Nuclear Factor-kappaB Pathway as a Therapeutic Target in Head and Neck Squamous Cell Carcinoma: Pharmaceutical and Molecular Validation in Human Cell Lines Using Velcade and siRNA/NF-κB.

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Abstract. Background: Nuclear factor-kappaB (NF-κB) is synthesized in the cytoplasm, complexed with its inhibitor, I-κB, and NF-κB is released in an activated (phosphorylated) form following phosphorylation of I-κB and proteasomal degradation of the NF-κB•p-κB complex. The free p-NF-κB can then be translocated to the nucleus where it effects transcriptional activation of genes leading to the synthesis of proteins that are generally pro-growth and anti-apoptosis.

Objective: To gain insight into the role of the NF-κB pathway in head and neck squamous cell carcinoma (HNSCC), we selected two HNSCC cell lines, SCC-15 of lingual origin and FaDu of pharyngeal origin, with constitutively activated (phosphorylated) NF-κB. We assessed the impact of interrupting the NF-κB pathway at the level of proteasomal degradation using Velcade (bortezomib), a proteasome inhibitor, and at the pretranslational level in the synthesis of NF-κB using a small interfering RNA (siRNA).

Results: Velcade produced a dose-dependent inhibition of cell growth in both cell lines. At 30 nM, Velcade inhibited cell growth in the SCC-15 cell line by 40%. In both cell lines, Velcade induced nuclear overexpression of p21WAF1, an inhibitor of G1 cell cycle progression, which appeared to be independent of p53 expression. Addition of siRNA augmented the inhibitory effects of Velcade in FaDu cells; siRNA/NF-κB alone led to a 48% decline in basal expression of NF-κB protein levels and effected a 25% inhibition of cell growth. In the presence of Velcade (30 nM), siRNA/NF-κB increased growth inhibition from 43 to 65%.

Conclusions: The mechanisms involved in growth inhibitory effects of Velcade on HNSCC cell lines include the NF-κB pathway, suggesting the possible therapeutic use of Velcade or other NF-κB pathway inhibitors (eg, curcumin). The data also suggest that combining siRNA/NF-κB with Velcade might achieve greater reduction in the growth of HNSCC in patients with constitutively activated NF-κB.

Keywords: squamous cell carcinoma, NF-κB, Velcade, siRNA

Introduction

Recent evidence implicates the NF-κB pathway in the pathobiology of HNSCC. Tamatani et al [1] showed that there was higher NF-κB binding activity and I-κB kinase alpha protein expression in head and neck carcinoma cells, compared to normal oral epithelial and salivary gland cells. Kato et al [2] correlated radiation resistance in human head and neck squamous cell carcinoma cell lines with activation of NF-κB and showed that gene transfer of a mutant I-κB inhibited NF-κB and sensitized the cells to radiation. Zhang et al [3] showed that overexpressed and translocated p-NF-κB is a marker for poor prognosis in patients with squamous cell carcinoma of the tonsil.
The goal of this study was to gain further insight into the role of the NF-κB pathway in HNSCC. Specifically, in two human HNSCC cell lines with constitutively activated (phosphorylated) NF-κB, we tested the impact of interrupting the NF-κB pathway at the level of translational synthesis of NF-κB using siRNA/NF-κB and at the level of proteasomal degradation/activation using Velcade. Our findings present pharmaceutical and molecular evidence for the NF-κB pathway as a therapeutic target in HNSCC with constitutively activated NF-κB.

Materials and Methods

**Human HNSCC cell lines.** FaDu and SCC-15 cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). These cell lines were derived from patients with head and neck squamous cell carcinomas of the pharynx and tongue, respectively. They were grown in Dulbecco’s Modified Eagle’s Medium-F12 (1:1 [Gibco-BRL, Gaithersburg, MD]), supplemented with 10% fetal bovine serum and cultured in 95% air/5% CO₂.

