Targeting constitutive and interleukin-6-inducible signal transducers and activators of transcription 3 pathway in head and neck squamous cell carcinoma cells by curcumin (diferuloylmethane)

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Numerous reports suggest that interleukin-6 (IL-6) promotes survival and proliferation of tumor cells through the phosphorylation of a cell-signaling protein, signal-transducer-and-activator-of-transcription-3 (STAT3). Constitutive activation of STAT3 in head and neck squamous cell carcinoma (HNSCC) and its role in proliferation of this tumor has been demonstrated. Thus, agents that can suppress STAT3 activation have potential for the treatment of HNSCC. In the present report, we demonstrate that most HNSCC cell lines had constitutively active STAT3 and that curcumin (diferuloylmethane), a pharmacologically safe agent in humans, inhibited STAT3 phosphorylation in a dose- and time-dependent manner. Nuclear translocation of STAT3 was also inhibited by curcumin. The inhibition of STAT3 activation by curcumin was reversible, although even 24 hr after curcumin removal, only partial reversal occurred. Besides inhibiting constitutive expression, curcumin also abrogated the IL-6-induced activation of STAT3 in HNSCC cells. When compared with AG490, a well-characterized JAK2 inhibitor, curcumin was more rapid (30 min vs. 4 hr) and more potent (25 μM vs. 100 μM) inhibitor of STAT3 phosphorylation. Curcumin was also a more potent inhibitor of HNSCC cell proliferation than AG490. Overall, our results demonstrated that curcumin is a potent inhibitor of constitutive and IL-6-induced STAT3 phosphorylation. This mechanism may be at least partially responsible for curcumin’s ability to suppress proliferation of HNSCC cells.

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Head and neck squamous cell carcinoma (HNSCC) accounts for the vast majority of malignant tumors in the upper aerodigestive tract. Worldwide, HNSCC is the sixth most common type of cancer. In 2005, there will be 29,370 new cases of HNSCC and 7,320 deaths due to this cancer in the USA. The association between tobacco and alcohol consumption and increased incidence of HNSCC suggests that primary prevention is feasible, while the relatively favorable prognosis for patients diagnosed with early stage HNSCC emphasizes the importance of early detection. Nonetheless, a number of socioeconomic and other factors hinder prevention or early discovery, and so patients with HNSCC are frequently present with advanced disease. Despite recent advances in surgery, radiation and chemotherapy, the prognosis for late-stage HNSCC remains dismal, with minimal improvement occurring in the last few decades. Moreover, the probability of a second primary upper aerodigestive tract malignancy remains high among long-term survivors.

The aforementioned facts and figures underscore the need of the development of a novel therapeutic approach for patients with HNSCC. One avenue that holds promise is to modulate the expression of proinflammatory and proangiogenic factors. The involvement of inflammation is particularly evident at sites prone to infection such as those found in the head and neck region. Growth of HNSCC is generally associated with an inflammatory component. Signal-transducer-and-activator-of-transcription-3 (STAT3), which is believed to be associated with transforming growth factor (TGF)-β, epidermal growth factor receptor (EGFR) signaling, is often constitutively activated in HNSCC. Inhibition of the EGFR pathway or TGF-α exclusion results in diminishing STAT3 DNA-binding activity. Moreover, inhibition of STAT3 function leads to growth inhibition of HNSCC. These results support the importance of signaling through STAT3 in HNSCC oncogenesis.

Although tyrosine kinase receptors such as EGFR are capable of inducing STAT3 phosphorylation, the reported incidence of active EGFR pathways in HNSCC varies significantly. Thus, it is possible that additional mechanisms participate in the constitutive activation of STAT3 in this malignancy. Sriuranpong et al. identified IL-6 as the major secretory ligand stimulating STAT3 activation by acting on the gp130 coreceptor in an autocrine/paracrine fashion in HNSCC. Engagement of cell surface cytokine receptors activated the JAK family of protein kinases, which in turn phosphorylated and activated latent cytoplasmic STAT3 protein to an active dimer, capable of translocating to the nucleus and inducing transcription of specific target genes. Some of these genes are involved in cell survival (Bcl-2 and Bcl-xL) and cell growth (cyclin D1). Expression of a dominant-negative STAT3 in HNSCC cells inhibited proliferation, cyclin D1 promoter activity and cellular levels of cyclin D1 mRNA and protein. The levels of the antiapoptotic Bcl-2 and Bcl-xL proteins were also diminished.

