (Table 3). Among the three subject groups, there were no significant differences in...
terms of age (mean age: HNSCC patients, 52 years; papilloma patients, 44 years; normal subjects, 55 years; Student’s t test, \( P > 0.18 \)), gender (Fisher’s exact test, \( P > 0.6 \)), or alcohol consumption (Fisher’s exact test, \( P > 0.2 \)). There was a difference in smoking history between patients with SCC (8 of 11) and normal subjects (3 of 12; Fisher’s exact test, \( P = 0.04 \)), and this difference was consistent with differences in the prevalence of smoking between patients with HNSCC and the general population. No subjects had a history of prior malignancy, immunodeficiency, autoimmune disorders, hepatitis, or HIV infection. Blood was drawn from control subjects and patients prior to treatment. Sera were collected by centrifuging whole blood at 3000 rpm for 15 min at 10°C by a Sorvall RT6000D centrifuge (DuPont, Wilmington, DE), and the aliquots were stored at −80°C until used in ELISA assay.

**ELISA for Quantitation of Cytokine Concentration in Supernatants from Cultured Human SCC and Serum from Patients and Normal Subjects.** ELISA kits for specific cytokines were used (R&D systems, Minneapolis, MN, and Endogen, Cambridge, MA) according to the manufacturer’s protocol. Each sample was tested in duplicate in each of two or more replicate experiments. After development of the colorimetric reaction, the absorbance at 450 nm was quantitated by an eight-channel spectrophotometer (Biotek Systems, Winooski, VT), and the absorbance readings were converted to pg/ml based upon standard curves obtained with recombinant cytokine in each assay. If the absorbance readings exceeded the linear range of the standard curves, ELISA assay was repeated after serial dilution of the supernatants. Each serum sample was tested in at least two independent ELISA experiments, and the data were calculated from mean of two to four tests for each sample.

**Determination of Tumor Volume.** Tumor volumes were calculated from computerized tomographic images using PowerTPS, the National Cancer Institute Radiation Oncology Treatment Planning System. For each patient, the tumor and involved lymph nodes were manually selected and contoured on each axial computed tomography image. Tumor areas were first determined in pixels by the intersection of the contoured region with the image matrix and then converted to slice volume by multiplying the number of selected pixels by the pixel area and the slice thickness. Total tumor volumes were then taken as the integral of the slice volumes divided by the total number of slices in the image set.

**Measurement of Acute-Phase Responses.** Fibrinogen, C-reactive protein, and sedimentation rate acute-phase responses were measured by standardized clinical laboratory methods in the Clinical Pathology Department of the Warren Grant Magnuson Clinical Center at NIH using serum obtained prior to treatment.

**Histopathological Study.** The cases were retrieved from the surgical pathology files of the National Cancer Institute. H&E-stained slides from in-house biopsies or from referring institutions (Kaiser Permanente, Kensington, MD) were reviewed in all of the cases. The pathological diagnosis, degree of differentiation, and infiltration of tumor by inflammatory cells were graded independently by Dr. Martha Quezado of the Surgical Pathology Section, Pathology Branch, National Cancer Institute. Slides from patients 1 and 11 were not available for the review at the time of this study, and the data were collected from the surgical pathology reports.

**Statistical Analysis.** Differences in gender, smoking, and alcohol consumption among SCC patients, papilloma patients, and normal subjects were tested by Fisher’s exact test using statistical software Prism 2.01. All of the other data were calculated, and statistical significance was tested by independent Student’s t test using software SigmaPlot Scientific Graph System (Jandel Scientific, San Rafael, CA).

**RESULTS**

**Secretion of Cytokines IL-1α, IL-6, IL-8, GM-CSF, VEGF, and Basic FGF by Established and Primary HNSCC Cell Lines.** The characteristics of the patients and UM-SCC cell lines studied are shown in Table 1. Cytokines IL-1α, IL-6, IL-8, GM-CSF, VEGF, and basic FGF were most commonly detected in culture supernatants from UM-SCC cell lines, as summarized in Table 4. Other important proinflammatory cytokines, such as IL-1β, TNF-α, and TGF-β, and immunoregulatory cytokines IL-2, IL-12, IFN-γ, IL-4, and IL-10 were not detected by ELISA in the UM-SCC supernatants. Among the six cytokines detected in the UM-SCC cell culture supernatants, IL-6, IL-8, and VEGF were detected in the highest concentrations.

