Development of Head and Neck Squamous Cell Carcinoma Is Associated With Altered Cytokine Responsiveness

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Abstract

Growth of head and neck squamous cell carcinoma (HNSCC) is generally associated with an inflammatory component. It is hypothesized that these tumor cells develop mechanisms to evade the growth inhibitory effects of cytokines that are present in the tumor microenvironment. This study determined the changes in responsiveness to inflammatory cytokines that accompany the transition of normal to transformed epithelial cells. Paired primary cultures of normal epithelial cells (NEC) and SCC cells were established from 16 patients. Receptor-mediated activation of signal transducer and activator of transcription and extracellular signal-regulated kinase pathways in response to cytokine treatments was identified by immunoblot analysis. Thymidine incorporation determined the impact of the cytokines on DNA synthesis. HNNEC and HNSCC displayed a prominent signaling in response to oncostatin M, interleukin-6, IFN-γ, and epidermal growth factor. Untreated HNSCC showed an elevated level of phosphorylated signal transducer and activator of transcription 3 and extracellular signal-regulated kinase ($P < 0.001$) compared with HNNEC, suggesting constitutively activated pathways. Moreover, HNSCC cells phosphorylated significantly more signal transducer and activator of transcription 1 in response to oncostatin M ($P = 0.002$) and IFN-γ ($P = 0.018$) treatments. DNA synthesis of SCC cells was less inhibited by cytokines produced by endotoxin-stimulated macrophages ($P = 0.016$) than that of NEC. Low-dose oncostatin M slightly enhanced proliferation of SCC, whereas that of NEC was suppressed ($P = 0.016$). This study identified significant alterations in signal transduction pathways engaged by cytokines and which are associated with loss of growth inhibition of HNSCC. Increased signal transducer and activator of transcription phosphorylation, along with constitutively phosphorylated extracellular signal-regulated kinase in HNSCC, suggest that these pathways as molecular markers are important in the malignant transformation process and are potential targets for treatment.
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Introduction

Growth of malignant lesions is invariably associated with the presence of inflammatory cells at the primary site (1). The involvement of inflammation is particularly evident at sites prone to infection such as those found in the head and neck region. The role that inflammation has on function of normal tissue, including epithelial, stromal, and endothelial cells as well as on supporting proliferation of established malignancies, is presently an active area of research (1-3). Members from the family of IFNs, tumor necrosis factors, and the hematopoietic cytokines, particularly that of the interleukin-6 (IL-6) group, have been found to be largely responsible for the suppressing proliferation of normal epithelial cells (NEC) in association with inflammation. As shown in various preclinical epithelial cell studies, the mode of action of these cytokines includes induction of cell cycle arrest, activation of differentiation, and initiation of apoptosis (4-6). Because of the preclinical promise, some of these inhibitory cytokines have been used in clinical trials as biological adjuvants in the treatment of malignancies (5-8). More recently, the IL-6-related cytokine, oncostatin M (OSM), produced by tumor-associated macrophages and lymphocytes (9), has been noted to have particularly prominent growth inhibitory properties on established epithelial cell lines and primary cell cultures from different disease sites (10-14). However, the fact that epithelial tumors do grow in the presence of inflammation suggests that the effects of the inflammatory cytokines on those cell types may be subject to alteration during the transforming process. The finding in part supports this notion that the expression of inflammatory cytokines and the function of the corresponding receptors in the epithelial cells are frequently modified in progressing malignancies (15, 16). The change in cytokine responsiveness is believed to result from genetic and epigenetic changes in expression of receptors, presence of autocrine factors, and deregulated activity of signaling proteins. These changes that occur during oncogenic conversion from normal to carcinoma cells have been interpreted to provide certain tumor cell types a proliferative or survival advantage (15).

OSM and other members of the IL-6 hematopoietic cytokine family function through cell surface receptors that are

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composed of a ligand binding subunit (α) and a common signal-transducing subunit (β), gp130 (17-19). The signal-transducing receptor subunits have associated with their cytoplasmic domain members of the Janus family tyrosine kinase (18, 20-22). On ligand binding, signaling is initiated by activation of Janus kinases, which then phosphorylate receptor subunits and recruited substrates including the signal transducer and activator of transcription (STAT; refs. 23-25). Similarly, IFN-γ receptor, when activated by ligand binding, preferentially phosphorylates STAT1 through the action of the receptor-associated Janus kinases (26). Activated STATs translocate to the nucleus where they mediate transcriptional induction of different genes (18). Besides STATs, all hematopoietic cytokine receptors, like most growth factor receptors, activate the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) pathway (22, 27). The ERK pathway is considered to promote survival and/or growth stimulating effects (28, 29). The immediate activation of the Janus kinase/STAT and mitogen-activated protein kinase/ERK pathways by ligand binding is a specific and quantitative measure for the action of the cytokine receptor system in the target cells and thus qualitatively and quantitatively defines the responsiveness of cells (15).

The signaling and effects of IL-6 cytokines have been characterized mainly in established cell lines. Much less is known about the level of responsiveness of normal, non-differentiated, and proliferation-competent human epithelial cells and the range of responsiveness among such cells from different individuals. No study has addressed the responsiveness of head and neck squamous cell carcinoma (HNSCC) to IL-6-type cytokines. Considering that HNSCC, due to their anatomic location, can be particularly susceptible to infection and inflammation, the action of inflammatory cytokines predicts a potentially important role in the tumor growth control. The cellular complexity of head and neck tissue with substantial presence of nonepithelial cell types inevitably will render functional analyses in intact carcinoma tissues inconclusive. Thus, the approach of short-term primary cultures of epithelial cells derived from confirmed normal sites and cancer lesions has been chosen. This study characterizes the responsiveness of head and neck NEC (HNNEC) to cytokines and growth factors that would be found in the local tumor environment and determines the effects of malignant transformation on cytokine signaling and the regulation of proliferation.

Results

Patient Demographics

From 25 surgical specimens that were processed for generating primary cell cultures, 16 specimens yielded matched sets of cultures representing NEC and SCC that could be analyzed comparatively for cytokine responsiveness. The other 9 specimens did not yield cultures for both NEC and SCC. Cells either failed to grow *in vitro* or were lost due to contamination. The demographic information on the 16 patients is given in Table 1. The following head and neck sites were represented: 6 oral cavity, 5 larynx, 3 hypopharynx, and 2 base of tongue. The patients enrolled in this study were stages I to IVa and none had any treatment prior to surgery.

