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STAT3 SIGNALING IS INDUCED BY INTERCELLULAR ADHESION IN SQUAMOUS CELL CARCINOMA CELLS

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Abstract

The signal transducer and activator of transcription-3 (STAT3) frequently activated during tumor progression has been linked to enhanced cell growth. In squamous cell carcinoma of the head and neck (HNSCC), STAT3 signaling has been shown to inhibit apoptosis and induce a more aggressive phenotype through the activation of specific signaling pathways. In the present study, we have examined the potential mechanism by which cell-cell contact initiates STAT3 activation. Using a panel of HNSCC cell lines, Ca^{+2} -dependent cell-cell adhesion and adherens junction formation in multicellular aggregates triggered phosphorylation of STAT3-Y705 and STAT1-Y701. This intercellular adhesion-induced STAT3 activation was mediated by JAK and Src signaling and partially by EGFR signaling. In addition, immunolocalization studies revealed initial formation of phosphorylated STAT3-Y705 at nascent E-cadherin cell junctions with eventual translocation to the nucleus in cell aggregates. Adhesion-mediated STAT activation in monolayer and cell aggregate cultures required functional E-cadherin. These results indicate that in HNSCC cells, cadherin-mediated intercellular adhesion induces STAT signaling that may modulate cell survival and resistance to apoptosis during tumor progression.

Keywords

STAT3; cadherin; intercellular adhesion

Introduction

Signal transducer and activator of transcription (STAT) members were first discovered as latent cytoplasmic transcription factors [1]. The STATs consists of seven members which are involved in a number of important cell signaling events and are altered following transformation, growth, differentiation and apoptosis [2]. Constitutive activation of STAT3 has been demonstrated in several human cancers, including breast cancer [3], prostate cancer [4], leukemia [5], lung cancer [6], thyroid cancer [7], and HNSCC [8]. STATs are activated by phosphorylation in response to cytokines (e.g. IL-6) and growth factors (e.g. epidermal growth factor (EGF) [9,10].

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Other studies have shown that the non-receptor tyrosine kinases (e.g. Src and JAK) are also involved in STAT activation [11–13]. STAT3 signaling is initiated through the phosphorylation of Tyr705, followed by formation of homo- and heterodimers through Src homology SH2 domain interactions. These complexes are then translocated to the nucleus where they drive transcription of specific genes through binding to STAT-specific response elements in the target gene promoter [1,14]. STAT3 has been demonstrated to up-regulate genes encoding apoptosis inhibitors (Bcl-xL, Mcl-1 and survivin) [15], cell-cycle regulators (cyclin D1 and c-Myc) [16]; and angiogenesis inducers (e.g. VEGF) [12], which are important for tumor progression.

During the process of metastasis, cancer cells undergo a series of intricate sequential events as they spread from the primary tumor to distant organ sites. Only cells that survive the steps exposed to apoptotic stimuli are able to establish distant tumor nests. For example, embolic tumor cells in the vascular compartment are subject to cell death at high frequency [17]. Given the well known induction of cellular signaling by cell-cell contacts, it is postulated that these adhesive interactions may modulate cellular activity during specific steps of cancer progression. There is increasing evidence from various types of epithelial cells that E-cadherin ligation at sites of cell-cell contact initiates specific cellular signaling events, including cell survival and proliferation [18–21]. STAT activation induced by intercellular contact potentially could enhance cell survival and provide resistance to apoptosis, thereby providing an advantage during the metastatic cascade.

Previous studies indicate that for squamous epithelial tumor cells, anchorage independent growth requires engagement of cadherin cell-cell adhesions [18]. Other work has also shown that cell-cell adhesion and high cell density can induce elevated STAT3 activation [22,23]. In the current study, we show that intercellular adhesion upregulates the key phosphorylation of STAT3 at Y705 and that this initially occurs at newly formed E-cadherin cell junctions. Subsequently, activated STAT3 translocated to the nucleus. E-cadherin was required for maximal induction of STAT3 activation triggered by cell-cell contact.

