Mechanisms of Immune Suppression in Patients with Head and Neck Cancer: Presence of CD34+ Cells Which Suppress Immune Functions within Cancers That Secrete Granulocyte-Macrophage Colony-Stimulating Factor

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ABSTRACT

Production of granulocyte-macrophage colony-stimulating factor (GM-CSF) by murine tumors has been shown to induce immune suppressive cells having homology with GM progenitor cells. The purpose of this study was to determine if human head and neck cancers secrete GM-CSF, if this is associated with an intratumoral presence of similar cells expressing the hematopoietic progenitor cell antigen CD34, and if such CD34+ cells suppress functions of intratumoral T cells. This was evaluated with fresh head and neck cancers, and in some instances regional lymph nodes and control tissue. Ten of the 14 squamous cell carcinomas (SCCs) studied secreted greater than 5 ng GM-CSF/g tissue. GM-CSF was not secreted in significant levels by either the other cancer types or by control normal muscle. Each of the high GM-CSF-secreting SCCs, but none of the cancers that did not secrete GM-CSF, contained cells expressing the hematopoietic progenitor cell antigen CD34 that had the capacity to grow into colonies in soft agar. Available regional lymph nodes from patients with high GM-CSF-producing cancers also contained CD34+ cells. Depletion of CD34+ cells from dissociated cancers increased interleukin 2 secretion by the intratumoral lymphocytes while addition of the CD34+ cells to dissociated cancers reduced interleukin 2 production, indicating that the presence of CD34+ cells within GM-CSF-producing head and neck SCCs results in suppressed functional competence of lymphocytes within the SCCs. These results show that GM-CSF-secreting SCCs contain cells expressing the hematopoietic antigen CD34 which are inhibitory to the capacity of lymphocytes within the SCCs to secrete interleukin 2.

INTRODUCTION

Clinically, patients with head and neck SCCs have been recognized to have profound defects in cellular immune responsiveness (1). Risk factors for development of this type of cancer include the initiating carcinogens in tobacco and possibly promoting factors in alcohol. Causes of the diminished immune responsiveness include "extrinsic" factors such as the malnourished state of the patients due to interference of the cancer with swallowing (2). In addition, patients with recurrent cancers have often had prior radiation therapy which, although localized to the head and neck, is well known to cause profound long-term alterations in the immune response. Attempts to define the cellular basis of the "intrinsic" factors in patients with head and neck SCCs that subvert immune responsiveness have, until the past decade, been limited to relatively nonspecific assessments of systemic immune capabilities.

More recent basic research studies have shown that cancers, including head and neck cancers, are vulnerable to immune effector cells such as natural killer cells, lymphokine-activated killer cells, and tumor-specific cytotoxic T lymphocytes (1-4). That head and neck cancers can evoke immune responses in vivo is suggested by the selective homing of CD8+ T cells into the cancer (5). Studies with other cancers have suggested that immune responses to cancers may also be important in maintaining cancer dormancy (6).

Although cancers are susceptible to immune-mediated destruction, patients with head and neck cancers and other cancers types have defects in their cellular immune functions, including the tumor-infiltrating T cells and natural killer cells (5, 7, 8). Some of this immune deficiency may be directly induced by soluble suppressive factors produced by the cancers (9, 10). Prostaglandins are among the suppressor factors that have been shown to be produced by some, although not all, head and neck cancers (9, 10). Cancers have also been shown to indirectly suppress immune functions by inducing immune suppressor cells. The immune suppressor cells that may be induced by head and neck cancers have not been extensively studied or described, but have been well documented in many other cancer types in both humans and animals. Some human cancers induce suppressive T cells that are inhibitory to antitumor T-cell reactivity (11). Other reports have shown suppression of T-cell function in cancer patients to instead be due to PGE2 that is secreted by mature monocytes/macrophages (12, 13). Our stud-

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3 The abbreviations used are: SCCs, squamous cell carcinomas; PGE2, prostaglandin E2; LLC, Lewis lung carcinoma; GM-CSF, granulocyte-macrophage colony-stimulating factor; TGFβ, transforming growth factor β; FACS, fluorescence-activated cell sorting; ELISA, enzyme-linked immunosorbent assay; IL-2, interleukin 2.
ies with the murine LLC model have shown that several immune suppressive populations can appear during the course of tumor growth, including PGE$_2$-producing macrophages during early phases of tumor growth (14). The significance of suppressor cells in limiting immunological destruction of tumor is underscored by the tumor regression, reduced metastasis, and increased host survival that occurs when suppressor T cells are reduced in tumor-bearing animals with low-dose chemotherapy, or when macrophage suppressive activity is blocked with prostaglandin synthesis inhibitors (15, 16).