The characteristics of the patients and primary SCC cell lines studied are shown in Table 2. Fig. 1 shows photomicrographs of the typical epithelial histology and morphology of representative tumors and primary cultures from patient 3 (Fig. 1, A–C) and patient 4 (Fig. 1, D–F). The squamous epithelial origin of the patient tumors was confirmed by immunohistochemical staining with pan cytokeratin, as shown in Fig. 1B. Cultured cells exhibited typical squamous epithelial morphology (Fig. 1, C and F). Table 5 shows that the primary HNSCC cultures produced the six cytokines in a range of concentrations.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Sample size (n)</th>
<th>Gender (M/F)</th>
<th>Age (yr) (Mean ± SE (range))</th>
<th>Alcohol use</th>
<th>Tobacco use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>11</td>
<td>8/3</td>
<td>51.9 ± 5.0 (24–77)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Papilloma</td>
<td>12</td>
<td>8/5</td>
<td>43.6 ± 3.6 (25–73)</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Normal</td>
<td>12</td>
<td>9/4</td>
<td>54.8 ± 4.5 (29–77)</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 3 Demographics of patients and control subjects

aThere is no statistically significant difference in gender between groups (Fisher’s exact test, \( P > 0.6 \)).

bThere is no statistically significant difference in ages between groups (t test, \( P > 0.18 \)).

cThere is no statistically significant difference in smoking history between groups (Fisher’s exact test, \( P > 0.2 \)).

dA significant difference is observed when comparing smoking history between SCC patients and control subjects (Fisher’s exact test, \( P = 0.04 \)).
similar to those detected in the supernatant of established UM-SCC cell lines. Cytokines IL-6, IL-8, and VEGF were produced in highest concentration by both established and freshly isolated HNSCC cultures.

**Detection of IL-1α, IL-6, IL-8, GM-CSF, and VEGF in HNSCC in Situ.** We examined whether the cytokines expressed in established and freshly isolated HNSCC cultures could be detected in HNSCC cells within tumor tissue in situ by immunohistochemical staining. Fig. 2, A–E, shows that HNSCC exhibit immunostaining with cytokine-specific antibodies for IL-1α, IL-6, IL-8, GM-CSF, and VEGF. A weaker staining was evidenced with IL-6, and the staining with IL-8-specific antibody exhibited a nonhomogeneous pattern (Fig. 2, B and C). The immunostaining of cytokines IL-1α, IL-6, IL-8, GM-CSF, and VEGF in Fig. 2, A–E was detected within keratin-positive malignant epithelium (Fig. 2, F–J), whereas significant staining with GM-CSF was also detected in the stroma of specimens (Fig. 2D). The strongest VEGF reactivity (Fig. 2E) was noted to be localized in areas of tumor that exhibited less epithelial differentiation and weaker keratin staining (Fig. 2J). The staining of tissue with cytokine antibodies was greater than that observed with isotype controls (Fig. 2, K–O). Furthermore, the binding of these cytokine-specific antibodies to HNSCC could be specifically inhibited by competition with recombinant cytokine in a dose-dependent manner.\(^4\) We conclude that cytokines IL-1α, IL-6, IL-8, GM-CSF, and VEGF can be detected in HNSCC in situ.

\(^4\) Z. Chen et al., manuscript in preparation.

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**Table 4** Secretion of cytokines by UM-SCC cell lines

<table>
<thead>
<tr>
<th>Cell lines(^d)</th>
<th>Proinflammatory</th>
<th>Proangiogenic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1α</td>
<td>IL-6</td>
</tr>
<tr>
<td>UM-SCC-1</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>UM-SCC-9</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>UM-SCC-11A</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>UM-SCC-11B</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>UM-SCC-22A</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>UM-SCC-22B</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>UM-SCC-38</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

\(a\) UM-SCC (University of Michigan) cell lines.

\(b\) Picograms/10⁶ cells/48 h. +, 1–10; ++, 10–100; ++++, 100–1,000; +++++, 1,000–10,000.

