Tumor necrosis factor α induces epithelial-mesenchymal transition and promotes metastasis via \( \text{NF-} \kappa \text{B} \) signaling pathway-mediated TWIST expression in hypopharyngeal cancer

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Abstract. Epithelial-mesenchymal transition (EMT) is an important mechanism in cancer metastasis. Tumor necrosis factor α (TNFα) can induce cancer invasion and metastasis associated with EMT. However, the underlying mechanisms are not entirely clear. Therefore, we investigated whether TNFα has an effect on EMT and invasion and metastasis in human hypopharyngeal cancer FaDu cells, and further explored the potential mechanisms. In the present study, we demonstrated that TNFα induced EMT in FaDu cells and promoted FaDu cell migration and invasion. TNFα-induced EMT was characterized by a change from well organized cell-cell adhesion and cell polarity to loss of cell-cell contacts, cell scattering and increased expression of vimentin and N-cadherin accompanied by a decrease in E-cadherin. Furthermore, we found that p65 translocated to the nucleus after TNFα stimulation and increased the nuclear expression of TWIST. We demonstrated that TNFα treatment also increased the expression of TWIST by activating the NF-κB signaling pathway. While p65 was inhibited by siRNA-65 or BAY11-7082 (inhibitor of NF-κB), TWIST expression was also decreased. Therefore, we conclude that TNFα induces EMT and promotes metastasis via NF-κB signaling pathway-mediated TWIST expression in hypopharyngeal cancer.

Introduction

The development of cancer is often associated with chronic inflammation, which suggests a strong relationship between inflammation and tumorigenesis (1). Tumor necrosis factor α (TNFα) is one of the key inflammatory mediators involved in inflammation-associated cancer (2). Although over the last few decades, a high dose of TNFα has been used as a cytotoxic agent, recent reports support the link between chronic low-level TNFα exposure and the acquisition of certain malignant phenotypes, such as increased growth, invasion and metastasis (2). In addition, TNFα is an important activator of the canonical NF-κB pathway. Upon stimulation, activated IKK-β phosphorylates the NF-κB inhibitor, IκBα, and triggers its rapid degradation resulting in the liberation of NF-κB. As a consequence, the NF-κB heterodimer translocates to the nucleus, binds to its cognate DNA motifs in the promoters, and induces expression of a myriad of genes implicated in the immune response, cell proliferation, angiogenesis, cell survival and invasion (3,4).

Epithelial-mesenchymal transition (EMT), which is a complex reprogramming process of epithelial cells, plays an important role in tumor invasion and metastasis (5). EMT is characterized as the morphologic alteration from an epithelial to a mesenchymal phenotype, including loss of epithelial cell markers, such as E-cadherin, α-catenin and γ-catenin, and a gain in mesenchymal components, such as vimentin, N-cadherin and fibronectin (6,7). In addition, studies show that a group of transcriptional factors regulate EMT, such as TWIST, Snail and Slug. Previous studies demonstrate that TWIST is implicated in metastasis by regulating EMT via AKT/GSK or NF-κB-mediated expression of Snail and TWIST in breast, renal and colon cancer (10-13). Collectively, the evidence suggests that TNFα may regulate the critical processes of tumor promotion and progression, including angiogenesis, oncogene activation and EMT.

Hypopharyngeal cancer is one of the most common head and neck squamous cell carcinomas (HNSCCs). More than 75% of patients with hypopharyngeal cancer are at an advanced stage at the time of diagnosis (14). Lymph node metastasis is present in 60-80% of patients and it directly affects the prognosis of this disease (15). Metastasis of tumors is a complex process, and various factors are involved in each step of metastasis (16). Thus, in the present study, we investigated whether TNFα induces EMT via an increase in TWIST...
expression in human hypopharyngeal cancer FaDu cells and thereby promotes FaDu cell metastasis. Next, we aimed to ascertain whether the NF-κB signaling pathway is activated and regulates TNFα-induced TWIST expression.

Materials and methods

Materials. Commercially available antibodies used were as follows: NFκbp65, TWIST, E-cadherin and N-cadherin (all from Abcam, UK); vimentin, p-IKK and p-IκBα (all from Cell Signaling Technology, Inc., USA); lamin B and actin (both from Santa Cruz Biotechnology, Inc., USA) and TNFα (Cell Signaling Technology, Inc.). p65siRNA(h) and Bay 11-7082 were both from Santa Cruz Biotechnology, Inc.

