The Effects of Reactive Species on the Tumorigenic Phenotype of Human Head and Neck Squamous Cell Carcinoma (HNSCC) Cells

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Abstract. *Sustained inflammation up-regulates the reactive species (RS) generating enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). While clinical data show that levels of iNOS and COX-2 are increased in epithelium during the transformation of dysplasia to overt head and neck squamous cell carcimoma (HNSCC), the mechanisms by which their overexpression contributes to HNSCC development have not been completely delineated. This study assessed the effects of RS on parameters associated with the HNSCC tumorigenic phenotype inclusive of activation of NF-κB (in situ immunostaining and reporter assay) and production of proinflammatory and proangiogenic proteins (ELISA analyses). Our data, which show both reactive oxygen and nitrogen species activated NF-κB, and that all RS donors evaluated increased HNSCC cellular production of vascular endothelial growth factor, IL-8 and epidermal growth factor receptor proteins, imply inflammation associated RS promote HNSCC by their abilities to modulate intracellular signaling and affect gene expression.*

Reactive species (RS), which include both reactive oxygen (ROS) and reactive nitrogen species (RNS), are naturally occurring cellular products generated during many processes including oxidative metabolism, intracellular signaling, and phagocytic activation (1). In general, RS have an unstable electron configuration, are cell permeable and demonstrate reactivities that are inversely proportional to their stabilities (1). During inflammation, both intra- and extracellular RS levels are appreciably increased as a result of enzyme up-regulation and activation (2). While the RS generated by activated phagocytes are ideally contained within the phagolysosome, some RS, including H_2O_2, superoxide (SO), and nitric oxide (NO) are released extracellularly (1, 2). Other enzymes, such as nitric oxide synthase (NOS) and cyclooxygenase (COX), that are present in phagocytes as well as epithelial and connective tissues, either generate RS directly (NOS) or release RS as a consequence of functional activity (COX) (3, 4). Clinical data have shown that the "high output", inflammation-induced isoforms of NOS and COX *i.e.* inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), are associated with development of colon, prostate, liver and head and neck squamous cell (HNSCC) cancers (5-10). Relevant to HNSCC pathogenesis, intraepithelial levels of iNOS and COX-2 increase during the transition from premalignant epithelial lesions to overt HNSCC (11-13). Another consideration is that cellular metabolism of the established HNSCC risk factors, tobacco and alcohol, by Phase I and II enzymes, provides yet another pathway which contributes to a RS-enriched oral microenvironment. These proinflammatory and carcinogen metabolizing enzyme-mediated increases in RS levels have the potential to create oxidative and nitrosative stress and the subsequent pro-tumorigenic consequences that include enhanced redox-mediated intracellular signaling and neovascularization (1, 14).

The NF-κB family of redox-responsive transcription activating factors is involved in the regulation of a number of genes that are associated with inflammation as well as

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cell survival and proliferation (15). NF-κB target genes include vascular endothelial growth factor (VEGF), proinflammatory and proangiogenic chemokines such as IL-8, adhesion molecules, antiapoptotic genes, COX-2, iNOS and MMP-9 (16). Upon stimulation by a variety of mediators including RS, cytosolic NF-κB is released from its inhibitory proteins (IκB) and the activated NF-κB subsequently undergoes nuclear translocation (16-18). Constitutive and/or aberrant NF-κB activation, which has the capacity to sustain the inflammatory response and promote cell survival and proliferation, is observed in many human diseases, including HNSCC (18-20). Recent studies by Pikarsky *et al.*, in which a murine model of inflammation associated hepatocellular carcinoma was used, demonstrated that NF-κB functions as a tumor promoter by virtue of its antiapoptotic effects (21).

