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ORIGINAL ARTICLE

Evidence that TNF-TNFR1-TRADD-TRAF2-RIP-TAK1-IKK pathway mediates constitutive NF-κB activation and proliferation in human head and neck squamous cell carcinoma

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Constitutively activated nuclear factor-κB (NF-κB) has been associated with a variety of aggressive tumor types, including head and neck squamous cell carcinoma (HNSCC); however, the mechanism of its activation is not fully understood. Therefore, we investigated the molecular pathway that mediates constitutive activation of NF-κB in a series of HNSCC cell lines. We confirmed that NF-κB was constitutively active in all HNSCC cell lines (FaDu, LICR-LON-HN5 and SCC4) examined as indicated by DNA binding, immunocytochemical localization of p65, by NF-κB-dependent reporter gene expression and its inhibition by dominant-negative (DN)-inhibitory subunit of NF-κB (IκBα), the natural inhibitor of NF-κB. Constitutive NF-κB activation in HNSCC was found to be due to constitutive activation of IκBα kinase (IKK); and this correlated with constitutive expression of phosphorylated forms of IκBα and p65 proteins. All HNSCC showed the expression of p50, p52, p100 and receptor-interacting protein; all linked with NF-κB activation. The expression of constitutively active NF-κB in HNSCC is mediated through the tumor necrosis factor (TNF) signaling pathway, as NF-κB reporter activity was inhibited by DN-TNF receptor-associated death domain (TRADD), DN-TNF receptor-associated factor (TRAF)2, DN-receptor-interacting protein (RIP), DN-transforming growth factor-β-activated kinase 1 (TAK1), DN-κ-Ras, DN-AKT and DN-IKK but not by DN-TRAF5 or DN-TRAF6. Constitutive NF-κB activation was also associated with the autocrine expression of TNF, TNF receptors and receptor-activator of NF-κB and its ligand in HNSCC cells but not interleukin (IL)-1β. All HNSCC cell lines expressed IL-6, a NF-κB-regulated gene product. Furthermore, treatment of HNSCC cells with anti-TNF antibody downregulated constitutively active NF-κB, and this was associated with inhibition of IL-6 expression and cell proliferation. Our results clearly demonstrate that constitutive activation of NF-κB is mediated through the TRADD-TRAF2-RIP-TAK1-IKK pathway, making TNF a novel target in the treatment of head and neck cancer.

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Introduction

Nuclear factor-κB (NF-κB) is a transcription factor that is activated in response to a variety of stimuli including stress, carcinogens, tumor promoters, inflammatory cytokines and growth factors (Aggarwal, 2004). This transcription factor primarily consist of RelA/p65, RelB, c-Rel, p105/p50, and p100/p52 subunits (Ghosh and Karin, 2002; Li and Verma, 2002; Pomerantz and Baltimore, 2002). In most cases, NF-κB resides in the cytoplasm as an inactive heterotrimer consisting of p50, p65 and IκBα. On activation, inhibitory subunit of NF-κB (IκBα) undergoes phosphorylation, ubiquitylation and degradation, thus leading to nuclear translocation of p50–p65 complex. Among all the activators, tumor necrosis factor (TNF) is perhaps the most potent activator of NF-κB and the pathway activated by this cytokine is better understood (Aggarwal, 2003). After TNF binds to its receptor (TNF receptor [TNFR]1 and TNFR2), TNFR2 sequentially recruits TNF receptor-associated death domain (TRADD), TNF receptor-associated factor (TRAF)2, receptor-interacting protein (RIP), TGF-β activated kinase (TAK)1 and IκBα kinase (IKK) proteins. The latter causes the phosphorylation of IκBα and NF-κB activation. On binding to the DNA, NF-κB regulates the expression of various gene products that control cell survival, proliferation, invasion, angiogenesis, metastasis, chemoresistance, radioresistance and inflammation (Aggarwal, 2004).

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Although rarely found in normal cells, various tumor cells have been shown to express constitutively active form of NF-κB (Shishodia and Aggarwal, 2004). More recently, constitutively active NF-κB activation has also been found in cancer patients and shown to correlate with prognosis of the disease (Buchholz *et al*., 2005; Izzo *et al*., 2006). Work from our laboratory and others have shown that NF-κB is constitutively active in head and neck squamous cell carcinoma (HNSCC) (Ondrey *et al*., 1999; Aggarwal *et al*., 2004), and play a critical role in the proliferation of the tumor cells (Aggarwal *et al*., 2004; LoTempio *et al*., 2005). Additionally, HNSCC have been shown to produce inflammatory cytokines such as interleukin (IL)-6 and IL-8 which are also regulated by NF-κB (Wolf *et al*., 2001; Wang *et al*., 2002; Thomas *et al*., 2004). IL-6 has been shown to acts as an autocrine growth factor for the HNSCC cells (Woods *et al*., 1998). Expression of vascular endothelial growth factor (VEGF) by HNSCC, a cytokine that mediates angiogenesis, is also connected with NF-κB signaling pathway (Bancroft *et al*., 2002). Molecular profiling of HNSCC also suggest the expression of multiple genes that are NF-κB regulated and are involved in growth, apoptosis and angiogenesis HNSCC (Dong *et al*., 2001; Loercher *et al*., 2004).

Why NF-κB is constitutively active in HNSCC is not fully understood. In the present report, we investigated the mechanism that is involved in constitutive activation of NF-κB. The results to be described show that TNF plays a major role in constitutive activation of NF-κB in HNSCC and that the inhibition of TNF cell-signaling pathway can suppress the proliferation of this tumor.

Results

HNSCC cell lines express constitutively active NF-κB

Like most other tumor cell lines, HNSCC cell lines have been shown to express constitutively active NF-κB (Aggarwal *et al*., 2004). To confirm that the HNSCC lines SCC4, LICR-LON-HN5 and FaDu expressed active NF-κB without extraneous cytokine induction, we performed electrophoretic mobility shift assay (EMSA) as described in Materials and methods (Figure 1a). For comparison, we analysed the embryonic kidney cell line A293 because this line has not shown constitutively active NF-κB in our previous experiments. All three HNSCC cell lines showed constitutive activation of NF-κB. The binding of the HNSCC nuclear protein to the NF-κB consensus oligonucleotide probe was dependent on the presence of p50 and p65, as indicated by a supershift assay in which antibodies against these proteins were added to the binding reaction (Figure 1b). DNA binding to wild-type oligonucleotide was not observed because it had been inhibited by the addition in the presence of an excess of unlabeled consensus oligonucleotide. In addition, the mutant oligonucleotide could not complete for the binding. These results indicate that the reaction was specific for the NF-κB consensus site.