Thus, pharmacologically safe and effective therapeutic agents that can block constitutive or inducible activation of STAT3 have a potential for HNSCC and other diseases. Curcumin (diferuloylmethane) has been well established as a dietary constituent with chemopreventive properties. Recently, we have shown that curcumin can suppress the proliferation of HNSCC cells, and this suppression correlated with suppression of nuclear factor-κB (NF-κB). Furthermore, curcumin has been shown to downregulate the expression of Bcl-xL and cyclin D1,10,11,12 which are also regulated by STAT3. There are no reports, however, on whether curcumin affects STAT3 pathway in HNSCC. In the present report, we set out to establish whether curcumin can also suppress constitutive or inducible activation of STAT3 in HNSCC.

Material and methods

Materials

The rabbit polyclonal antibodies to STAT3 and mouse monoclonal antibodies against phospho-STAT3 (sc-8059), which detects STAT3 phosphorylated at tyrosine residue 705, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit-HRP conjugate was purchased from Bio-Rad ( Hercules, CA). Goat anti-mouse HRP was purchased from Transduction Laboratories (Lexington, KY), and goat anti-rabbit Alexa 594 was purchased from Molecular Probes (Eugene, OR). Hoechst 33342 and MTT were purchased from Sigma-Aldrich (St. Louis, MO). Curcumin, with purity greater than 98%, was purchased from...
LKT laboratories (St. Paul, MN) and was prepared as a 20 mM solution in dimethyl sulfoxide and then further diluted in cell culture medium. DMEM-F12, fetal bovine serum (FBS), 0.4% trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Life Technologies (Grand Island, NY). AG490, with purity ≥98% by HPLC, was purchased from Calbiochem (San Diego, CA). Bacteria-derived recombinant human IL-6 was kindly provided by Sandoz Pharmaceutical (East Hanover, NJ). LSAB + kit, liquid DAB and hematoxylin were purchased from DAKO Cytomation (Carpentaria, CA).

Cell culture

Human HNSCC cell lines MDA 1986 (cervical nodal metastasis of tongue cancer), Tu 686 (squamous cell carcinoma from the base of tongue), Tu 167 (floor of mouth squamous cell carcinoma line), MDA 686LN (poorly differentiated lymph node metastasis from the base of tongue) and JMAR (squamous cell carcinoma from the floor of mouth) were obtained from Dr. Gary Clayman (our institution). The characterization of these cell lines has been described previously. All HNSCC cell lines were cultured in DMEM containing 10% FBS, nonessential amino acids, pyruvate, glutamine (1%) and vitamins (2%). Multiple myeloma cell line U266 cells were obtained from American Type Culture Collection. U266 cells were cultured in RPMI 1640 containing 10% FBS. Culture media were also supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. Occasionally, cells were tested for mycoplasma contamination by Hoechst staining and by custom PCR and were discarded if found positive.

Western blot

For detection of STAT proteins, whole-cell extracts were prepared by lysing curcumin-treated cells in lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, pH 8.0, 0.1% Triton-X100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mM PMSF and 4 mM NaVO4). Lysates were then spun at 14,000 rpm for 10 min to remove insoluble material and resolved on a 7.5% acrylamide gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk and probed with anti-STAT antibodies (1:1,000 overnight) at 4°C. The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 hr and finally examined by chemiluminescence (ECL, Amersham Pharmacia Biotech. Arlington Heights, IL).

Immunocytochemistry for pSTAT3 localization

HNSCC cells were plated on a glass slide and allowed to adhere overnight before being treated with curcumin. The Avidin-Biotin complex method was used to detect pSTAT3 immunolocalization. Briefly, curcumin-treated cells were fixed with cold acetone. After a brief washing in PBS, slides were blocked with 5% normal goat serum for 1 hr and then incubated with mouse monoclonal anti-human pSTAT3 antibody (1:100 dilution). After overnight incubation, the slides were washed and then incubated with biotinylated anti-mouse antiserum and then with horseradish peroxidase streptavidin conjugate (LSAB + Kit). Sections were rinsed and color was developed using 3,3-diaminobenzidine hydrochloride (DAB) as chromogen. Finally, sections were rinsed in distilled water, counterstained with Mayer’s hematoxylin and mounted for evaluation with DPX. Pictures were captured using Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX) and MetaMorph version 4.6.5 software (Universal Imaging, Downingtown, PA).

Immunocytochemistry for STAT3 localization

HNSCC cells were plated on a glass slide and allowed to adhere overnight before being treated with curcumin. The immunofluorescence method was used to detect STAT3 immunolocalization as described earlier.