**Inhibition studies.** Pharmaceutical agents used as potential inhibitors of cell growth (proliferation) included Velcade (bortezomib; Millenium Pharmaceuticals, Cambridge, MA), obtained from the pharmacy of Geisinger Medical Center. Velcade was dissolved in distilled water. The stock solution was diluted in culture medium and added to each of the cell lines to give final desired concentrations. (Separate experiments tested the impact of the vehicle alone on each of the cell lines, and no effects were identified.) After 2 days of incubation with and without Velcade, viable cells in each well were determined colorimetrically (Cell Titer 96 Aqueous One Solution Proliferation Assay, Promega, Madison, WI). The inhibition rate (%) was calculated as the control proliferative result minus the treated proliferative result, divided by the control proliferative result (times 100).

**siRNA transfection procedure.** Human NF-κB p65 siRNA was purchased as a pre-validated single sequence siRNA duplex (Cell Signaling Technology, Beverly, MA). After growing for 24 hr in 12-well or 24-well dishes, FaDu cells (which were selected because of their relatively rapid growth rate and short time to confluence) were transfected at 50-70% confluence. NF-κB p65 siRNA was used in the transfection, carried by TransIT-siQUEST transfection reagent (Mirus Bio Corporation, Madison, WI). After 24-hr transfection, the medium was replaced by fresh culture medium, and 24 hr later, cells were ready for treatment. Initially, a dose-response experiment was carried out to test the impact of transfection with 0, 50, 100, or 150 nM of siRNA/NF-κB p65 on the protein level of NF-κB. Subsequently, 100 nM of NF-κB p65 was used in transfection experiments to test the effect of this siRNA on the growth of FaDu cells with and without Velcade.

**Western blotting.** Control and pharmaceutical-agent-treated HNSCC cells were harvested and sonicated. Whole cell or nuclear homogenates (30 µg total protein per lane) were separated on 6-12% SDS PAGE and transferred onto PVDF membranes. Primary antibodies used in the immunostaining procedure included those against phosphorylated proteins, p-Akt [Ser 473], p-NF-κB p65 [Ser536], (Cell Signaling Technology, Beverly, MA), and other proteins (I-κB, p53, p21WAF1, and actin, Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was horseradish-peroxidase-linked. Immunoreactive proteins were visualized by a chemiluminescence-Western blotting system (Amersham).

**Statistics.** In vitro inhibitory rates were expressed as mean ± SE. An unpaired t-test was used to compare the mean inhibitory rates between 2 cell lines or between non-siRNA treated cells versus siRNA treated cells (p < 0.05 was considered significant).

Results

**Inhibitory effects of Velcade.** Dose-response patterns of inhibition were seen in SCC-15 and FaDu cells after incubation with Velcade for 2 days (Fig. 1). The dose-response pattern was more pronounced in the SCC-15 cell line; Velcade produced 40% and 60% inhibition of cell growth at 30 nM and 90 nM concentrations, respectively. There was modest
growth inhibition in the FaDu cell line with increasing concentrations of Velcade.

**Protein alterations following Velcade treatment assessed by Western blotting.** In both cell lines, Velcade induced increased protein expression of p21WAF1, a factor that inhibits cell growth by interfering in G1 cell cycle progression [4]. This effect was more pronounced in the SCC-15 cell line compared to the FaDu cell line. This apparent upregulation occurred despite a constitutive deficiency of p53 in SCC-15 cells (Fig. 2). Similarly, p-NF-κB showed greater nuclear expression following Velcade treatment, particularly in the SCC-15 cell line, which was accompanied by a discernible increase of I-κB protein (Fig. 2), suggesting blockade of nuclear proteasome activity.

**NF-κB protein levels following transfection of FaDu cells at graded concentrations of siRNA/NF-κB.** Increasing concentrations (0, 50, 100, and 150 nM) of siRNA/NF-κB transfected into FaDu cells resulted in a progressive decrease in the protein concentration of NF-κB (Fig. 3). These were quantified by densitometry as 0, 41%, 55%, and 65% reduction of NF-κB protein on Western blots at 0, 50, 100, and 150 nM, respectively, versus the concurrent unexposed (0 nM) control. ERK 1/2 was used as an internal reference control.

**Additive effects of siRNA against NF-κB and Velcade in FaDu cells.** We tested the impact of adding small interfering RNA (siRNA), with or without Velcade, on inhibition of cell growth in the FaDu cell line. Velcade alone, at 30 nM, resulted in 43.4% inhibition in cell proliferation (Fig. 4, upper panel). Testing siRNA against NF-κB (siRNA/NF-κB) alone led to 25.3% inhibition in cell proliferation (Fig. 4, upper panel) and 48% decline in basal protein level of NF-κB expression (Fig. 4, lower panel).
Addition of siRNA/NF-κB to Velcade at 30 nM concentration caused an additive response, with increase of growth inhibition from 43.4% to 64.5%.