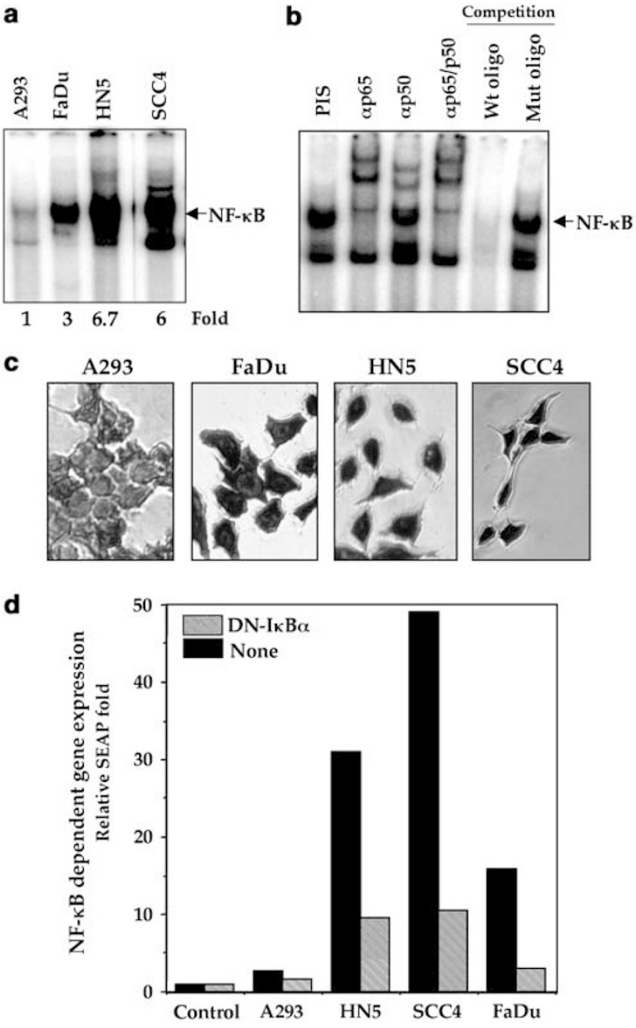

Figure 1 Head and neck cell lines express constitutive NF-κB. (**a**) Nuclear extracts prepared from 2×10^6 FaDu, LICR-LON-HN5 (HN5), SCC4 and A293 cells were tested for DNA binding by EMSA as described in Materials and methods. The levels of active NF-κB varied among cell lines, but HNSCC cells expressed substantially higher levels of active NF-κB than did A293 cells. (**b**) The binding of NF-κB to DNA is specific and consists of the p50 and p65 subunits, as indicated by a supershift assay. Nuclear extracts were preincubated with preimmune rabbit serum, anti-p65, anti-p50, anti-p65/anti-p50, cold oligonucleotide or mutated NF-κB binding site oligonucleotide for 15 min before the DNA binding reaction and then assayed by EMSA. (**c**) Immunocytochemical staining of cells with anti-p65 antibody using an HRP-tagged secondary antibody, as described in Materials and methods, indicated that p65 is localized in the nucleus of HNSCC cells but not A293 cells. HN5, LICR-LON-HN5. (**d**) NF-κB-dependent reporter gene expression is consistent with EMSA results. Cell culture media were tested for SEAP activity, as described in Materials and Methods, 48 h after transfection with either pBasic-SEAP, NF-κB-SEAP or NF-κB-SEAP plus pCMV-DN-IκBα plasmids. A293 cells had 2.7-fold, LICR-LON-HN5 (HN5) had 31.0-fold, SCC4 had 49.0-fold and FaDu had 15.9-fold higher basal NF-κB levels than did pBasic-SEAP. Introduction of the pCMV-DN-IκBα vector suppressed NF-κB-SEAP expression by 59% in A293, 70% in LICR-LON-HN5, 79% in SCC4 and 81% in FaDu cell lines.

We also visualized the location of the p65 subunit of NF-κB with immunocytochemical staining using a polyclonal antibody directed against the p65 subunit

of NF-κB (Figure 1c). The p65 protein was definitively localized in the nuclei of LICR-LON-HN5, SCC4, and, to a lesser extent, FaDu cells, consistent with DNA binding activity. The overall staining intensity in HNSCC cells appeared to differ substantially from that in A293 cells. A293 cells expressed far less p65 overall, and essentially none was located in the nucleus. Our experiments confirmed that although the level of NF-κB expression varied among HNSCC cell lines, they all expressed constitutively active NF-κB.

Neither the presence of p65 in the nucleus nor its DNA binding indicates that NF-κB is transcriptionally active, and whether NF-κB is transcriptionally active in HNSCC has not been demonstrated. Therefore, we determined the transcriptional activity of NF-κB using the secretory alkaline phosphatase (SEAP) reporter-gene assay system as discussed in Materials and methods (Figure 1d). The NF-κB reporter plasmid had no significant activity in A293 cells but had 5.8-fold higher activity in the FaDu cell line, 11.5-fold higher in LICR-LON-HN5 and 18.0-fold higher in SCC4 than that in A293. Inclusion of the IκBα dominant-negative (DN) plasmid in the transfections resulted in a dramatic downregulation of the expressed NF-κB-SEAP, clearly indicating that the observed SEAP expression was regulated through NF-κB (Figure 1d). Transfection using a null vector containing the SEAP reporter gene without an NF-κB promoter site was used as a negative control in each cell line. The fold difference in NF-κB activity was calculated, and null-SEAP expression was compared with NF-κB-SEAP expression. As a control for transfection efficiency, pSV-β-galactosidase was added to each transfection reaction, and the resulting SEAP values were normalized against the expressed β-galactosidase activity.

HNSCC cell lines express high levels of constitutive phosphorylated forms of IκBα and p65

NF-κB activity is regulated by the inhibitory protein IκBα. Under quiescent conditions, the IκBα protein is sequestered in the cytoplasm by its association with the NF-κB complex. After being stimulated with cytokines or other reagents, IκBα is phosphorylated by an activated IKK which subsequently targets IκB proteins for degradation via the proteosome complex (Ghosh and Karin, 2002; Li and Verma, 2002; Aggarwal, 2004). The resulting loss of the association between IκBα and NF-κB allows the translocation of NF-κB subunits to the nucleus, where DNA binding and transcriptional activation ensue. Typically, unstimulated cells express low or undetectable basal levels of phosphorylated IκBα or phosphorylated p65. The p65 protein is usually not found at any substantial level in the nucleus of unstimulated cells.

All three HNSCC cell lines had substantial levels of phosphorylated IκBα within the cytoplasmic protein fraction (Figure 2a). Treatment with the proteosome inhibitor *N*-acetyl-leu-leu-norleucinal (ALLN) increased the detectable level of phosphorylated cytoplasmic protein. Phosphorylated p65 was also observed in the cytoplasmic and nuclear fractions of HNSCC cells (Figure 2b and c). FaDu cells expressed the highest basal levels of cytoplasmic total p65 and IκBα, but the highest level of p65 was observed in the nuclear fraction of SCC4 cells (Figure 1a). This observation is consistent with the immunostaining results (Figure 1c). We then determined the basal expression of other relevant NF-κB subunits (Pomerantz and Baltimore, 2002). SCC4 cells expressed substantially more p105, p50, p100 and p52 than did A293 cells. FaDu and LICR-LON-HN5 cells also expressed higher levels of these proteins than did A293 cells. All three HNSCC cell lines expressed higher levels of RelB than did A293 cell lines (Figure 2d, bottom panel). RIP, which has been closely linked with TNF-induced NF-κB activation, was expressed at equal levels in all cell lines studied (Figure 2e).

IκBa kinase is constitutively active in HNSCC

We determined the basal activity of the IKK complex in HNSCC cells using an *in vitro* kinase assay as described in Materials and methods. IKK activity is essential for the activation of NF-κB through the phosphorylation and subsequent degradation of IκBα (Li *et al*., 2000; Ghosh and Karin, 2002; Li and Verma, 2002). The glutathione-*S*-transferase (GST)-IκBα substrate was phosphorylated by the immunoprecipitated IKK complex in all HNSCC cells, with no extraneous stimulation (Figure 3a). The IKK complex was less active in A293 cells, which was consistent with the observed lower quantity of phosphorylated IκBα in these cells (Figure 2a). IKK-α and IKK-β proteins were expressed in all cell lines.

The kinase that activates IKK is not known, but AKT may be responsible (Ozes *et al*., 1999; Romashkova and Makarov, 1999). We found that only one HNSCC line, LICR-LON-HN5, expressed constitutively active phosphorylated AKT, as determined by Western blot analysis using antiphospho-AKT (Figure 3b). It was interesting that A293 cells also expressed a substantial quantity of constitutively active phosphorylated AKT.