While epidemiologic data clearly show that tobacco and alcohol use contributes to HNSCC development, the role of chronic inflammation and poor oral hygiene in oral cancer progression is more speculative. A common feature of these established and putative HNSCC risk factors is increased RS generation by activated carcinogen metabolizing and proinflammatory (COX-2, iNOS) enzymes (2, 12, 13, 22). While sustained inflammation and associated increased RS levels have been correlated with development of several human cancers (13, 22), the molecular mechanisms by which RS can promote HNSCC tumorigenesis have not been completely characterized. Accordingly, this study evaluated the effects of pathophysiological relevant levels of RS, which would be present in persons at high risk for HNSCC development, *i.e.* alcohol and tobacco use, poor oral hygiene and associated inflammation, on promotion of the HNSCC tumorigenic phenotype by measuring extent and downstream consequences of redox-mediated signaling in HNSCC cells.

Materials and Methods

Reagents. Reagents used for cell treatments included tumor necrosis factor α (TNFα, Invitrogen, Carlsbad, CA, USA), H_2O_2 (Sigma, St. Louis, MO, USA), 2,2'-(hydroxynnitrosohydrazino)*bis*-ethanamine (NOC18, CalBiochem, San Diego, CA, USA) and 3-morpholinosydnonimine (SIN-1, CalBiochem). Anti-p65 antibody was purchased from Chemicon Division of Millipore (Temecula, CA, USA). Transfection reagents included NF-κB specific reporter gene construct, SEAP (secreted embryonic alkaline phosphatase) detection and Clonfectin transfection reagent (Clontech, Mountain View, CA, USA). ELISA development kits were from R&D Systems (Minneapolis, MN, USA). VEGF, EGFR and secondary antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). β-Actin antibody was from AB Cam (Cambridge, MA, USA). Antibodies were detected by ECL Plus (GE Healthcare, Biosciences Corp., Piscataway, NJ, USA).

Cell culture. Four cell lines (SCC4, SCC9, SCC25, SCC2095), derived from human squamous cell carcinomas of the tongue, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Previous studies in our laboratory have confirmed that these cell lines retain features of intact oral mucosa inclusive of functional Phase I and II carcinogen metabolizing, iNOS and COX-2 enzymes (23-25). For expansion, cells were cultured in their optimal medium (DMEM/Ham's F12 Medium 90%, heat-inactivated fetal bovine serum, 10%, GIBCO, Grand Island, NY, USA) at 37˚C, 5% CO_2. To reduce interfering effects from serum components, cells were challenged in sera-free medium (Base medium) using TNFα, H_2O_2, NOC18 or SIN-1. Functional comparisons were made relative to the same cell line, matched sera starved control culture.

Effect of RS challenge on cell viability, NF-κB activation, reporter gene expression, and intracellular and extracellular protein production. The treatment protocols to assess these experimental parameters were similar. To reduce interfering effects from serum components, cells were challenged in sera-free medium (Base medium). Twenty-four hours prior to challenge cultures were grown in Base medium. At time 0, fresh Base medium was added, and treatments included: Base medium (negative control), TNFα (100U/ml), H_2O_2 (100 μM), NOC18 (25 μM) or SIN-1 (100 μM). While TNFα itself does not directly generate RS, TNFα was included as a cell challenge reagent for several reasons. TNFα mediated signaling results in RS generation, potentially *via* a cytosolic phospholipase A2-linked cascade (26). Further, increased levels of TNFα (as would be generated by tumor associated macrophages), and/or sustained TNFα mediated signaling has been implicated in the development of many human diseases, inclusive of cancer (27). Finally, there is crosstalk between TNFα mediated NF-κB activation and the modulatory effects of NF-κB activation on RS accumulation following TNFα exposure (27, 28). Fresh treatment, but not media, was added every 24 hours, with harvests occurring at the 24, 48, 72, and 96 hour time points. For select experiments (reporter gene assays), cell harvests were limited to the 24 hour time point.

Determination of RS challenge effects on cell number and viability. In order to determine appropriate dosing levels, initial experiments were conducted to assess the effects of RS challenge on cell number and viability. Cell numbers were determined by hemocytometer counting and viability assessed by cell capacity to exclude Trypan blue (Trypan Blue Exclusion Dye; Sigma). Previous studies conducted in our laboratory have shown a good correlation between Trypan blue exclusion and lactate dehydrogenase release for viability assessment (29).

