Epidermal Growth Factor Receptor-independent Constitutive Activation of STAT3 in Head and Neck Squamous Cell Carcinoma Is Mediated by the Autocrine/Paracrine Stimulation of the Interleukin 6/gp130 Cytokine System

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ABSTRACT

The aberrant growth of head and neck squamous cell carcinoma (HNSCC) is often associated with the constitutive activation of signal-transducer-and-activator-of-transcription-3 (STAT3), which is believed to result from the persistent stimulation of EGF receptors that are highly expressed in squamous cell carcinoma (SCC) cells. To investigate the mechanism underlying STAT3 deregulation in HNSCC, we examined the interplay of the STAT3 and epidermal growth factor receptor (EGFR) signaling pathways using a panel of HNSCC cell lines. Although STAT3 was active in most HNSCC cell lines, only 3 of 10 HNSCC cell lines were moderately to strongly positive for activated EGFR. Even in the EGFR-positive cell lines, STAT3 activation was not dependent on EGFR activation, as STAT3 tyrosine phosphorylation levels persisted after treatment with AG1478, a chemical inhibitor of EGFR activity. Furthermore, we found that conditioned medium harvested from HNSCC cells could induce STAT3 tyrosine phosphorylation in immortalized keratinocytes regardless of the status of EGFR signaling. In contrast, blocking the cytokine gp130 coreceptor abolished STAT3 tyrosine phosphorylation in HNSCC cells and that induced by the conditioned medium. Immunodepletion studies suggested interleukin 6 (IL6) as the major autocrine/paracrine factor for STAT3 activation, which coincided with high levels of secretion of IL6 into the culture medium by these cancer cells. Treatment with a specific inhibitor of Janus kinase, AG490, in HNSCC cells led to a reduction of active STAT3 and caused significant growth retardation and apoptosis. Thus, constitutive activation of STAT3 in HNSCC may use an autocrine/paracrine-activating loop mediated by IL6 and other cytokines acting through the gp130 receptor family, which may confer both proliferative and survival potential in this malignancy.

INTRODUCTION

Cytokine signaling pathways play critical roles in controlling lineage commitment of multiple cell types during embryogenesis and organ development by transmitting extracellular signal into the nucleus (1). To accomplish these intricate processes, STAT3 family proteins serve both as cytoplasmic signal transducers and transcriptional activators controlling gene expression (2). Optimal STAT activity is essential for normal cellular functions, and deregulation of the STAT pathway can contribute to a number of human diseases. For example, loss of function of STAT1 leads to increased susceptibility to mycobacterial infection (3). Conversely, a gain of function of STATs is often associated with cellular transformation and oncogenic potential (4, 5). Although deregulation of STAT is frequently observed in a variety of human malignancies (6), the molecular mechanisms by which cancer cells maintain STATs in a persistently activated state are still poorly understood.

The activation of STATs often involves a ligand-receptor interaction (7). For example, binding of cytokine to their cognate receptors leads to the recruitment and phosphorylation of JAK-1 and JAK-2 (8). Activated JAKs then phosphorylates STAT proteins at specific tyrosine residues, which, in turn, promote the homo- and heterodimerization of STATs through reciprocal phosphotyrosine-SH2 domain interactions (9, 10). Activated STAT dimers then translocate to the nucleus where they bind to consensus DNA sequences and transactivate target genes. Additionally, STAT1 and STAT3 contain a serine residue (serine-727) located in the carboxyl-terminal transcriptional-activating domain, the phosphorylation of which is thought to enhance DNA-binding activity of activated STATs (11, 12). Many studies have suggested an interplay between protein kinase C and mitogen-activated protein kinase families in the regulation of this serine phosphorylation on STATs (13–16).