NF-κB-dependent gene expression is inhibited by DN plasmids of the TNF/TNFR1 pathway

Individual elements of the normal NF-κB activation pathway were systematically investigated by co-transfecting the NF-κB reporter vector (SEAP) and a collection of plasmids expressing DN proteins into the HNSCC cell lines. Similar results were found in all three cell lines. The results for LICR-LON-HN5 cells are reported as an example for all cell lines tested. NF-κB-dependent reporter expression was modulated in response to DN gene expression. To compensate for variations in reporter expression due to the addition of DNA, an equal quantity of a null plasmid (pCMV-flag) was co-transfected with the NF-κB-SEAP plasmid in the positive control sample. All transfections included the pSV-β-gal plasmid. SEAP values were normalized against the observed β-galactosidase results for each sample. In addition, the cell lines were transfected with, pBasic-SEAP, a null-SEAP plasmid that lacked

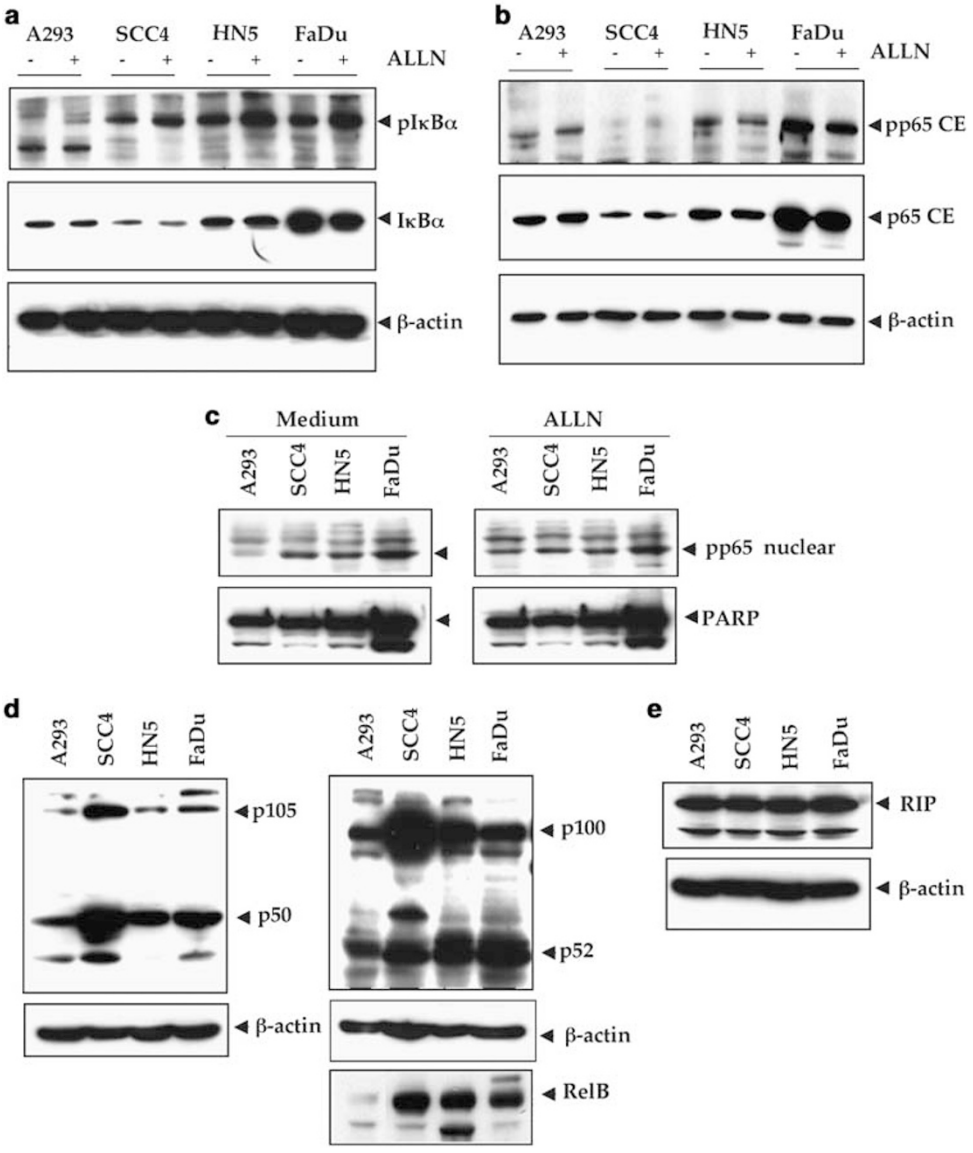

Figure 2 HNSCC cells express high levels of constitutively phosphorylated IκBα and p65. Cytoplasmic fractions were prepared from 3×10^6 cells that were either untreated or treated with the proteosome inhibitor ALLN (50 ng/ml for 30 min), as described in Materials and Methods, and then analysed by Western blot using the stated antibodies. Blots were tested for the presence of the phosphorylated protein and then stripped and reprobed for total protein and *β*-actin. (**a**) The cytoplasmic lysates (50 *μ*g protein) were analysed for phosphorylated IκBα (pIκBα) and IκBα, with *β*-actin as a loading control. Expression of phosphorylated IκBα was higher in HNSCC cells than in A293 cells. (**b**) This expression increased with the addition of ALLN, a reagent that blocks the normal degradative pathway of the protein. In addition, cytoplasmic lysates (50 *μ*g protein) were tested for phosphorylated p65 and p65, with *β*-actin as a loading control. HN5, LICR-LON-HN5. Cytoplasmic extracts are referred as CE. (**c**) Nuclear fractions (30 *μ*g protein) were tested for phosphorylated p65 (pp65), with poly (ADP-ribose) polymerase (PARP) as a loading control, by Western blot analysis. HN5, LICR-LON-HN5. (**d**) Whole-cell lysates (50 *μ*g protein) prepared from 3×10^6 cells were tested for other known subunits of the NF-κB complex: p105, p50, p100, p52 and RelB. HNSCC cells consistently expressed higher levels of these subunits than did A293 cells. HN5, LICR-LON-HN5. (**e**) Whole-cell lysates prepared as described above were tested for the expression of RIP; RIP was expressed in all cell lines. HN5, LICR-LON-HN5.

the NF-κB binding domain as a control for basal SEAP expression. All NF-κB-SEAP values were compared with those of the negative control pBasic-SEAP in each cell line.

The addition of a DN-IκBα vector inhibited NF-κB-dependent reporter expression by 76% in LICR-LON-HN5 cells (Figure 4a). Overexpression of the DN mutant plasmids of IKK-*β* inhibited NF-κB-dependent reporter expression by 57% in LICR-LON-HN5 cells. The inhibition in TNF-induced A293 cells was 40% with the IKK-*β* DN plasmid.

In the LICR-LON-HN5 cell line, inhibition of k-Ras downregulated NF-κB-dependent SEAP expression by 57%, whereas the observed inhibition with DN-AKT was 88%. It was interesting that DN-TRAF5 and DN-TRAF6 plasmids failed to downregulate NF-κB-dependent SEAP

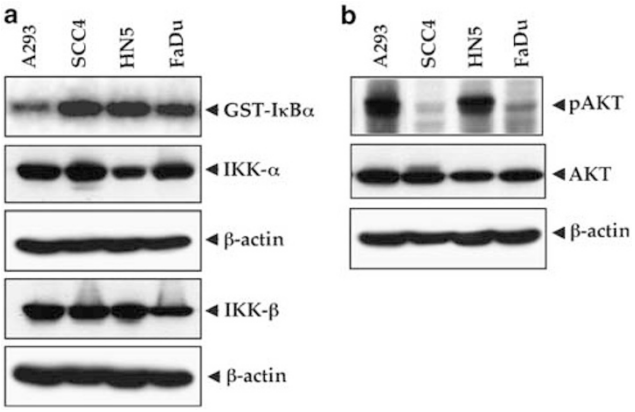

Figure 3 Protein kinases are constitutively active in HNSCC. (**a**) HNSCC cells express constitutively active IKK. Whole-cell lysates were prepared as described in Materials and methods and then assayed for IKK activity using the previously described protocol with the GST-IκBα substrate. Total levels of IKK-α, IKK-β and β-actin were determined by Western blot analysis of the same whole-cell extracts. HN5, LICR-LON-HN5. (**b**) Whole-cell extracts were analysed for phosphorylated AKT (pAKT) and total AKT, with β-actin as a loading control, by Western blot. The blots were initially probed with an antibody against the phosphorylated protein and then stripped and reprobed with an antibody to the total protein and β-actin. Phosphorylated AKT was present in all HNSCC cells but at substantially lower levels in FaDu and SCC4 cells than in LICR-LON-HN5 (HN5) cells.

expression in any cell line. Conversely, the introduction of a DN-TRAF2 plasmid dramatically inhibited SEAP expression in all cell lines tested. Consistent with these observations, TNF-induced NF-κB reporter activity was significantly inhibited by DN-TRAF2 but much less by DN-TRAF5 and DN-TRAF6 plasmids (Figure 4b). SEAP expression in HNSCC cells was inhibited by 89% in response to the introduction of a DN-TRADD plasmid. The results for TNF-induced NF-κB-dependent reporter expression in LICR-LON-HN5 cells were consistent with those in A293 cells with respect to the addition of DN-TRADD, DN-TRAF2, DN-TRAF5, DN-TRAF6 and DN-IKK. However, neither DN-AKT nor DN-k-Ras had an inhibitory effect on TNF-induced NF-κB-dependent SEAP expression in A293 cells (data not shown).

