Abrogation of IL-6-mediated JAK signalling by the cyclopentenone prostaglandin 15d-PGJ₂ in oral squamous carcinoma cells

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Cyclopentenone 15-deoxy-Δ¹²,¹⁴-prostaglandin J₂ (15d-PGJ₂) exerts antineoplastic effects on various types of human cancer. We recently showed that treatment with 15d-PGJ₂ induces apoptosis accompanied by downregulation of the oncogenic signal transducer and activator of transcription 3 (Stat3) signalling in human oral squamous cell carcinoma (SCC) cells. The current study examines the effects of 15d-PGJ₂ on the epidermal growth factor receptor (EGFR) and Janus Kinase (JAK)-mediated signalling pathways. Inhibition of Stat3 by 15d-PGJ₂ was abolished by exogenous stimulation with transforming growth factor alpha (TGF-α), but not interleukin 6 (IL-6), supporting a selective effect of 15d-PGJ₂ on IL-6-mediated signalling. Importantly, 15d-PGJ₂ selectively abrogated constitutive and IL-6-mediated JAK phosphorylation without affecting EGFR-activated levels. Moreover, the inhibitory effect of 15d-PGJ₂ on JAK signalling required the reactive α,β-unsaturated carbon within the cyclopentenone ring. Targeting of JAK signalling using a specific JAK inhibitor also abolished Stat3 phosphorylation and resulted in apoptosis in oral SCC cells. Our findings provide the first evidence for 15d-PGJ₂—mediated downregulation of constitutive and IL-6-induced JAK signalling in cancer and support that JAK inhibition and suppression of EGFR-independent Stat3 activation by 15d-PGJ₂ represent a promising approach for induction of apoptosis in oral SCC cells.

Keywords: 15d-PGJ₂; IL-6; JAK; Stat3; oral squamous cell carcinoma

Signal transducer and activator of transcription (STAT) proteins are latent cytoplasmic transcription factors that typically become activated in response to extracellular signals such as growth factors and cytokines (Darnell Jr, 1997; Bromberg and Darnell Jr, 2000). Upon binding to their corresponding cell membrane receptors, cytokines and growth factors induce STAT tyrosine phosphorylation, mediated by cytokine receptor-associated tyrosine kinases (i.e. Janus Kinase family members, including Jak1, Jak2, Jak3 and Tyk2) and growth factor receptors with intrinsic kinase activity (e.g. epidermal growth factor receptor, EGFR), respectively (Darnell Jr, 1997; Bromberg and Darnell Jr, 2000). In addition, the Src family of nonreceptor tyrosine kinases has also been shown to phosphorylate STAT proteins (Bromberg and Darnell Jr, 2000). Activated STAT molecules form homo- or heterodimers and translocate to the nucleus, where they bind to the promoter region of specific target genes, thus regulating their transcription (Darnell Jr, 1997; Bromberg and Darnell Jr, 2000).

STATs have been identified as critical regulators of various normal cellular processes (Darnell Jr, 1997; Levy, 1999). However, persistent STAT activation has been convincingly implicated in oncogenesis (Bowman et al, 2000; Bromberg and Darnell Jr, 2000; Bromberg, 2001, 2002). It has been established that abnormal activation of STAT molecules (especially involving Stat3 and Stat5) stimulates cell proliferation and prevents apoptosis in a number of human tumours, including leukaemia, multiple myeloma, breast, prostate and non-small-cell lung cancer (Bromberg, 2002). Therefore, disruption of aberrant STAT activation in tumours, which typically depends on deregulation of specific upstream tyrosine kinases, has been proposed as a valid molecular target for cancer therapy (Bowman et al, 2000; Turkson and Jove, 2000; Bromberg, 2001, 2002; Buettner et al, 2002).