Table 1. Patient Demographics, Age, Disease Site, and Pathologic Stage Based on the American Joint Committee on Cancer 6th Edition Are Listed for the 16 Cases Used in This Study

Patient	Age	Gender	Site	Stage	Grade
1	81	F	FOM	I	PD
2	46	M	Base tongue	II	MD
3	72	F	Hypopharynx	IVa	WD
4	52	M	Larynx	II	MD
5	52	F	Larynx	III	MD
6	69	M	Hypopharynx	IVa	MD
7	68	M	Larynx	III	WD
8	58	M	FOM	I	PD
9	69	M	Oral tongue	III	MD
10	68	F	Oral tongue	I	MD
11	77	M	Larynx	II	WD
12	57	F	Oral tongue	III	WD
13	67	M	Larynx	II	WD
14	65	M	Base tongue	II	PD
15	77	M	Oral tongue	I	PD
16	45	F	Hypopharynx	III	MD

Abbreviations: FOM, floor of mouth; grades for the tumors: WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

Cytokine Response Pattern

The procedure to isolate and culture primary epithelial cells was successful in producing proliferating cultures that consisted almost exclusively of cells with epithelial morphology (Fig. 1A). Each NEC preparation, regardless of head and neck origin, yielded cells with essentially identical morphology. The corresponding SCC preparations often consisted of cells that were morphologically indistinguishable from the normal counterpart. In some cases, the cells were heterogeneous in size ranging from larger to smaller than the corresponding NEC (Fig. 1A). Immunocytochemical staining with anti-cytokeratin antibodies confirmed that each of NEC and SCC preparations contained >90% of cytokeratin-positive epithelial cells.

When the primary cultures reached ~70% confluence, subcultures from the passage 1 cells were established in 24-well culture plates. These subcultures were used to identify the responsiveness to leukemia inhibitory factor (LIF), IL-6, OSM, epidermal growth factor (EGF), and IFN-γ. The level of phosphorylation of STAT and ERK after 15-minute treatment served as indicator for the ligand-inducible receptor activity. The comparison of the response patterns of NEC and SCC provided a measure of transformation-associated changes in receptor signaling that were detectable in tissue cultures. The comparison of basal level phosphorylation of ERK and STATs indicated whether the transformation was also associated with an activation of signaling reaction that was independent of treatment with exogenous ligands.

The representative case of hypopharyngeal SCC cells and the corresponding NEC in Fig. 1B reveals the salient features of the cytokine response pattern of epithelial cells from head and neck origin and some of the alterations of the pattern that were observed in HNSCC cells. The quantitative values for the cytokine and growth factor–induced phosphorylation of STAT3 (Fig. 2A), ERK-1/2 (Fig. 2B), and STAT1 (Fig. 3) were compiled for the 16 cases. The response pattern of NEC was highly consistent among independent cell preparations made

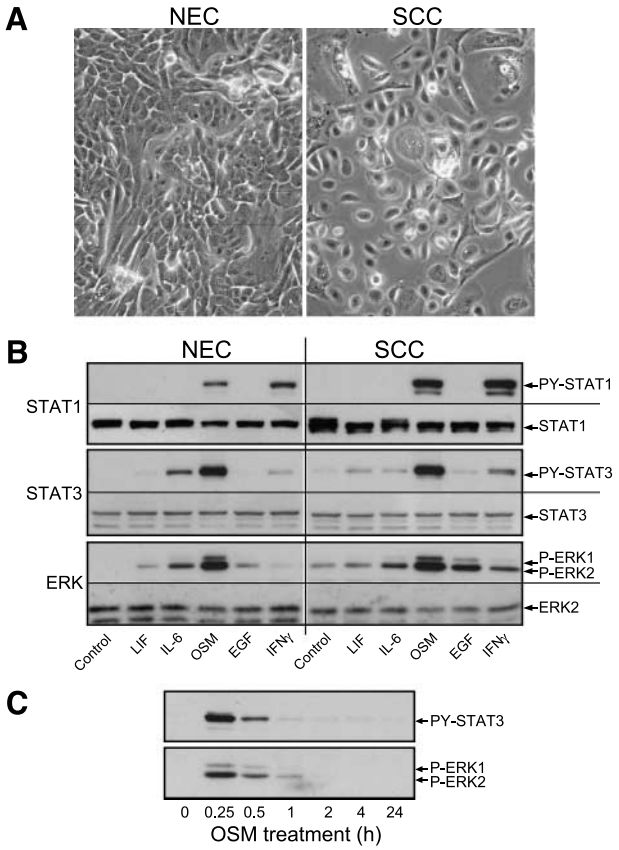

FIGURE 1. Activation STAT1, STAT3, and ERK in cytokine-treated HNNEC and HNSCC. **A.** Morphology of proliferating cell culture of HNNEC and corresponding HNSCC derived from patient 3. Phase microscopic images were taken at ×40 magnification. **B.** Cells from patient 3 were treated for 15 minutes with the cytokines as indicated. Phosphorylated and total STAT and ERK were determined by immunoblotting. Representative enhanced chemiluminescence exposures of the coanalyzed NEC and SCC samples are reproduced. **C.** Time course of signaling in NEC.

from different head and neck locations. The following features characterized the pattern: Unstimulated cells showed a low to nondetectable basal phosphorylation of STAT1, STAT3, and ERK (Fig. 1B). LIF treatment yielded a barely detectable activation of STAT3 and ERK. IL-6 was more effective than LIF but consistently less than OSM. The specificity of OSM response was indicated by the high-level phosphorylation of STAT1, STAT3, and ERK (Figs. 1B, 2A and B, and 3A). The response of NEC to EGF was evident by the phosphorylation of ERK and, in some cases, by a minor phosphorylation of STAT3. Although STAT3 activation by EGF was variable among cell preparations from different donors, it was consistently less than STAT3 activated by OSM. Lastly, IFN-γ produced in all NEC cultures a maximal activation of STAT1 with minor elevation of phosphorylated STAT3 and ERK (Figs. 1B, 2, and 3).