It has been shown that ubiquitylation of RIP is required for TNF induced NF-κB activation (Ea *et al*., 2006). Ubiquitinated RIP then interacts with transforming growth factor-β-activated kinase 1 (TAK1) through TAK1-binding protein (TAB1) to activate IKK complex (Li *et al*., 2006). Therefore, we tested the role of RIP and TAK1 in the constitutive activation of NF-κB in HNSCC cells. Figure 4c shows that DN-TAK1 (K63A mutant) (Blonska *et al*., 2005) inhibited the activation of NF-κB in a dose-dependent manner in HN5 cells. We found that another DN-TAK1 (K63W mutant) also inhibited, though weakly, the constitutive NF-κB activation (data not shown). Whether RIP has any role in the constitutive activation of NF-κB in HNSCC was also examined. We found that DN-RIP (K337R mutant) that lacks K63 polyubiquitylation site (Li *et al*., 2006) inhibited the constitutive activation of NF-κB in a dose-dependent manner in HN5 cells (Figure 4c). TNF-induced activation of NF-κB SEAP was also inhibited by DN-RIP and DN-TAK1 (Figure 4d).

The inhibitory effects of blocking TRADD, TRAF2, RIP and TAK1 activity on the constitutive activation of NF-κB in HNSCC cells strongly implicates the TNFR1/TNF-α pathway as the autocrine mechanism for persistent NF-κB activation in these cells. Both receptor activator of NF-κB (RANK) and IL-1β are known to signal through TRAF6. Blocking TRAF6 activity with the expression of a DN proteins failed to inhibit NF-κB-dependent SEAP expression to any great extent. Our results do not implicate either RANK or IL-1β as candidates for an autocrine activation loop in the HNSCC cells studied in this investigation.

TNF-α, TNFR1, receptor activator of NF-κB ligand, RANK and IL-6 transcripts are expressed in HNSCC

Although TNF, IL-1β and receptor activator of NF-κB ligand (RANKL) can potently activate NF-κB in most cells, our results suggest that the TNF-mediated pathway is involved in the constitutive activation of NF-κB in HNSCC. Whether TNF and its receptors are expressed in HNSCC was determined, with Raw 264.7 cells used as the positive control. The results (Figure 5a) show that all HNSCC cell lines expressed TNF transcripts. A293 cells lacked TNF expression. Our results also show that all cell types expressed TNFR1 transcripts, as determined by reverse transcription–polymerase chain reaction (RT–PCR) amplification (Figure 5a). Whether expression of the TNFR1 transcript correlates with expression of receptor protein, was investigated. We examined the expression of TNF receptor 1 in HNSCC cells by flow cytometry. We found that TNFR1 was expressed by all HNSCC cell lines (Figure 5b). However, the TNFR2 transcript, which is normally restricted to immune and endothelial cells, was expressed most prominently in FaDu and SCC38 cell lines. SCC38 cells did not express constitutive levels of NF-κB, as indicated by EMSA, and FaDu cells expressed lower levels than did LICR-LON-HN5 and SCC4 cells (Figure 1a).

All cell types expressed transcripts for RANKL with the exception of the osteoclastic cell line Raw 264.7. The HNSCC and Raw 264.7 cell lines expressed RANK, but no RT–PCR amplification was observed in the A293 cell line (Figure 5c). An RT–PCR analysis of HNSCC and A293 cells using oligonucleotide primers to amplify IL-1β indicated that no substantial levels of IL-1β were present in any cell type tested, although a weak signal was observed in Raw 264.7 cells (Figure 5d).

An RT–PCR analysis of HNSCC and A293 cells using oligonucleotide primers to amplify IL-6 indicated that RNA transcripts were present in all cell types (Figure 5e and f). The indicated PCR products were not generated in the absence of reverse transcriptase, confirming that amplification of these products was due to the presence of a specific target mRNA.

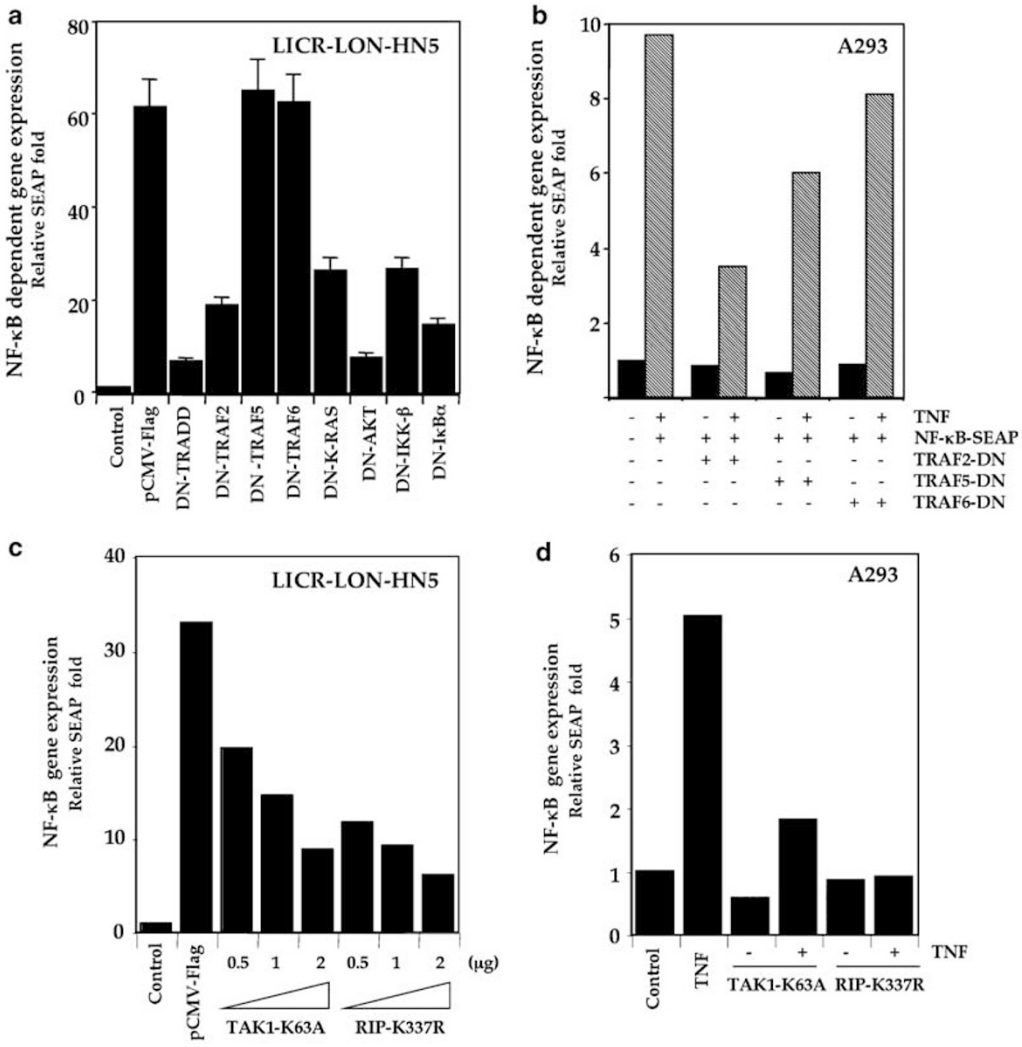

Figure 4 NF-κB-dependent gene expression is inhibited by DN proteins of the TNF/TNFR1 pathway. Co-transfections of the NF-κB-dependent expression vector NF-κB-SEAP (1.0 μg) with either an irrelevant pCMV-flag plasmid (0.5 μg) or a specific DN plasmid (0.5 μg) or as concentration indicated and the transfection control plasmid pSV-β-gal (0.25 μg) were performed using the Fugene 6 transfection reagent as described in Materials and methods. (**a** and **c**) The effects of a panel of DN genes on NF-κB-dependent SEAP expression were analysed in LICR-LON-HN5 cells as described in Materials and methods. The results are normalized against the observed β-galactosidase activity and the SEAP expression of pBasic-SEAP (control). (**b** and **d**) The effect of a panel of DN genes on TNF-induced NF-κB-dependent SEAP expression in A293 cells.

