The Nonsteroidal Anti-inflammatory Drug Sulindac Causes Down-Regulation of Signal Transducer and Activator of Transcription 3 in Human Oral Squamous Cell Carcinoma Cells

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Abstract

The nonsteroidal anti-inflammatory drug sulindac exerts a significant antineoplastic effect on several types of human cancers including oral squamous cell carcinoma (SCCa). Because constitutive activation of signal transducer and activator of transcription 3 (Stat3) has been linked to carcinogenesis of various tumors including head and neck SCCa, we studied whether sulindac treatment affects the Stat3 signaling pathway in oral SCCa cells. Western blot experiments showed that short-term treatment of cells with sulindac resulted in a large reduction of phosphorylated Stat3, without significantly affecting Stat3 protein levels. In contrast, 3 days of sulindac treatment eliminated both phosphorylated and unphosphorylated Stat3 protein levels. Also, sulindac treatment exerted a significant time-dependent cell growth-inhibitory effect on oral SCCa cells under the same conditions shown to induce Stat3 down-modulation. The sulfone metabolite of sulindac, which lacks cyclooxygenase-inhibitory activity, did not affect either Stat3 expression or Stat3 phosphorylation. Antisense oligonucleotide treatment against peroxisome proliferator-activated receptor γ did not attenuate the ability of sulindac to down-regulate Stat3. Our results suggest that down-modulation of Stat3 can be induced by sulindac treatment, thus possibly contributing to the antineoplastic effect of this drug.

Introduction

A plethora of studies have established the antineoplastic effect of NSAIDs,1 such as sulindac (reviewed in Ref. 1). Sulindac exerts a significant time- and dose-dependent growth-inhibitory effect on cell lines derived from various types of human cancers including oral SCCa. Induction of apoptosis and alterations in the rate of cell proliferation account for the observed cell growth-inhibitory effect of sulindac (3, 4). However, the specific underlying molecular mechanisms, traditionally attributed to the ability of sulindac to inhibit the enzyme COX, remain largely unknown despite numerous suggested theories (reviewed in Ref. 5). Although STAT (signal transducers and activators of transcription) proteins are involved in oncogenesis (reviewed in Ref. 6), the potential association between the anticancer properties of sulindac and STAT signaling has not been investigated. STAT proteins are latent cytoplasmic transcriptional factors that directly participate in both (a) transmitting a signal from the cell membrane to the nucleus and (b) driving the transcription of specific genes in the nucleus (reviewed in Ref. 7). Inappropriate activation of STAT signaling has been shown to induce specific target genes that promote tumor formation by stimulating cell proliferation and preventing apoptosis (8, 9). Constitutive activation of Stat3 has been associated with tumorigenesis of various types of human cancer, such as multiple myeloma (9) and non-small cell lung cancer (10). In head and neck SCCa, there is evidence that aberrant tumor growth factor-α/epidermal growth factor receptor signaling leads to constitutive activation of Stat3, which is accompanied by increases in Stat3 protein expression and Stat3 tyrosine phosphorylation (11, 12). A critical role of Stat3 activation for head and neck SCCa carcinogenesis has been documented, because the targeting of Stat3 results in significant growth inhibition and induction of apoptosis both in vitro and in vivo (11, 12).

In this study, we explored the possibility that sulindac’s antineoplastic effect on oral cancer cells is mediated through Stat3 down-modulation. We observed that sulindac treatment leads first to a significant decrease in Stat3 phosphorylation and eventual elimination of Stat3 expression. This is the first time that the antineoplastic effect of NSAIDs is linked to the abrogation of Stat3 signaling pathway.

Materials and Methods

Cell Lines and Cell Culture. All of the experiments were performed using established cell lines of human oral SCCa (SCC-4, -9, -15, and -25) obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in a 1:1 mixture of Ham’s F12 and DMEM containing 10% fetal bovine serum, 100 units of penicillin, 100 μg/ml streptomycin, and 0.4 μg/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO) at 37°C in a 5% CO2 air atmosphere. Cells were subcultured by disaggregation with trypsin (0.1%-EDTA (0.01%) in PBS at pH 7.5.

Sulindac Treatment, Protein Lysate Preparation, and Western Blotting. Cells were plated in 6-well plates at a density of 5 × 104 cells/well and were allowed to grow to 80% confluency. Sulindac sulfide or sulindac sulfone (Biomol Research Laboratories, Inc., Plymouth Meeting, PA), dissolved in 100% DMSO, were added to the medium at a concentration of 150 μM. The final concentration of DMSO did not exceed 0.1%. After incubation for various time periods, the cells were washed twice with cold PBS, lysed in radioimmunoprecipitation assay buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, sodium salt, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 mM DTT, and 1 mM sodium orthovanadate] for 10 min, and scraped. The extracts were centrifuged at 40,000 × g for 15 min at 4°C. Protein concentrations were measured and equalized using Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer’s instructions.