Materials and Methods

Cell lines and culture conditions

HaCaT immortalized keratinocytes and a panel of oral squamous carcinoma cell lines (HSC-2, HSC-3, LMF-4 and UM-SCC-10A) have been described previously [24–26]. Cells were routinely maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc. Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Hyclone).

To investigate the possible influence of adherens junction formation on STAT pathway in oral squamous carcinoma cells, three different models were utilized. For the calcium-switch assay, cells were first grown as a confluent monolayer in normal levels of Ca²⁺ to optimize cell-cell contacts [27]. Cultures were then serum-starved overnight in DMEM and cell-cell contacts were disrupted by treatment with 4 mM EGTA in Ca²⁺-free DMEM for 45 min at 37°C. Thereafter, intercellular interactions were allowed to recover in the presence of fresh DMEM medium containing Ca²⁺ (1.8 mM). At different time points after calcium restoration, cells were harvested and processed for immunoblotting or for immunofluorescent staining.

To generate multicellular aggregates (MCAs), 2×10⁶ cells were plated on polyhydroxyethyl-methacrylate (poly-HEMA)-coated (Sigma) 10 cm dishes in DMEM supplemented with 0.5% FBS [18]. Single cell suspension cultures were prepared in semisolid medium consisting of 0.5% FBS in DMEM containing 1.5% methylcellulose (Sigma) at 6×10⁵ cells per 10 cm poly-HEMA-coated dish.

For studies using cell pellets, suspensions of HSC-3 cells ($2 \times 10^5/\text{ml}$) in 5 ml DMEM were placed in 15 ml conical polypropylene centrifuge tubes (CLP) and subjected to centrifugation at a constant acceleration of $900 \times g$ for 5 min at 4°C to form compact cell pellets. The compacted cell pellets were then incubated at 37°C for the indicated time and processed for immunoblotting. In parallel, control cells were kept as a single cell suspension as described above during this period.

Western blotting

Cells were processed by lysis in 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), 0.5% sodium deoxycholate, 1 mM sodium vanadate, plus a protease inhibitor cocktail (Roche Molecular Biochemicals, Basel, Switzerland) on ice. The lysates were centrifuged at 13,000 rpm for 15 min at 4°C and the resultant pellets were discarded. In each assay, 15 μg of protein from clarified cell extract were resolved on a 6 or 12% polyacrylamide-SDS gel and were transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad laboratories, Hercules, CA). The membranes were blocked with 5% non-fat dry milk in TBS-T (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) for 1 h at RT, followed by incubation with primary antibodies overnight at 4°C , then 1 h at RT with HRP-conjugated secondary antibodies. Blots were washed in TBS-T after antibody incubations and bands were visualized with ECL (Amersham Bioscience).

Antibodies

Rabbit pAbs specific for the tyrosine-705 phosphorylated form of STAT3 and total STAT3 were purchased from Cell Signaling Technology (Danvers, MA). Rabbit mAbs specific for the serine-727 phosphorylated form of STAT3 was purchased from Epitomics (Burlingame, CA). Mouse mAbs specific for STAT1, tyrosine-701 phosphorylated form of STAT1, STAT5, and tyrosine-694 phosphorylated form of STAT5, were purchased from BD Transduction Laboratories (San Jose, CA). Function blocking anti-E-cadherin antibody, clone She-78-7 was from Zymed Laboratories (San Francisco, CA). HRP-conjugated antibodies specific for rabbit IgG or mouse IgG, and FITC- or rhodamine-conjugated antibodies specific for rabbit IgG or mouse IgG were purchased from Jackson ImmunoResearch (West Grove, PA).