HNSCC cells express TNF and IL-6 proteins and anti-TNF antibody suppresses constitutive NF-κB activation

The expression of the TNF transcript alone is not sufficient to suggest that HNSCC expresses TNF protein. Thus, we determined the expression of TNF protein by Western blot analysis. The results clearly demonstrate that SCC4 cells expressed both the transmembrane (26 kDa) and processed (17 kDa) forms of TNF protein. NF-κB activation is also known to regulate the expression of IL-6. Whether HNSCC cells can produce IL-6 was examined. We found that SCC4 cells produced IL-6 protein (Figure 6b).

Whether constitutive activation of NF-κB in HNSCC was due to the autocrine expression of TNF was examined using antibodies against TNF. Treatment of cells with anti-TNF antibodies downregulated constitutive NF-κB activation in SCC4 (Figure 6c) and LICR-LON-HN5 (Figure 6d) cells, as examined by EMSA. Under these conditions, preimmune serum (PIS) had no effect (Figure 6c and d, left panels). Anti-TNF antibody also inhibited NF-κB-mediated reporter gene transcription in a dose-dependent manner in both cell lines (Figure 6e). Whether downregulation of NF-κB by anti-TNF antibody modulates the expression of IL-6, was also examined. We found that treatment of cells with anti-TNF antibodies downregulated the expression of IL-6 in SCC4 cells but control antibodies (PIS) had no effect (Figure 6f).

Proliferation of HNSCC cells is suppressed by anti-TNF antibody

We have shown that TNF is an autocrine growth factor for human cutaneous T-cell lymphoma (Giri and

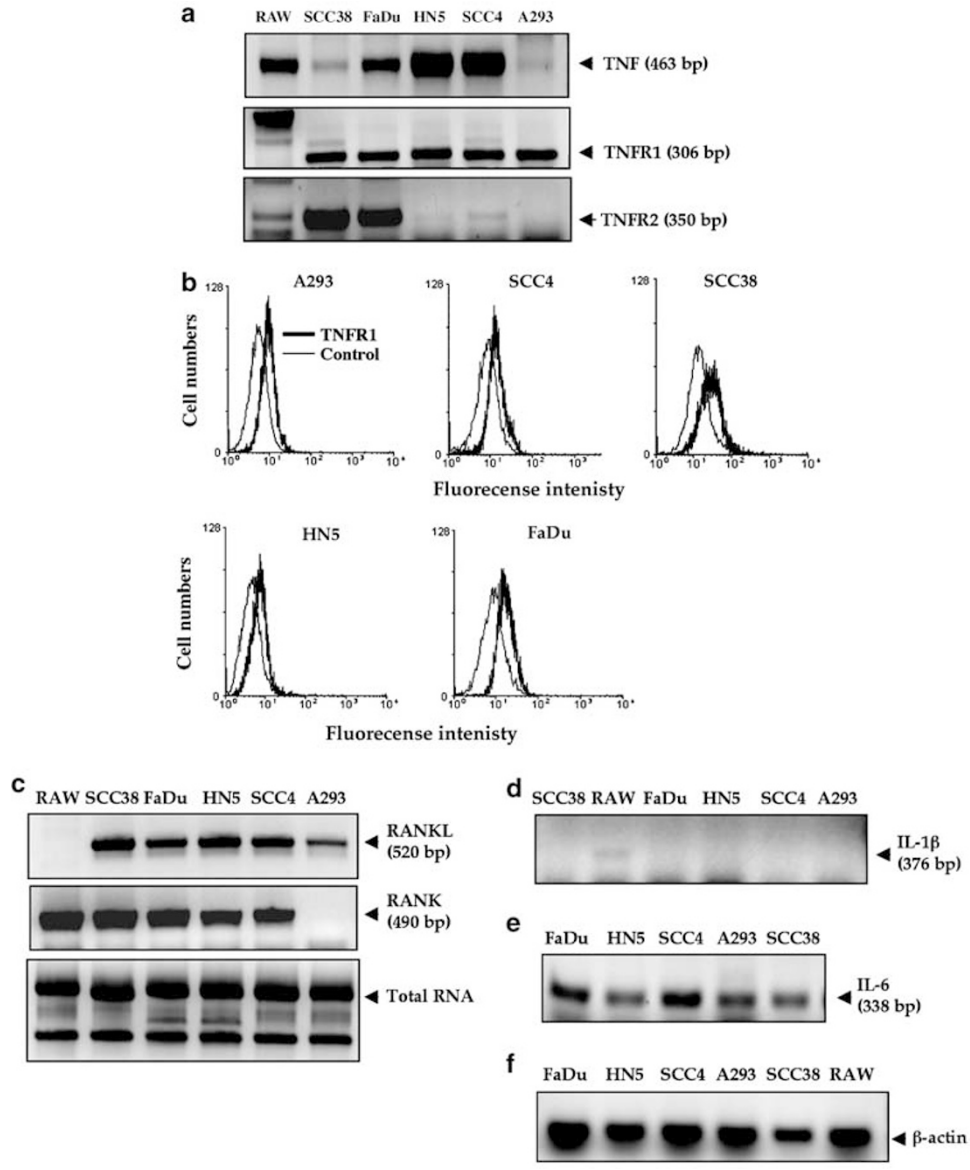

Figure 5 TNF, TNFR1, RANKL, RANK and IL-6 transcripts are present in HNSCC. Total RNA was prepared as described in Materials and methods. RT–PCR was performed on total HNSCC RNA (1.0 μg) with SuperScript One-Step RT–PCR reagents, as described, using various specific oligonucleotide primers. Primer sequences and PCR conditions are listed in Materials and methods. (**a**) RT–PCR analysis of HNSCC and A293 using oligonucleotide primers to amplify TNFR1 and TNF. All cell types appeared to contain TNFR1 transcripts, but only HNSCC cell lines expressed transcripts for TNF. HN5, LICR-LON-HN5. For panel (**b**) cells were preincubated with 10% goat serum for 20 min and washed, and then monoclonal rabbit IgG anti-TNFR1 antibodies were added. Following 1 h incubation at 4°C, the cells were washed and incubated for an additional 1 h in a FITC-conjugated goat anti-rabbit IgG antibodies. The cells were analysed using a FACSCalibur flow cytometer. (**c**) RT–PCR analysis of HNSCC and A293 cells using oligonucleotide primers to amplify RANKL and RANK. All cell types expressed transcripts for RANKL, but only HNSCC cell lines included transcripts for RANK. (**d**) RT–PCR analysis of HNSCC and A293 cells using oligonucleotide primers to amplify IL-1β. No significant levels of IL-1β were detected in any cell type, although a weak signal was observed in SCC4 cells. HN5, LICR-LON-HN5. (**e**) RT–PCR analysis of HNSCC and A293 cells using oligonucleotide primers to amplify IL-6. All cell types tested expressed IL-6 transcripts. HN5, LICR-LON-HN5. (**f**) RT–PCR of HNSCC and A293 cells, with β-actin as a control. HN5, LICR-LON-HN5.

Aggarwal, 1998), glioblastoma (Aggarwal *et al*., 1996) and B cells (Estrov *et al*., 1993). As HNSCC expressed TNF that leads to constitutive activation of NF-κB, it is possible that TNF expression leads to proliferation of HNSCC. We found that indeed anti-TNF antibody inhibited the proliferation of two different HNSCC cell lines, whereas PIS did not, indicating that TNF is an autocrine growth factor for HNSCC (Figure 7).