Discussion

The NF-κB pathway represents a paradigm illustrating the DNA-to-protein and protein-to-DNA circuitry responsible for cell growth and maintenance of cellular integrity [5,6]. Its role in the maintenance of cellular integrity includes protection against xenobiotic injury and ensuring adequate nutrition and oxygenation through angiogenic mechanisms [2,7-9]. Therefore, patients’ tumors that show constitutive activation of the NF-κB pathway, such as HNSCC [1,3,10], may have an advantage in terms of metastatic potential and innate resistance to chemotherapy and radiation therapy [2,11]. Major components in the NF-κB pathway exemplifying this DNA-protein-DNA circuitry include: (1) transcription of mRNA/NF-κB at the genomic (DNA) level; (2) translational synthesis of NF-κB protein; (3) complexing of free NF-κB, either phosphorylated or non-phosphorylated with its inhibitor, IκB; (4) phosphorylation of IκB by inhibitor-kappa kinase (IKK) leading to its degradation by cytoplasmic and nuclear proteasomes with the release of phosphorylated NF-κB for nuclear translocation; and (5) activation of transcription of multiple genes following the complexing of free p-NF-κB protein with DNA binding sites and leading to the synthesis of anti-apoptotic proteins (eg, bcl-2, bfl-1, and bcl-xL [5,6,12-15]), growth promoting proteins (eg, cyclin D1) [5,16]), and angiogenic cytokines (eg, vascular endothelial growth factor [8,9]). Such circuitry also exposes potential targets amenable to therapeutic intervention with small molecular inhibitors, which include siRNA/NF-κB, IKK antagonists (eg, curcumin, nonsteroidal anti-inflammatory agents, resveratrol, and a farnesyl transferase inhibitor [5,16-20]), and Velcade (bortezomib) to block proteasomal degradation and release of activated p-NF-κB and to interfere in p-NF-κB/DNA binding [21-24]. These are illustrated in Fig. 5.

In the present study, we applied Velcade against two HNSCC cell lines with constitutively phos-
phorylated NF-κB (p-NF-κBp65 [Ser 536]). Velcade induced a dose-response, growth inhibitory effect in both cell lines, but with greater impact on the SCC-15 lingual cell line. At the molecular level there was a dramatic increase in nuclear p-NF-κB and a discernible increase in I-κB, consistent with blockade of the nuclear proteasome and interference in DNA/p-NF-κB binding. The upregulation of
p21\textsuperscript{WAF1} expression in the nuclear compartment of the SCC-15 cell line was greater than that seen in the FaDu cell line following Velcade treatment. Parenthetically, we have noted a similarly variable increase in the expression of p21\textsuperscript{WAF1} in the nuclear compartment following Velcade exposure in 3 breast cancer cell lines [23]. Because the marked increase in p21\textsuperscript{WAF1}, an inhibitor of G1 cell cycle progression [4], occurred in the SCC-15 cell line in the absence of detectable p53 expression, it evidently involved p53-independent induction [25-27]. Both of these molecular events could explain the greater growth inhibitory impact of Velcade on the SCC-15 cell line vis-à-vis the FaDu cell line.

In a separate experiment, when siRNA/NF-\kappa B was added to the FaDu cell line with and without Velcade exposure, it effected a decrease in growth rate with a correlative decrease in NF-\kappa B protein expression. Moreover, in conjunction with Velcade it produced an additive growth inhibitory effect. Although the FaDu cell line was selected for the transfection experiments with siRNA/NF-\kappa B based on its more rapid growth rate and shorter time to confluence, it provided a unique opportunity to test the validity of a combinatorial therapeutic approach to the NF-\kappa B pathway in HNSCC. That is to say, FaDu cells demonstrated relative resistance to the inhibitory effects of Velcade alone (Fig. 1); FaDu cells showed not only constitutive activation (phosphorylation) of NF-\kappa B but also nuclear translocation in the form of a distinctive band in nuclear extracts on Western blots (Fig. 2); FaDu cells contain mutant p53 [28], which is consistent with our finding of high expression in nuclear extracts (Fig. 2). The implications of the latter are relevant to the poor clinical outcome in patients with HNSCC who show p53 overexpression [29,30]. In short, the ability to have a significant impact on the growth rate of FaDu cells, despite all of these adverse factors, supports the validity of a combinatorial therapeutic approach directed against a constitutively activated NF-\kappa B pathway in HNSCC.