MTT assay

The antiproliferative effects of curcumin and AG490 against HNSCC cells were determined by the MTT dye uptake method, as described earlier. Briefly, the cells (2,000/well) were seeded in triplicate in a 96-well plate in the presence or absence of indicated test samples in a final volume of 0.1 ml for 24 hr at 37°C, and then examined for cell viability.

Results

In the present report, we investigated the effect of curcumin on constitutively active and IL-6-inducible STAT3 phosphorylation in HNSCC cell lines. We used 5 different well-characterized HNSCC cell lines. The dose and duration of curcumin treatment to examine STAT3 phosphorylation had no effect on the cell viability of various cell lines (data not shown). Phospho-STAT3 antibody, which detects STAT3 phosphorylated at tyrosine residue 705, was used.

Curcumin inhibits constitutive STAT3 phosphorylation in HNSCC cells

Four of the five HNSCC cell lines we used expressed phosphorylated STAT3 (Fig. 1a). JMAR22 cells did not express the phosphorylated form of STAT3. Unlike pSTAT3, all the HNSCC cell
lines did not express phosphorylated STAT1 or STAT5 (data not shown).

Curcumin inhibited the constitutively active STAT3 in the MDA 1986LN cell line in a dose- (Fig. 1b) and time- (Fig. 1c) dependent manner. The inhibition appeared as early as 15 min and at a concentration as low as 10 μM. Curcumin at 25 μM for 30 min completely inhibited STAT3 phosphorylation. Curcumin treatment did not alter the overall expression of STAT3 protein.

Curcumin inhibits STAT3 nuclear translocation in HNSCC cells

Under resting conditions, and in the nonphosphorylated state, STAT3 is retained in the cytoplasm. It translocates to the nucleus when phosphorylated. Phosphorylation induces STAT3 dimerization, thus permitting its translocation into the nucleus. To confirm that curcumin suppresses nuclear translocation of phosphorylated STAT3, HNSCC cells were grown on a glass slide and were either treated with curcumin or vehicle alone, immunostained with antibody to phosphorylated STAT3 and then visualized by DAB as described in methods. Figure 2a clearly demonstrates that curcumin prevented the translocation of the pSTAT3 to the nucleus in MDA1986LN and MDA686LN cells, consistent with the curcumin-induced inhibition of STAT3 phosphorylation. Consistent with the western blot results for STAT3 phosphorylation, immunostaining showed that JMAR cells did not have any nuclear STAT3 staining.

Whether curcumin modulates the nuclear localization of STAT3 was examined by using nonphosphorylated STAT3 antibody and fluorescence (Alexa 595)-labeled second antibody. These results indicate that STAT3 is present in the nucleus of MDA1986LN and MDA686LN cells, and that curcumin-treatment suppresses the nuclear presence of STAT3 (Fig. 2b).

Curcumin-induced inhibition of STAT3 phosphorylation is reversible in HNSCC cells

We next determined whether curcumin-induced inhibition of STAT3 phosphorylation was reversible. MDA 1986LN cells were first treated for 60 min with curcumin, and then the cells were washed twice with PBS to remove curcumin. The cells were then cultured in fresh medium for various lengths of time, and the levels of phosphorylated STAT3 were measured. Curcumin induced the suppression of STAT3 phosphorylation (Fig. 3a), but once curcumin was removed, phosphorylated STAT3 gradually increased (Fig. 3b). The reversal was partial by 24 hr and did not involve changes in STAT3 levels (data not shown).

Curcumin inhibits IL-6-inducible STAT3 phosphorylation in HNSCC cells

Since IL-6-induced signals are mediated through STAT3 phosphorylation, we next determined the effect of IL-6 and curcumin on STAT3 phosphorylation in JMAR cells, which did not constitu-
Curcumin is more effective than AG490 in inhibiting STAT3 phosphorylation

AG490 is a well-characterized inhibitor of STAT3 phosphorylation.\(^5\) It has certain structural features that are similar to curcumin’s (see Fig. 5a). When we compared the effect of AG490 on constitutive phosphorylation of STAT3 in cells with the effect of curcumin, we found that curcumin was more effective. A 100 \(\mu\)M dose of AG490 was needed to completely inhibit STAT3 phosphorylation (Fig. 5b) and 4 hr was required for that dose of AG490 to completely inhibit STAT3 phosphorylation (Fig. 5c). In comparison, treatment of cells with 25 \(\mu\)M curcumin for 30 min was sufficient to inhibit constitutive STAT3 phosphorylation (see Fig. 1b and 1c).