Immunofluorescent staining

For staining, monolayer cultures grown on coverslips were washed twice in PBS at indicated times prior to fixation. MCAs were attached to poly-L-lysine coated coverslips. Next, cells were then briefly fixed with 4% formaldehyde in PBS, permeabilized with 0.5 % Triton-X-100 and blocked with 10% goat serum in PBS. Samples were incubated in primary antibodies overnight at 4°C , followed by 1 h at RT with fluorescent secondary antibodies. Nuclei were further stained for 30 min with 5 $\mu\text{g}/\text{ml}$ Hoechst 33342 in PBS. Samples were mounted in Vectashield mounting media (Vector Laboratories, Burlingame, CA) and viewed on a Zeiss Axiovert 200M microscope with an AxioCam high resolution CCD camera (Zeiss, Jena, Germany), using AxioVision 4.3 software (Zeiss, Jena, Germany).

Results

Cell-cell adhesion induces STAT3 activation

To define the potential mechanism responsible for intercellular mediated STAT3 phosphorylation, we initially used a calcium-switch adhesion assay with monolayer cultures of HSC-3 squamous cell carcinoma cells. As shown in Fig. 1A, STAT-Y705 phosphorylation was at low levels in untreated serum-starved cells and following adherens junction disruption by EGTA treatment. Upon initiation of cell-cell contacts in Ca^{2+} containing media, cells

responded in a time-dependent manner with STAT-Y705 phosphorylation showing initial activation by 40 min, reached a plateau by 60 min, and declined by 100 min.

To examine whether acquired cell-cell adhesion independent of cell-matrix interactions was sufficient to induce STAT activation, we performed forced cell-cell contact and multicellular aggregation studies. To achieve this, serum-starved HSC-3 cells were detached from culture dishes non-enzymatically and were either incubated as single cell suspensions or as compact cell pellets following centrifugal cell packing for various time points. In a different approach, cells were cultured on non-adhesive poly-HEMA substrates to allow formation of multicellular aggregates (MCA) or kept as single cells in medium containing methylcellulose medium. Cell lysates were then analyzed by immunoblotting with specific antibodies to phospho-STAT3. Cells subjected to centrifugal forced cell-cell contact showed high levels of STAT3 phosphorylation by 1 h and approached maximal levels after 2 h of incubation (Fig. 1B). Elevated STAT3 activation was long-lived and remained at high levels for at least 4 h. In contrast, cells incubated as single cell suspensions did not display increased STAT3 activity during this time period. Similarly, cells permitted to form multicellular aggregates on non-adhesive plates were also found to induce an increase of STAT3-Y705 phosphorylation when compared to suspended single-cells (Fig. 1C). Activated STAT3-Y705 correlated with the formation of cell aggregates was detected by 6 h of MCA culture and the elevated levels of activation persisted for over 24 h of culture.

For comparison, we evaluated human immortalized keratinocytes (HaCaT cells) and a panel of squamous cell carcinoma cell lines (UMSCC10A, HSC-2, and LMF-4) (Fig. 2). HaCaT keratinocyte cells and HSC-2 cells generated limited activation of STAT3 in the calcium-switch assay compared to most of the HNSCC cells (Fig. 2A,B). In contrast, UMSCC10A cells similar to HSC-3 cells strongly activated STAT3 after about 40 min following Ca^{2+} restoration. However, unlike HSC-3 cells activation persisted beyond 100 min but then began to recede at about 120 min (Fig. 2C and data not shown). Interestingly, the highly metastatic LMF4 cell line, originally derived from the parental HSC-3 cells [25] generated the highest level of STAT3-Y705 activation (Fig. 2D).

We next tested whether formation of intercellular adhesion also lead to activation of STAT1-Y701, STAT3-S727 or STAT5-Y694 under similar experimental conditions. Ca^{2+} restoration of adherens junctions produced only modest induction of phosphorylated STAT1-Y701 or STAT3-S727 (Fig. 3A) or STAT5-Y694 (not shown). The time course of STAT1-Y701 phosphorylation after calcium restoration was limited in intensity and closely followed that of STAT3-Y705 phosphorylation. STAT3-S727 phosphorylation was actually attenuated by calcium restoration, showing an inverse relationship to STAT3-Y705 signaling. In contrast, STAT5 phosphorylation levels were negligible and were unaltered following induction of cell-adhesion but could be effectively activated by EGF stimulation (data not shown). In MCA, STAT3-Y701 activation was significant but limited and appeared to reach maximum activation at 12 h where it approached a plateau and activity decayed by 24 h (Fig. 3B). On the contrary, cells incubated as single cell suspensions did not display increased STAT activity during this time period. Altogether these results indicate that intercellular adhesion in epithelial cells preferentially induces intracellular signaling pathways leading to the activation of STAT3-Y705, but limited or no activation of STAT1 or STAT5.