Among STAT family members, STAT3 is the most frequently associated with deregulated cell growth and neoplasia (6). Constitutive activation of STAT3 has been demonstrated in many cancers, such as breast cancer (17, 18), leukemia (19, 20), lymphoma (21), lung cancer (22), thyroid cancer (23), and head and neck cancer (24). These cancers are frequently associated with aberrant activation of growth factor receptor tyrosine kinases. For example, activated receptor tyrosine kinase is often seen in thyroid cancer (23), activating mutations of c-kit receptor in acute myeloid leukemia and gastrointestinal tumors (25), and EGFR activation in breast cancer (26). In at least some of these cancers, STAT3 activation appears to be a consequence of receptor tyrosine kinase activation. In HNSCC, constitutively active STAT3 has been shown to be associated with TGFα and EGFR signaling (24, 27, 28). Inhibition of the EGFR pathway or TGFα exclusion results in diminishing STAT3 DNA-binding activity. Moreover, inhibition of STAT3 function leads to growth inhibition of HNSCC (24, 27, 28). These results support the importance of signaling through STAT3 in HNSCC oncogenesis.

Although tyrosine kinase receptors like EGFR are capable of inducing STAT3 phosphorylation (7), reported incidences of active EGFR pathways in HNSCC range from 5% to 90% (29–35). Thus, it is possible that additional mechanisms may participate in the constitutive activation of STAT3 in this malignancy. To investigate this possibility, we analyzed the status of STAT3 activation in a panel of HNSCC cells. As expected, almost all cell lines tested exhibited levels of active STAT3 higher than that of normal keratinocytes. However, 30% of the cell lines studied showed moderate to high EGFR activity. Furthermore, inhibition of EGFR stimulation by specific inhibitors did not prevent STAT3 activation in HNSCCs. Interestingly, we found...
that the conditioned medium harvested from HNSCC was capable of inducing STAT3 activation in control keratinocytes through the cyto-
kine receptor gp130 family, independent of the EGFR pathway.
Furthermore, we identified IL6 as the major secretory ligand stimu-
lating the STAT3 activation by acting on the gp130 coreceptor in an
autocrine/paracrine fashion. We also obtained evidence that interfer-
ning with this cytokine pathway of STAT3 activation can inhibit cell
growth and promote apoptosis of HNSCC cells.

MATERIALS AND METHODS

Cell Culture. Head and neck cancer cell lines [HN4, HN6, HN8, HN12,
HN13, HN17, HN19, HN22, HN30, and HN31 (36)] and HaCaT cells (37), an
immortalized human skin keratinocyte cell line, were cultured in DMEM
supplemented with 10% FCS, penicillin, and streptomycin. HFSKs were
cultured in defined keratinocyte-serum-free-medium (Invitrogen) with supple-
ments containing insulin, EGF, and fibroblast growth factor. Conditioned
media from cell lines were prepared by incubating subconfluent cultured cells
for 24 h in DMEM without supplements. Harvested conditioned media were
then filtered through a 0.22-µm low protein-binding polyethylenimine mem-
brane filter. An EGFR inhibitor, N-(3-chlorophenyl)-6,7-dimethoxy-4-quina-
zolinamine (AG1478), and a JAK inhibitor, 2-cyano-3-(3,4-dihydroxyphenyl)-
N-benzyl)-2-propanenamide (AG490; Calbiochem) were prepared and used
according to the manufacturer’s instructions.

EMSA. Nuclear extractions were prepared by using a NE-PER Nuclear and
Cyttoplasmic Extraction Reagent (Pierce). EMSAs were carried out as described
previously using 32P-labeled double-stranded oligonucleotide m67SIE (12).
The m67SIE sequences were as follows: upper strand, CTAGATTTCGGCCG-
TAAT; lower strand, CTAGATTTCGGGAGAATG. Protein-DNA com-
plexes were separated by electrophoresis on 4% polyacrylamide gel in Tris-
borate EDTA buffer.