Cell culture and transfection. The human hypopharyngeal cancer FaDu cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin and 100 mg streptomycin at 37°C in a humidified atmosphere composed of 95% air and 5% CO2. Detailed experimental procedures of the cell transfection were previously described (9).

Observation of morphological changes. The morphological changes in the FaDu cells were observed using an inverted microscope. Images were captured using a Leica microscope image system (Leica, Germany).

Immunofluorescence. The cells were cultured on chamber slides, serum starved for 12 h, then exposed to TNFα (10 ng/ml) for the indicated times. Cells were washed 3 times with PBS, fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.3% Triton X-100 for 10 min. After blocking with bovine serum albumin for 2 h at room temperature, cells were incubated with antibodies against E-cadherin, N-cadherin, p65 or TWIST (1:100 dilution) at 4°C overnight. Slides were washed 3 times with PBS and incubated with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) secondary antibodies for 1 h at room temperature. The nuclei were stained with 4',6-diamidine-2'-phenylindole (DAPI) for 2 min. Samples were examined using confocal microscopy (Leica, Germany) to analyze expression of E-cadherin, N-cadherin, TWIST and p65.

Western blotting. Detailed experimental procedures of western blot analysis of gene expression were previously described (17). Western blot analysis was performed with antibodies against TWIST (1:100), E-cadherin (1:200), N-cadherin (1:200), p65 (1:400), vimentin (1:1,000), p-IKK (1:1,000), p-IκBα (1:1,000), lamin B (1:1,000) and β-actin (1:3,000).
Wound healing assay. The FaDu cells were plated onto 6-well plates at a concentration of 5\times10^5 cells/well, and were serum starved for 12 h. Tumor cells were then treated with or without TNFα (10 ng/ml). Cell monolayers were carefully wounded by scratching with a sterile plastic pipette tip. The cells were then washed twice with cooled PBS before observation. For each wound, the images were captured at 0 and 24 h in the same fields after treatment.

Transwell chamber assay. FaDu cells were pretreated with or without TNFα (10 ng/ml) for 24 h, and 3\times10^4 cells were plated in the upper chamber. Detailed experimental procedures of the invasion and migration assays were previously described (9).

Statistical analysis. Data are expressed as means ± standard deviation (SD), and statistical significance was assessed by the analysis of variance test. All statistical tests employed in the present study were two-sided. P-values <0.05 were considered statistically significant results. Statistical calculations were performed using SPSS software package, version 13.0 (SPSS Inc., USA).

Results

TNFα induces EMT. To explore whether TNFα has an effect on the morphology of FaDu cells, FaDu cells were treated with TNFα (10 ng/ml). We found that the morphology of the FaDu cells following treatment with TNFα was altered from well organized cell-cell adhesion and cell polarity to loss of cell-cell contacts and cell scattering (Fig. 1A). Cells underwent a significant change in morphology from a cobblestone morphology to exhibiting mesenchymal spindle-like features. Next, we observed the expression of EMT molecular markers using immunofluorescence and western blotting, and found that the expression of the epithelial marker E-cadherin was downregulated, whereas the mesenchymal markers, vimentin and N-cadherin, were significantly upregulated in the FaDu cells (Fig. 1B and C). This phenomenon indicates that TNFα induces EMT in human hypopharyngeal cancer FaDu cells.

TNFα increases cancer cell motility, migratory and invasive abilities. To confirm that the change in morphology has an effect on the function of the cells, wound healing and Transwell chamber assays were used to measure cellular motility, migratory and invasive abilities. After 24 h following exposure to TNFα, the speed of motility of the FaDu cells was found to be more rapid than that of the control group; the former was closer to the center of the wound area than the control group (Fig. 2A). Promotion of migration and invasion in the FaDu cells by TNFα was also confirmed by the Transwell chamber assay. The numbers of cells that had migrated in the control and TNFα treatment groups were 124±15 and 276±38, respectively (P<0.05) (Fig. 2B). In the in vitro invasion assay,
we found that the number of invasive cells in the TNFα treatment group was 95±3, significantly more than the number in the control group (63±6, P<0.05) (Fig. 2B). These results suggest that TNFα increased cellular motility, migratory and invasive abilities in the FaDu cells.

**TNFα induces TWIST expression in the FaDu cells.** Given that TWIST plays an important role in promoting the migration and invasion of HNSCC cells by regulating EMT, we aimed to ascertain whether expression of TWIST is altered in TNFα-induced EMT by western blotting. We found that TWIST expression was increased in a time-dependent manner in the FaDu cells following exposure to TNFα (Fig. 3). The results indicate that TNFα induces TWIST expression in the FaDu cells.