Western blot analysis was performed using phospho-Stat3 (Tyr 705) antibody (1:500 dilution; Cell Signaling Technology, Beverly, MA) according to the manufacturer’s instructions. Blots were stripped [20 mM DTT, 2% SDS, and 67.5 mM Tris-HCl (pH 6.7)] and then reprobed sequentially with Stat3 (Tyr 705) antibody (1:1000 dilution; Cell Signaling Technology) and with actin antibody (1:500 dilution; Sigma Chemical Co.). Densitometric analyses were performed using the Image Quant program of a Molecular Dynamics laser densitometer.

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2 The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; Stat, signal transducer and activator of transcription; PPAR, peroxisome proliferator-activated receptor; COX, cyclooxygenase; SCCa, squamous cell carcinoma.
Design, Synthesis, and Transfection of Antisense/Sense Oligonucleotides. Antisense and sense phosphorothioate oligonucleotides were designed using the PPARγ cDNA nucleotide sequence. The oligonucleotides were synthesized by Oligos etc. (Wilsonville, OR), as follows: PPARγ antisense (5'-CTCTTTGGTCAACATGGTCTA-3'); PPARγ sense (5'-ATGACCTG- GGTGACGACAGAG-3'). The transfection reagent Lipofectin (Life Technologies, Inc., Paisley, Scotland) was used to introduce oligonucleotides into the carcinoma cell lines. For these studies, 5 × 10^4 cells/well were seeded in six-well plates and incubated under normal conditions to 60% confluency. Cells were transfected using 0.2 μM oligonucleotides and 5 μg of Lipofectin according to the manufacturer’s instructions.

To confirm the efficacy of PPARγ antisense treatment, the effect of the treatment on mRNA and protein levels of PPARγ was assessed. After 24 h of treatment with PPARγ antisense or sense oligonucleotides, the medium was removed and replenished with normal growth medium for 24 h. Total RNA was then isolated using the TRIZOL Reagent (Life Technologies, Inc.) and quantitative real-time reverse transcription-PCR was performed using PE Biosystems protocols to estimate the mRNA levels of PPARγ. The forward and reverse primers, selected using Primer Express software (PE Biosystems, Foster City, California), were 5'-TATGGAGCCAGTGAAATCCAGAG-3' and 5'-TCGTCATTGGCTTGATCA-3', respectively. To assess the protein levels of PPARγ, protein was extracted and Western Blotting was performed using a PPARγ antibody (sc-1984, 1:200 dilution; Santa Cruz Biotechnology, Inc.), as described above.

Subsequent to 24 h of treatment with PPARγ antisense or sense oligonucleotides, cells were treated for 48 h with 150 μM sulindac sulfide or sulindac sulfone dissolved in 100% DMSO and added to the medium. Protein lysate preparation and Western blot analysis for assessment of the protein levels of phosphorylated Stat3 and Stat3 followed as described above. Appropriate normal growth medium and Lipofectin controls were included.

Cell Growth Inhibition. Cells were plated in 24-well plates at a density of 5 × 10^4 cells/well in normal growth medium. After 24 h, DMSO at a concentration of 0.1% or 150 μM of sulindac sulfide or sulindac sulfone dissolved in 100% DMSO (Biomol Research Laboratories, Inc., Plymouth Meeting, PA), were added to the growth medium. The final concentration of DMSO did not exceed 0.1%. After incubation for 24, 48, or 72 h, either treated or untreated cells were removed enzymatically and counted using a Coulter counter (Coulter Model ZI; Coulter Corporation, Miami, FL). The percentage of growth was determined, setting as 100%, the growth of cells treated only with the vehicle (0.1% DMSO). All of the analyses were performed in triplicate.

Results

Effect of Sulindac on Stat3 Phosphorylation and Expression. Western blotting experiments confirmed the expression of Stat3 protein in all four oral SCCa cell lines tested. Phosphorylated Stat3 was also detected in all four cell lines (Fig. 1). A significant reduction of phosphorylated Stat3 could be detected as early as 45 min after the initiation of treatment of SCC9 cells with 150 μM of sulindac sulfide. Longer treatment, up to 48 h, did not induce further reduction in phosphorylated Stat3 levels. In contrast, treatment of cells for 72 h with 150 μM of sulindac sulfide resulted in the elimination of phosphorylated Stat3 (Fig. 1A).