Immunoblotting. Cultured cells were harvested at the times indicated in
each experiment, washed with PBS and lysed in 62.5 mM Tris, 2% SDS, 10%
glycerol (SDS sample buffer) with aprotinin, leupeptin, pepstatin, and 4-(2-
aminoethyl) benzenesulfonyl fluoride. Cell suspensions were briefly sonicated,
and the protein concentration for each cell lysate was determined by using DC
protein assay (Bio-Rad, Hercules, CA). Fifty to 100 µg of total protein from
the whole-cell lysate was loaded onto each lane during gel electrophoresis.
Immunoblotting was performed by using 0.1 M Tris (pH7.5), 0.09% NaCl,
0.05% Tween-20 with 5% nonfat dry milk as a blocking and antibody dilution
buffer. Working concentrations of antisera were as follows: STAT3, 1:1000
(Cell Signaling Technology); phosphoSTAT3-tyrosine-705, 1:1000 (Cell Sig-
naling Technology); phosphoSTAT3-serine-727, 1:1000 (Cell Signaling Tech-
nology); phospho-ERK, 1:1000 (Santa Cruz Biotechnology); ERK1, 1:1000
(Santa Cruz Biotechnology); ERK2, 1:1000 (Santa Cruz Biotechnology); phos-
photyrosine 4G10, 1:1000 (Upstate Biotechnology); phosphor-ERK, 1:1000
(Santa Cruz Biotechnology); phosphoSTAT3-serine-727, 1:1000 (Cell Signaling Tech-
nology) following the manufacturer
as indicated in each experiment.

RT-PCR. Total RNAs were isolated by using the Trizol reagent (Invitro-
gen) following the manufacturer’s instructions. Two micrograms of total RNA
were used for each RT reaction using the Superscript II reagent (Invitrogen).
PCRs were performed by using Jumpstart Readymix REDTaq DNA poly-
merase (Sigma) with 30 amplifying cycles. Sequences of primers homologous
were used for each RT reaction using the Superscript II reagent (Invitrogen).
levels of phosphorlated, active STAT3, confirming the EMSA findings (Fig. 2; upper two panels; Lanes 11–14). It should be noted that under EGFR stimulation, we were able to detect STAT3 phosphorylation in HFSKs, a finding not observed in the EMSA. This may stem from different sensitivities of detection between EMSA and immunoblotting. Nonetheless, the above findings demonstrate the presence of constitutively active STAT3 in HNSCC and confirm similar findings reported in a different panel of HNSCC cells (24).

**HNSCCs Use a STAT3 Regulatory Mechanism Independent from the EGFR Signaling Pathway.** We first investigated whether HNSCC cells use the EGFR signaling pathway to stimulate STAT3 by exploring the relationship between STAT3 phosphorylation and the status of EGFR activation in this HNSCC cell panel. All cells expressed EGFR, as judged by its immunodetection with anti-EGFR-specific antibodies (Fig. 2, bottom panel). Of interest, some of these cell lines vastly overexpressed EGFR, such as HN6 and, to a lesser extent, HN13 and HN12. This correlated well with the presence of heavily tyrosine phosphorylated band at $M_r \sim 170,000$, likely corresponding to phosphorylated EGFR, when using antiphosphotyrosine immunoblot analysis of the same HNSCCs extracts and using EGFR-stimulated human keratinocytes as a control (Fig. 2, second panel from the bottom). This was further confirmed by antiphosphorylated EGFR-specific immunoblots (data not shown), which together revealed that in unstimulated culture conditions, only 3 of 10 HNSCC cell lines tested (HN12, HN13, and HN6) showed moderate to high levels of activated EGFR, whereas the remaining HNSCC cells contained relatively little activated EGFR (Fig. 2; second panel from the bottom; compare Lanes 4, 5, and 2 with Lanes 1, 3, and 6–10). In contrast to EGFR, moderate to high levels of STAT3 tyrosine phosphorylation was found in most of the cells in the panel (Fig. 2; top panel; Lanes 1–11), even if the total levels of STAT3 were nearly identical among all cell lines (Fig. 2, third panel). Furthermore, this pattern of pSTAT3-Y705 had no correlation with the EGFR-activation status. For example, the cell line HN6, which exhibited high levels of activated EGFR, showed only moderate expression pSTAT3-Y705. In HN19, HN30, and HN31, low levels of EGFR activation were observed, whereas these same cell lines exhibited relatively high pSTAT3-Y705 levels. This evidence raised the possibility of STAT3 regulation independent of EGFR activity in these HNSCC cell panel. Interestingly, pSTAT3-S727 expression patterns did not parallel those of pSTAT3-Y705 or activated EGFR. This finding further suggests a complex STAT3 regulatory mechanism in HNSCC.