\(c\) Proinflammatory cytokines, IL-1β, TNF-α, TGF-β, and immunoregulatory cytokines IL-2, IL-12, γ-IFN, IL-4, and IL-10 were also screened in UM-SCC cell lines. The cell lines did not produce significant detectable levels of the cytokines.
Elevated IL-6, IL-8, and VEGF Cytokine Levels in Serum from Patients with HNSCC. On the basis of the findings that proinflammatory and proangiogenic cytokines may be expressed by HNSCC in vitro and in the local tumor environment in situ, we prospectively examined and compared the levels of these cytokines in the serum of unaffected subjects and patients with HNSCC and benign squamous papilloma of the upper aerodigestive tract using ELISA. Serum from 11 patients with HNSCC, 12 patients with laryngeal squamous papilloma, and 12 unaffected age- and gender-matched control subjects were obtained (Table 3). Among the three subject groups, there were no significant differences in terms of age, gender, or alcohol consumption. The difference in smoking history between SCC (8 of 11) and normal subjects (3 of 12) was consistent with the increased prevalence of smoking history in patients with HNSCC when compared with the general population. Fig. 3 shows that cytokines IL-6, IL-8, and VEGF were detected in higher concentration in the serum of patients with HNSCC when compared with either patients with squamous papilloma or control subjects ($t$ test, $P < 0.05$). When we compared the serum cytokine levels between patients with papilloma and control subjects, we did not find significant differences in the serum concentration of the three cytokines (Fig. 3). Within the limited sample size studied, we were unable to detect differences between smoking and nonsmoking subjects (data not shown). Concentrations of cytokines IL-1$\alpha$, GM-CSF, and basic FGF were detected at the limits of sensitivity of the assays, and no significant differences were detected in serum of patients with HNSCC, squamous papilloma, or normal subjects (data not shown).

Relationship of Proinflammatory and Proangiogenic Cytokine Expression to Tumor Volume, Differentiation, and Inflammation. We examined whether the expression of proinflammatory and proangiogenic cytokines in the patients with HNSCC was associated with differences in tumor volume, differentiation, and inflammation. Table 6 displays the concentration of cytokines IL-6, IL-8, and VEGF in serum, along with the stage, volume, and the presence of differentiation and inflammation in the tumor for each patient. The relationship of the serum cytokine concentration to tumor volume in patients with HNSCC was examined. Tumor volume was calculated from the computed tomography/magnetic resonance imaging scans obtained for treatment planning, and the relationship of serum cytokine concentration to both primary and metastatic tumor volume in the lymph nodes was determined. A weak relationship between primary tumor volume and VEGF concentration (Fig. 4, upper panel, linear regression $R^2 = 0.420$) and IL-8 concentration (Fig. 4, lower panel, linear regression $R^2 = 0.214$) was detected. There was no correlation between IL-6 concentration and tumor volume. No significant relationship was found between the cytokines with tumor volume in metastatic lymph nodes or total tumor volume consisting of the sum of volumes of the primary and lymph node tumor volumes (Table 6). When we examined whether the differences in cytokine levels observed in serum were associated with differences in the other pathological correlates, such as differentiation or presence of leukocytes, no relationship was evident (Table 6). Leukocytes were detected within the tumor specimens of all of the patients with HNSCC.

Increase in IL-1- and IL-6-dependent Acute Phase Responses in Patients with HNSCC. We examined whether there was evidence for induction of acute phase proteins in the patients with upper aerodigestive neoplasms. Fig. 5 shows that patients with HNSCC exhibit an increase in the mean fibrinogen, C-reactive protein, and the erythrocyte sedimentation rate when compared with patients with papilloma or unaffected subjects ($t$ test, $P < 0.05$). There were no significant differences in the values of fibrinogen, C-reactive protein, and the erythrocyte sedimentation rate observed between papilloma patients and normal subjects or within the groups between smoking and nonsmoking subjects (data not shown). The increase in acute-phase proteins detected in the patients with HNSCC showed no correlation with tumor volume and was not associated with concurrent infection, fever, or elevation in WBC count (data not shown). There were no consistent changes in the concentration of several other acute-phase components tested, including $\alpha$-1 anti-trypsin, $\alpha$-2 macroglobulin, complements factors C3/C4 or factor B, haptoglobin, or albumin (data not shown, $P > 0.05$).
DISCUSSION