Intercellular adhesion-induced STAT3-Y705 phosphorylation is mediated through multiple upstream pathways

To understand the mechanism of STAT3-Y705 activation induced by cell-cell interaction, we explored the potential role of the major pathways known to contribute to STAT3 tyrosine phosphorylation, JAK, Src and EGFR. HSC-3 cells grown to confluence were pretreated for 1 h with a range of optimal inhibitor concentrations for EGFR (1 μM AG1478), JAK (100

μM AG490) and Src kinase (1 μM SU6656). Cells were then processed for the calcium-switch assay and STAT3-Y705 phosphorylation at 60 min was monitored. As shown in Fig. 4A, inhibition of JAK and Src significantly blocked phosphorylation of STAT3-Y705, whereas the vehicle alone remained without effect. While further detailed analyses are necessary to define the precise contribution of these signaling pathways, the results indicate the involvement of both JAK and Src family kinases. Under our experimental conditions, we frequently noticed a variable degree of inhibition by the EGFR inhibitor AG1478. For example, in Fig. 4A and B, only a slight inhibition is seen for STAT3-Y705 phosphorylation with 1 μM AG1478. However, use of 10 μM effectively blocked phosphorylation of STAT3-Y705 (Fig. 4B). Similar doses of AG1478 exposures were simultaneously tested to verify inhibitor potency and specificity. As depicted in Fig. 4C, 1 or 10 μM AG1478 affected phosphorylation of EGFR and substantially inhibited the EGF-induced STAT3 activation in HSC-3 cells. Since inhibition with AG1478 was variable in a dose-related manner we tested a second inhibitor, PD168393, to further validate the specificity of EGFR inhibition affecting STAT3-Y705 during Ca^{2+} -induced intercellular adhesion. PD168393 is a highly selective inhibitor of EGFR known to irreversibly bind the receptor via a covalent modification of a cysteine residue present in the ATP binding pocket [28]. As indicated in Fig. 4D, 1 μM PD168393 blocked STAT3-Y705 that was comparable to inhibition with 10 μM AG1478.

Intercellular adhesion induces junctional and nuclear localization of STAT3-Y705

Upon activation, phosphorylated STAT forms dimers and translocates to nucleus, whereby it binds to specific DNA sequences and induces transcription of specific genes. To examine the localization of phosphorylated STAT3-Y705 observed during cell-cell contact formation, cells subjected to calcium-switch or MCA assay conditions were then analyzed by immunostaining. In untreated, serum-starved HSC-3 monolayer cells, E-cadherin junctions were visible (Fig. 5). Phosphorylated STAT3-Y705 was visible only as weakly stained and diffusely localized granules throughout the cytoplasm. Following chelation of Ca^{2+} with EGTA, intercellular adhesion was disrupted leading to further lower levels of pSTAT3 staining accompanied by loss of cadherin junctions (Fig. 5). However, after exposure to Ca^{2+} -containing medium for 60 min, adherens junctions were gradually restored as visualized by strong E-cadherin staining (Fig. 5). There were still some areas of junctional discontinuity where cell-cell adhesions had not yet reformed. In these nascent cell-cell contacts, phosphorylated STAT3-Y705 staining intensified along the edges of juxtaposed cells colocalizing with the reconstituted E-cadherin adhesions. Moreover, as compared to quiescent monolayer cells, enhanced but variable staining of phosphorylated STAT3-Y705 could be detected diffusely distributed throughout the cytoplasm. However, under these short-term assay conditions, STAT3-Y705 was not readily detected in the DAPI-stained nucleus.