To gain further insight into the roles of EGFR signaling in STAT3 activation, we chose to challenge HNSCC cells with a specific EGFR inhibitor, AG1478, and monitor the status of phosphorylation of STAT3 together with that of ERK, which represents a downstream target for the EGFR pathway. Four HNSCC cell lines were selected based on their EGFR status in unstimulated culture conditions. In HN6 and HN13 cells, which exhibit high basal levels of activated EGFR, serum deprivation did not significantly alter the levels of active EGFR, although pERK was significantly down-regulated in HN6 (Fig. 3A; upper two panels; compare Lanes 1 versus 2 and 7 versus 8). Increasing amounts of AG1478 led to a dramatic decrease in EGFR activation in parallel with down-regulation of pERK (Fig. 3A; upper two panels; Lanes 3–6 and 9–12), supporting the effectiveness of the inhibitor in preventing EGFR signaling. As expected, in HN6 cells pSTAT3-Y705 decreases correlated well with the reduction of active EGFR (Fig. 3A; third panel from the bottom; Lanes 2–6). In contrast, HN13 cells showed persistent pSTAT3-Y705 levels even after treatment with AG1478 (Fig. 3A; third panel from the bottom; Lanes 8–12). In HN19 and HN30, cells with lower basal levels of active EGFR, we observed persistent pSTAT3-Y705 levels despite a similar down-regulation of active EGFR by the treatment with AG1478 (Fig. 3B; top panel and third panel from the bottom; Lanes 2–6 and 8–12). The level of pSTAT3-S727 in all cells tested decreased with AG1478 treatment in a similar pattern to the down-regulation observed in active EGFR (Fig. 3, A and B; second panel from the bottom; Lanes 2–6 and 8–12). Taken together, these results clearly indicate the existence of STAT3 regulatory mechanism in HNSCC that are independent of EGFR signaling.

**EGFR-independent STAT3 Activation in HNSCC Cells Is Mediated through an Autocrine/Paracrine Mechanism.** Because STAT3 phosphorylation is not solely dependent on EGFR-induced pathways in all HNSCC cells, we asked whether the STAT3 activation in these cells may derive from the release of another growth factor acting on an autocrine/paracrine mechanism. To test this possibility, CM from HNSCC cultures were harvested and added to HaCaT cells, which contain relatively low basal level of STAT3 phosphorylation. CM from HaCaTs, HN6, and HN19 cells had minimal or no stimulatory effects on ERK or STAT3 (Fig. 4, Lanes 5, 7, and 11), whereas CM from HN13 and HN30 strongly induced phosphorylation of STAT3-Y705 and STAT3-S727 and ERK (Fig. 4, Lanes 9 and 13). Pretreatment with AG1478 blocked STAT3-S727 and ERK phosphorylation by CM from HN13 and HN30 (Fig. 4; compare Lanes 9 versus 10 and 13 versus 14). Strikingly, AG1478 failed to prevent STAT3-Y705 phosphorylation by CM of
HN13 and HN30 cells under identical conditions. These findings strongly indicate the existence of a secretory factor(s) from HNSCC cells, which are capable of inducing STAT3 tyrosine phosphorylation through a non-EGFR-dependent pathway.