The comparison of normal and tumor cells indicated that, in 11 of 16 HNSCC cases, an increased basal level of phosphorylated ERK was detectable, whereas the amount of total ERK proteins was not appreciably different between NEC and SCC (e.g., Fig. 1B). The magnitude of the enhanced ERK phosphorylation varied substantially among SCC preparations (Fig. 2B). Despite the elevated basal phosphorylation, in all HNSCC cultures, ERK phosphorylation was increased by treatment with the cytokines and EGF noted to be effective in NEC.

In 5 of 11 of the HNSCC cases with elevated basal level of phosphorylated ERK, an increased basal level of phosphorylated STAT3 was found as well (Figs. 1B and 2A). Of note is that in none of the SCC cases was a basal phosphorylation of STAT1 detectable. A minor LIF response was detectable in all HNSCC cases and often was ~2-fold higher than in NEC. In contrast, the magnitude of IL-6 response was reduced. In 14 of 16 of the HNSCC cases, there was an enhanced activation of STAT1 in response to OSM and IFN-γ treatments. The effect of OSM and IFN-γ on STAT1 was probably in part due to a more effective recruitment of this factor, because the level of total STAT1 in most of the HNSCC cells did not differ appreciably from that of the corresponding normal cells. A few SCC cultures showed, however, a <2-fold increase of immune detectable STAT1 that could contribute to the higher amounts of phosphorylated STAT1 (e.g., Fig. 1B). The comparison of

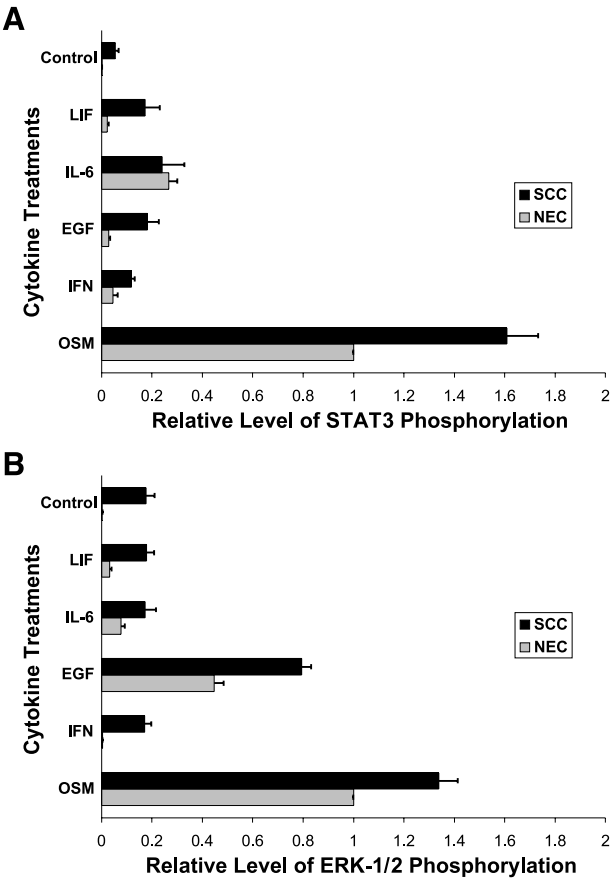

FIGURE 2. Relative changes in STAT3 and ERK signaling between HNSCC and corresponding HNNEC. Densitometric analyses of STAT3 phosphorylation (**A**) and ERK phosphorylation (**B**) in untreated control and in cells in response to15-minute treatment with LIF, IL-6, IFN-γ, or OSM cytokine treatments were compared between NEC and the corresponding SCC in all 16 cases.

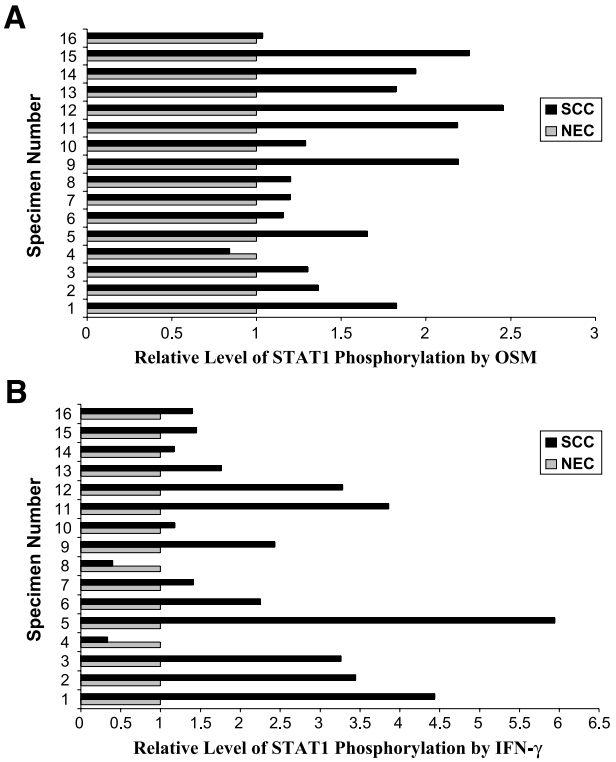

FIGURE 3. Comparison of the amount of STAT1 phosphorylated in response to OSM (**A**) and IFN-γ (**B**) treatments. The level of STAT1 phosphorylation was determined for all 16 paired sets of NEC and SCC by immunoblotting as shown in Fig. 1. The quantitative values of the SCC samples were expressed relative to the NEC (defined as 1.0). The average fold increase of STAT1 phosphorylation by OSM in SCC was 2.37; STAT1 phosphorylation by IFN-γ cells was 2.61.

HNNEC and HNSCC cells also indicated in 10 of the 16 cases an enhanced EGF response, as is evident by the more prominent activation of STAT3 and ERK (Fig. 2B).