In this study, we show that established and primary SCC lines from patients with head and neck cancer can produce proinflammatory cytokines IL-1α, IL-6, IL-8, and GM-CSF and proangiogenic cytokines VEGF and basic FGF. In an extended survey of other important proinflammatory and immunoregulatory cytokines, we did not detect secretion of IL-1β, IL-2, IL-4, IL-10, IL-12, TNF-α, or TGF-β in any of the UM-SCC cell lines. Several of the proinflammatory cytokines detected in this study, including IL-1α, IL-6, IL-8, and GM-CSF, have been detected in tumor homogenates, primary cultures, established HNSCC lines, and in serum from patients by ourselves and others (18–22). In addition, we show that cytokines IL-1α, IL-6, IL-8, GM-CSF, and VEGF could be detected in situ in HNSCC within the local tumor environment. The detection of these cytokines by immunohistochemical methods is consistent with similar findings for IL-1α, and IL-6 by others (22), and results we obtained demonstrating localization of IL-1α, IL-6, and GM-CSF mRNA in HNSCC in situ (28). We conclude that the six cytokines selectively produced by HNSCC possess proinflammatory, proangiogenic, and immunoregulatory activities that are consistent with pathological alterations observed in patients with these neoplasms. These cytokines may play an important role in promoting tumorigenesis.

We compared the concentrations of these cytokines in the serum of patients with HNSCC, benign squamous papilloma, and unaffected age- and gender-matched controls in a prospective study to explore whether elevated levels of these cytokines may be detected in serum of HNSCC patients in vivo. We obtained evidence that cytokines IL-6, IL-8, and VEGF, as well as proinflammatory cytokine-induced, acute-phase proteins, are detected at higher concentrations in the serum of patients with HNSCC but not in patients with benign squamous papilloma or unaffected control subjects. Within the repertoire of proinflammatory and proangiogenic cytokines detected in the supernatants of established and primary HNSCC cell lines, the most abundant cytokines detected were IL-6, IL-8, and VEGF, which is consistent with the elevation of these cytokines detected in the

![Fig. 2 Detection of IL-1α, IL-6, IL-8, GM-CSF, and VEGF in HNSCC by immunohistochemistry in situ. IL-1α (A), IL-6 (B), IL-8 (C), GM-CSF (D), and VEGF (E) were detected in primary tumor specimens from HNSCC by immunohistochemical staining, as described in "Materials and Methods." Positive staining of tumor with pan cytokeratin antibody in adjacent sections is demonstrated (F–J) but is not observed with matched isotype control antibodies (K–O). Specimens from A, F, K and C, H, M were counterstained with hematoxylin to demonstrate nuclear structures. The magnification of all light photomicrographs was ×400, except for E, J, O, which were taken at ×200.](image-url)
Cytokines in Head and Neck Squamous Cell Carcinoma

The evidence that HNSCCs express proinflammatory and proangiogenic cytokines in vitro and in situ suggests that HNSCCs are an important source for the elevated serum levels of proinflammatory and proangiogenic cytokines. Presently, we obtained evidence that increased serum levels of IL-8 and VEGF are correlated with tumor volume (Fig. 4). Conversely, preliminary results of an analysis of the effect of surgery or chemo- and radiation therapy upon cytokine levels indicate that serum cytokine levels decrease in posttreatment patients. However, HNSCC cells may not be the only source of the elevated serum cytokine levels in patients. In the immunohistochemical analysis of cytokines IL-6, IL-8, and GM-CSF, we also detected areas of staining of cytokine IL-6 in fibroblasts and IL-8 and GM-CSF in tumor-infiltrating leukocytes within tumor stroma, which was negative for cytokeratin staining. This evidence indicates that the serum levels of cytokines may also depend in part upon individual host inflammatory responses within the tumor, which may explain some of the variation in serum cytokine concentration observed among individual patients with HNSCC. However, we did not observe a well-defined relationship between serum cytokine levels and the degree of tumor differentiation, presence of inflammatory infiltrating cells, or lymph node metastasis in this pilot study (Table 6). Development of more sensitive and quantitative measurement of cytokines in tissue could complement serum cytokine analysis. The concentration of cytokines detected in serum in this study between or within groups did not appear to be associated with gender, age, or alcohol or tobacco use. When we compared the serum cytokine levels between smokers and nonsmokers in the three groups, no significant difference was found, although this was limited by the number of patient samples in this study.