Discussion

Most tumor cell types, and not normal cells, express constitutively active NF-κB, through a mechanism that is not fully understood. In the present report, we found constitutive activation of NF-κB in three different HNSCC cell lines. NF-κB was transcriptionally active and was inhibited by DN-TRADD, DN-TRAF2,

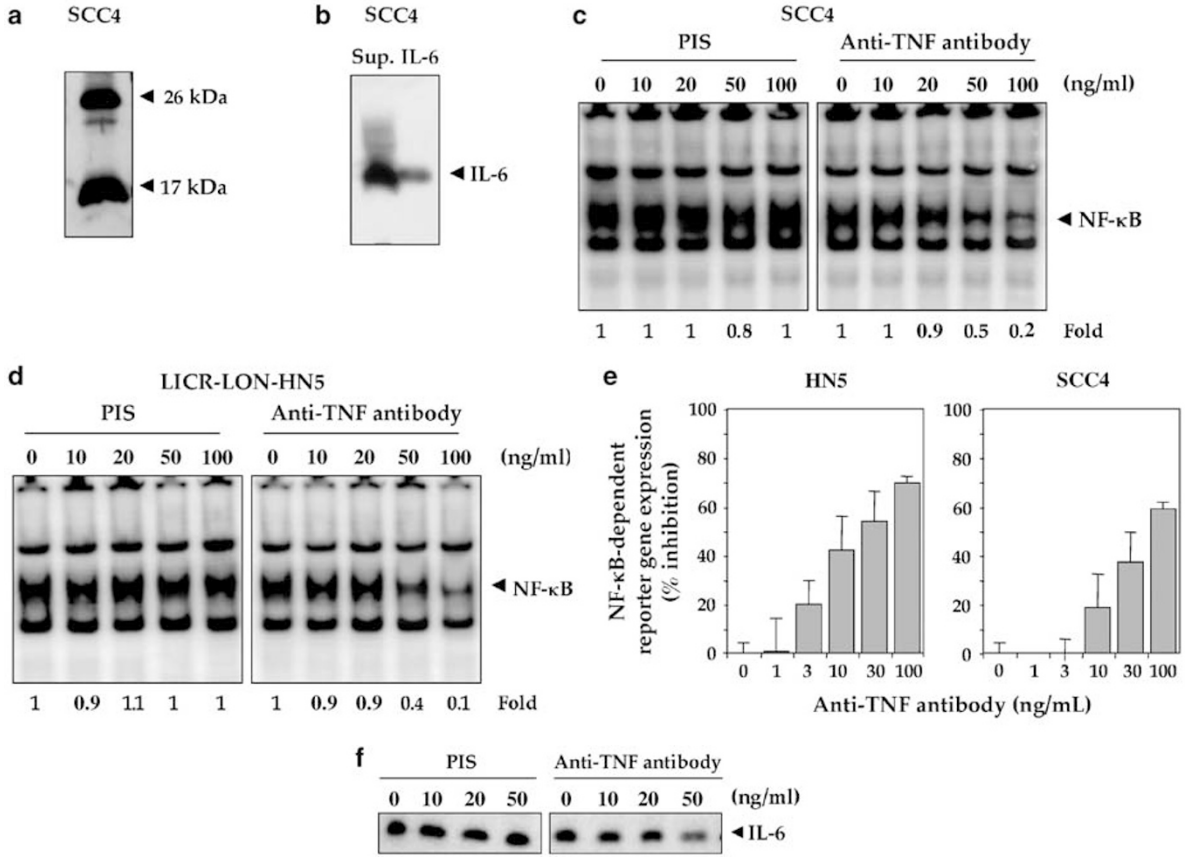

Figure 6 TNF protein is expressed in HNSCC cells, and anti-TNF antibody inhibits constitutive NF-κB activation. For (**a**) whole-cell extracts were prepared from HNSCC cells, resolved on 15% SDS–PAGE gel, and probed with anti-TNF antibodies. For (**b**) HNSCC cells are incubated with culture media for 24 h and then supernatant was resolved on 12% SDS–PAGE gel, and probed with antibody to IL-6. For (**c** and **d**), cells were incubated with the indicated concentrations of anti-TNF antibody for 72 h, nuclear extracts were prepared, and EMSA was performed to assay NF-κB activity. (**e**) TNF neutralizing antibody inhibits constitutive NF-κB reporter activity in HNSCC. Plated 1×10^5 cells were transfected with NF-κB-reporter plasmid (SEAP) for 24 h and incubated with anti-TNF antibody for 24 h before undergoing the SEAP assay as described in Materials and methods. (**f**) Cells were incubated with the indicated concentrations of anti-TNF antibody for 72 h, and then supernatant were resolved on 12% SDS–PAGE gel, and probed with antibody to IL-6.

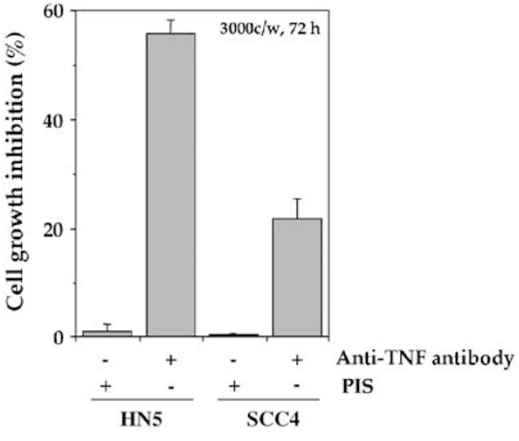

Figure 7 Anti-TNF antibody inhibits the proliferation of HNSCC cells. Three thousand cells were seeded in 96-well plates and incubated in triplicate with anti-TNF antibody or PIS for 72 h before undergoing the MTT assay as described in Materials and methods.

DN-RIP, DN-TAK1, DN-k-Ras, DN-AKT and DN-IKK but not DN-TRAF5 or DN-TRAF6. Constitutive NF-κB activation was also associated with the autocrine expression of NF-κB-regulated gene product. Furthermore, suppression of TNF cell signaling pathway in HNSCC cells downregulated the constitutively active NF-κB, and proliferation of cells (Figure 8).

Using DNA binding and immunocytochemical analysis, we and others have previously shown that NF-κB is constitutively active in HNSCC (Ondrey *et al*., 1999; Aggarwal *et al*., 2004). Our results, for the first time, now demonstrate that HNSCC cells express transcriptionally active NF-κB. However, why NF-κB is constitutively active in HNSCC cells is unknown. We found that constitutive activation of IKK leads to phosphorylation of IκBα, which could lead to NF-κB activation. We detected the phosphorylated form of IκBα even without ALLN, suggesting that the rate of IκBα synthesis is faster than the rate of IκBα degradation in HNSCC cells. ALLN increased the

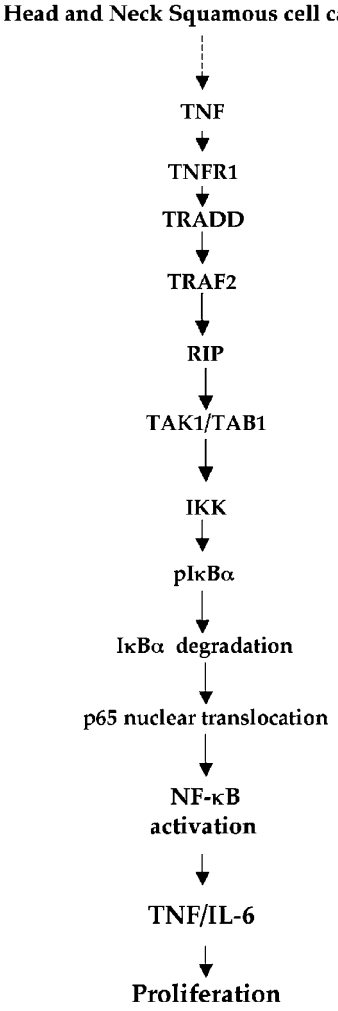

Figure 8 Proposed pathway of the constitutive activation of NF-κB in HNSCC cells. The scheme includes potential avenues of the activation of the IKK complex, which ultimately leads to NF-κB activation and cell proliferation. Active NF-κB induces the transcription of many genes associated with neoplastic conditions, such as antiapoptotic, proproliferative and proinflammatory genes.

phosphorylated levels of IκBα, but only slightly. DN-IKK inhibited NF-κB-mediated reporter activity, suggesting that this pathway is functionally active in HNSCC.