We next examined the localization of STAT3-Y705 in HSC-3 cell aggregates. Immunofluorescent staining of the MCA at 6 h after plating showed traces of phosphorylated STAT3-Y705 localization at intercellular contact regions of adjacent cells, and also distributed within the cytoplasm (Fig. 6A). By 12 h, phosphorylated STAT3-Y705 was frequently found to be translocated to the nucleus (Fig. 6A), and remained strongly evident as cells formed compact aggregation by 24 h (not shown). Electrophoretic mobility shift assay from MCA lysates detected STAT3 binding activity, suggestive of nuclear translocation (data not shown). Junctional and nuclear localization was further confirmed by confocal microscopy at 6 and 12 h, respectively (Fig. 6B). Taken together, these results show that as MCA form over time there is induction of phosphorylated STAT3-Y705 at cell-cell contacts that is followed by its nuclear translocation.

Role of E-cadherin in cell-cell adhesion-mediated STAT3 activation

The above results suggest that critical cell-adhesion molecules may be involved in triggering signaling components leading to STAT3 activation. To determine whether E-cadherin receptor engagement during cell-cell contact was required in STAT3 activation, an E-cadherin functional blocking study was initially performed in the calcium-switch assay. As compared to treatment with normal mouse IgG, the presence of HECD1, blocking antibody to E-cadherin contributed to a modest suppression of STAT3 activation in HSC-3 cells (Fig. 7A). Next, in a similar approach single cell suspensions were cultured in the presence of a potent blocking anti-E-cadherin antibody (SHE78-7) or normal IgG for 6 h on poly-HEMA coated dishes. In contrast to IgG treated cells, the presence of the blocking antibody hindered homophilic interactions and prevented HSC-3 cells from forming compact cell aggregates (Fig. 7B). As revealed by immunoblotting, phosphorylation of STAT3-Y705 was partially inhibited and was associated with the failure to form cell aggregates (Fig. 7C). These observations indicate that E-cadherin engagement during cell-cell adhesion appears to be required for efficient STAT3 activation.

Discussion

STAT family proteins are activated by multiple receptor and nonreceptor tyrosine kinases in response to various cytokines, hormones and growth factors [29]. There is increasing evidence of the potential role of STAT1, STAT3 and STAT5 in oncogenic pathways [30]. Importantly, STAT3 and STAT5 are known to act as potent anti-apoptotic signaling pathways [31,32]. Previous studies had shown that high cell density and confluency can regulate STAT3 activation in both HNSCC [22] and breast carcinoma [33]. However, it remains unclear how cell-cell contact leads to activation of STAT3. We observed that STAT3 signaling was activated during formation of cadherin mediated cell-cell contacts in a short-term calcium-switch monolayer and long-term multicellular aggregation (MCA) assays.

When we analyzed STAT expression and function using the calcium-switch assay, restoration of cell-cell adhesion induced phosphorylation of STAT3-Y705 and STAT1-Y701 but not STAT5-Y694. In a similar observation, STAT3-Y705 and STAT1-Y701 were phosphorylated when cells formed MCA indicating that cell-cell contact was sufficient for STAT activation. However, the degree of phosphorylation of STAT1 was significantly weaker than STAT3-Y705, suggesting that intercellular adhesion activates preferentially STAT3. We observed that in monolayer assay, STAT3 phosphorylation was rapid and transient in contrast to MCA where phosphorylation remained stable and persisted over 24 h. For monolayer cells, multiple molecular signals are derived from both cell-cell and cell-ECM adhesion receptors. Although we do not have direct evidence it is possible cell-ECM adhesion responsive signals may play role in regulating STAT3 activation. Conversely, loss of attachment to ECM may deregulate STAT3 signaling. Interestingly, integrin $\alpha 1$ ligation has been shown to activate PTEN [34], which is known to dephosphorylate STAT3-Y705 [35].