Contrasting with some tumor types, head and neck cancers have been suggested not to induce lymphoid immune suppressive cells (7). However, studies with experimental animal tumors have indicated that some tumors induce immune suppressor cells that are neither suppressor T cells nor macrophages, and whose presence is induced by tumor production of GM-CSF (17–20). These suppressor cells are an immature population of bone marrow-derived cells which appear as a result of the myelopoietic stimulation that is induced by tumor-produced GM-CSF. We have shown that in LLC tumor bearers, this myelopoiesis-associated suppressor population becomes the dominant immune suppressor cell as LLC tumor growth progresses, appearing first in the bone marrow and then also becoming visible within the tumor (18, 19, 21). Extensive phenotypic and functional characterizations have shown that these GM-CSF-induced suppressor cells resemble GM progenitor cells and mediate their suppressive effects in part by producing TGFβ (18, 19, 22, 23). The in vivo functional significance of such suppressor cells in influencing the course of tumor development was suggested when LLC-induced suppressor bone marrow cells enhanced growth and metastasis of cooincubated LLC tumor cells (18).

Many tumor types, such as human lung cancer, breast cancers and hepatocellular carcinomas, and an equally broad spectrum of murine tumors, express GM-CSF mRNA and protein (17, 24–27). Despite the demonstration in animals that tumor production of GM-CSF results in the induction of suppressor cells, no studies have evaluated whether human head and neck cancers secrete GM-CSF or if this induces an intratumoral influx of cells that are homologous to the immune suppressive GM progenitor cells that have been described for GM-CSF-producing animal tumors. The objective of this study was to determine if human head and neck cancers secrete GM-CSF and if this is associated with the presence of immune suppressive cells within the cancer that resemble GM progenitor cells. Our results show that a high frequency of human head and neck SCCs secrete GM-CSF and that these cancers contain cells resembling GM progenitor cells whose presence results in reduced function of intratumoral T lymphocytes.

**MATERIALS AND METHODS**

**Human Head and Neck Cancers.** Fresh nonnecrotic human head and neck cancer specimens, involved regional lymph nodes, and adjacent normal sternocleidomastoid muscle biopsies were obtained immediately after surgical removal. Most of these cancers, whose histologies are described in Table 1, were SCCs. The head and neck cancer specimens were prepared for analysis by separating tumor tissue from necrotic tissue and then mincing the tissue into 2–4-mm fragments. The fragments were either directly placed into culture for measurement of GM-CSF secretion, or were dissociated into single-cell suspensions. For analyses requiring single-cell suspensions of cancer specimens, the fragments were enzymatically dissociated by incubation for 2 h in a mixture of 1 mg/ml collagenase type IV-0.5 mg/ml hyaluronidase type V-0.1 mg/ml DNase type I (Sigma, St. Louis, MO). Single-cell suspensions of the dissociated tumor were centrifuged in a Ficoll-Hypaque gradient (1.077 g/liter), recovered from the interface, washed, and either FACS analyzed or seeded into culture. The culture medium used for all studies was RPMI 1640 containing 10% endotoxin-free defined fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, 0.02 μg 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 5 × 10⁻⁵ M 2-mercaptoethanol and 2 mM l-glutamine (Sigma). For two of the head and neck SCC specimens studied, cancer lines were established by culturing fragments and selectively trypsinizing explants to enrich for cancer cells. Once cancer cultures became established, they were maintained in culture medium and passaged twice weekly.

**FACS Analysis.** Dissociated tumor samples were stained by direct immunofluorescence using fluorescein isothiocyanate-conjugated CD34 antibody (QBEND/10 clone; BioSource, Camarillo, CA) or a fluorescein isothiocyanate-conjugated isotype control antibody. After 30 min of staining on ice in the dark, the cells were washed and the percentage of positive staining cells was measured using a FACS 420 (Becton Dickinson, Sunnyvale, CA). Positive cells were quantified using marker settings established to exclude 95% of the cells that were incubated with the isotype control antibody. Data shown are FACS histograms and the percentage of cells identified by these analyses to be positive.

**GM-CSF Secretion.** To measure GM-CSF secretion, minced fragments (2–4 mm) of cancer specimens were individually cultured with 1 ml culture medium for 24 h. The supernatants were then collected and the tumor pieces were weighed. The amount of GM-CSF in the supernatant was quantitated by ELISA, having a sensitivity of 20 pg/ml using a combination of capture and secondary antibodies that was paired for this application (PharMingen, San Diego, CA). Results were expressed as ng GM-CSF secreted per g tumor tissue (mean ± SD).