Sulfide treatment of SCC9 cells for 45 min also caused a small decrease in Stat3 protein expression levels. However, this could not account for the decrease in phosphorylated Stat3, as determined by densitometric analysis. Longer treatment for 24 h and 48 h further reduced Stat3 protein levels, whereas 72 h of treatment eliminated Stat3 expression (Fig. 1A). In contrast, the levels of actin protein remained stable throughout the treatment, indicating that the effect on Stat3 was not caused by a nonspecific reduction of protein expression (Fig. 1A).

The aforementioned results were very similar to those obtained using SCC25 cells (data not shown). Likewise, Stat3 phosphorylation and expression were eliminated by 72 h of sulindac treatment with 150 μM in all four of the SCCa cell lines tested (Fig. 1B).

On the other hand, treatment of cells for 72 h with 150 μM of sulindac sulfone, which is devoid of COX inhibitory activity, did not significantly change the protein levels of either Stat3 or phosphorylated Stat3 (Fig. 2).

Effect of PPARγ Antisense Treatment on Stat3 Down-Modulation Induced by Sulindac. We assessed the mRNA and protein levels of PPARγ in oral SCCa cells treated with PPARγ antisense oligonucleotides by quantitative real-time reverse transcription-PCR and Western blot. The relative PPARγ mRNA expression levels after treatment with normal growth medium, Lipofectin, sense, and antisense oligonucleotides were 1, 1.07, 0.84, and 0.16, respectively. Protein expression of PPARγ was abolished after PPARγ antisense oligonucleotide treatment, but was not changed after treatment with PPARγ sense oligonucleotides or Lipofectin alone, confirming the effectiveness of the PPARγ antisense treatment (Fig. 3A).

Because we have previously demonstrated that up-regulation of PPARγ is an important mediator of the antineoplastic effect of sulindac on oral SCCa cells (2), we next tested whether PPARγ might also mediate the decrease in Stat3 phosphorylation and expression induced by sulindac. Oral SCC9 cells pretreated for 24 h with PPARγ antisense oligonucleotides responded to 48 h of sulindac sulfide treatment with significant reduction of Stat3 and phosphorylated Stat3 protein levels. This reduction was very similar to that exhibited in response to sulfide treatment by control cells pretreated with PPARγ sense oligonucleotides, Lipofectin, or normal medium (Fig. 3B). Similar results were obtained using SCC25 cells (data not shown). Thus, PPARγ antisense treatment does not attenuate sulfide’s ability to down-modulate expression of both Stat3 and phosphorylated Stat3.

Effect of Sulindac on Cell Growth. Sulindac sulfide treatment (150 μM) resulted in a statistically significant (P ≤ 0.05) time-dependent reduction in cell growth (Fig. 4). Treatment of cells with 150 μM sulindac sulfone also resulted in a significant (P ≤ 0.05) cell growth reduction, which, however, was smaller than that induced by sulfide (data not shown).

Discussion

Although the antineoplastic effect of NSAID sulindac on several types of human cancer has been well documented in the literature, the responsible underlying molecular mechanisms remain largely un-
NSAID Sulindac Causes Down-Regulation of Stat3

In vitro experiments have established the antitumor efficacy of Stat3 targeting, which has been shown to attenuate Bcl-X₁ protein expression leading to enhancement of apoptosis (11, 12). Accordingly, down-modulation of Stat3 protein expression and phosphorylation may represent a thus-far uninvestigated mechanism through which NSAID sulindac may exert its antineoplastic effect. Interestingly, treatment of oral SCCa cells, under the same conditions shown to induce Stat3 down-modulation (i.e., 150 μM of sulindac sulfide for 24 to 72 h), also caused significant cell growth inhibition, suggesting that the targeting of Stat3 by sulindac has functional consequences by decreasing cell number. These observations lend support to the hypothesis that sulindac’s cell growth-inhibitory effect may be mediated through Stat3 down-modulation. The molecular pathways that underlie these phenomena and the role that specific apoptotic molecules, such as members of the Bcl-2 family, play warrant further exploration.