Why IKK is constitutively active in HNSCC is not clear. Numerous kinases have been linked to IKK activation. We found that both RIP and TAK1 are required for the constitutive activation of NF-κB in HNSCC and that induced by TNF. However, we also found evidence to suggest that AKT causes constitutive NF-κB activation in HNSCC cells. First, AKT was constitutively maximally active in at least one of the HNSCC cell lines (LICR-LON-HN5). Second, DN-AKT inhibited NF-κB-mediated reporter activity in all HNSCC cell lines. Third, the pharmacologic inhibitors of phosphatidylinositol 3-kinase suppressed NF-κB-mediated reporter activity in all cell lines (data not shown). We also found that DN-Ras blocked the constitutively active NF-κB reporter in HNSCC. Evidence from our laboratory and others showed that Ras is needed for NF-κB activation (Finco *et al*., 1997; Mayo *et al*., 1997; Takada *et al*., 2004).

NF-κB activation by RANKL and IL-1β has been found to be mediated through TRAF6 (Cao *et al*., 1996; Darnay *et al*., 1999). Neither DN-TRAF-6 nor DN-TRAF5 blocked constitutive NF-κB in HNSCC cells. These results indicate that NF-κB activation in HNSCC is not mediated through the RANKL- or IL-1β-activated pathways. We evaluated HNSCC cells for expression of RANKL and IL-1β and found that they expressed TNF and RANKL but not IL-1β. All cell lines also expressed TNFR1 and RANK receptors. Although all HNSCC cells expressed RANKL and RANK, our results suggest that this pathway has only a minor role in constitutive NF-κB activation. As DN-TRAF2, DN-RIP and DN-TAK1, all suppressed both constitutive and TNF-inducible NF-κB activation, thus suggesting that the TNF-mediated pathway is involved in HNSCC. The constitutive expression of TNF and TNFR1 also points to the involvement of the TNF pathway. Furthermore, we found that both the processed and transmembrane forms of TNF protein were expressed in HNSCC cells, and treatment with anti-TNF antibody downregulated constitutive NF-κB activation and NF-κB regulated gene products.

We found that inhibition of the engagement of TNF to its receptor using anti-TNF antibody inhibited the proliferation of cells, indicating that TNF is an autocrine growth factor in HNSCC. NF-κB-regulated gene product IL-6 was also expressed in HNSCC cells. These results are in agreement with those of a previous report (Duffey *et al*., 1999). IL-6 has also been shown to promote the proliferation of HNSCC (Woods *et al*., 1998). We and others (Aggarwal *et al*., 2004; Loercher *et al*., 2004) have shown that cyclooxygenase-2 and several other NF-κB-regulated products are constitutively expressed in HNSCC and could promote HNSCC cell proliferation. That TNF is an autocrine growth factor for HNSCC is consistent with its effects in other tumors, including B-cell lymphoma (Estrov *et al*., 1993), glioblastoma (Aggarwal *et al*., 1996) and cutaneous T-cell lymphoma (Giri and Aggarwal, 1998).

Antibodies against TNF (adalimumab and infliximab) and a soluble decoy receptor to TNF (etanercept) have been approved for the treatment of rheumatoid arthritis and inflammatory bowel disease (Aggarwal *et al*., 2006). Our results suggest that these TNF blockers may also be effective in the treatment of HNSCC. Small molecules such as thalidomide and curcumin have also been shown to downregulate TNF expression and signaling by inhibiting the NF-κB activation pathway (Singh and Aggarwal, 1995; Majumdar *et al*., 2002). On the basis of the results presented here, these blockers may be useful in the treatment of HNSCC and other tumors.

Materials and methods

Materials

Bacteria-derived human recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 U/mg, and

nonessential amino acids, sodium pyruvate, vitamins, Dulbecco's modified Eagle medium (DMEM), MEM and fetal bovine serum (FBS) were obtained from Invitrogen (Grand Island, NY, USA). ALLN was purchased from Calbiochem (San Diego, CA, USA). The labeled streptavidin-biotin (LSAB)+ system, liquid 3,3′-diaminobenzidine substrate chromogen system, serum-free protein-blocking solution, and antibody diluent were purchased from Dako (Carpinteria, CA, USA). Lab Tech II chamber slides were obtained from Nalge Nunc International (Nasperville, IL, USA).

Antibodies

Anti-β-actin antibody (1:10 000) was obtained from Sigma-Aldrich (St Louis, MO, USA). The antibodies anti-p65, anti-p50, anti-IκBα, anti-poly (adenosine diphosphate (ADP)-ribose) polymerase (PARP), anti-p100, anti-p105, anti-relB, anti-RIP, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and diluted at 1:2000. Phosphospecific anti-IκBα (Ser32), anti-p65 (Ser536) and anti-AKT (Ser473) were purchased from Cell Signaling (Beverly, MA, USA) and diluted at 1:1000. Anti-IKK-α and anti-IKK-β antibodies were provided by Imgenex (San Diego, CA, USA). Anti-TNF antibodies were obtained from BD Biosciences Pharmigen (San Diego, CA, USA). Anti-IL-6 antibody was kindly supplied by Dr T Kishimoto from Osaka University, Japan.

Cell culture

A293 (human embryonic kidney), RAW 264.7. (mouse macrophage cell line) and FaDu (human squamous cell carcinoma) cells were obtained from American Type Culture Collection (Manassas, NJ, USA). LICR-LON-HN5 and SCC4 human squamous cell carcinoma cells were obtained from Dr MJ O'Hare (Haddow Laboratories, Institute of Cancer Research, Sutton, Surrey, UK). A293 cells were cultured in DMEM supplemented with 10% FBS. LICR-LON-HN5, SCC4 and SCC38 cells were cultured in DMEM containing 10% FBS, 100 μM nonessential amino acids, 1 mM pyruvate, 6 mM L-glutamine and 1 × vitamins. The culture medium was also supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

Cellular extract buffers

Lysis buffer A contained 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (pH 7.9), 10 mM KCl, 0.1 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA), 0.1 mM ethylene glycol-bis-(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1.0 mM dithiothreitol (DTT), 1.0 mM phenylmethylsulphonyl fluoride (PMSF), 2 mg/ml leupeptin, 2 mg/ml aprotinin and 0.5 mg/ml benzamidine. Lysis buffer B (nuclear extract buffer) contained 20 mM HEPES (pH 7.9), 400 mM NaCl, 1.0 mM EDTA, 1 mM EGTA, 1.0 mM DTT, 0.5 mM PMSF and the protease inhibitors stated above and in the same concentrations. Whole-cell extract buffer contained 20 mM HEPES (pH 7.9), 250 mM NaCl, 0.5 mM EGTA, 0.4 mM EDTA, 6.7 mM NaF, 1.3 mM Na_3VO_4, 0.5 mM PMSF, 1.0% NP-40, 2 mg/ml leupeptin and 2 mg/ml aprotinin.

Electrophoretic mobility shift assay

To determine NF-κB activation, we performed an EMSA as described previously (Chaturvedi *et al*., 2000). In brief, cellular nuclear extracts were incubated with ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (12 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat, 5′-TTGTTACAA**GGGACTTT**CCGCTG**GGGACTTT**CCAGGGA-GGCGTGG-3′ (boldface indicates NF-κB binding sites) for 30 min at 37°C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TTGTTACAA**CTCACTTT**CCGCTG**CTCACTTT**CCAGGGAGGCGTGG-3′, was used to determine the specificity NF-κB and DNA binding. The binding specificity was also evaluated by competition with the unlabeled oligonucleotide. For supershift assays, cellular nuclear extracts were incubated with antibodies against either p50 or p65 of NF-κB for 15 min at 37°C, and the complex was analysed by EMSA. Preimmune serum (PIS) was included as a negative control. The dried gels were visualized, and radioactive bands were quantitated by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) using Imagequant software.

Kinase assay

To determine the levels of active IKK or c-Jun N terminal kinase (JNK) present in cells, IKK and JNK assays were performed by a method described previously (Manna *et al*., 2000). In brief, the IKK complex and JNK from whole-cell extracts were precipitated with antibodies against either IKK-α or JNK, respectively. This was followed by treatment with protein A/G-Sepharose beads (Pierce, Rockford, IL, USA). After a 2 h incubation, the beads were washed with lysis buffer and then assayed in a kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 2 mM DTT, 20 mCi [γ-^{32}P] ATP, 10 mM unlabeled ATP and 2 μg of substrate GST-IκBα (aa1–54) or GST-jun. The samples were incubated at 30°C for 20 min, and the reaction was terminated by boiling the sample with sodium dodecyl sulfate (SDS) sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-polyacrylamide gel electrophoresis (PAGE), the gel was dried, and the radioactive bands were visualized with a PhosphorImager. To determine the total amounts of IKK-α, IKK-β, or JNK in each sample, 50 μg of the whole-cell protein was resolved on 7.5% SDS–PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK-α, anti-IKK-β, or anti-JNK antibodies.