**Immunohistology.** Multiple areas throughout the tissue samples were immunohistologically analyzed to obtain a collective representation of the cells expressing CD34 or producing GM-CSF. Specimens were cryosectioned, fixed, and incubated with antibody to GM-CSF (PharMingen), CD34 antibody, or with isotype control antibody (Biosource). To visualize cells producing GM-CSF or expressing CD34, sections were stained with antibody to GM-CSF (PharMingen, San Diego, CA). Results were expressed as % positive.

**Growth of Immunomagnetically Isolated CD34+ Cells in Soft Agar.** The capacity of CD34+ cells to grow into granulocytic and/or monocytic colonies in soft agar was measured. CD34+ cells were immunomagnetically isolated from freshly dissociated head and neck cancer specimens, using a procedure previously shown to result in a high degree of progenitor cell purity without diminishing their capacity for growth (28). Briefly, dissociated tumors were adjusted to 10⁷/ml and mixed with CD34 antibody-coated magnetic beads (Advanced
Magnetic, Cambridge, MA) at 4°C for 15–20 min. The cells that
complexed with the beads were separated with a magnetic plate
at a perpendicular angle to gravity. After 3–5 min, the cells
remaining in suspension were removed by pipetting and dis-
carded. The cells that remained in the flask attracted to the
magnetic plate (CD34+ population) were released into suspen-
sion, washed, and again magnetically isolated from the cells in
suspension. Various numbers of cells in the CD34+ fraction
were added to 35- × 10-mm tissue culture dishes with 1 × 10^6
irradiated (25 Gy) spleen cells as feeder cells in 1 ml semisolid
RPMI 1640 supplemented medium. The medium was supple-
mented with 20% fetal bovine serum, 0.3% agar (Bacto-Agar;
Difco, Detroit, MI), and a mixture of 20% (v/v) GCT super-
tant (Sigma) plus 10% supernatant from GM-CSF-secreting
head and neck cancer lines as sources of CSFs. Cultures were
incubated at 37°C for 6 days, after which colonies (>50 cells)
were counted. The cells which composed the colonies were
identified histologically after fixing colonies with paraf-
formaldehyde and methanol, and staining with hematoxylin and
eosin.

**IL-2 Production by Intratumoral Lymphocytes.** The
effect of the presence of CD34+ cells on IL-2 production by
lymphocytes of autologous cancers was measured after immu-
nomagnetic separation of CD34+ cells from freshly dissociated
head and neck cancer specimens, as described above. The posi-
tively selected CD34+ cell population was added to aliquots of
unfractionated dissociated tumor to also establish CD34+-en-
riched tumor cultures. Thus, the following dissociated cancer
cultures were prepared to assess the effects of CD34+ cells on
IL-2 production by intratumoral T cells: (a) CD34-depleted
cultures; (b) CD34-enriched cultures; and (c) unfractionated
cultures, when sufficient cells were available. FACS analysis
of a fraction of each population showed the absence of CD34+
cells in the CD34-depleted cultures and a proportionate increase
(by 5–10%) of CD34+ cells in the CD34-enriched cultures. The
remaining cells of each of these three populations were incu-
bated on CD3 antibody- (Biodesign, Kennebunkport, ME)
coated tissue culture plates to induce IL-2 production (29). After
2 days, culture supernatants were collected and the IL-2 content
was measured at least in triplicate using ELISA (Endogen,
Boston, MA, or Incstar, Stillwater, MN). The significance of the
difference between values was determined using Student’s *t*
test.

**RESULTS**

**GM-CSF Secretion by Head and Neck Cancers.**
Freshly excised specimens of human head and neck cancers
were obtained, with specimens from 18 patients being of suffi-
cient size to measure GM-CSF secretion and the frequency of
cells staining strongly and moderately for GM-
CSF, GM-CSF production by normal muscle biopsies (sterno-
clidomastoid muscle from the operative field) from 9 of the
studied patients was also measured and was found to be insig-
ificant (values for several patients in Table 2).

Whether the cancer cells were capable of secreting GM-
CSF was determined by establishing two head and neck cancer
cell lines from GM-CSF-secreting cancers used in the studies
described in Fig. 1. The amount of GM-CSF secreted by these
cells was measured after over 3 months of passage to assure loss
of infiltrating cells. Both of these cancer lines secreted GM-
CSF, with cells from patients HN13 and HN18 secreting 451 pg
and 598 pg GM-CSF, respectively, per 10^6 cells during 18 h of
culture. In addition, head and neck SCC tissues were analyzed
for cancer cell production of GM-CSF by immunostaining cryo-
sections with anti-GM-CSF antibodies. Shown in Fig. 2 is a high
frequency of cells staining strongly and moderately for GM-
CSF in sections from a cancer that was shown by ELISA to
secrete GM-CSF. Also shown is negatively staining tissue from
a cancer shown by ELISA not to secrete GM-CSF. These data
support the results of ELISA analyses showing that head and
neck cancers can secrete GM-CSF.