Our studies also revealed that, unlike STAT3-Y705, STAT3-Ser727 phosphorylation tended to decrease following restoration of cell-cell adhesion. Although our results do not identify the mechanism, Ser-727 appears to play supplementary role to STAT3-Y705. Phosphorylation of Ser-727 in the C-terminal transcriptional activation domain in STAT3, enhances stable formation of STAT3/STAT3-DNA complexes [36] and the transcriptional activity of STAT3 [37]. However, these regulatory events are less understood in comparison to STAT tyrosine phosphorylation. Our results suggest that there are events induced by specific intercellular adhesion leading to independent STAT1 and STAT3 activation.

In response to growth factor and cytokines, activated STATs translocate to the nucleus and regulate gene expression following their binding to specific promoter DNA sequences thereby

controlling cell proliferation and survival [13,38]. The subcellular distribution of phosphorylated STAT3-Y705 upon formation of cell-cell contact shows close correlation with the biochemical result. During Ca^{2+} -mediated cell-cell contact formation, phospho-STAT3 predominantly resided at the cell junctions. This localization appeared transient and we were unable to detect significant translocation to the nucleus by immunofluorescence assay. On the contrary, for cells that formed aggregates in suspension, we observed that STAT3 was initially phosphorylated at cell junctions and in the neighboring cytoplasm, which became strongly localized to nucleus as cells formed compact MCA. As it is established that activated STAT translocates to the nucleus to regulate gene expression, this suggests that cells forming intercellular adhesion are competent for inducing functional STAT signaling.

The major upstream tyrosine kinases that can activate STAT signaling includes JAKs [39], Src [40], and EGFR [6,41]. JAK and Src have been previously implicated in cell confluence-mediated STAT3 activation, although Src appears to be selectively operative in lower cell-density cultures [33]. Based on the inhibitor study, our results were unable to show a dominant specific pathway for intercellular adhesion-mediated STAT3 activation, suggestive of the complexity in the signaling involved. Our study reveals that both the non-receptor kinase JAK and Src appear contribute to cell-cell contact-induced STAT3 activation. Based on results from specific inhibitor assays (AG14789 and PD168393), the EGFR pathway also appears to contribute to and enhance STAT3 activation. This latter observation, however deviates from existing reports that cell-cell adhesion-mediated STAT3 signaling is independent of EGFR pathway [22]. EGFR inhibitors appeared to have targeted transient and variable events that occur during the initial formation of cell-cell contact in the Ca^{2+} -switch assay. In MCA experiments, contribution by EGFR signaling was not readily observed, suggesting that this mechanism is operational in early adhesion events. Previous studies were carried out in long term assays performed by varying cell density and prolonged culture. The disparity could therefore be in part attributed to the different experimental conditions and cell lines used. Our results nonetheless demonstrate that besides JAK and Src, EGFR signaling pathway could function as a cumulative mechanism to STAT3 activation. However, the molecular role of each specific pathway is not clear since EGFR-mediated STAT activation could require sequential activation of Src and JAK, acting as a single signaling component [42]. ErbB-2, another EGFR family receptor kinase has been shown to activate STAT3 in a Src and JAK2 dependent manner [43]. The complexity of these signaling events will require additional work to sort out the role of individual pathways that are activated during early intracellular adhesion. In addition, the recent finding by Gutkind and collaborators [44] that constitutive STAT3 activation by autocrine/paracrine signaling in HNSCC cells raises the possibility that intercellular adhesion may be directly modulating these events.

On the premise of cell-cell contact dependent STAT phosphorylation, an appealing hypothesis was to test the possibility that E-cadherin engagement is the trigger for STAT signaling. In our study, perturbation of E-cadherin function by antibodies significantly blocked the cell-cell contact-mediated transient STAT3 activation as well as hindered compact cell aggregate formation that resulted in inhibition of STAT3 phosphorylation. Signaling induced by cell-cell adhesion molecules is complex. Several classes of molecules on the cell surface that form zonula adherent junctions, desmosomal assembly, tight junction and gap junctions could contribute to intercellular adhesion and signaling even though E-cadherin ligation might regulate the initial step of intercellular linkage [45].