Statistical evaluation of the data indicated that phosphorylation of STAT3 was significantly higher in the untreated HNSCC ($P < 0.001$) and HNSCC treated with LIF ($P = 0.038$), EGF ($P = 0.038$), IFN-γ ($P = 0.011$), or OSM ($P = 0.002$) when compared with their corresponding HNNEC cultures (Fig. 2A). Phosphorylation of STAT1 was also significantly higher in response to OSM ($P = 0.002$) and IFN-γ ($P = 0.018$) treatments (Fig. 3A and B). The data on ERK-1/2 phosphorylation indicated that significantly more ERK-1/2 were phosphorylated already in the untreated HNSCC ($P < 0.0001$) and that the level was also higher in HNSCC treated with LIF ($P = 0.011$) and IFN-γ ($P = 0.0003$; Fig. 2B).

The low responsiveness of various epithelial cell types to IL-6 and LIF (as seen for HNNEC; Fig. 2B) has previously been correlated with a low to nondetectable expression of the ligand binding subunits, IL-6Rα and LIFRα, as judged from transcript analyses by reverse-transcription and PCR and by immunoblot analysis of cellular proteins for LIFRα (15). In contrast, the elevated basal phosphorylation of ERK and STAT3 in HNSCC conceivably could represent, among others, the result of genetic changes causing the constitutive activation of signaling pathways or the action of autocrine factors (24). The paucity of cellular material available from the primary culture systems precluded a characterization of the former possibility. The latter possibility, namely, an autocrine activity as potentially exerted by secreted cytokines, was assessed by two approaches: One approach was to treat SCC cultures with function-neutralizing anti-gp130 antibodies to determine the role of gp130 in elevated phosphorylation of ERK and STAT3. The other approach was to test 3-day conditioned medium from SCC cultures with high basal levels of phosphorylated ERK for its ability to induce signaling in NEC. However, both approaches failed to document significant activating effects of autocrine components that act extracellularly (data not shown).

These experiments could not rule out stimulatory activity, such as an autocrine factor, that acted in SCC at the intracellular level and thus was inaccessible to extracellular probing agents. Because the assessment of the responsiveness of the epithelial cells relied on 15-minute cytokine treatment that generates the maximal signaling reaction (Fig. 1B), the response pattern thus established was not comparable with the one expected for an autocrine factor that acted in a chronic manner. Therefore, to evaluate whether prolonged stimulation of NEC with OSM, the most effective cytokine on these cells, would in principle result in a pattern of phosphorylation of STAT3 and ERK as found in untreated SCC, primary NEC cells were incubated in the presence of OSM for 24 hours (Fig. 1C). Phosphorylation of STAT3 and ERK was maximal within 15 minutes. ERK phosphorylation returned to pretreatment level by 2 hours, whereas phosphorylation of STAT3 was maintained at low but above basal level. This level was comparable with that observed in some of the untreated SCC (Fig. 1B). The results indicated that an autocrine IL-6-related activity could account for an increased STAT3 activation in SCC but not for elevated ERK activity.

Effects of Cytokines on DNA Synthesis

Based on the altered responsiveness of HNSCC cells, we assessed whether treatment of the cells with OSM, or the physiologically relevant mixture of inflammatory mediators as provided by endotoxin-stimulated lung macrophages (CMM), would differentially affect the proliferation of the epithelial cells. CMM contains a complex mixture of mediators, including, among others, IL-6, LIF, OSM, tumor necrosis factor-α, IL-1β, IL-8, IL-10, and granulocyte-colony stimulating factor at concentrations from 1 to 300 ng/mL. HNNEC and HNSCC cells were treated with serially diluted CMM (Fig. 4) or OSM (Fig. 5).

Treatment of HNNEC with normal growth medium containing serially diluted CMM reduced DNA synthesis in a dose-dependent manner with a maximal reduction of 40% observed at the highest concentration tested (Fig. 4). In separate sets of cultures, NEC cells from the second and third passages from different donor tissues were maintained in normal growth medium up to 9 days to establish the growth rate. Doubling times varied substantially, ranging from 33 to 50 hours.

Proliferation rates of HNSCC maintained in normal growth medium did not significantly differ from those of the corresponding NEC. This was also evident by the comparable incorporation of [^{3}H]thymidine. In our experimental setting, the incorporation of radioactivity (counts per minute; mean ± SE; $n = 11$) for control cultures of HNNEC was 154,883 ± 13,376,

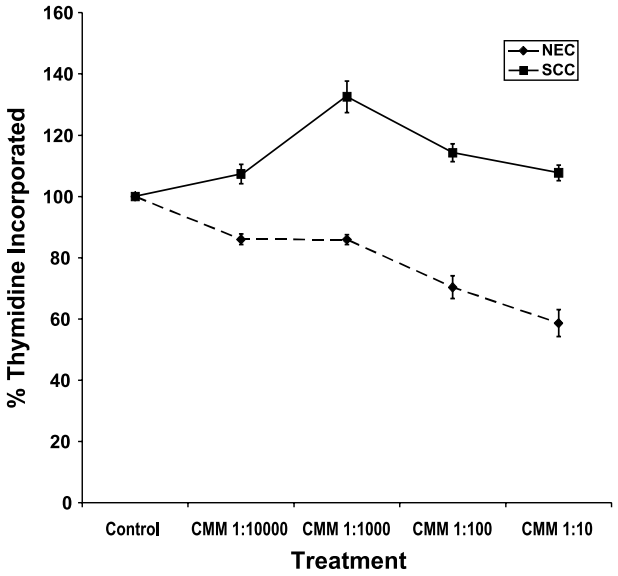

FIGURE 4. Comparison of the effect CMM has on the DNA synthesis of HNNEC and HNSCC. SCC and NEC were treated with serial dilutions of CMM. [^{3}H]Thymidine incorporation of the untreated controls in each assay was defined as 100% and all other data were expressed relative to the control. *Bars,* SE of 11 HNSCC and 10 HNNEC cases.

and for HNSCC, it was 164,143 ± 6,889. When considering only the five HNSCC cases that exhibited constitutive activation of both ERK and STAT3 pathways, a slightly higher thymidine incorporation was measured (183,120 ± 5,294 counts per minute). Treatment of HNSCC cells with the same CMM dose gradient as applied to HNNEC did not lower thymidine incorporation below that of the control-treated cultures (Fig. 4). In fact, treatment of the cells with a 1:1,000 dilution of CMM even increased DNA synthesis by 30%. The response of HNSCC at this and higher CMM concentrations differed significantly from that of the corresponding HNNEC cultures ($P = 0.016$).