In head and neck squamous cell carcinoma (SCC), there is evidence that Stat3 constitutive activation is linked to cancer development and growth (Grandis et al, 2000; Song and Grandis, 2000; Kijima et al, 2002). Importantly, targeting of Stat3, through transfection of dominant-negative constructs or application of antisense oligonucleotide treatment, results in significant growth inhibition (Grandis et al, 1998; Song and Grandis, 2000). Furthermore, Stat3 antisense gene therapy leads to increased tumour apoptosis in vivo, which is associated with decreased Bcl-Xl protein expression (Grandis et al, 2000; Song and Grandis, 2000; Kijima et al, 2002).

Cyclopentenone prostaglandins, especially 15-deoxy-Δ¹²,¹⁴-prostaglandin J₂ (15d-PGJ₂), have been shown to exert antineoplastic effects on various types of human cancer (Kim et al, 1993; Ahn et al, 1998; Clay et al, 1999; Keelan et al, 1999; Butler et al, 2000; Chang and Szabo, 2000; Clay et al, 2001; Straus and Glass, 2001). These effects, frequently attributed to activation of peroxisome proliferator-activated receptor gamma (PPARγ), have been recently proven to be at least partially mediated through PPARγ-independent pathways (Clay et al, 2001, 2002; Straus and
Glass, 2001; Hsiang and Straus, 2002; Liu et al., 2003). Similarly, we recently demonstrated that 15d-PGJ2 inhibits cell growth and induces apoptosis in oral SCC utilising PPARγ-independent mechanisms (Nikitakis et al., 2002). Moreover, we suggested that the effects of 15d-PGJ2 on oral SCC cells may be related to its ability to downregulate Stat3 (Nikitakis et al., 2002). In the present study, we investigated the effect of 15d-PGJ2 on tyrosine kinases that regulate growth and survival of oral SCC cells, including JAKs and EGFR. Our results indicate that 15d-PGJ2 targets JAK signalling independent of EGFR signalling and suggest that inhibition of IL-6-mediated JAK signalling and suppression of EGFR-independent Stat3 activation may represent a novel therapeutic approach in oral cancer.

MATERIALS AND METHODS

Cell lines and cell culture

All experiments were performed using established human oral SCC cell lines (SCC-4, -9, -15 and -25) obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in a 1:1 mixture of Ham’s F12 and Dulbecco’s modified Eagle’s medium (DMEM) containing 10% foetal bovine serum (FBS), 100 U of penicillin, 100 μg ml⁻¹ streptomycin and 0.4 μg ml⁻¹ hydrocortisone (Sigma Chemical Co., St Louis, MO, USA) at 37°C in a 5% CO₂ atmosphere. Cells were subcultured by disaggregation with trypsin (0.1%)-EDTA (0.01%) in phosphate-buffered saline (PBS) at pH 7.5.

Western blot analysis

Cells were plated in six-well plates at 5×10⁴ cells well⁻¹ and were allowed to grow to 80% confluency. A measure of 10, 20 or 40 μM 15d-PGJ2 (Cayman Chemical, Ann Arbor, MI, USA) dissolved in 100% DMSO, 50 μg AG490 (Calbiochem, San Diego, CA, USA) dissolved in 100% DMSO, 100 μg rIL-6 (Calbiochem, San Diego, CA, USA) or SOCS3 (M-20) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to the precleared supernatant and incubated overnight at 4°C. Lysates were then incubated with protein A magnetic beads (New England Biolabs, Beverly, MA, USA) for 2 h. Beads were washed and resuspended in sample loading buffer (187.5 mM Tris-HCl (pH 6.8), 6% (w/v⁻¹) SDS, 30% glycerol, 150 mM DTT, 0.03% (w/v⁻¹) bromophenol blue and 2% β-mercaptoethanol). Western blot analysis was performed using phospho-tyrosine-specific monoclonal antibody 4G10 (1 μg ml⁻¹) (Upstate Biotechnology). Membranes were reprobed with Jak1 (1 : 1000), EGFR (1 : 1000) or SHP2 (1 : 2500) antibodies as controls.