Because we have previously shown that the growth inhibitory effect of sulindac on human oral SCCa cell lines is, at least partially, mediated through PPARγ (2), we investigated next whether PPARγ is also involved in sulindac-induced Stat3 down-modulation. Although a direct association between these two molecules has not been reported, cross-talk between PPAR and STAT pathways has been suggested. Ricote et al. (13) have shown that PPARγ inhibits gene expression in activated macrophages by antagonizing the nuclear factor-κB, AP-1, and STAT signaling pathways. However, only Stat1 and not Stat3 was tested. Moreover, Zhou and Waxman (14, 15) established the ability of Stat5b to inhibit the transcriptional activity of PPARα, PPARγ, and PPARδ and suggested the possibility for a more general inhibitory cross-talk between STAT proteins and nuclear receptors. Direct protein-protein interactions or indirect mechanisms, such as competition for common coactivators or modulation of inhibitors of transcriptional activity, may be responsible for STAT-PPAR cross-talk (14, 15). However, PPARγ antisense treatment did not reduce sulfide’s ability to cause Stat3 down-modulation. Moreover, the sulfone metabolite of sulindac, which despite lacking COX-2 inhibitory effect induces a PPARγ-mediated cell growth inhibition and apoptosis induction on oral SCCa cells, did not alter expression and phosphorylation of Stat3. Taken together, these results suggest that sulindac-induced Stat3 down-modulation is independent of the drug’s ability to function as PPARγ ligand, most probably being related to the COX-2 inhibitory effect of sulindac.

Another possible mechanism through which sulindac may down-modulate Stat3 could involve the inhibition of the production of molecules that may trigger pathways that lead to the activation of Stat3. The cytokine IL-6 could serve as such a molecule through its ability to induce activation of Stat3 (16), which has been linked to up-regulation of the antiapoptotic protein Bcl-X₁ in multiple myeloma cells (9). This pathway may be of particular importance for oral cancer, because Hong et al. have demonstrated that IL-6 stimulation in oral cancer cells led to increases in both Stat3 phosphorylation and cell growth (17). They also showed that treatment with the pan-COX inhibitor ketorolac decreased IL-6 levels and reduced tumor cell growth. In accordance with these observations, sulindac’s ability to down-modulate Stat3 may be the result of the inhibitory effect that sulindac exerts on IL-6 (18). However, the mechanism by which

![Fig. 2. Sulindac sulfone does not affect phosphorylation and production of Stat3 in oral SCCa cells. Oral SCCa cells, derived from cell lines SCC4, 9, 15, or 25, were treated for 72 h with normal medium (Untreated) or with 150 μM sulindac sulfone (Sulfone-treated). Cells were lysed, blotted with antibody to phosphorylated Stat3 (Phospho Stat3), stripped, and reprobed with antibody to Stat3.](Image 59x474 to 281x624)

![Fig. 3. In A, PPARγ antisense treatment eliminates PPARγ protein expression. Oral SCC9 cells were treated for 24 h with normal medium (C), Lipofectin (L), PPARγ antisense oligonucleotides (A), or PPARγ sense oligonucleotides (S), followed by 24 h of incubation with normal medium. Subsequently, cells were lysed and sequentially blotted with antibody to PPARγ and to actin, respectively. In B, PPARγ antisense treatment does not affect sulindac sulfide’s ability to inhibit Stat3 phosphorylation and production. Oral SCC9 cells were pretreated for 24 h with normal medium (Control), Lipofectin, PPARγ sense oligonucleotides (Sense), or PPARγ antisense oligonucleotides (Antisense). Subsequently, cells were treated for 48 h with normal medium (–) or with 150 μM sulindac sulfide (+). Cells were lysed, blotted with antibody to phosphorylated Stat3 (Phospho Stat3), stripped, and reprobed with antibody to Stat3.](Image 69x476 to 271x741)

![Fig. 4. Oral SCC9 and SCC25 cells were treated with vehicle (0.1% DMSO) or 150 μM sulindac sulfide and cell growth was assessed by counting cells in a Coulter counter at indicated time points. The growth of cells treated only with the vehicle (0.1% DMSO) was set as 100% for each time point.](Image 344x88 to 524x220)
sulindac causes IL-6 down-modulation necessitates further investigation. The aforementioned model of Stat3 down-modulation through sulindac-induced inhibition of IL-6 (and possibly other Stat3 activating molecules) can explain not only the observed reduction in Stat3 phosphorylation but also the decrease in Stat3 expression. The latter is justified by the existence of an autoregulatory loop for Stat3, according to which IL-6-induced Stat3 activation results in increased Stat3 gene expression (19).

Whether the elimination of Stat3 protein expression by sulindac treatment is secondary to the inactivation of Stat3 or is the result of a more direct effect of sulindac on Stat3 expression remains to be elucidated. Independent of the exact molecular mechanism, the down-modulation of Stat3 expression and phosphorylation by sulindac is of particular importance, because it offers an alternative strategy for disrupting Stat3, an oncogene that constitutes a valid molecular target for cancer therapy.

References
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