Both HNNEC and HNSCC cells responded to OSM in a dose-dependent manner with a decrease in thymidine incorporation. A treatment for 40 hours at the maximal concentration of 100 ng/mL OSM reduced DNA synthesis by 50% (Fig. 5). At lower concentrations ranging from 0.1 to 1 ng/mL, OSM seemed to be less inhibitory or even to exert a minor stimulatory action on DNA synthesis in the HNSCC when compared with the corresponding NEC cultures. This minor stimulatory effect has been detected in 14 of the 16 HNSCC cases that also presented an increased STAT1 (Fig. 3A). None of the HNNEC cultures showed a comparable stimulation. At the dose of 0.1 ng/mL OSM, the relative thymidine incorporation by HNSCC and HNNEC reached statistically significant difference ($P = 0.016$) but not at the other dose level.

Discussion

This comparative study of normal and transformed head and neck epithelial cells has indicated several significant alterations in the signaling pathway and cytokine responsiveness that accompany the tumorigenic process. Changes found with high frequency in SCC include an enhanced basal activity (phosphorylation) of the ERK pathway, an elevated activation of STAT1 signaling by OSM and IFN-γ, an attenuation of IL-6 responsiveness, and a less suppressed DNA synthesis by inflammatory cytokines. These changes represent not only novel markers for the regulatory capabilities of HNSCC but also potential explanations for the ability of SCC to proliferate in presence of inflammation. The future goals will be (*a*) to elucidate the precise molecular mechanisms that link signaling reactions activated by OSM and other inflammatory mediators with the proliferation control characteristic for NEC and SCC and (*b*) to establish what extent these changes have a causative role in clinical progression of the cancer.

The findings of this study draw attention to following two questions: How do these alterations in signaling arise and what is the functional consequence of these alterations to the biology of head and neck tumors? Whereas deviations in responsiveness to cytokines by altered signaling at various levels are generally observed in transformed cell types (15), the high frequency by which the same changes (i.e., activation of ERK and STAT) occur in independent HNSCC cases suggests that these may have been functionally selected. Whereas technical limitations intrinsic to primary epithelial cell culture systems precluded us from identifying the cause of the constitutive ERK and STAT phosphorylation, potential mechanisms could include deregulating mutations of signaling kinases or the introduction of autocrine stimulatory activities. The higher activation of STAT proteins by cytokine treatments often in the absence of a detectable change in STAT protein levels, as seen in most HNSCC cases, suggests a more effective recruitment of the STAT proteins to the signal-transducing components by the cytokine receptors. In some cases, a transformation-associated changes in the level of STAT proteins, such as STAT1 (Fig. 1B), probably also contribute to the apparent increased STAT signal in HNSCC. Moreover, increased amounts of receptor proteins

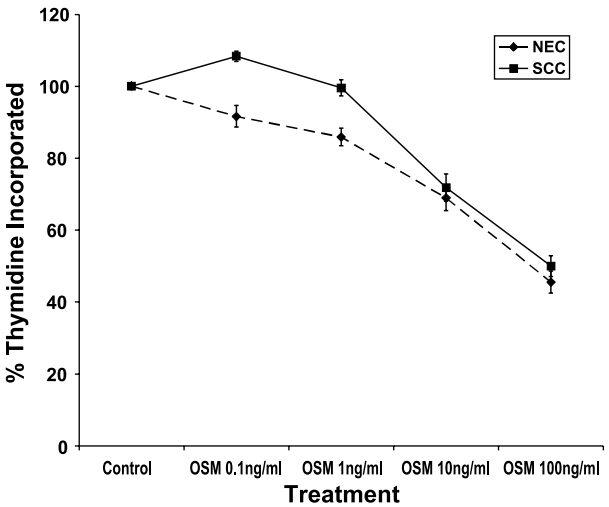

FIGURE 5. Comparison of the effect OSM treatments has on the DNA synthesis of HNNEC and HNSCCA. SCC and NEC were treated with serial dilution of OSM. Incorporation of [^{3}H]thymidine in untreated NEC in each set was defined as 100% and all other data were expressed relative to the control. *Bars,* SE of 11 HNSCC and 10 HNNEC cases.

could also explain an enhanced overall signaling reaction as noted for LIF (15) but not the preferential recruitment of specific signaling pathways. Loss of receptor function, such as the specific reduction of IL-6 responsiveness has tentatively been attributed to a reduced expression of IL-6Rα. The function of the gp130 subunit seems not to be attenuated based on the maintenance of OSM response (Fig. 1B). With the currently available immunoreagents for receptor proteins, we have not yet been able to quantitate receptor proteins in the limited amounts of extracts from the primary head and neck epithelial cell cultures.

Because the signaling capability of the receptor systems was tested by short-term (15 minutes) treatment with cytokines and EGF, the level of STAT and ERK phosphorylation reflects to a large extent the activity of the receptor-proximal protein tyrosine kinases and enzymes of the immediate downstream signaling cascade such as of the mitogen-activated protein kinase pathways. The effects that are bought about by a long-term cytokine treatment, such as enhanced or reduced proliferation, involve a broader array of signaling reactions that include not only the immediate mediators STATs and ERKs but also many secondary effectors downstream of STATs and ERKs, such as signal-attenuating phosphatases, kinase inhibitors of the SOCS family, and coactivators or corepressors for signal-mediating transcription factors, including those for STATs. Clearly, the role of these components needs to be determined in the definition of the regulatory phenotypes of HNSCC.

The consequence of the altered signaling reactions is interpreted in the context of growth regulation in HNSCC. A possible causal relationship is suggested by the finding that DNA synthesis is less suppressed in cytokine-treated HNSCC (Figs. 4 and 5). Activation of mitogen-activated protein kinase/ERK is often connected to growth promotion and/or enhanced survival (28, 30-32). Thus, the increased ERK phosphorylation as noted in HNSCC cases would predict a more effective protection of the cells from the inhibitory action of the cytokines signals. Besides activation of ERK, OSM and IL-6 are also effective in activating STAT3. The effect STAT3, alone or in the combination with activated ERK on growth control in HNSCC, is, however, less predictable. Several studies have linked activated STAT3 alone to increased cell proliferation and oncogenic action (33, 34), whereas others have noted suppression of proliferation in cells with STAT3 activated through cytokine receptor action (20, 35). Similarly unclear is the role of STAT1 in tumorigenesis. Activated STAT1, such as through the action of the IFN-γ or OSM, is believed to promote growth arrest and apoptosis, yet the level of STAT1 is increased in many tumor cells (10, 36, 37). Our data on HNSCC suggest that the control of DNA synthesis, and thus proliferation, by OSM is dependent on both the combination and the activation levels of ERK and STATs. The relative magnitude and duration of these pathways to be activated are in turn a function of the cytokine dose and signaling capability of the receptor system. These processes may account for the switch from stimulation to suppression of DNA synthesis when treating HNSCC cultures with low or high concentration of OSM.