Cell growth inhibition

Cells were plated at 5×10⁴ cells well⁻¹ in 24-well plates containing normal growth medium. After 24 h, DMSO at 0.1% or AG490 at 25, 50 or 100 μM was added. The final concentration of DMSO did not exceed 0.1%. Following incubation for 24, 48 or 72 h, cells were enzymatically removed and counted using a Coulter Counter (Coulter Model ZI, Coulter Corporation, Miami, FL, USA). All analyses were performed in triplicate.

Measurement of apoptosis

Cells were treated with either 0.1%, DMSO or AG490 at 50 or 100 μM for 48 h. Cells were washed twice with cold PBS and resuspended in 1× binding buffer. Early apoptotic changes were identified by Annexin V-FITC and PI staining (BD Biosciences). The extent of apoptosis was assessed by relative fluorescence intensity using a FACSCan and Cell Quest software (Becton Dickinson) as described (Nikitakis et al., 2002). All analyses were performed in duplicate.

Statistical analysis

A two-way analysis of variance (ANOVA) test and Tukey test were used to assess the presence of statistically significant differences between groups (SigmaStat 3.0, SPSS Inc, Chicago, IL, USA); P<0.05 was considered statistically significant.

RESULTS

Inhibition of IL-6-mediated Stat3 phosphorylation by cyclopentenone prostaglandin 15d-PGJ2

In our previous study, we reported that treatment with 15d-PGJ2 (20 μM) suppresses the levels of constitutively phosphorylated Stat3 (Nikitakis et al., 2002). To assess if 15d-PGJ2 modulates upstream signalling leading to Stat3 repression, we tested the effect of 15d-PGJ2 on IL-6-mediated Stat3 phosphorylation. In the absence of pretreatment with 15d-PGJ2, IL-6 stimulation induced Stat3 phosphorylation (Figure 1A). However, in the presence of 10 μM 15d-PGJ2, IL-6-induced Stat3 tyrosine phosphorylation was attenuated as early as 45 min. Pretreatment with 20 or 40 μM 15d-PGJ2 resulted in further reduction of IL-6-stimulated Stat3 phosphorylation (Figure 1B). This decrease in Stat3 tyrosine phosphorylation could not be attributed to a reduction in total cellular Stat3 levels, which remained stable despite the various treatments (or combinations thereof).

Immunoprecipitation

Cells (5×10⁴ well⁻¹) were allowed to grow to 80% confluency and rIL-6 was added to normal medium at 25 ng ml⁻¹ concentration. Alternatively, cells were treated with 20 μM 15d-PGJ2, 40 μM CAY10410 or 50 μM AG490, dissolved in 100% DMSO. Cells were then preincubated with 15d-PGJ2 and subsequently treated with rIL-6. The final concentration of DMSO did not exceed 1%. Following incubation for various time periods, the cells were lysed in RIPA buffer as described above. Immunoprecipitation of Jak1, Jak2, EGFR and SHP2 were performed using 1 mg of whole cell lysate. Jak1 antibody (4 μg ml⁻¹) (Upstate Biotechnology, Lake Placid, NY, USA), EGFR antibody (1 : 100) (Cell Signalling Technology) or SHP2 (5 μg ml⁻¹) (BD Biosciences Pharmingen, San Diego, CA, USA) were added to the precleared supernatant and incubated overnight at 4°C. Lysates were then incubated with protein A magnetic beads (New England Biolabs, Beverly, MA, USA) for 2 h. Beads were washed and resuspended in sample loading buffer (187.5 mM Tris-HCl (pH 6.8), 6% (w/v⁻¹) SDS, 30% glycerol, 150 mM DTT, 0.03% (w/v⁻¹) bromophenol blue and 2% β-mercaptoethanol). Western blot analysis was performed using phospho-tyrosine-specific monoclonal antibody 4G10 (1 μg ml⁻¹) (Upstate Biotechnology). Membranes were reprobed with Jak1 (1 : 1000), EGFR (1 : 1000) or SHP2 (1 : 2500) antibodies as controls.
15d-PGJ2 abrogates the activation of JAKs