Immunostaining to assess NF-κB activation. HNSCC cells were plated on 8-well chamber slides and grown overnight in complete media. Cultures were washed and incubated in Base media for 48 hours. Cells were challenged for 1 hour with either base media (negative control), TNFα (100U/ml), H_2O_2 (100 μM), NOC-18 (25 μM) or SIN-1 (100 μM). Cells were subsequently fixed and stained with an anti-p65 antibody (Chemicon) specific for the nuclear translocation sequence which is only accessible following dissociation from IκB.

Transient transfection to determine whether NF-κB activation elicited functional consequences. Once the cultures had achieved approximately 70% confluence, cells were transfected with 1 μg DNA:1 μl CLONfectin using either a NF-κB reporter gene (SEAP)

or control reporter with no known transcription factor binding site. Transfected cells were grown for 24 hours in optimal medium before a 24 hour challenge in base medium alone (to establish baseline levels of NF-κB activation) or with one of the following TNFα (100U/ml), H_2O_2 (100 μM), NOC18 (25 μM) or SIN-1 (100 μM). DNA from the transfected cells was purified using Trizol (Invitrogen). Following Trizol addition to the cell pellet, the RNA layer was removed, the DNA was precipitated and washed and DNA concentration determined by UV spectrometry.

Monitoring of SEAP release to confirm NF-κB functional activity. Media samples were collected at 24 hours and stored at –20˚C until analysis. Analyses were conducted on a Perkin Elmer LS50B fluorimeter using the Fluorescent SEAP Detection Kit (BD Biosciences, San Jose, CA, USA). SEAP concentrations released into the media (the fluorescent product indicative of NF-κB-mediated gene expression) were determined by comparison to a standard curve derived from pure enzyme (secreted embryoynic alkaline phosphatase) and data was normalized to plasmid DNA concentrations in the cell lysates following a 24-hour treatment. Plasmid DNA was amplified by PCR using primers specific for the pUC ORI sequence of the vector using the following primer sequences: pUC Forward, 3127: GAGGCACCTATCTCAGCG ATCT, pUC Reverse, 3503: CATGTAACTCGCCTTGATCCT and the resulting amplicon is 376bp. PCR products were purified using the Qiagen PCR Product Purification Kit (Valencia, CA, USA), and single band amplification was confirmed by gel electrophoresis. Plasmid DNA concentration was determined by spectrometry in accordance with Beer's Law. The millimolar extinction coefficient of the amplicon was calculated to be $3.55x10^6$ based on the nearest neighbor model (Ambion Technical Resources, Austin, TX, USA).

Evaluation of HNSCC release of IL-8 and VEGF. IL-8 and VEGF ELISAs (R&D Systems) were used to assess HNSCC culture release of IL-8 and VEGF. Media samples were collected from triplicate flasks at 24, 48, 72 and 96 hours post-treatment. Data were normalized to cell number, presented as nanograms (IL-8) or picograms (VEGF) protein per million cells and represented as mean±S.E.M. The rationale to evaluate HNSCC cell production of these two proteins is as follows. VEGF, which is a complete angiogenic cytokine, is also regulated, at least partially, by NF-κB activation (30). Furthermore, increased tumor production of VEGF is poor prognostic indicator in persons with HNSCC (15, 31, 32). Due to its potent induction of neutrophil chemotaxis, IL-8 is an effective pro-inflammatory cytokine that also functions as a pro-angiogenic protein (33, 34).

Immunoblot analyses to determination of intracellular levels of VEGF and EGFR. Cells were treated as previously described, and samples obtained at the 24, 48, 72, and 96 hour time points. Proteins were purified using M-PER (Pierce, Rockford, IL, USA) reagent, protein concentrations were determined by Bio-Rad Protein Determination (Bio-Rad, Hercules, CA, USA) and 20 μg of protein was used for immunoblot analysis. Samples were run using a 7.5% SDS-PAGE and probed for VEGF (1:800, Santa Cruz Biotechnology), EGFR (1:800, Santa Cruz) and β-actin (1:6666, AB Cam, Cambridge, MA, USA). As immunoblots detected both the VEGF dimer (~49 kDA) and monomer (~24 kDa) (35) both bands were quantified. Immunoblot data was analyzed using Kodak 1D v3.6 gel analysis software (Rochester, NY, USA). Protein expression was normalized to β-actin levels and compared to the matched, same cell line unstimulated cells.