It will be important to determine whether elevated levels of proinflammatory and proangiogenic cytokines in serum of patients with HNSCC at the time of diagnosis can allow monitoring of patients for therapeutic response and recurrence and help with defining prognosis. The present small pilot study offers encouragement, because we have observed that four of five patients who have developed progressive disease (patients 4, 6, 7, and 9) showed significantly elevated serum levels of one or more of IL-6, IL-8, or VEGF. This is in accordance with reports that elevated serum levels of proinflammatory and proangiogenic cytokines are correlated with advanced stage, metastatic disease or large tumor burden in other types of cancer. In these other cancers, increased serum IL-6 levels correlated well with the disease status and prognosis, including patients with epithelial ovarian cancer (29, 30), metastatic renal cell carcinoma (31), colorectal cancer (32), esophageal squamous cell carcinoma (33), and cervical cancer (34). Elevated serum IL-8 was found in patients with colorectal cancer (32), hepatocellular carcinoma (35), metastatic melanoma (36), and endometrial cancer (37). Increased serum VEGF level has been found in patients with metastatic renal cell carcinoma (31), colorectal cancer (32), esophageal squamous cell carcinoma (33), and cervical cancer (34). Elevated serum IL-8 was found in patients with colorectal cancer (32), hepatocellular carcinoma (35), metastatic melanoma (36), and endometrial cancer (37). Increased serum VEGF level has been found in patients with invasive breast carcinoma (38) and is associated with the poor outcome in small-cell lung cancer (39). Our laboratory is continuing to monitor serum cytokine levels in the patients enrolled in this clinical study and will investigate the relevance of serum cytokine levels as an indicator for prognosis, effectiveness of therapy, and disease recurrence.

The detection of elevated expression of proinflammatory and proangiogenic cytokines (IL-1α, IL-6, IL-8, GM-CSF, and

Fig. 3 Concentration of proinflammatory and proangiogenic cytokines IL-6, IL-8, and VEGF in serum from patients with HNSCC, papilloma, and normal subjects by ELISA. The levels of cytokines, IL-6, IL-8, and VEGF in serum from age- and gender-matched patients with HNSCC, papilloma, and normal subjects were measured by ELISA. The mean concentration of cytokine detected in replicate samples in serum of patients with cancer ( ), papilloma ( ), and normal subjects ( ) is shown. Solid bars, mean concentration (±SEM) of samples for each group, a significant difference by t test between affected and normal subjects at P < 0.05. The sample size and individual serum cytokine values of patients with HNSCC are shown in Table 6.
VEGF in cultured squamous cell carcinoma lines in vitro and in tumor specimens from cancer patients suggests that such cytokine expression may play a role in the increased pathogenicity of HNSCC by providing a growth advantage. We have observed that rIL-1α, rIL-6, and rIL-8 can promote growth of UM-SCC lines in vitro, which is consistent with our detection of proinflammatory and proangiogenic cytokines in human SCC cell lines and in patient serum. Moreover, we have evidence from oral and cutaneous SCC murine models, that murine SCCs constitutively produce a similar repertoire of proinflammatory and proangiogenic cytokines, consisting of IL-1α, IL-6, KC, and IL-8/gro homologue, and GM-CSF (16, 17). Such high cytokine-producing lines exhibit a selective advantage in the rate of tumor growth and metastasis in vivo (15, 16).

IL-1α, IL-6, IL-8, or VEGF may have effects on other non-neoplastic cells that could enhance survival and growth of SCC cells through a paracrine mechanism. The diverse local and systemic inflammatory responses observed in patients with SCC (1–14) are consistent with the broad proinflammatory and proangiogenic activity that have been reported for the cytokines detected in HNSCC. Thus, IL-1α, IL-6, KC, an IL-8/gro homologue, and GM-CSF (16, 17). Such high cytokine-producing lines exhibit a selective advantage in the rate of tumor growth and metastasis in vivo (15, 16).