It is well established that members of the JAK family of kinases play an essential role in transducing signals from IL-6 type family of receptors to downstream effectors including STATs (Heinrich et al., 2003). In order to test the possibility that JAKs contribute to aberrant Stat3 activity in oral SCC cells, we examined the effect of AG490, a selective JAK inhibitor (Meydan et al., 1996; Nielsen et al., 1997), on Stat3 phosphorylation. AG490 (50 μM) inhibited constitutive Stat3 phosphorylation during treatment for up to 24 h (Figure 1C). Moreover, treatment with the EGFR ligand, TGF-α (20 ng ml⁻¹) reversed the AG490-mediated Stat3 repression (Figure 1D), while 100 μM treatment with a selective EGFR inhibitor, PD153035, abolished TGF-α-mediated Stat3 phosphorylation. (Figure 1E).

15d-PGJ2 does not inhibit EGFR-mediated Stat3 signalling

Given that autocrine and paracrine activation of the EGFR signalling pathway by TGF-α has been linked to Stat3 activation in head and neck SCC cells (Grandis et al., 2000; Song and Grandis, 2000), the possible involvement of EGFR activity in the inhibitory effect of 15d-PGJ2 on Stat3 was examined. To assess the effect of 15d-PGJ2 on EGFR-mediated activation of Stat3, oral SCC cells were stimulated with exogenous TGF-α in the presence or absence of 15d-PGJ2. Pretreatment with 15d-PGJ2 (20 μM) failed to inhibit
JAK and Stat3 activation following treatment with 15d-PGJ2

In order to determine the physiologic consequence of JAK inhibition in oral SCC cells, cells were treated with a selective JAK inhibitor. Treatment with AG490 resulted in a significant dose-dependent increase in apoptosis (Figure 5B). Moreover, the antineoplastic effects of AG490 in oral SCC cells also correlated with the abrogation of Stat3 constitutive phosphorylation at 24, 48 and 72 h (Figure 5C).

Effect of 15d-PGJ2 on negative regulators of Stat3 signalling

Because negative regulators of STAT signalling, including suppressors of cytokine signalling (SOCS) and Src homology 2 domain-containing protein phosphatases (SHPs), may contribute to the 15d-PGJ2-mediated repression of JAK and Stat3 phosphorylation, the effect of 15d-PGJ2 on SOCS3 expression and SHP2 activity was examined. SOCS proteins are transcriptional targets of STATs and function in a classic negative feedback loop to inhibit further STAT activation by interacting with either JAK catalytic or receptor sites (Starr and Hilton, 1998, 1999). In this regard, treatment with IL-6 for 1 h potently induced SOCS3 protein expression (Figure 6A). In contrast, treatment with 15d-PGJ2 (20 μM) for 1 h did not have an effect on SOCS3 protein expression (Figure 6A). Moreover, we assessed the effect of 15d-PGJ2 on SHP2 phosphorylation. Treatment with 15d-PGJ2 (20 μM) for 10 min did not upregulate SHP2 phosphorylation in oral SCC cells (Figure 6B).