Statistical analyses. Cell number, viability and ELISA data were analyzed by 2-way ANOVA followed by Bonferroni *post hoc* test using GraphPad Prism v4.03 (GraphPad Software Inc., San Diego, CA, USA). Transfection and immunoblot data were analyzed by Chi-square test with Yates correction. The level of significance was established at the 95% confidence interval ($\alpha \leq 0.05$).

Results

Effects of RS challenge on cell number and viability. Cell numbers (Figure 1a) were not significantly altered by challenge with TNFα, NOC18 or SIN-1 at any time points between 24 and 72 hours. In contrast, H_2O_2 challenge at levels comparable to those detected in the vicinity of activated macrophages (36, 37) resulted in a significant decrease in cell number at 48, 72 and 96 hours post-treatment ($p<0.001$, 0.01, 0.001, respectively). Viability data demonstrate retention of comparable viabilities through 72 hours of challenge with TNFα, NOC18 or SIN-1 (Figure 1.b), with significant reduction in viability only apparent following 96 hours of treatment ($p<0.05$, 0.001, 0.05 respectively). Similar to the cell number data, H_2O_2 treatment resulted in significant reduction in viability following 48, 72 and 96 hours of treatment ($p<0.001$ at all time points). With the exception of H_2O_2, these results revealed a HNSCC cellular tolerance for RS challenge for periods up to 72 hours as manifest by no significant changes in cell number or viability. Despite the evidence of cell selection associated with H_2O_2 challenge, the selected (0.1 mM) H_2O_2 concentration was used in subsequent experiments for the following reasons. As *in vitro* cultured cells become "acclimated" to an oxidative environment, higher, pro-oxidant agent concentrations are often employed to elicit intracellular effects (38-40). In addition, the 100 μM H_2O_2 concentration is pathophysiologically relevant, and is comparable to NF-κB activating levels of H_2O_2 used by other investigators in cultured human cells (38).

RS challenge induces IκB dissociation and results in NF-κB activation. Immunostaining of cell cultures treated with RS for one hour demonstrated the ability of RS to activate NF-κB as shown by dissociation between NF-κB and IκB (Figure 2). Positive cytosolic staining is indicative of IκB dissociation and all treatments demonstrated positive staining. The observed trend for IκB dissociation among all four cell lines evaluated was NOC18>H_2O_2>TNFα>SIN-1>Base medium.

Reporter gene activity was assessed following 24 hours of treatment and compared to untreated cells in Base media (Figure 3). Matched cell cultures, transfected with a control