**P65 expression is upregulated and translocated into the nucleus along with TNFα-induced TWIST expression.** Given that TNFα induces p65 activation in the canonical NF-κB pathway, we investigated whether p65 is activated upon TNFα-induced TWIST expression in the FaDu cells. We measured the p65 expression by western blotting, and the results showed that p65 expression following exposure to TNFα was increased in a time-dependent manner (Fig. 3). This suggests that P65 was upregulated together with TNFα-induced TWIST expression in the FaDu cells. To further elucidate how TNFα induces p65 activation upon TNFα-induced TWIST expression, we examined p-IKK and p-IκBα expression. Western blot analysis showed that p-IKK and p-IκBα expression was increased in the TNFα-activated p65-expressing cells, respectively (Fig. 3). Next, we tested the nuclear p65 and TWIST expression by immunofluorescence. Compared to the control group, we observed that the levels of nuclear p65 and TWIST were increased in the TNFα treatment group (Fig. 4A). To further confirm the upregulation of nuclear TWIST and p65 expression, cytoplasmic and nuclear fractions in the FaDu cells were isolated upon treatment with TNFα. We also observed that TNFα-activated nuclear expression of p65 and TWIST was significantly increased by western blotting (Fig. 4B). These results suggest that TNFα activates P65 expression and triggers a dynamic interaction between nuclear translocation of p65 and nuclear expression of TWIST.

![Figure 4](image_url)
Downregulation of p65 expression inhibits TNFα-induced TWIST expression in FaDu cells. To further explore whether the alteration of p65 expression has any effect on TNFα-induced TWIST expression, FaDu cells were pretreated with BAY11-7082 (inhibitor of NF-κB) was used. FaDu cells were then treated with TNFα for 5 h. The results showed that BAY11-7082 inhibited p65 expression and blocked TNFα-induced TWIST expression. (A) siRNA-p65 was transfected into the FaDu cells that were treated with TNFα for 5 h. The transfected cells were incubated for 24 h and used for analysis by western blotting. (B) siRNA-p65 was transfected into the FaDu cells that were treated with TNFα for 5 h. The transfected cells were incubated for 24 h and used for analysis by western blotting. We found that p65 expression was decreased in the siRNA-p65 group, and TWIST expression was also decreased in the siRNA-p65 group. TNFα, tumor necrosis factor α.

Discussion

TNFα, a pro-inflammatory cytokine predominantly produced by macrophages, is a key molecule in the regulation of the inflammatory processes in tumor promotion. Clinically, increased expression of TNFα is present in various preneoplastic and malignant diseases. TNFα has been reported to be elevated in the blood serum of patients diagnosed with advanced stage breast tumors and to be correlated with an increased number and size of metastatic sites (18,19). In addition, TNFα is frequently detected in human cancers with poor prognosis, such as ovarian, renal and breast cancers (20). Furthermore, TNFα has been shown to promote the growth and invasiveness of colon and prostate cancer epithelial cells in vitro and in vivo (18,21).

It is known that TNFα is involved in EMT and enhances transforming growth factor-β1 (TGFβ-1)-induced EMT in multiple cancer cell types (18). EMT is an important step during primary tumor metastasis. Although increasing evidence indicates that TNFα induces EMT and promotes cancer migration and invasion (19,22), the effect of TNFα on HNSCC remains undetermined.

In the present study, we found that TNFα induced morphological alterations in FaDu cells, and their morphology switched from a tightly packed growth pattern to scattered and fibroblast-like colonies. At the same time, we found that expression of the epithelial marker E-cadherin was downregulated, whereas that of mesenchymal markers vimentin and N-cadherin was significantly upregulated. These results indicate that TNFα induces EMT.

The most distinguished characteristic of EMT is the morphologic alteration from an epithelial to a mesenchymal phenotype, which is often accompanied by the dissolution of epithelial tight junctions, loss of cellular adhesion, downregulation of the expression of various epithelial markers, but acquired expression of mesenchymal components, resulting in loss of cell polarity, cell-basement adhesion, and cell-cell contact and the acquisition of migratory and invasive abilities (7). When epithelial cells undergo EMT, which is thought to contribute to the invasive ability of cancer cells (5), EMT and its accompanying reduction in E-cadherin expression have been shown to be essential for the extravasation of cancer cells into secondary organs (23). Thus, we aimed to ascertain whether TNFα-induced EMT has an effect on HNSCC cell motility, migratory and invasive abilities. We found that TNFα increased cellular motility, migratory and invasive abilities.