NF-κB-dependent reporter gene expression assay/SEAPassay

The NF-κB-dependent reporter gene expression assay was performed (Manna *et al*., 2000) according to the manufacturer's protocol, as previously described (Clontech, Palo Alto, CA, USA).

DN mutants of pRK-TNFR1-associated death domain protein (TRADD)-myc, pcDNA3-TNFR-associated factor (TRAF)-2-myc, pCR-TRAF-5-flag, pCR-TRAF-6-flag, pCR-IKK-β-flag, pAKT, pk-RAS N17, pMyc-RIP (K377R) (Li *et al*., 2006), pFlagTAK1 (K63A) (Blonska *et al*., 2005) and pCMV-IκBα were introduced, and their effects on NF-κB-dependent reporter gene transcription were analysed using a SEAP assay as previously described (Manna *et al*., 2000). In brief, A293 cells (5×10^5 cells/well) were plated in six-well plates and transiently transfected with Fugene 6 reagent (Roche Molecular Biochemicals, Mannheim, Germany), pNF-κB-SEAP (0.5 μg) and pSV-β-gal (0.25 μg) (Promega Corporation, Madison, WI, USA). To determine the expression of TNF-induced reporter genes, A293 cells were transfected with 0.5 μg of SEAP expression plasmid, 0.25 μg of β-gal plasmid, and 0.5 μg of either pCMV-flag1 DNA or a DN plasmid for 24 h. Thereafter, cells were incubated for 24 h with 1 nM TNF. HNSCC cell lines were transfected with 1.0 μg of SEAP expression plasmid, 0.25 μg of β-gal plasmid, and 0.5 μg of pCMV-flag1 plasmid or a DN plasmid. The A293 cell

culture medium was harvested after 24 h of TNF treatment, and the HNSCC cell culture medium was harvested 48 h after transfection. The media were then analysed for SEAP activity according to the protocol essentially as described by the manufacturer (Clontech), using a 96-well fluorescence plate reader (Victor^3, Perkin-Elmer, Shelton, CT, USA) with excitation set at 360 nm and emission at 460 nm. Transfection efficiencies were normalized by assaying the β-galactosidase enzyme activity of transfected cellular lysates according to the manufacturer's protocol (Promega, Madison, WI, USA) and dividing the observed SEAP value by the resultant value of β-galactosidase (mU). In addition, NF-κB-dependent SEAP expression was compared with SEAP activity produced from the null pBasic-SEAP vector (Clontech) transfected into the identical cell line.

Immunocytochemical analysis to determine NF-κB nuclear localization

The nuclear localization of p65 was visualized using the LSAB+ horseradish peroxidase (HRP) system (Dako).

Cells were plated (1×10^5 cells/ml) onto Lab Tech II glass chamber slides (Nalge Nunc International) and incubated overnight for adherence. The slides were washed three times with phosphate-buffered saline (PBS) and air-dried for 1 h. The cells were fixed for 30 min with 4% paraformaldehyde/PBS (pH 7.4) solution, washed three times with PBS, and treated with 0.2% TritonX-100/PBS for 30 min, washed with PBS again, and blocked with Dako protein serum-free blocking solution for 1 h before being incubated overnight at 4°C with a polyclonal anti-p65 antibody diluted to 1:200 in Dako antibody diluent. The cells were then washed three times with PBS and treated with Dako LSAB+ HRP secondary antibody cocktail for 1 h at ambient temperature. After being washed, the cells were blocked with 0.3% H_2O_2 in methanol for 30 min and then washed again. The cells were treated with Dako LSAB+ streptavidin HRP reagent for 1 h at ambient temperature before being washed, treated with liquid 3,3′-diaminobenzidine substrate-chromogen for 5 min, and washed again with distilled water. The nucleus was stained with hematoxylin (Dako). Stained slides were mounted with DPX mounting fluid and analysed with a Labophot-2 microscope (Nikon, Tokyo, Japan). Images were captured with a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX, USA) using MetaMorph software, version 4.6.5 (Universal Imaging Corp., Dowingtown, PA, USA).

Western blot analysis

Thirty to fifty micrograms of cellular lysates were prepared as described (Takada *et al*., 2003). Proteins were resolved on SDS–PAGE gel (7.5–15.0%). After undergoing electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with either 5% nonfat milk or 3% bovine serum albumin in PBS, and probed with the antibody of interest.

RT–PCR

RT–PCRs were carried out in a 25 μl volume using SuperScript One-Step RT–PCR reagents according to the manufacturer's protocol (Invitrogen). In brief, 1 μg of total RNA was added to the reaction mix and incubated for 30 min at 50°C, followed by 2 min at 95°C to denature the cDNA, 40 cycles of amplification at 95°C for 1 min, and annealing of primers at 72°C for 2 min. The PCR products were resolved on 1.5% tris acetate EDTA/agarose gel with ethidium bromide and visualized using FluorChem IS-8900 software with Alpha Ease FC stand-alone software version 4.0 (Alpha Innotech, San Leandro, CA, USA).

RT–PCR oligonucleotide primers

TNF-α: 5′-AGTTCTATGGCCCAGACCCT-3′ (S), 5′-CGGACTCCGCAAAGTCTAAG-3′(AS), annealing temperature 55°C, 463 bp; RANK: 5′-GGGAAAGCACTCACAGCTAATTTG-3′(S), 5′CAGCTTTCTGA-ACCCACTGTG-3′ (AS), annealing temperature 55°C, 490 bp; RANK ligand (RANKL): 5′-CGTTGGATCACAGCACATCAG-3′(S), 5′-AGTATGTTGCATCCTGATCCG-3′ (AS), annealing temperature 55°C, 520 bp; TNFR1: 5′-TACATTGCAGCCTC-TGCCTC-3′ (S), 5′-AGAGCTTGGACTTCCACCGT-3′(AS), annealing temperature 50°C, 306 bp; TNFR2: 5′-ACATCAGACGTGGTGGTGCAA-3′ (S), 5′-CCAACTGGAAGAGCCAAGTC-3′ (AS), annealing temperature 50°C, 350 bp; IL-6: 5′-GTGTCCTCATTGAATCCAGATTGG-3′ (S), 5′-AGCTCAGCTATGAACTCC-TTCTC-3′ (AS), annealing temperature 65°C, 338 bp; IL-1β: 5′-GCAGACAACTGCA-CTACAGGCTC-3′ (S), 5′-GCTCTGCTTGTGAG-GTGCTGATG-3′(AS), annealing temperature 55°C, 376 bp; and β-actin: 5′-GGGTCAGAAGGATTCCTATG-3′ (S), 5′-GGTCTCAAACATGATCTGGG-3′ (AS), annealing temperature 60°C, 230 bp.

3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium. Bromide Assay

The effect of anti-TNF on cell growth was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method as previously described (Takada *et al*., 2004).

Cell surface expression of TNFR1

For analysis of cell surface expression of TNFR1, cells were harvested and suspended in Dulbecco's PBS containing 1% FBS and 0.1%. sodium azide. The cells were preincubated with 10% goat serum for 20 min and washed, and then monoclonal rabbit IgG anti-TNFR1 antibodies were added. Following 1-h incubation at 4°C, the cells were washed and incubated for an additional 1 h in a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibodies. The cells were analysed using a FACSCalibur flow cytometer and CellQuest acquisition and analysis programs (BD Biosciences, San Jose, CA, USA).

Abbreviations

ALLN, *N*-acetyl-leu-leu-norleucinal; IκB, inhibitory subunit of NF-κB; IKK, IκBα kinase; JNK, c-Jun N terminal kinase; MAPK, mitogen-activated protein kinase; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; TRAF, TNF receptor-associated factor; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; TNF, tumor necrosis factor; TRADD, TNF receptor-associated death domain; RIP, receptor-interacting protein; TAK1, transforming growth factor-β-activated kinase 1; TAB1, TAK1-binding protein.

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