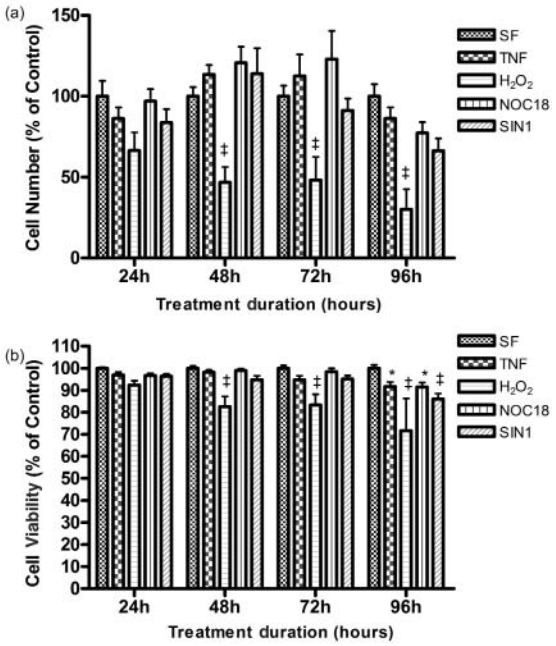

Figure 1. *Cell viability and number. Cell number (a) and cell viability (b) were determined by enumeration and trypan dye exclusion, respectively. Cell number (a) revealed only H_2O_2 treatment significantly decreased cell number, with significant reductions noted at the 48, 72 and 96 hour time points. Significant reductions in viability (b) were noted at 48, 72 and 96 hours following H_2O_2 treatment. At 96 hours, cultures treated with TNF, H_2O_2, NOC18 and SIN1 all showed significant decreases in viability. n=12 for each treatment group for 24 and 48 hour treatments with exception of 24 hour H_2O_2 (n=11), n=8 at 72 hours, n=5 at 96 hours. *p<0.05, ‡p<0.001.*

reporter gene containing no known transcription factor binding element (results not shown), did not demonstrate any reporter gene activity. The NF-κB reporter gene assay, determined by detection of fluorescent labeled SEAP protein released into the media, showed that while all four treatments increased SEAP release above the control transfectants, only H_2O_2 and SIN-1 significantly increased NF-κB-regulated gene expression (Figure 3).

Sustained RS challenge affects HNSCC release of IL-8 and VEGF. Results from the IL-8 ELISA assays show changes in secreted protein levels (average stimulated±S.E.M. *vs.* unstimulated±S.E.M.) in response to RS treatment which varied in accordance with time of harvest and RS challenge agent (Figure 4a). TNFα treatment induced a significant increase in secreted IL-8 after 24 hours of exposure (645±168 ng/10^6 *vs.* 350±100 ng/10^6), $p<0.001$. This increase was diminished at 48 hours but returned significantly at 72 hours (1529±352 ng/10^6 *vs.* 1368±438 ng/10^6, $p<0.05$). At 96 hours, TNFα treatment did not significantly impact HNSCC IL-8 secretion. H_2O_2 treatment led to a significant increase of secreted IL-8 protein after 24 hours (373±40 ng/10^6 *vs.* 350±100 ng/10^6, $p<0.05$). Between 48 and 96 hours there was no significant increase. SIN-1 treatment resulted in a significant increase of IL-8 protein only at 96 hours (2612±1081 *vs.* 1445±473 ng/10^6, $p<0.05$). NOC18 challenge resulted in modest, insignificant increases in HNSCC IL-8 secretion at 24, 72, and 96 hour time points.

Repeated RS exposure altered HNSCC VEGF secretion in a time and agent-specific fashion (Figure 4b). TNFα treatment caused a significant increase only at 96 hours (591±84 pg/10^6 *vs.* 385±85 pg/10^6, $p<0.05$). In contrast H_2O_2 treatment showed a highly significant increase in VEGF at all times between 24 and 96 hours (at 24h 399±158 pg/10^6 *vs.* 98±23 pg/10^6, at 48h 1811±762pg/10^6 *vs.* 197±34 pg/10^6, at 72h 10,502±8,841 pg/10^6 *vs.* 273±47 pg/10^6, at 96h 13,105±5,272 pg/10^6 *vs.* 390±145 pg/10^6, $p<0.001$ at each time point). SIN-1 only significantly increased VEGF secretion at the 96 h time point (790±221 pg/10^6 *vs.* 385±85 pg/10^6, $p<0.05$). Similar to its effects on IL-8 release, NOC18 exposure resulted in modest, insignificant increases in HNSCC VEGF secretion at 24, 72, and 96 hour time points

As shown by the ranges in standard errors, there were cell-line associated differences with regard to the mean IL-8 and VEGF protein released. Notably, all four cell lines, isolated from four different patients' HNSCC tumors, showed similar RS induced responses with regard to IL-8 and VEGF secretion.