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Table 6  Serum cytokine levels, tumor load, and pathological characteristics of patients with head and neck squamous cell carcinoma

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cytokines</th>
<th>Tumor size (ml)</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-6</td>
<td>IL-8</td>
<td>VEGF</td>
</tr>
<tr>
<td>1</td>
<td>2.7</td>
<td>29.5</td>
<td>779.7</td>
</tr>
<tr>
<td>2</td>
<td>28.5</td>
<td>24.4</td>
<td>900.6</td>
</tr>
<tr>
<td>3</td>
<td>7.5</td>
<td>27.6</td>
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</tr>
<tr>
<td>4</td>
<td>7.2</td>
<td>130.9</td>
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</tr>
<tr>
<td>5</td>
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<td>699.1</td>
</tr>
<tr>
<td>6</td>
<td>68.1</td>
<td>194.5</td>
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</tr>
<tr>
<td>7</td>
<td>129.6</td>
<td>0.0</td>
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</tr>
<tr>
<td>8</td>
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<tr>
<td>9</td>
<td>4.8</td>
<td>3.3</td>
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</tr>
<tr>
<td>10</td>
<td>0.5</td>
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<td>374.2</td>
</tr>
<tr>
<td>11</td>
<td>1.9</td>
<td>63.6</td>
<td>234.9</td>
</tr>
</tbody>
</table>

* Cytokines were detected in patients’ serum and presented as pg/ml.

** Differentiation was classified as well differentiated, ++++; moderately differentiated, ++; poorly differentiated, +; and undifferentiated, 0.

† The inflammatory infiltrate cells were evaluated as: intense, ++++; moderate, +++; mild, +; or absent. NA, not applicable.

Fig 4 Correlation between serum VEGF and IL-8 levels and tumor load in patients with HNSCC. Linear regression analysis of cytokine serum levels (pg/ml) of VEGF (upper panel) and IL-8 (lower panel) as a function of primary tumor volume (cm³) in patients with HNSCC. Cytokine concentration was plotted against tumor volume and was determined from computed tomography scan. Rs shown in each figure were determined by linear regression analysis.

VEGF) in cultured squamous cell carcinoma lines in vitro and in tumor specimens from cancer patients suggests that such cytokine expression may play a role in the increased pathogenicity of HNSCC by providing a growth advantage. We have observed that rIL-1α, rIL-6, and rIL-8 can promote growth of UM-SCC lines in vitro, which is consistent with our detection of proinflammatory and proangiogenic cytokines in human SCC cell lines and in patient serum. Moreover, we have evidence from oral and cutaneous SCC murine models, that murine SCCs constitutively produce a similar repertoire of proinflammatory and proangiogenic cytokines, consisting of IL-1α, IL-6, KC, an IL-8/gro homologue, and GM-CSF (16, 17). Such high cytokine-producing lines exhibit a selective advantage in the rate of tumor growth and metastasis in vivo (15, 16).

IL-1α, IL-6, IL-8, or VEGF may have effects on other non-neoplastic cells that could enhance survival and growth of SCC cells through a paracrine mechanism. The diverse local and systemic inflammatory responses observed in patients with SCC (1–14) are consistent with the broad proinflammatory and proangiogenic activity that have been reported for the cytokines detected in HNSCC. Thus, IL-1α, IL-6, and GM-CSF have been reported to play important roles in the initiation of local inflammation, activation of CD4+ and B-lymphocyte responses, as well as systemic acute-phase responses. For example, chronic elevation of IL-6 can promote immune unresponsiveness and induction of wasting, cachexia, and hypercalcemia, all of which are observed in patients with SCC who have a poor prognosis (32, 33, 40). 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 SCCs (2, 4, 5 32, 35, 36). VEGF is thought to be a major angiogenic growth factor in animal and human cancer (41–43) that provides sufficient vascularization for tumor growth expansion (42–44). Thus, there is considerable evidence that IL-1α, IL-6, IL-8, GM-CSF, and VEGF cytokines promote tumorigenesis in vivo.

The constitutive expression of cytokines in SCC cells could result from activation of pathways involving IL-1α, other con-


6 G. Thomas, unpublished observations.
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Expression of Proinflammatory and Proangiogenic Cytokines in Patients with Head and Neck Cancer


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