The control of epithelial cell proliferation in response to CMM is even more complex; besides OSM and IL-6, other potent effectors such as tumor necrosis factor, IL-1, IL-8, and prostaglandins act on the target cells (Fig. 4). The intracellular signals communicated by these factor, including stress mitogen-activated protein kinase, nuclear factor-κB, and G proteins, will certainly influence the effects expected for STAT and ERK that are activated by IL-6 cytokines. The results revealed that the transforming process has led in most SCC to a modification that attenuated the suppressing signaling function. The responsible mediators and the effect of transforming process on the function of those remain to be identified.

The analysis of epithelial cells in primary tissue cultures allowed us to define the signaling capability and the qualitative and quantitative responsiveness to defined inflammatory cytokines of the cells as a function of transformation from HNNEC to HNSCC. This approach purposely removed the influence that tissue milieu has on the epithelial cells *in situ*. However, to extrapolate the results gained in tissue culture to regulatory properties in tumor, we have to consider the nature of the tumor environment. In the majority of patients who develop a HNSCC, tobacco and alcohol consumption is a relevant component that contributes to the local milieu. Chronic injury and repair in the upper aerodigestive tract caused by tobacco and alcohol use involves inflammatory reaction that in turn coordinates stimulation and suppression of epithelial cell growth. Moreover, the growth of malignancies adds appreciably to the local inflammatory reaction. These injury and tumor-related inflammatory processes are involving the suppressive action of those cytokines as defined *in vitro* (10, 38). Hence, we hypothesize that HNSCC *in vivo* have a similarly altered signaling process as found in the same cells in primary culture; thus, the cells are less subject to growth suppression than normal cells. An important goal of future studies will be to establish the correlation of *in vitro* established phenotype based on marker proteins with the manifestation of the same markers in tumors. This will allow a more specific prediction as to the influence of inflammation on tumor progression.

Materials and Methods

Tissue Procurement

Postsurgical specimens from mucosal-bearing head and neck sites were obtained through the institutional review board–approved tissue procurement protocol CIC 00-91. From each specimen, the diagnosing pathologist selected residual tissue representing confirmed normal epithelium and corresponding SCC. These tissue samples were immediately transferred to laboratory analysis that was covered by the institutional review board–approved protocol CIC 00-17.

Primary Cell Cultures

Preparation of primary epithelial cell cultures from normal and carcinoma tissue was carried out by a modified tissue dissociation procedure (39). Briefly, the specimens were rinsed with PBS containing antibiotics penicillin, streptomycin, and amphotericin B at a 1:1,000 dilution. The epithelial layer was separated from the specimens with a scalpel, cut in to 2 to 3 mm pieces, and partially disassociated by digestion with 1%

trypsin in PBS containing 1 mmol/L EDTA for 15 minutes at 37°C. The pieces were then placed in 6 cm tissue culture dishes in serum-free, hormonally defined keratinocyte medium containing bovine pituitary extract, cholera toxin, and recombinant human (rhu) EGF (Life Technologies, Carlsbad, CA). Whereas this culture condition favored the outgrowth of epithelial cells (proliferation of primary epithelial cells is growth factor dependent), low level of cocultured fibroblasts could occur. Essentially all fibroblasts were removed from the epithelial cell cultures by selective release by brief digestion with 0.5% trypsin for 5 minutes at 37°C. Greater than 90% homogenous epithelial cells cultures, as determined by cytokeratin staining, were routinely obtained after 2 to 3 weeks. Subcultures of the first and second passages were used to determine the cytokine response profile and DNA synthesis of the cells, respectively. Because the amount of starting tissue material as well as the proliferation of the epithelial cells from the tissue samples often differed appreciably, simultaneous treatment of control and carcinoma cells from the same patient was not always possible. In all cases of paired samples with different growth, cell extracts were stored at −70°C for combined immunoblot analyses. The determination of thymidine incorporation was carried whenever the individual cell preparations of the second passage became available. In most cases, the normal as well as tumor epithelial cell cultures gradually lost proliferative activity after the second to fourth passages, what limited, or even precluded further biochemical analyses of those cell cultures.

Resident pulmonary macrophages were mechanically extracted from residual tumor-free lung tissue and purified by centrifugation on Histopaque (Life Technologies). After adhesion to plastic tissue culture support, 3×10^6 macrophages per milliliter RPMI containing 10% FCS were treated for 16 hours with 1 μg/mL lipopolysaccharides. The concentrations of cytokines in CMM were determined by multiplex immunobead flow cytometry (Luminex, Inc., Austin, TX).

Cytokine Treatments for Analysis of Signaling

Cells from passage 1 of the primary cultures of NEC and SCC were plated into 24-well cluster plates. When the cultures reached ~90% confluence, they were incubated for 2 hours in serum-free and factor-free RPMI followed by incubation for 15 minutes with the same medium containing 100 ng/mL rhu IL-6, rhu OSM (Amgen Corporation, Seattle, WA), rhu LIF (Wyeth Pharmaceuticals, Cambridge, MA), rhu EGF (Invitrogen, Carlsbad, CA), or rhu IFN-γ (Roche Applied Science, Indianapolis, IN). Dose-response analyses have indicated that 100 ng/mL of each cytokine was ~10 times above the concentration required to trigger maximal receptor signaling. However, a dose of 100 ng/mL was needed to sustain maximal stimulation during long-term treatment such as when measuring the cytokine effects on gene induction and proliferation. Thus, a treatment dose of 100 ng/mL was chosen to ensure that maximal receptor signaling did occur under all selected culture conditions. Treated cells were washed with PBS and lysed in the culture well with radioimmunoprecipitation assay buffer containing 0.1 mmol/L orthovanadate and 1:100 diluted protease inhibitor cocktail (Calbiochem, San Diego, CA).