**Fig. 1** Human head and neck cancer secretion of GM-CSF and intra-
tumoral CD34+ cell content. Fragments of cancer specimens were
individually cultured for 24 h, after which supernatants were removed
and their GM-CSF content was measured by ELISA. Top, Data shown
is the mean of triplicates (ng GM-CSF/g tissue) ± SD. Enzymatically
dissociated cancer fragments were centrifuged through a discontinuous
density gradient, recovered from the interface, and stained by direct
immunofluorescence using fluorescein isothiocyanate-conjugated
CD34. Bottom, The proportion of CD34+ staining cells was measured
by FACS and shown as percentage of positive-staining cells.

**Fig. 2** Eosin-stained sections with anti-GM-CSF antibodies. Shown in Fig. 2 is a high
frequency of cells staining strongly and moderately for GM-
CSF in sections from a cancer that was shown by ELISA to
secrete GM-CSF. Also shown is negatively staining tissue from
a cancer shown by ELISA not to secrete GM-CSF. These data
support the results of ELISA analyses showing that head and
neck cancers can secrete GM-CSF.
98 CD34 Cells in Head and Neck Cancers

CD34+ Cells within GM-CSF-Producing Human Head and Neck SCCs. Since our prior studies with murine tumors showed GM-CSF-producing tumors induce an intratumoral influx of immune suppressive cells resembling GM progenitor cells, the human head and neck tumors were analyzed for the presence of cells that express the human progenitor cell antigen CD34. This was accomplished both by FACS and immunohistochemical analysis of enzymatically dissociated tumor samples. Examples of FACS analyses for 4 of these specimens that contained CD34+ cells and 1 that did not contain CD34+ cells (from patient HN11) are shown in Fig. 3. Summarized in Fig. 1 is the percentage of CD34+ cells within the 18 tested dissociated cancers. The group of cancer specimens that contained CD34+ cells was the same group of SCCs that secreted the high levels of GM-CSF. Linear regression analysis did not show a highly significant correlation between the absolute amount of GM-CSF the tumors secreted and the number of intratumoral CD34+ cells ($r^2 = 0.65$), but all 10 of the SCCs that secreted more than 5 ng GM-CSF/g tissue contained the higher content of CD34+ cells. To determine if the presence of CD34+ cells may have been associated with the number of infiltrating T cells and, in fact, the result of the occurrence, they were measured. The results of these FACS analyses showed that the CD34+ cell content was not significantly correlated with the absolute amount of GM-CSF secreted by the cancer, the frequency of CD4+ plus CD8+ T cells within the cancer was measured. The results of these FACS analyses showed that the CD34+ cell content was not associated with the number of infiltrating T cells and, in fact, the