Fig. 4. Secreted factors from HNSCC cells induce STAT3 tyrosine phosphorylation independent of the EGFR pathway. CM from a near confluent plate of the indicated HNSCC cell lines or HaCaT cells were harvested after 24 h incubation with DMEM without supplements. HaCaT cells were serum starved for 24 h with or without 30 min of AG1478 pretreatment, incubated for 10 min with the conditioned media, and harvested for cell lysates. The addition of EGF to serum-deprived HaCaT cells led to phosphorylation of ERK and STAT3 at both tyrosine and serine residues (compare Lanes 2 versus 3) and served as a positive control. Pretreatment of HaCaT cells with AG1478 prevented the stimulatory effects of EGF (Lane 4). Immunoblots for total cellular ERK and STAT served on the same filters used for the pERK and pSTAT3 blots, respectively. The addition of CM from HN13 and HN30 markedly induced STAT3 phosphorylation (pSTAT3-Y705 and pSTAT3-S727 panels; Lanes 9 and 13). Prior treatment with AG1478 could prevent only ERK and pSTAT3-S727 activation, but not STAT3 tyrosine phosphorylation induced by the CM (Lanes 3 versus 4, 9 versus 10, and 13 versus 14).

Autocrine/Paracrine STAT3 Stimulation in HNSCC Occurs via the Cytokine Signaling Coreceptor gp130 Family. To identify the potential autocrine/paracrine factor(s) in HNSCC that is responsible for EGFR-independent STAT3 stimulation in HNSCC, we screened possible candidates previously shown to stimulate STAT3 (8). By RT-PCR analysis for expression of nine known cytokines, we found relatively higher expression levels of IL6 and LIF in HN13 and HN30 when compared with HaCaT, HN6, and HN19 cells, which correlated with the profile of stimulatory activities of CM from these HNSCC and HaCaT cells (Fig. 5A; right upper panel, Lanes 1–5; right lower panel, Lanes 6–10). CM from these cells were also assayed for IL6 protein. The results of these ELISAs coincided well with the RT-PCR expression profiles for IL6 (Fig. 5A; right lower panel; compare band intensities with quantitative numbers below the panel). Additional analyses for IL6 protein in CM from four additional HNSCC cell lines, HN4, HN6, HN8, and HN12, showed a high level of IL6 secretion, which was comparable with that of HN13 and HN30 CM (data not shown). Other cytokines tested included IL2 and IL10, the levels of which in CM were below the lower limits of detection by ELISA of 15.6 and 9.4 pg/ml, respectively.

IL6 and LIF share the same coreceptor for binding and stimulating cytokine signaling, the gp130 family coreceptor. To address whether the two cytokines were responsible for the activation of STAT3 in HNSCCs, we asked whether blocking the gp130 receptor could prevent STAT3 activation in HaCaT cells by their CM. Indeed, pretreatment of HaCaT cells with anti-gp130 dramatically inhibited tyrosine phosphorylation of STAT3 by CM from these HNSCC and HaCaT cells, as shown by the immunoblot (arrowhead 1pY panel). Inhibition of ERK by AG1478 corresponded well with EGFR status (pERK panel). The level of pSTAT3-Y705 in HN6 cells decreased with AG1478, whereas, in contrast, HN13 cells displayed persistent pSTAT3-Y705 levels despite EGFR inhibition (pSTAT3-Y705 panel). The level of pSTAT3-S727 showed some correlation with active EGFR in both cell lines (pSTAT3-S727 and pY panels). HN19 and HN30 cells, which exhibit low levels of active EGFR, showed higher sensitivity to AG1478 as demonstrated by almost complete inhibition of EGFR at 1–10 μM concentration (pY panel). The pSTAT3-Y705 levels in both cell lines were resistant to AG1478 treatment (pSTAT3-Y705 panel). Again, pSTAT3-S727 showed a response similar to that of active EGFR (pSTAT3-S727 and pY panels).
anti-gp130 to serum-deprived HN13 or HN30 cells dramatically down-regulated pSTAT3-Y705 (Fig. 5C), although the pSTAT3 level began to increase as the blocking effect of anti-gp130 wore off in long-term culture (Fig. 5C, Lanes 6 and 12). These results indicate that autocrine/paracrine factors secreted by HNSCC cells can mediate STAT3 activation through the cytokine gp130 family of coreceptors. 