AG490 induces growth inhibition and apoptosis in oral SCC cells

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induced Stat3 downregulation. Moreover, 15d-PGJ2 attenuated JAK
Jak2 by 15d-PGJ2 preceded the inhibitory effects on IL-6-mediated
phosphorylation in the presence or absence of IL-6 treatment,
exogenous ligands had opposite effects on 15d-PGJ2 activity in that
a the Stat3 signalling complex, including the IL-6/JAK and the TGF-
particular importance. Moreover, 15d-PGJ2 possesses the ability to
growth inhibition of AG490 (\(\leq 0.05\)) with respect to
preferential target of 15d-PGJ2 is the IL-6/JAK signalling pathway,
of which downmodulation results in the inhibition of Stat3
activation and induction of apoptosis.
Stat3 constitutive activation has been previously linked to
aberrant TGF-\(\beta\)/EGFR autocrine or paracrine stimulation in
head and neck SCC (Grandis et al, 2000; Song and Grandis, 2000; Leong
et al, 2002). However, recent evidence points to the contribution of
Src in EGFR-mediated Stat3 activation (Xi et al, 2003) and
serves a novel molecular target in oral SCC cells. This
hypothesis is further corroborated by our findings that targeting of
JAK activity by the specific kinase inhibitor AG490 abrogates
constitutive Stat3 activation and induces growth inhibition
accompanied by apoptotic cell death in oral SCC cells.
In that JAKs play an essential role in driving oncopgenic Stat3
signalling (De Vos et al, 2000; Ni et al, 2000; Zhang et al, 2000; Li
and Shaw, 2002; Rane and Reddy, 2002), the ability of 15d-PGJ2 to
donimum IL-6/JAK signalling may also in part, explain the
antineoplastic properties of this agent against other types of
human cancer. For example, 15d-PGJ2 has been shown to have
potent PPAR\(\gamma\)-independent proapoptotic effects on breast cancer
cells (Clay et al, 2001, 2002), which exhibit constitutive Stat3
activation linked to cooperative activity of Src and JAK family
tyroisine kinases (Garcia et al, 2001).
Further investigations should focus on elucidating the mechan-
isms by which 15d-PGJ2 inhibits JAK. The fact that exogenous
stimulation with IL-6 was unable to reverse 15d-PGJ2-mediated
JAK inhibition suggests that the target of 15d-PGJ2 is the
kinase, but not the ligand. Consistent with this view, we have not
observed downregulation of IL-6 levels after treatment with 15d-
PGJ2 (unpublished data). Instead, JAK inhibition may be the result
of direct interaction between 15d-PGJ2 and JAK, especially
considering the ability of 15d-PGJ2 molecules to bind to and
modify specific proteins through their reactive \(\alpha,\beta\)-unsaturated
carbonyl group within the cyclopentenone ring (Straus and Glass,
2001). In view of the latter, our data highlighting the requirement
of the reactive cyclopentenone ring system for 15d-PGJ2-mediated
repression of JAK and Stat3 constitutive phosphorylation are of
particular importance. Moreover, 15d-PGJ2 possesses the ability to

**DISCUSSION**

In an attempt to pinpoint the molecular target of 15d-PGJ2 in oral
SCC, we examined the effect of 15d-PGJ2 on critical regulators of
the Stat3 signalling complex, including the IL-6/JAK and the TGF-
\(\beta\)/EGFR pathways. Induction of these two signalling pathways
with exogenous ligands had opposite effects on 15d-PGJ2 activity in that
only TGF-\(\beta\), but not IL-6, stimulation could reverse 15d-PGJ2-
induced Stat3 downregulation. Moreover, 15d-PGJ2 attenuated JAK
phosphorylation in the presence or absence of IL-6 treatment,
without modestly increased EGFR constitutively phosphorylated
levels. It is noteworthy that the downmodulation of both Jak1 and
Jak2 by 15d-PGJ2 preceded the inhibitory effects on IL-6-mediated
Stat3 activation. Based on these observations, we suggest that the

**Figure 5** AG490 reduces cell growth and induces apoptosis in oral SCC
cells. (A) Oral SCC9 and SCC25 cells were treated with vehicle (0.1%
DMSO) or AG490 at 25, 50 and 100 \(\mu\)M and cell growth was assessed
following 24, 48 and 72 h of treatment using a Coulter Counter. Data
are expressed as mean values \(\pm\) s.d. for representative cells. There was a
significant growth inhibitory effect of AG490 (\(P\leq 0.05\)) with respect to
both dose and time using a two-way analysis of variance (ANOVA)
test and Tukey test. (B) Representative oral SCC cells (SCC25) were
treated with vehicle (0.1% DMSO) or AG490 at 50 \(\mu\)M, and 100 \(\mu\)M for 48 h.
Annexin V-FITC assay revealed induction of apoptosis in cells treated with
AG490 compared to vehicle. (C) Oral SCC9 cells were treated with
vehicle (0.1% DMSO) or AG490 at 100 \(\mu\)M and Stat3 constitutive
phosphorylation was assessed following 24, 48 and 72 h of treatment.
Cells were blotted with phospho-Stat3 antibody (Y705) and subsequently
stripped and reprobed with Stat3 antibody.