Table 1  Head and neck cancers

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>TNM</th>
<th>Histology</th>
<th>Site</th>
<th>Smoking</th>
<th>Alcohol</th>
<th>Prior therapy</th>
<th>Multiple tumors</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN1</td>
<td>62</td>
<td>M</td>
<td>T3N0</td>
<td>SCC, poorly dif.</td>
<td>Larynx (supraglottic)</td>
<td>No, 25 yr</td>
<td>None</td>
<td>Yes</td>
<td>No</td>
<td>NED</td>
</tr>
<tr>
<td>HN2</td>
<td>49</td>
<td>M</td>
<td>T3N0</td>
<td>SCC, moderately dif.</td>
<td>Larynx (supraglottic; false and true cord)</td>
<td>Heavy</td>
<td>Heavy</td>
<td>No</td>
<td>No</td>
<td>NED</td>
</tr>
<tr>
<td>HN3</td>
<td>46</td>
<td>M</td>
<td>T1N0</td>
<td>SCC, well dif.</td>
<td>Right floor of mouth</td>
<td>Heavy</td>
<td>Heavy</td>
<td>Yes</td>
<td>Yes</td>
<td>Recurrent 1st</td>
</tr>
<tr>
<td>HN4</td>
<td>40</td>
<td>M</td>
<td>T1N0</td>
<td>Adenocarcinomatous, moderately dif.</td>
<td>Ethmoid sinus</td>
<td>None</td>
<td>Moderate</td>
<td>No</td>
<td>No</td>
<td>NED</td>
</tr>
<tr>
<td>HN5</td>
<td>63</td>
<td>M</td>
<td>T1N0</td>
<td>SCC</td>
<td>Pharyngeal wall</td>
<td>Heavy</td>
<td>Heavy</td>
<td>No</td>
<td>No</td>
<td>NED</td>
</tr>
<tr>
<td>HN6</td>
<td>67</td>
<td>F</td>
<td>T1N0</td>
<td>SCC, poorly dif.</td>
<td>Larynx (pyriform sinus)</td>
<td>Heavy</td>
<td>Small</td>
<td>Yes</td>
<td>Yes</td>
<td>Recurrent 1st</td>
</tr>
<tr>
<td>HN7</td>
<td>45</td>
<td>M</td>
<td>T1N0</td>
<td>SCC, moderately dif.</td>
<td>Trachea (2nd occurrence)</td>
<td>Heavy</td>
<td>Small</td>
<td>Yes</td>
<td>No</td>
<td>Recurrent 1st, DWD</td>
</tr>
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<td>HN8</td>
<td>62</td>
<td>M</td>
<td>T1N0</td>
<td>SCC, moderately dif.</td>
<td>Larynx (supraglottic)</td>
<td>Heavy</td>
<td>Heavy</td>
<td>No</td>
<td>No</td>
<td>NED</td>
</tr>
<tr>
<td>HN9</td>
<td>70</td>
<td>F</td>
<td>T3N0</td>
<td>SCC</td>
<td>Tongue</td>
<td>None</td>
<td>None</td>
<td>No</td>
<td>No</td>
<td>NED</td>
</tr>
<tr>
<td>HN10</td>
<td>48</td>
<td>F</td>
<td>T1N0</td>
<td>Carcinoma ex-pleomorphic adenoma (salivary gland)</td>
<td>Parapharyngeal space</td>
<td>Moderate</td>
<td>Moderate</td>
<td>No</td>
<td>No</td>
<td>NED</td>
</tr>
<tr>
<td>HN11</td>
<td>73</td>
<td>M</td>
<td>T1N0</td>
<td>SCC</td>
<td>Larynx (false cord)</td>
<td>Heavy</td>
<td>Heavy</td>
<td>No</td>
<td>No</td>
<td>NED</td>
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<tr>
<td>HN12</td>
<td>48</td>
<td>M</td>
<td>T1N0</td>
<td>High grade intestinal-type adenocarcinoma</td>
<td>Left ethmoid maxillary sinus</td>
<td>Heavy</td>
<td>Small</td>
<td>No</td>
<td>No</td>
<td>NED</td>
</tr>
<tr>
<td>HN13</td>
<td>54</td>
<td>F</td>
<td>T1N0</td>
<td>SCC, poorly dif.</td>
<td>Larynx</td>
<td>Heavy</td>
<td>Heavy</td>
<td>No</td>
<td>Yes</td>
<td>NED</td>
</tr>
<tr>
<td>HN14</td>
<td>62</td>
<td>M</td>
<td>T1N0</td>
<td>SCC, moderately dif.</td>
<td>Larynx (true cord)</td>
<td>Heavy</td>
<td>Moderate</td>
<td>No</td>
<td>No</td>
<td>NED</td>
</tr>
<tr>
<td>HN15</td>
<td>59</td>
<td>F</td>
<td>T1N0</td>
<td>SCC, moderately dif.</td>
<td>Tongue</td>
<td>Heavy</td>
<td>Heavy</td>
<td>No</td>
<td>No</td>
<td>NED</td>
</tr>
<tr>
<td>HN16</td>
<td>62</td>
<td>M</td>
<td>T1N0</td>
<td>SCC, moderately dif.</td>
<td>Larynx (supraglottic)</td>
<td>Heavy</td>
<td>Heavy</td>
<td>No</td>
<td>No</td>
<td>NED</td>
</tr>
<tr>
<td>HN17</td>
<td>61</td>
<td>M</td>
<td>T3N1</td>
<td>SCC, moderately dif.</td>
<td>Mandible</td>
<td>Heavy</td>
<td>Heavy</td>
<td>No</td>
<td>No</td>
<td>NED</td>
</tr>
<tr>
<td>HN18</td>
<td>66</td>
<td>M</td>
<td>T1N0</td>
<td>SCC, moderately dif.</td>
<td>Larynx (pyriform sinus)</td>
<td>No, 23 yr</td>
<td>Small</td>
<td>No</td>
<td>No</td>
<td>NED</td>
</tr>
</tbody>
</table>

* diff., differentiated; NED, no evidence of disease; DWD, dead with disease.