Western Blot Analysis

Replicate aliquots of cell lysates containing 10 or 20 μg of protein were electrophoresed on 7.5% to 10% polyacrylamide gels. The proteins were transferred to protean membranes (Schleicher & Schuell, Keene, NH). Immediately after transfer, the membranes were stained with Ponceau red to verify loading and membrane transfer of equal amounts of protein per sample. In each experimental series, two replicate blots were cut horizontally at the ~60-kDa size position; the upper sections were probed for phosphorylated and total STAT3 and the lower sections for phosphorylated ERK and total ERK. Separate blots were used to probe for phosphorylated and total STAT1. Unless limited by the available cell material, replicate separations and immunoblot analyses instead of reprobing of membranes were applied due to difficulties in complete removal of the antibodies from the first round of reaction. The membranes were reacted with antibodies to phosphospecific forms or ERK-1/2, STAT1, and STAT3 (Cell Signaling Technology, Inc., Beverly, MA) and total forms of ERK-1/2, STAT1, and STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were incubated with the appropriate peroxidase-conjugated secondary antibodies (ICN Biomedical, Aurora, OH) and the antibody binding was visualized by enhanced chemiluminescence reaction (Amersham Biosciences, Piscataway, NJ). In each experimental series, immunoblots were exposed to X-ray films for various lengths of time (1 second to 30 minutes) to obtain images that are in the linear range of signal detection by the scanner.

Densitometric Analysis

The chemiluminescence images of immunoblots were scanned with a high-resolution desktop scanner. The digital images were quantified with Image Quant Software 5.0 (Amersham Biosciences). The net pixel value for each protein band that lied within the linear range of detection was normalized to the coanalyzed standard and used to calculate the relative difference to the untreated control cells in each experimental series. To compare the responses between NEC and SCC from individual patients as well the responses among different patients, the OSM-induced activation of STAT1, STAT3, and ERK-1/2 of the NEC in each paired set was used as an internal reference. The net pixel values determined for the phosphorylated signaling proteins in OSM-treated cells were defined as being equal 1.0. In each set of NEC and SCC, the pixel values of the basal level phosphorylation and those induced by cytokine treatments (where detectable) were then expressed relative to the OSM reference.

Thymidine Incorporation

To determine the effect of cytokines on DNA synthesis, NEC and SCC cells from passage 2 were seeded into 24-well culture plates (5×10^4 cells per well). After 24 hours, duplicate cultures were treated with full growth medium containing serially diluted rhu OSM or conditioned medium from lipopolysaccharide-activated primary human pulmonary macrophages (CMM). The use of growth medium was necessary, because in the absence of growth factors, the growth of epithelial cells was arrested. Twenty-four hours later, 1 μCi of

[^{3}H]thymidine (Amersham Biosciences) was added to each culture and incubation continued for additional 16 hours. Cells were released by trypsin and collected onto paper filter by the cell harvester (Tomtec, Hamden CT). The amount of incorporated tritium was measured by a scintillation counter (Trilux microbeta, Perkin-Elmer Wallac, Turku, Finland). The mean of the net values of the duplicate wells was expressed relative to the incorporation determined for the control cultures in each of the series, which was defined as 100%. Proliferation rates (doubling times) of epithelial cells were determined by seeding cells at a density 5×10^3 cells/cm^2 (equivalent to ~5% confluence) and maintained for 6 to 9 days in full growth medium alone or containing 1:10 diluted CMM, 100 ng/mL OSM, or 5 μg/mL function-neutralizing monoclonal anti-human gp130 antibody (R&D Systems, Minneapolis, MN). Media were changed every third day. Cells were released by trypsin digestion, resuspended in trypan blue dye–containing PBS, and counted using a hematocytometer.

Statistical Evaluation

All statistical analyses were done in an exploratory manner at the significance level of 0.05. The exact nonparametric inferences were employed for all the hypothesis tests. For each possible biomarker (P-STAT3, P-ERK, and P-STAT1) and treatment (one control and five cytokines) combination, the relative increase in phosphorylation of the SCC compared with its matched baseline from the same patient was tested using the matched pairs sign test. To test the decreasing or increasing trend of DNA syntheses within a treatment (OSM or CMM) by different doses of the treatment, the matched pairs sign test and Page's *L* test were used because samples from the same individual were used across the different dose levels.

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References

1. Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420:860–7.

2. Adami HO, Boffetta P, Homey B. Chemokines: agents for the immunotherapy of cancer? J Intern Med 2002;251:455–66.

3. Brigati C, Noonan DM, Albini A, Benelli R. Tumors and inflammatory infiltrates: friends or foes? Int Immunopharmacol 2001;1:1651–67.

4. Grohmann U, Puccetti P. The immunosuppressive activity of proinflammatory cytokines in experimental models: potential for therapeutic intervention in autoimmunity. Curr Drug Targets Inflamm Allergy 2002;1:77–87.

5. Hernberg M, Virkkunen P, Bono P, Ahtinen H, Maenpaa H, Joensuu H. Interferon α-2β three times daily and thalidomide in the treatment of metastatic renal cell carcinoma. J Clin Oncol 2003;21:3770–6.

6. Sikorski M, Zrubek H. Recombinant human interferon γ in the treatment of cervical intraepithelial neoplasia (CIN) associated with human papillomavirus (HPV) infection. Eur J Gynaecol Oncol 2003;24:147–50.

7. Bray D, Yu SZ, Koprowski H II, et al. Combination nonviral interleukin 2 gene therapy and external-beam radiation therapy for head and neck cancer. Arch Otolaryngol Head Neck Surg 2003;129:618–22.

8. Dawson DE, Gapany M, Burgess RC, Boesen PV, Headley DB. Tumor necrosis factor and chemotherapeutic agents. Potentiation of cytotoxicity with interferon γ. Arch Otolaryngol Head Neck Surg 1992;118:1168–71.