Curcumin is more effective than AG490 in inhibiting the proliferation of HNSCC

Next, we compared the antiproliferative activity of AG490 with that of curcumin. As shown in Figure 6, curcumin was more potent than AG490 in suppressing the proliferation of MDA 1986 LN cells. The results indicate a 50% cell growth inhibitory concentration was 30 \(\mu\)M for curcumin and 100 \(\mu\)M for AG490. These results coincide with their relative effects on STAT3 phosphorylation.

Discussion

Growth of 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. Various studies have shown significant alterations in signal transduction pathways engaged by cytokines that are associated with loss of growth inhibition in HNSCC. Increased STAT3 phosphorylation, along with constitutively phosphorylated extracellular signal-regulated kinase in HNSCC, suggest that these pathways are important as molecular markers in the malignant transformation process and are potential targets for treatment. Furthermore, there are very few studies addressing the responsiveness of HNSCC to IL-6-type cytokines. Considering that HNSCC, because of their anatomic location, can be particularly susceptible to infection and inflammation, the action of inflammatory cytokines predicts a potentially important role in tumor growth control.

We found that curcumin abrogated both constitutive and IL-6-induced phosphorylation of STAT3. None of these cell lines expressed activated STAT1 or STAT5. Moreover, our previous studies have shown that curcumin had no effect on the phosphorylation status of either STAT1 or STAT5.\(^4\) Curcumin-induced inhibition of STAT3 phosphorylation was reversible. It was a more rapid and more potent inhibitor of STAT3 phosphorylation than AG490, and this inhibition correlated with curcumin’s anti-proliferative effects against HNSCC cells.

Four of 5 HNSCC cell lines we tested expressed constitutively active STAT3. Others have also shown that STAT3 is constitutively active in HNSCC cells.\(^6\) and IL-6 induces proliferation of HNSCC cells.\(^7\) Constitutive activation of STAT3 recently has been observed in many tumor cells, and dysregulation of the STAT3 signaling pathway has been implicated in malignant transformation.\(^18-21\) The oncogenic significance of activated STAT3 molecules is due to their effects on numerous parameters of the development and progression of malignancy, such as apoptosis, cell proliferation, angiogenesis and immune system evasion.\(^22-24\)

Constitutively active STAT3 has been implicated in the induction of resistance to apoptosis,\(^6\) possibly through the expression of Bcl-X\(_L\) and cyclin D1.\(^25,26\) The effects of curcumin against STAT3...
phosphorylation shown here are similar to that previously reported against multiple myeloma cells.\textsuperscript{14}

In our study, curcumin completely eliminated the constitutively phosphorylated form of STAT3. Previously, we have shown that curcumin downregulates the expression of cyclin D1 and Bcl-xL in HNSCC cells.\textsuperscript{11,12,14} These two genes are known to be regulated by both STAT3 and NF-κB.\textsuperscript{24,26} In addition, the downregulation of NF-κB by curcumin in HNSCC has been reported.\textsuperscript{10,27} Thus, it is possible that curcumin downregulates the expression of cyclin D1 and Bcl-xL through downregulation of both NF-κB and STAT3 activation. We found that suppression of STAT3 phosphorylation was reversible, returning to nearly control values within 24 hr.

HNSCC cell lines have been shown to secrete IL-6.\textsuperscript{28} Additionally, IL-6 was detected at higher concentrations in the serum of patients with oral squamous cell carcinoma compared with sex- and age-matched disease-free subjects.\textsuperscript{29} The tissues of oral cancer patients with lymph node metastasis exhibited increased expression levels of IL-6 transcripts.\textsuperscript{30} Also, IL-6 secreted by oral cancer cells plays a significant role in bone invasion.\textsuperscript{31} and it has also been linked with radioresistance of HNSCC patients.\textsuperscript{32} We found that exogenous IL-6 induced the proliferation of HNSCC cells (data not shown). Additionally, in the HNSCC cell line (JMAR) that did not express constitutively activated STAT3, IL-6 induced the phosphorylation of STAT3. The constitutive activation of STAT3 in HNSCC has been shown to be mediated by the autocrine/paracrine stimulation of the IL-6,\textsuperscript{8} and this confers both proliferative and survival potential in this malignancy. We found that curcumin also abolished the IL-6-induced STAT3 phosphorylation in HNSCC cells.