Table 2  GM-CSF production and CD34+ cell content of cancers and regional lymph nodes

<table>
<thead>
<tr>
<th>Tumor code</th>
<th>Specimen</th>
<th>GM-CSF secreted* (ng/g tissue)</th>
<th>CD34+ cells%</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN8</td>
<td>Larynx, SCC</td>
<td>8.9 ± 0.3</td>
<td>13.8</td>
</tr>
<tr>
<td>HN8</td>
<td>Jugulocarotid node</td>
<td>20.2 ± 2.0</td>
<td>13.5</td>
</tr>
<tr>
<td>HN8</td>
<td>Jugulodigastric node</td>
<td>10.9 ± 4.6</td>
<td>14.2</td>
</tr>
<tr>
<td>HN10</td>
<td>Lower jugular node</td>
<td>0.0 ± 0.4</td>
<td>13.0</td>
</tr>
<tr>
<td>HN10</td>
<td>Salivary gland, carci. pleomorphic adenoma</td>
<td>0.1 ± 0.1</td>
<td>2.1</td>
</tr>
<tr>
<td>HN10</td>
<td>Cervical node</td>
<td>2.0 ± 3.8</td>
<td>0.7</td>
</tr>
<tr>
<td>HN11</td>
<td>Normal muscle</td>
<td>0.3 ± 0.3</td>
<td>10.1</td>
</tr>
<tr>
<td>HN11</td>
<td>Tongue, SCC</td>
<td>12.6 ± 1.8</td>
<td>14.8</td>
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<tr>
<td>HN11</td>
<td>Normal muscle</td>
<td>2.1 ± 0.2</td>
<td>10.4</td>
</tr>
<tr>
<td>HN12</td>
<td>Tongue, SCC</td>
<td>25.9 ± 7.5</td>
<td>10.6</td>
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<td>HN12</td>
<td>Left upper jugular node</td>
<td>7.0 ± 3.3</td>
<td>6.2</td>
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<tr>
<td>HN12</td>
<td>Normal muscle</td>
<td>2.4 ± 1.4</td>
<td>7.4</td>
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<tr>
<td>HN12</td>
<td>Larynx, pyriform sinus, SCC</td>
<td>16.9 ± 3.0</td>
<td>9.2</td>
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<tr>
<td>HN12</td>
<td>Jugulodigastric node</td>
<td>0.0 ± 0.2</td>
<td>7.4</td>
</tr>
</tbody>
</table>

* GM-CSF secreted by tissue fragments is expressed as ng GM-CSF/g tissue (mean ± SD).

The proportion of CD34+ cells in enzymatically dissociated tissue is expressed as percentage of positive cells from flow cytometric analysis of each sample.
Fig. 2  Immunostaining of cancers for GM-CSF. Cryosections of SCCs were stained with immunoperoxidase for GM-CSF, and then counterstained with ethyl green. Shown are cells staining positive for GM-CSF production in sections from patient HN13 (left) and a lack of staining in a section from patient HN16 (right). Top row, × 400; bottom row, × 1000.

high GM-CSF-secreting cancers contained fewer T cells (6.8 ± 3.3%) than did the low GM-CSF-secreting cancers (12.9 ± 3.4%).

Regional lymph node specimens containing metastatic cancer were also available in sufficient size from four patients with high GM-CSF-producing SCCs and from one patient with a low GM-CSF-producing adenoma to measure both GM-CSF production and CD34+ cell content (Table 2). Node samples from three of the four patients with high GM-CSF-producing primary cancers also secreted high levels of GM-CSF. A node from one of these three patients and the node sample from the fourth patient with a GM-CSF-producing primary cancer did not secrete GM-CSF. Evaluation of these nodes for presence of CD34+ cells showed that the nodes that secreted high levels of GM-CSF also had a significant number of CD34+ cells. In addition, the nodes that did not secrete GM-CSF but which were obtained from patients with GM-CSF-secreting SCCs contained CD34+ cells. The node from the patient with a low GM-CSF-producing cancer did not secrete significant levels of GM-CSF and did not have significant levels of CD34+ cells. Although these results are from a relatively small number of samples, they showed that CD34+ cells could be found in GM-CSF-secreting SCCs and in nodes involved with metastatic cancer.