**Figure 6** Effect of 15d-PGJ2 on negative regulators of Stat3 signalling.
(A) Effect of 15d-PGJ2 on SOCS3 expression. SCC9 cells were either
treated with 20 \(\mu\)M 15d-PGJ2 (PG) or IL-6 (25 ng/ml) for 1 h. Cells were
blotted with SOCS3 (M-20) antibody and subsequently stripped and
reprobed with actin control antibody. (B) Effect of 15d-PGJ2 on SHP2
phosphorylation. SCC9 cells were treated with 20 \(\mu\)M 15d-PGJ2 (PG) for
10 min. Cell lysates were immunoprecipitated with SHP2 antibody and
Western blotted for phosphorylated (PY) and total SHP2.

In an attempt to pinpoint the molecular target of 15d-PGJ2 in oral
SCC, we examined the effect of 15d-PGJ2 on critical regulators of
the Stat3 signalling complex, including the IL-6/JAK and the TGF-
\(\beta\)/EGFR pathways. Induction of these two signalling pathways
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phosphorylation in the presence or absence of IL-6 treatment,
without modestly increased EGFR constitutively phosphorylated
levels. It is noteworthy that the downmodulation of both Jak1 and
Jak2 by 15d-PGJ2 preceded the inhibitory effects on IL-6-mediated
Stat3 activation. Based on these observations, we suggest that the
induce reactive oxygen species, which have been shown to contribute to the biological activities of cyclopentenone prosta-
glandins, including cytotoxic effects of 15d-PGJ2 (Kondo et al.,
2001; Li et al., 2001; Lennon et al., 2002). The possible involvement of
redox-sensitive mechanisms in the function of 15d-PGJ2 as an
antineoplastic agent that induces cell growth inhibition and IL-6/ JAK/Stat3 suppression in oral SCC cells is currently under
investigation.

Alternatively, 15d-PGJ2, directly or through production of other
mediators, may induce negative regulators of JAK activity, such as
SOCS or SHP2. In this respect, stimulation with 15d-PGJ2 has been
shown to induce the transcription of SOCS1 and SOCS3 and to
activate SHP2, which in turn suppress inflammatory interferon
signalling mediated by the JAK/STAT pathway in primary
astrocytes (Park et al., 2003). However, we did not observe upregulation of SOCS3 and SHP2 during 15d-PGJ2-mediated
repression of JAK phosphorylation in oral SCC cells. Although
our findings suggest that the inhibition of JAK/STAT signalling by
15d-PGJ2 may require other mechanisms in oral SCC cells, the
possible involvement of other negative regulators of JAK activity
(Starr and Hilton, 1998; Starr and Hilton, 1999) warrants further
investigation.

Negative regulation of Stat3 has also been associated with
activation of mitogen-activated protein kinases (MAPKs), includ-
ing ERKs, JNKs and p38 MAPK, possibly involving suppression of
JAK activity (Sengupta et al., 1998; Bode et al., 1999; Lim and Cao,
1999; Ahmed and Ivashkiv, 2000; Bode et al., 2001). These
observations, along with recent findings supporting activation of
MAPKs by 15d-PGJ2 in a number of cells (Wilmer et al., 2001;
Lennon et al., 2002), provide another possible explanation for 15d-
PGJ2 effects on Stat3 signalling.

Although the mechanisms by which 15d-PGJ2 causes suppres-
sion of the IL-6-mediated JAK signalling in oral SCC necessitate
further investigation, our findings provide a novel molecular
explanation for the antineoplastic properties of cyclopentenone
prostaglandins, which may facilitate their optimal use as
therapeutic agents.

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