AG490 is considered an inhibitor of JAK2,\textsuperscript{15} a kinase that phosphorylates STAT3, was less potent in inhibiting STAT3 phosphorylation than curcumin. Although 50\% inhibitory dose was not calculated, longer exposure (4 hr vs. 30 min) and higher dose (100 µM vs. 25 µM) of AG490 was needed to suppress STAT3 phosphorylation. Similarly, while exposure of cells to 30 µM curcumin for 24 hrs inhibited the proliferation of HNSCC cells by 50\%, 100 µM AG490 was needed for equivalent effect (Fig. 6). Several other kinases have been implicated in the phosphorylation of STAT3, including members of the src family (hck, src), Erb2, ALK, PKC-d, c-fos and EGFR.\textsuperscript{33–36} There are reports that curcumin can inhibit JAK2,\textsuperscript{41} Src,\textsuperscript{42} Erb2,\textsuperscript{43} and EGFR.\textsuperscript{44} A study by Kim et al.\textsuperscript{45} has shown that curcumin activate a protein tyrosine phosphatase SHP-2, which can then inactivate JAK1/2, thus inhibiting the activation of JAK/STAT pathway. Uddin et al.\textsuperscript{46} also recently showed that curcumin induces the suppression of the con-

![Figure 5](image_url)
Also, LoTempio et al. have shown that curcumin can suppress growth of HNSCC both in vitro and in vivo using nude mice xenograft tumors.27 Since curcumin can effectively inhibit activation of both STAT3 and NF-kB, it should be expected that curcumin should suppress cell proliferation more efficiently than specific inhibitors of either transcription factor alone. Since there is considerable evidence implicating STAT3 in the transformation of cells, inhibition of STAT3’s activity as a transcriptional activator is a prime anticancer target. First, all Src-transformed cell lines have persistently activated STAT3 and dominant-negative STAT3 blocks transformation.22,47 Dominant-negative STAT3 has also been shown to induce apoptosis in cells with constitutively active STAT3.16 Second, STAT3-C, a constitutively active mutant dimerized by cysteine-cysteine bridges instead of pTyr-SH2 interaction, can transform cultured cells so that they form tumors when injected into mice.48 Indeed, STAT3 functions in fibroblast development to resist apoptosis.49 Third, besides HNSCC,3 multiple myeloma,3 hepatocellular carcinoma,50 and lymphomas and leukemia51 have constitutively active STAT3. Because there is no reported mutation in STAT3 that results in persistent activation, the only putative mechanism to account for the constitutive activity of STAT3 is dysregulation of signaling molecules or mutation or deletions in the protein that negatively regulate STAT3 (e.g., PIAS3 or SOCS).50 For instance SOCS-1, a negative regulator of cytokine signaling is frequently silenced by methylation in various tumors.52,53 Thus, constitutively active STAT3 can contribute to oncogenesis by protecting cancer cells from apoptosis. This implies that suppression of STAT3 activation by agents such as curcumin, as described here, could facilitate apoptosis.

Various new pharmacologic agents are currently under clinical development for treatment of malignant tumors, including agents that either block growth-promoting signaling cascades or trigger apoptotic signals in neoplastic cells. Given that growing evidence implicates a number of important STAT3 target genes in the formation of tumors,23,55,56 inhibition of STAT3 through pharmacological blockade of upstream molecules such as Src and JAK may reduce tumor formation.

Whether doses of curcumin used in our study are achievable in vivo is not clear. It is difficult, however, to correlate the bioavailability of any drug in vitro to that in vivo, as the metabolism of the drug in vitro may not be the same as in vivo. Curcumin has been shown by us and others to be remarkably effective in vivo inspite of the fact that serum concentration does not exceed beyond low micromolar or even nanomolar range.27,56 Curcumin has been shown to undergo glucuronidation in liver and intestine that is inhibitors of bioperine.59 The presence of bioperine has been shown to enhance the bioavailability of curcumin by 2,000-fold in humans.59 The use of liposomal curcumin has also been shown to be quite effective in vivo in the treatment of pancreatic cancers.50 Recently, synthetic analogues of curcumin were reported, which were more potent than native curcumin and showed impressive activity in the breast cancer xenotransplant model.61,62 In conclusion, the ability to suppress STAT3 phosphorylation, inhibit IL-6 signaling and inhibit proliferation of HNSCC cells, combined with curcumin’s well-established pharmacological safety,52,56 provides a rationale for the potential use of curcumin as therapeutic or chemopreventive agent for HNSCC patients.

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