In addition to providing evidence of the role of the NF-\kappa B pathway in HNSCC, our preclinical studies raise the possibility of using multiple agents to inhibit the NF-\kappa B pathway at more than one point, in patients whose tumors constitutively express activated NF-\kappa B. Specifically, agents that inhibit either IKK, or proteosomal degradation of p-I\kappa B-NF-\kappa B complexes, thereby blocking the release of activated NF-\kappa B for nuclear translocation or that inhibit the translational synthesis of NF-\kappa B might be used in combination as adjunctive therapies (Fig. 5). Among the IKK antagonists are non-steroidal anti-inflammatory drugs (NSAID) such as aspirin, sulindac, and celecoxib [5,16], apparently independent of their ability to inhibit cyclooxygenases (COX’s). It is noteworthy that celecoxib, a COX-2 inhibitor, inhibited growth of all HNSCC cell lines derived from primary and metastatic head and neck cancer regardless of whether or not they showed COX-2 expression [31]. A computer-assisted literature search of the National Library of Medicine MEDLINE database from 1966 to April 2005 revealed no articles on clinical trials with these agents and HNSCC. Other IKK antagonists include curcumin, resveratrol, and farnesyl transferase inhibitors [17-20]. Curcumin has been shown to be effective against HNSCC cell lines with constitutively active NF-\kappa B and IKK, inhibiting cell proliferation and inducing apoptosis through suppression of IKK-mediated NF-\kappa B activation and NF-\kappa B-regulated gene expression [17]. In a phase I clinical trial, Cheng et al [32] found that there was no treatment-related toxicity up to 8 g/day of oral curcumin. Moreover, 2 of 7 patients with oral leukoplakia showed histological improvement [32]. Similarly, resveratrol has been shown to be a potent inhibitor of both NF-\kappa B-dependent gene expression, and these effects are associated with inhibition of activation of I\kappa B kinase [19,33]. Elatter and Virji [34] reported on the ability of resveratrol to inhibit growth of human oral squamous carcinoma cells. Finally, the rationale for using a farnesyl transferase inhibitor to suppress the activation of IKK [20] is strengthened by our observations in tonsillar squamous cell carcinoma. We detected the immunohistochemical expression of the alpha subunit common to farnesyl transferase and geranylgeranyl transferase in the cytoplasmic compartment of 11 of 11 cases of squamous cell carcinoma of the tonsil. Moreover, the highest intensity score in each case was moderate to strong (2 to 3+ on a scale of 0-3+). Thus there appears to be consistent expression of
the farnesylation pathway in this subtype of HNSCC and therefore a target for use of a farnesyl transferase inhibitor (Brown et al, unpublished data).

In regard to the clinical application of Velcade in HNSCC, van Waes and co-workers [35] recently reported preliminary results in an ongoing phase I clinical trial assessing the radio-sensitizing potential of bortezomib in head and neck cancer. Although the use of a specific siRNA to target the translational activation of transcription factor NF-(kappa)B; AP-1 and IL-8 and VEGF by human head and neck squamous cell carcinoma involves coactivation by MEK-MAPK and IKK-NF-kappaB signal pathways. Clin Cancer Res 2001;7:435-442.

In summary, in vitro studies in 2 head and neck squamous cell carcinoma cell lines provide evidence that the constitutively activated NF-kappaB pathway plays a role in promoting tumor cell growth. The findings suggest that it may be worth considering therapies that incorporate several inhibitors of the NF-kappaB pathway into prospective clinical trials in patients with tumors that show constitutive activation of the NF-kappaB pathway.

Acknowledgment. The authors thank Dr. Conrad Schuerch for constructive comments on this manuscript. The authors thank Ms. Sharon Coup for secretarial support and help with the graphics.

References