While possible roles of these complex classes of cell adhesion molecules cannot be excluded our results show that E-cadherin is involved in initiating events that lead to contact-induced activation of STAT3 in HNSCC. It is likely that activation of STAT3 during cell-cell adhesion proceeds via a two-step process. First, the initial linkage by E-cadherin involves formation of cadherin homodimers and this is then followed by formation of nascent zonula adherens

junctions, involving interactions with the cytoskeleton. Importantly, such structures are also known to trigger downstream signaling events. For example, E-cadherin engagement induces EGFR signaling [46] and Rac1 activation [47]. In addition, N-cadherin has been shown to induce ligand-independent FGFR activation [48].

What is the physiological significance of STAT3 activation induced by E-cadherin mediated intercellular adhesions? Signals produced by intercellular adhesion-mediated STAT activation might be important for prolonged survival and growth of tumor cell aggregates that commonly are present in HNSCC lesions in vivo. Here many of the cells in tumor nests lack direct interaction with surrounding extracellular matrix and these cells could depend on survival signaling generated by cell-cell contacts. Importantly, E-cadherin mediated cell-cell adhesion has been implicated in anchorage-independent cell growth and survival [18]. Maintenance of cadherin mediated cell-cell interaction not only promotes cell survival, but enhances resistance to chemotherapeutic agents [20,49]. It is reasonable to suggest that E-cadherin dependent intercellular adhesion may contribute to SCC cell's resistance against anoikis through the activation of STAT3 besides previously reported signaling pathways via EGFR and ERK activation [50]. Additionally, as discussed above EGFR signaling induced by E-cadherin mediated intercellular adhesion could initiate and modulate STAT3 activation through autocrine/paracrine mechanism [44]. As a result, the understanding of the STAT3 signaling might lead to the development of rationally designed therapies that target one of the molecular components.

The current findings provide evidence that HNSCC cell-cell adhesion, likely mediated in part by E-cadherin, induces primarily STAT3 and to a lesser extent STAT1 activation. Selective targeting of cell-cell adhesion induced signaling and the STAT pathway during tumor metastasis may represent a valid therapeutic approach. Future studies are needed to elucidate the complicated crosstalk of cell surface molecules in cell-cell adhesion and how these interactions induce a complex array of important signaling pathways. In addition, understanding the potentially important role of STAT signaling on the tumor progression may contribute effective therapeutic strategies in head and neck squamous cell carcinoma.

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Figure 1.

Cell-cell interactions induce STAT3-Y705 phosphorylation. (A) Confluent HSC-3 monolayer cells were starved overnight and processed for Ca^{2+} -switch assay as described in Methods and Materials. Cell lysates were then analyzed by immunoblotting for phosphorylated STAT3-Y705 and total STAT3 as indicated. (B) HSC-3 cells cultured as above were harvested and processed for centrifugal cell packing. Cells were incubated at 37°C as cell pellets or left as a single cell suspension for the indicated times. Cell lysates were prepared and analyzed for phosphorylated STAT3-Y705 by immunoblotting. (C) HSC-3 cells were cultured as single cells (SC) or as multicellular aggregates (MCA) (see Materials and Methods) for the indicated times. Cell lysates were prepared and immunoblotted for phosphorylated-STAT3-Y705. As a control serum-starved monolayer cells were treated with EGF (15 ng/ml) for 30 min.

Figure 2.

Induction of STAT3-Y705 activation by cell-cell adhesion. Cell monolayer cultures of (A) HaCaT, (B) HSC-2, (C) UMSCC10A and (D) LMF4 were processed for Ca²⁺-switch assay as in Figure 1A and cell lysates were analyzed for STAT3-Y705 and total STAT3 by immunoblotting.

Figure 3.