Characterization of Intratumoral CD34+ Cells. The results described above showing that cancer production of GM-CSF was associated with an intratumoral presence of cells that express the human progenitor cell antigen CD34 prompted studies to evaluate the identity of these CD34+ cells. Immuno-histological analyses were first performed to confirm the presence of CD34+ cells and to visualize their distribution within the cancers. An example of CD34+ cells within a GM-CSF-secreting cancer is shown in Fig. 4. These CD34+ cells stained intensely and typically appeared in small clusters, contrasting with the occasionally observed endothelial cells which stain diffusely with CD34 (30). To evaluate the possibility that the CD34+ cells could be myeloid progenitor cells, the capacity of CD34+ cells isolated from head and neck SCCs to grow into colonies in soft agar containing CSFs was determined. In the presence of CSFs, the isolated CD34+ cells grew into colonies with an efficiency of 46 ± 8% (mean ± SE of results from two cancer samples). Fig. 5 shows the appearance of colonies that formed from one of these head and neck SCCs. Most of the colonies that formed (>70%) consisted of both monocytes and granulocytes. The remaining colonies contained either granulocytes or monocytes. In the absence of CSFs, isolated CD34+ cells failed to form colonies, supporting the possibility that a
large proportion of the intratumoral CD34⁺ cells are GM progenitor cells.

IL-2 Production by T Lymphocytes from Dissociated Cancer after Depletion of CD34⁺ Cells. Our prior studies in a murine tumor model showed that GM-CSF-producing tumors induced intratumoral infiltration of cells that resembled GM progenitor cells and were immune suppressive (18, 21, 22). Since human head and neck cancers that produce GM-CSF contained cells expressing the progenitor cell antigen CD34 (Fig. 1), the possibility was examined that these cells might be immune suppressive to the activity of the intratumoral T cells. Primary SCC specimens from 5 of the 18 above-described head and neck cancer patients were large enough for these analyses. This was accomplished by determining if depletion of CD34⁺ cells from dissociated cancer would result in an increased capacity of the intratumoral T cells to be induced by immobilized CD3 in to produce IL-2. In the absence of stimulation with immobilized CD3, IL-2 was not secreted by the dissociated cancer (not shown). After incubation for 2 days on immobilized CD3, IL-2 secretion was induced in each of the unfractionated dissociated cancer cultures (Table 3). In each of the 4 analyzed GM-CSF-producing samples that contained CD34⁺ cells, IL-2 production was approximately doubled when CD34⁺ cells were depleted as compared to the cultures to which CD34⁺ cells were added. Addition of the CD34⁺ cells to unfractionated dissociated tumor cultures further diminished by almost one half their capacity to be induced to produce IL-2. By contrast, similar treatment with CD34-coated magnetic beads had no effect on IL-2 secretion by an analyzed tumor sample that did not contain CD34⁺ cells. These studies suggest that in the presence of CD34⁺ cells the capacity of the intratumoral T cells to be induced to secrete IL-2 is reduced, and that removal of these CD34⁺ cells enhances IL-2 production.

DISCUSSION

Many human and animal tumor types have been shown to produce GM-CSF (24, 25, 31) which can modulate antitumor immune responses both positively and negatively (17, 26, 32). The demonstration that GM-CSF can have potent immune stimulatory effects has led to the inclusion of GM-CSF in strategies to develop tumor vaccines (32, 33). For example, vaccines composed of irradiated tumor cells engineered to secrete GM-CSF have been shown to stimulate long-lasting antitumor immunity in murine tumor models (32). Negative immune regulatory effects of GM-CSF have also been reported, but are typically indirect and are mediated by the populations of immune suppressive cells that can be induced by GM-CSF, including mature suppressor macrophages (17). A second population of immune suppressor cells is induced as a result of GM-CSF stimulation of myelopoiesis and appears to resemble GM progenitor cells (17, 18, 20). However, human head and neck cancers have not been previously studied for whether they secrete GM-CSF or whether this might be associated with the presence of immune suppressor cells that resemble the GM progenitor cells.

The results of the present study have shown that approximately two thirds of the evaluated head and neck SCCs, but none of the non-SCCs, secrete GM-CSF and contain cells expressing the hematopoietic progenitor cell antigen CD34. The SCCs that did not secrete GM-CSF did not contain significant numbers of CD34⁺ cells. Cancer-containing lymph nodes that were regional to GM-CSF-secreting primary SCCs also contained CD34 cells and most were shown to produce GM-CSF. The source of the GM-CSF was most likely the cancer within the nodes, although this has not yet been rigorously studied. In a few instances, there were differences in GM-CSF production
between the cancer samples and involved node. This may be due to the variability in the extent or distribution of cancer within the node.