A Ligand for the gp130 Receptor Family, IL6, Is the Major Autocrine/Paracrine Factor for STAT3 Stimulation in HNSCC Cells. As described above, HNSCC cells produce ligands capable of stimulating the gp130 family receptor (39). From their expression patterns, IL6 and LIF are the most likely candidates for mediating the autocrine/paracrine STAT3 activation. We next assessed the contribution of IL6 and LIF to STAT3 activation by immunodepletion experiments. Incubating different concentrations of CM from HNSCC cells with anti-IL6 before addition to HaCaT cells resulted in attenuation of STAT3 activation, which was significant in HN13 and nearly complete in HN30 cells (Fig. 6A, Lanes 2–7 and 9–14). The effectiveness of the immunodepletion experiment was confirmed by measuring levels of IL6 protein in CM of HN13 and HN30 after precipitation with protein-G agarose with or without anti-IL6. The levels of IL6 protein in CM from both cells were reduced from 274.5 to 11.5 pg/ml in HN13 CM and from 785.9 to 13.2 pg/ml in HN30 CM after anti-IL6 treatment. With anti-LIF immunodepletion, there was only a moderate decrease in STAT3 activity as a result of treatment with CM from HN13, and no decrease was observed in cells treated with HN30 CM (Fig. 6B, Lanes 2–7 and 9–14). Because IL6 is highly expressed in HNSCC cells, these findings suggest that IL6 may represent the major autocrine/paracrine factor stimulating STAT3 activation, acting through the gp130 receptor. In addition, LIF seems to also participate in this autocrine/paracrine stimulation, as suggested by the partial decrease of STAT3 activation in HN13 cells.
Inhibition of STAT3 Activation by the JAK Pathway Leads to Decreased Proliferation and Survival Potential of HNSCC Cells.

Constitutive activation of STAT3 has been shown in a variety of cancer types, including leukemias, lymphomas, breast cancer, and head and neck cancer (6). In the latter case, inhibition of STAT3 by an antisense approach reduced the proliferative potential of HNSCC cells (24). Because our findings support the activation of STAT3 by a cytokine-dependent mechanism, and gp130 promotes the recruitment and activation of JAK tyrosine kinases thereby phosphorylating STAT3, we explored the possibility of interfering with STAT3 activation in HNSCC by taking advantage of the recent development of inhibitors of the JAK pathway, AG490 (40, 41). Initially, we tested whether AG490 could prevent activation of STAT3 by CM from HNSCC cell lines. When treated with AG490 30 min before the addition of CM, serum-deprived HaCaT cells demonstrated lower STAT3 tyrosine phosphorylation (Fig. 7A; upper panel; Lanes 4 versus 5 and 6 versus 7), but the JAK inhibitor had no effect on EGF- or CM-mediated ERK activation, which served as a specificity control (Fig. 7A; second panel from the bottom; Lanes 2–7). Furthermore, AG490 inhibited endogenously active STAT3 in HNSCC cells at concentrations of 50 μM for HN13 cells and 10–50 μM for HN30 cells (Fig. 7B, Lanes 4, 7, and 8). These doses of AG490 effectively inhibited HNSCC proliferation, as demonstrated in a growth curve spanning 72 h (Fig. 7C and D). It is interesting to note that the magnitude of the decline in the growth rate correlates well with inhibition of STAT3 activation. Interestingly, HN30 seemed to be more sensitive to AG490 than HN13. Cell-cycle analysis by propidium iodide staining revealed an apoptotic response in both HN13 and HN30 cells, with the fraction of cells in the sub-G0/G1 population rising from 6 to 24% in HN13 cells and 7 to 50% in HN30 cells (Table 1). These results underscore the crucial contributions of STAT3 in HNSCC cell proliferation and survival and raise the possibility that JAK may represent a suitable target for drug development in HNSCC.