9. Fujii T, Hayashi S, Hogg JC, et al. Interaction of alveolar macrophages and airway epithelial cells following exposure to particulate matter produces mediators that stimulate the bone marrow. Am J Respir Cell Mol Biol 2002; 27:34–41.

10. Kortylewski M, Mackiewicz A, Schniertshauer U, et al. Interleukin-6 and oncostatin M-induced growth inhibition of human A375 melanoma cells is STAT-dependent and involves upregulation of the cyclin-dependent kinase inhibitor p27/Kip1. Oncogene 1999;18:3742–53.

11. Li C, Ahlborn TE, Kraemer FB, Liu J. Oncostatin M-induced growth inhibition and morphological changes of MDA-MB231 breast cancer cells are abolished by blocking the MEK/ERK signaling pathway. Breast Cancer Res Treat 2001;66:111–21.

12. Klausen P, Pedersen L, Jurlander J, Baumann H. Oncostatin M. Interleukin 6 inhibit cell cycle progression by prevention of p27kip1 degradation in HepG2 cells. Oncogene 2000;19:3675–83.

13. Grant SL, Begley CG. The oncostatin M signaling pathway: reversing the neoplastic phenotype? Mol Med Today 1999;5:406–12.

14. Brown TJ, Lioubin MN, Marquardt H. Purification and characterization of cytostatic lymphokines produced by activated human T lymphocytes. Synergistic antiproliferative activity of transforming growth factor β1, interferon-γ, and oncostatin M for human melanoma cells. J Immunol 1987;139:2977–83.

15. Blanchard F, Kinzie E, Wang Y, et al. FR901228, an inhibitor of histone deacetylases, increases the cellular responsiveness to IL-6 type cytokines by enhancing the expression of receptor proteins. Oncogene 2002;21: 6264–77.

16. Lu C, Kerbel RS. Cytokines, growth factors and the loss of negative growth controls in the progression of human cutaneous malignant melanoma. Curr Opin Oncol 1994;6:212–20.

17. Taga T, Kishimoto T. Gp130 and the interleukin-6 family of cytokines. Annu Rev Immunol 1997;15:797–819.

18. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signaling and its regulation. Biochem J 2003;374:1–20.

19. Nishimoto N, Shima Y, Yoshizaki K, Kishimoto T. Oncostatin M. Leukemia inhibitory factor, and interleukin 6 induce the proliferation of human plasmacytoma cells via the common signal transducer, gp130. Blood 1994;84: 3040–6.

20. Kim H, Baumann H. Transmembrane domain of gp130 contributes to intracellular signal transduction in hepatic cells. J Biol Chem 1997;272:30741–7.

21. Wang Y, Robledo O, Kinzie E, et al. Receptor subunit-specific action of oncostatin M in hepatic cells and its modulation by leukemia inhibitory factor. J Biol Chem 2000;275:25273–85.

22. Schiemann WP, Graves LM, Baumann H, et al. Phosphorylation of the human leukemia inhibitory factor (LIF) receptor by mitogen-activated protein kinase and the regulation of LIF receptor function by heterologous receptor activation. Proc Natl Acad Sci U S A 1995;92:5361–5.

23. Darnell JE Jr. Transcription factors as targets for cancer therapy. Nat Rev Cancer 2002;2:740–9.

24. Savarese TM, Campbell CL, McQuain C, et al. Coexpression of oncostatin M and its receptors and evidence for STAT3 activation in human ovarian carcinomas. Cytokine 2002;17:324–34.

25. Levy DE, Darnell JE Jr. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 2002;3:651–62.

26. Fu XY, Plate J, Chong AS, Xu X. IFN-γ induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. Cancer Res 1998;58:2832–7.

27. Hibi M, Yamanaka Y, Takahashi-Tezuka M, et al. IL-6 cytokine family and signal transduction: a model of the cytokine system. Immunity 1996;5: 449–60.

28. Handra-Luca A, Bilal H, Bertrand JC, Fouret P. Extra-cellular signal-regulated ERK-1/ERK-2 pathway activation in human salivary gland mucoepidermoid carcinoma: association to aggressive tumor behavior and tumor cell proliferation. Am J Pathol 2003;163:957–67.

29. Lui VW, Thomas SM, Zhang Q, et al. Mitogenic effects of gastrin-releasing peptide in head and neck squamous cancer cells are mediated by activation of the epidermal growth factor receptor. Oncogene 2003;22:6183–93.

30. Mishima K, Yamada E, Masui K, et al. Overexpression of the ERK/MAP kinases in oral squamous cell carcinoma. Mod Pathol 1998;11:886–91.

31. Kyriakis JM. Making the connection: coupling of stress-activated ERK/MAPK (extracellular-signal-regulated kinase/mitogen-activated protein kinase) core signaling modules to extracellular stimuli and biological responses. Biochem Soc Symp 1999;64:29–48.

32. O'Neill E, Kolch W. Conferring specificity on the ubiquitous Raf/MEK signaling pathway. Br J Cancer 2004;90:283–8.

33. Bromberg JF, Wrzeszczynska MH, Devgan G, et al. Stat3 as an oncogene. Cell 1999;98:295–303.

34. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin Cancer Res 2002;8:945–54.

35. Chung TD, Spiotto MT. STAT3 mediates IL-6-induced growth inhibition in the human prostate cancer cell line LNCaP. Prostate 2000;42:186–95.

36. Komyod W, Kauffmann ME, Bosserhoff A, Heinrich PC, Behrmann I, Kortylewski M. Interferon-γ-mediated growth regulation of melanoma cells: involvement of STAT1-dependent and STAT1-independent signals. J Invest Dermatol 2004;122:414–22.

37. Calo V, Migliavacca M, Bazan V, et al. STAT proteins: from normal control of cellular events to tumorigenesis. J Cell Physiol 2003;197:157–68.

38. Hutt JA, DeWille JW. Oncostatin M induces growth arrest of mammary epithelium via a CCAAT/enhancer-binding protein δ-dependent pathway. Mol Cancer Ther 2002;1:601–10.

39. Sacks PG. Cell, tissue and organ culture as *in vitro* models to study the biology of squamous cell carcinomas of the head and neck. Cancer Metastasis Rev 1996;15:27–51.

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Wade G. Douglas, Erin Tracy, Dongfeng Tan, et al.

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