Intercellular adhesion mediated phosphorylation of STAT1-Y701 and STAT3-S727 in HSC-3 cells. Cultures of HSC-3 cells were processed as in Fig. 1A and C for calcium switch assay (A) or for MCA (B). As a positive control, serum-starved cells were stimulated with 15 ng/ml EGF for 30 min (A). Cell lysates were prepared and analyzed by immunoblotting as indicated for pSTAT-Y701 or pSTAT-S727.

Figure 4.

Effect of inhibitors on intercellular adhesion mediated STAT3-Y705 phosphorylation. (A) Serum-starved monolayer HSC-3 cells were pretreated with inhibitors for epidermal growth factor (EGF) receptor (AG1478), Janus-activated kinase (AG490), and Src-family tyrosine kinase (SU6656), or DMSO vehicle alone for 1 h prior to and during performance of the calcium-switch assay. After calcium-induced cell-cell contact for 60 min, cell lysates were processed for immunoblotting with antibodies to pSTAT3-Y705 and total STAT3. (B) Serum-starved cells were processed as in (A) in the absence or presence of 1 and 10 μM AG1478 or DMSO alone for 60 min. Cell lysates were collected and analyzed for pSTAT3-Y705 and total STAT3 by immunoblotting. (C) Serum-starved HSC-3 cells were treated with 15 ng/ml of EGF in the absence or presence of 1 and 10 μM AG1478 or DMSO alone for 60 min. Cell lysates were collected and analyzed for pEGFR and pSTAT3-Y705 by immunoblotting. Membranes

were stripped and reprobed for total EGFR and STAT3. (D) Serum-starved cells were processed as in (A) or in the presence of 1 μ M PD168393. Cell lysates were collected and analyzed for pSTAT3-Y705 and total STAT3 by immunoblotting.

Figure 5.

Localization of phosphorylated STAT3-Y705 during Ca²⁺-induced cell-cell contact. Immunofluorescence analysis of HSC-3 cells cultured to 100% confluence and subjected to Ca²⁺ switch assay. Cells were either left untreated or treated with EGTA for 45 min and then exposed to Ca²⁺-containing media for 60 min. Cells were then fixed, permeabilized and processed for immunostaining with antibodies to E-cadherin (red) and phosphorylated STAT3-Y705 (green); DAPI was used to stain nuclei (blue). Magnified images of the cell are indicated by the dashed boxes. Arrow indicates junctional localization of E-cadherin and phospho-STAT3-Y705. Bars, 20 μm; 5μm

Figure 6.

Localization of phosphorylated STAT3-Y705 in cell aggregates. (A) HSC-3 cells were grown as MCA on poly-HEMA coated plates. For each time point MCA were harvested and collected on poly-L-lysine coated coverslips, fixed, permeabilized and stained with antibodies to pSTAT-Y705. (B) pSTAT3-Y705 was visualized by confocal microscopy in MCA after 6 and 12 h of culture. Note: for clarity, the nuclear DAPI stain is presented in red for contrast with the FITC-conjugated pSTAT3-Y703 staining. Bar, 20 μ m.

Figure 7.

Inhibition of cell-cell contact by function-blocking E-cadherin antibody inhibits STAT3 phosphorylation. (A) Serum-starved confluent HSC-3 cells were processed for Ca^{2+} -switch assay in presence of control IgG or function blocking E-cadherin antibody (She78-7) for 30 or 60 min. Cell lysates were prepared and analyzed for phosphorylated STAT3-Y705 and total STAT3. (B) HSC-3 cells were detached non-enzymatically, permitted to form MCAs in the presence of 5 $\mu\text{g}/\text{ml}$ E-cadherin functional blocking antibody or control isotype IgG. Phase-contrast microphotographs of cells after 6 h of MCA culture. Bar, 20 μm . (C) Corresponding STAT3 phosphorylation status is shown as assessed by immunoblotting with anti-pSTAT antibody of the cell extracts. Results are representative of three independent experiments.