DISCUSSION

Recent progress in understanding the molecular genetic changes of HNSCC carcinogenesis has led to the identification of critical tumor suppressor genes and proto-oncogenes involved in the progression of HNSCC (42, 43). Few studies, however, have described signal transduction abnormalities in HNSCC. Among the signaling aberrations present in HNSCC, constitutive STAT3 activation seems to be one of the critical pathways in HNSCC oncogenesis (44). In the present study, we investigated the mechanisms that contribute to STAT3 activation in HNSCC cells. Indeed, constitutively active STAT3 was observed under steady-state culture conditions in most HNSCC cells, as shown by both DNA-binding activity and tyrosine phosphorylation of STAT3, whereas immortalized skin keratinocytes (HaCaTs) and the normal keratinocytes (HFSK), displayed relatively low to undetectable levels of active STAT3. Thus, as in agreement with previous reports (24, 27), these data suggest the existence of mechanisms promoting and maintaining a constitutively active state of STAT3 in HNSCC.

Classically, STAT family proteins, including STAT3, are activated by phosphorylated JAK (7). However, several receptor and nonreceptor tyrosine kinases are also capable of directly or indirectly activating STAT proteins. Because overexpression and amplification of EGFR have been described in many cancers, including HNSCC, it is conceivable that maintenance of STAT3 activation in HNSCC could be accomplished by similar mechanisms. Indeed, STAT3 activation has

Table 1 Summary of cell-cycle distribution of HNSCC cells treated with AG490

<table>
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<tr>
<th></th>
<th>%Sub-G0/G1</th>
<th>%G0/G1</th>
<th>%G2/M</th>
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<tr>
<td>HN13</td>
<td>6.3 ± 3.8</td>
<td>74.5 ± 3.7</td>
<td>9.9 ± 0.1</td>
<td>7.9 ± 0.5</td>
</tr>
<tr>
<td>HN13 + AG490</td>
<td>24.2 ± 1.1</td>
<td>50.5 ± 0.6</td>
<td>12.1 ± 0.6</td>
<td>5.2 ± 1.2</td>
</tr>
<tr>
<td>HN30</td>
<td>7.1 ± 0.9</td>
<td>70.4 ± 1.9</td>
<td>8.9 ± 2.0</td>
<td>10.8 ± 1.3</td>
</tr>
<tr>
<td>HN30 + AG490</td>
<td>50.3 ± 2.2</td>
<td>27.7 ± 2.4</td>
<td>10.7 ± 0.7</td>
<td>2.9 ± 0.2</td>
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been shown to be closely linked to active EGFR signaling (24, 27, 28). Our results also confirmed a similar association in one HNSCC cell line, HN6, which exhibited overtly active EGFR tightly linked to the elevation of pSTAT3-Y705. However, the majority of HNSCC cells we analyzed did not display highly active EGFR signaling. Moreover, when excluding the contribution of EGFR signaling to STAT3 activation through the use of AG1478, we still observed persistent STAT3 tyrosine phosphorylation in most HNSCC cells. These results clearly indicate the existence of an EGFR-independent pathway leading to STAT3 constitutive activation in HNSCC cells. In the search for the underlying molecular events, we observed that HNSCC cells, with or without active EGFR, use an autocrine/paracrine STAT3 activation mechanism mediated through the gp130 family of cytokine receptors. Together, the above findings support the idea that constitutive activation of STAT3 can be maintained in HNSCC by at least two independent mechanisms, the EGFR and the gp130 receptor pathways.