That the head and neck cancer cells within the surgical specimens were a significant source of the secreted GM-CSF was supported by the demonstration that the head and neck cancer lines that we established from GM-CSF-producing specimens were also capable of secreting GM-CSF. In addition, immunohistochemical analysis of cryosections from several of the cancers shown by ELISA to secrete GM-CSF also showed a high number of cancer-like cells expressing GM-CSF.

As to whether the CD34+ cells are in fact GM progenitor cells was supported by the capacity of isolated CD34+ cells to grow into colonies composed of granulocytes and monocytes in the presence of CSFs but not in the absence of a CSF source. Of principal significance is our demonstration that in the presence of these CD34+ cells, the capacity of the SCC-derived lymphocytes to be activated to secrete IL-2 is reduced. Additionally, removal of the CD34+ cells from the tumor enhances the capacity of the intratumoral T cells to be induced to secrete IL-2. Although immune suppressive cells that are homologous to GM progenitor cells have been shown to be induced by GM-CSF-secreting animal tumors (17, 18, 20), this has not been previously suggested for human cancers. However, studies with normal human bone marrow have shown the presence of an immune suppressive cell population that copurifies with myeloid progenitor cells (34). Our speculation is that this is the same population of cells as we have shown to be present within the GM-CSF-secreting cancers.

The patients with GM-CSF-secreting SCCs were not evaluated for whether the tumor-derived GM-CSF might have increased the frequency of GM progenitor cells in the marrow, although other studies have indicated myeloid stimulation in patients with other types of GM-CSF-secreting cancers (27, 31). Interestingly, head and neck cancers have also been shown to secrete high levels of PGE2, which can function as a negative regulator of both immune competence and myelopoiesis (9, 35). Thus, it is possible that production of PGE2 by the cancers might function to restrict the frequency of CD34+ cells which might otherwise be further increased if PGE2 production were not increased or if prostaglandin synthesis inhibitors were being administered.

Presence of immune suppressor cells that interfere in host anti-tumor immune competence has been suggested to facilitate
the growth and metastasis of cancer (15, 16, 36, 37). Expression of GM-CSF by progressively growing murine tumors has been associated with increased metastasis (22, 25). The studies presently being described did not demonstrate any clear correlation between tumor stage and either the capacity of the tumor to secrete or induce immune suppression by secreting immune suppressive mediators (9, 10), or indirectly induce immune suppression in head and neck cancer patients, whose immune functions have previously been shown to be impaired (5, 7). Cancers have been shown to directly suppress immune competence by secreting immune suppressive mediators (9, 10), or indirectly induce immune suppression by inducing immune suppressor cells, including the more frequently studied suppressor macrophages or T cells (11, 13). Although we do not know if these other suppressor cascades are present in GM-CSF-secreting cancers that contain CD34+ cells, it is unlikely that they are all mutually exclusive and most likely combinations of suppressive mechanisms contribute to immune suppression in patients with head and neck SCCs.

This report is the first demonstration of CD34+ cells within head and neck SCCs and of the reduced intratumoral T-cell function in the presence of these CD34+ cells. Other types of cancers have not been previously evaluated for the presence of CD34+ GM progenitor cells having immune suppressive properties. We are currently continuing these studies to further define whether the intratumoral CD34+ cells are present in GM-CSF-secreting cancers other than SCCs. Ongoing studies are also aiming to delineate the mechanisms by which CD34+ cells mediate their suppression and whether they directly inhibit functions of intratumoral T cells or if the suppressor cells are a different population that is either induced by the CD34+ cells or is a progeny of the CD34+ cells. These latter possibilities have not been tested, but their likelihood is diminished by our demonstration that depletion of CD34+ cells is sufficient to restore the capability of intratumoral T cells to produce IL-2. Our parallel studies with the murine LLC tumor model have shown that the GM progenitor cells mediate their suppressive activity at least in part by producing TGFβ. The possibility that the intratumoral CD34+ cells of human head and neck SCCs also mediate suppression by producing TGFβ is currently being evaluated. Since a variety of cancers have been shown to secrete GM-CSF, it is possible that the presence of CD34+ cells that inhibit intratumoral T-cell functions is not unique to head and neck SCCs and may occur in other GM-CSF-secreting human cancers.

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REFERENCES


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* Dissociated cancers that had been shown to secrete high levels of GM-CSF (HN1, HN7, HN9, and HN18) or low levels of GM-CSF (HN16) were adjusted to 10^6 cells/ml and then either immunomagnetically depleted of CD34+ cells or enriched with immunomagnetically isolated CD34+ cells. IL-2 secreted by cell populations after incubation on CD3-coated dishes is expressed as IU IL-2 secreted per ml ± SD of at least 4 determinations. *, P < 0.05; **, P < 0.001.

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