TWIST, is known as an essential regulator of the aggressive phenotype of EMT (24). Once TWIST is activated, it recruits histone deacetylases to the E-box elements within the E-cadherin promoter, resulting in transcriptional silencing of E-cadherin expression and increased motility, migration and invasion.

Figure 5. Downregulation of p65 expression inhibits TNFα-induced TWIST expression in FaDu cells. (A) FaDu cells were pretreated with NF-κB inhibitor (BAY11-7082) for 2 h before TNFα stimulation, and FaDu cells were then treated with TNFα for 5 h. p65 and TWIST expression was determined by western blotting. The results showed that BAY11-7082 inhibited p65 expression and blocked TNFα-induced TWIST expression. (B) siRNA-p65 was transfected into the FaDu cells that were treated with TNFα for 5 h. The transfected cells were incubated for 24 h and used for analysis by western blotting. p65 expression was decreased in the siRNA-p65 group, and TWIST expression was also decreased in the siRNA-p65 group. TNFα, tumor necrosis factor α.

Figure 6. Schematic illustration of TNFα-induced EMT and promotion of metastasis via NF-κB signaling pathway-mediated TWIST expression in hypopharyngeal cancer. I) IKK is activated after TNFα stimulation. II) IkBα is phosphorylated, NF-κB (p65) dissociates from IkBα and is translocated to the cell nucleus. III) Nuclear NF-κB (p65) binds to its target gene TWIST, which is involved in EMT, migration, invasion and tumor growth. TNFα, tumor necrosis factor α; EMT, epithelial-mesenchymal transition.
Thus, further investigation is warranted to determine whether AKT/GSK-3β signaling pathway is required for TNFα-induced EMT in HNSCC cells. Indeed, the present study showed that expression of TWIST was significantly elevated in TNFα-induced EMT. The results indicate that TNFα induced EMT by mediating TWIST expression consequently increasing cell motility, migration and invasion.

TNFα is one of the most important pro-inflammatory cytokines produced in the tumor microenvironment. Several lines of evidence demonstrate that TNFα and/or the NF-κB signaling pathway plays a key role in the regulation of EMT (26). The contribution of NF-κB signaling to the initiation and progression of cancer is clearly documented (26-28).

Given that TNFα can activate the NF-κB signaling pathway and provides a mechanistic link between inflammation and cancer, we hypothesized that the NF-κB signaling pathway may be involved in TNFα-induced TWIST expression. In the present study, we found that p-IKK, p-IκBα and p65 expression was increased upon TNFα-induced TWIST expression. The results indicate that the canonical NF-κB signaling pathway was activated. Furthermore, p65 translocated to the nucleus after TNFα stimulation increased the nuclear expression of TWIST. The change in p65 expression was consistent with TWIST.

In order to further determine whether the alteration in p65 expression has an effect on TNFα-induced TWIST expression, we analyzed and found that when p65 was inhibited by siRNA-65 or BAY11-7082, TWIST expression was also attenuated. The results indicate that the alteration in p65 expression affected TNFα-induced TWIST expression. Taken together, these data revealed that the NF-κB signaling pathway is involved in TNFα-induced EMT, and p65 activation regulates TWIST expression in TNFα-induced EMT. A recent study showed that TNFα induces EMT via upregulation of TWIST in breast cancer cells (26). TNFα can also upregulateSlug, which imparts breast cancer cells with a stem cell-like phenotype (30). Further research demonstrated that inflammation induces invasion and metastasis via NF-κB-mediated stabilization of Snail (12).

In contrast, several studies reported that EMT induced by TNFα requires the AKT/GSK-3β signaling pathway and revealed that AKT/GSK-3β-mediated stabilization of Snail is required for TNFα-induced EMT in colorectal cancer (10,11). Thus, further investigation is warranted to determine whether TWIST cooperates with other transcriptional factors, such as Snail and Slug regarding the regulation of TNFα-mediated EMT in FaDu cells.

In summary, we demonstrated that TNFα induces EMT via increased TWIST expression in human FaDu cells and promotes hypopharyngeal cancer migration and invasion. Furthermore, we elucidated that the NF-κB signaling pathway was activated in FaDu cells, which regulated TNFα-induced TWIST expression. The detailed mechanism is illustrated in Fig. 6. We conclude that TNFα induced EMT and promoted metastasis via NF-κB signaling pathway-mediated TWIST expression in hypopharyngeal cancer.

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References