In addition to this autocrine/paracrine STAT3 activation, we also observed the ability of CM from HNSCC to induce ERK phosphorylation in immortalized keratinocytes in an EGFR-dependent manner. This finding is consistent with the secretion and release of EGFR ligand(s), including TGFα, from HNSCC cells (Ref. 24 and data not shown). In this regard, it is interesting to note that serine phosphorylation on STAT3 in HNSCC cells correlated with the presence of an active ERK, a downstream effector of EGFR signaling, and that this serine phosphorylation was independent of the status of STAT3 tyrosine phosphorylation. Furthermore, our results revealed an inverse correlation between STAT3 DNA-binding activities and the levels of pSTAT3-S727 in many HNSCC cells (compare Fig. 1 with the two upper panels in Fig. 2), which may be consistent with recent observations describing the existence of a negative regulatory role for STAT3-S727 phosphorylation on STAT3 activity (14, 45). Thus, these results suggest the existence of a complex interaction between the signaling routes initiated by the EGFR and the cytokine signaling, the relative contribution of which to the STAT3 transcriptional activity is still not fully understood and will be the focus of future investigations.

The cytokine IL6 displays pleiotropic functions, including proinflammatory and proangiogenic activities. Consistent with the above notion, cytokine production by HNSCC cells has often been regarded as a marker of paracrine interaction between a developing tumor and the surrounding stroma (46, 47). In our study, under homotypic culture conditions, the autocrine/paracrine activity of IL6 was demonstrated to be crucial for HNSCC cells to maintain constitutive activation of STAT3, in addition to its classical role in tumor-stroma communication. Our data demonstrating that IL6 is produced by HNSCC cells are also supported by previous studies on cultured cells and clinical tumor specimens (46–48). We cannot exclude, however,
the possibility that other cytokines acting on the gp130 receptor family, such as leptin, may also cooperate in STAT3 activation in HNSCC. Additionally, certain HNSCC cells, such as HN19, despite releasing significant amount of IL6 protein, could not activate STAT3 in the HaCaT cells, suggesting the presence of a possible IL6 antagonist, like soluble forms of gp130 in its condition medium (49). If so, it is unclear which alternative mechanisms, such as other tyrosine kinase receptors or G-protein coupled receptors, promote the STAT3 activation in this particular cell line. Nonetheless, all available results together are consistent with the idea that IL6 is the major cytokine acting on the gp130 receptor in most HNSCC cells in an autocrine and paracrine fashion and underscore the importance of the role of cytokine signaling in these tumor types.

Previous studies using antisense treatment and transfection of dominant negative STAT3 have revealed a key role for STAT3 in HNSCC cell growth (24). In this study, we took advantage of the recent development of a small molecule inhibitor for JAK, AG490, to target this tyrosine kinase, the immediate upstream activator of STATs, in HNSCCs. Treatment with the JAK inhibitor led not only to a marked growth inhibition, but also resulted in the apoptosis of HNSCC cells, findings that occurred in parallel with down-regulation of active STAT3. Thus, we can hypothesize that the IL6/gp130 autocrine/paracrine loop maintaining a constitutive activation of STAT3 may confer both proliferative and survival advantages to malignant HNSCC cells.

Currently, clinical trials aimed at inactivating EGFR are under evaluation for the treatment of head and neck cancer (30, 50, 51). Given the fact that high levels of EGFR activation are documented in only a fraction of patients with HNSCC, it is likely that, in many circumstances, inactivation of EGFR signaling alone could be ineffective as a treatment strategy. Our present study suggests that constitutive activation of the STAT3 pathway may be a more common mechanism of HNSCC oncogenesis than EGFR activation. In addition, the finding that HNSCC cells use an IL6/gp130 autocrine/paracrine mechanism to activate STAT3 in an EGFR-independent manner suggests that the disruption of IL6/gp130 cytokine signaling, or inactivation of their ability to stimulate the STAT3 pathway, for example by blocking JAK activity, may represent novel therapeutic targets for pharmacological intervention in squamous malignancies of the head and neck.

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