RS modulation of intracellular proteins (VEGF and EGFR) is also agent and duration dependent. Comparable to changes in secreted VEGF protein levels, immunoblot analyses demonstrated increases in cellular VEGF in response to RS challenge (Figure 5a and b). Significant increases in intracellular VEGF protein were observed at the 48 hour harvest in response to TNFα or NOC18 ($p<0.05$). At 72 hours, H_2O_2, NOC18 and SIN-1 significantly increased intracellular VEGF ($p<0.05$). EGFR immunoblots also revealed significant effects from RS challenge (Figure 5a and c). At 24 hours NOC18 showed significant decreases in EGFR protein levels ($p<0.05$). After 48 hours NOC18 led to an increase of EGFR that was also detected after 72 hours of challenge ($p<0.05$ for both). TNFα and H_2O_2 treatment both significantly increased EGFR protein at 48 hours, while at 96 hours SIN-1 induced a significant increase ($p<0.05$ for all points).

Discussion

The development of HNSCC reflects multiple genetic and epigenetic perturbations that include loss of tumor suppressor function, failure of terminal differentiation, development of a pro-angiogenic phenotype and

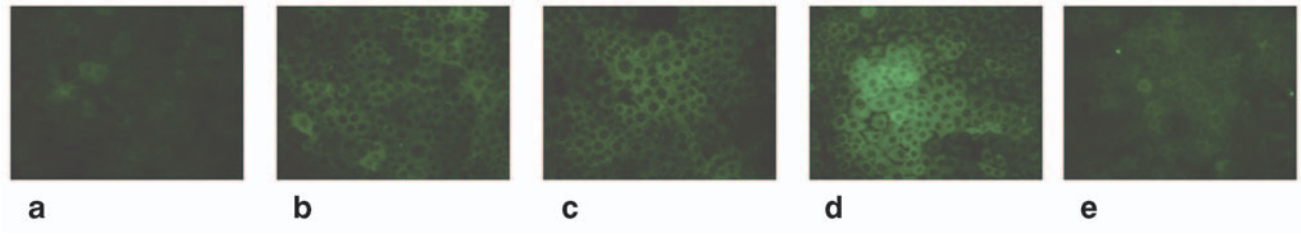

Figure 2. *Immunostaining of nuclear translocation sequence of p65 subunit of NF-κB. Cells were plated on 8-chamber well slides in serum free base medium (SF) for 48 h prior to 1 hour challenge with either SF control medium (a), 100 U/ml TNF (b), 0.1 mM H_2O_2 (c), 25 μM NOC18 (d), or 100 μM SIN1 (e). Cells were fixed and dissociated p65 was immunohistochemically detected to assess the effects of RS on NF-κB activation. Qualitative results demonstrate NOC18> H_2O_2> TNF> SIN1> SF with regard to extent of NF-κB activation. Representative image shown, 400x image scale.*

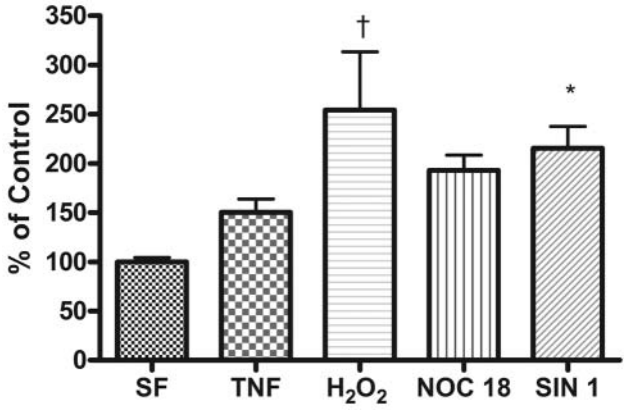

Figure 3. *NF-κB activity monitored by gene reporter construct. SCC 2095 cells transiently transfected with an NF-κB specific reporter gene construct were challenged with either base medium or reactive species generators. Following 24 hours of treatment, media were collected and reporter gene activity determined. Activity was normalized to the concentration of PCR amplified reporter gene vector. Results show significant increase of NF-κB initiated transcription in response to H_2O_2 ($p<0.01$) and SIN1 ($p<0.05$). $n=6$ for all treatment groups, *$p<0.05$, †$p<0.01$.*

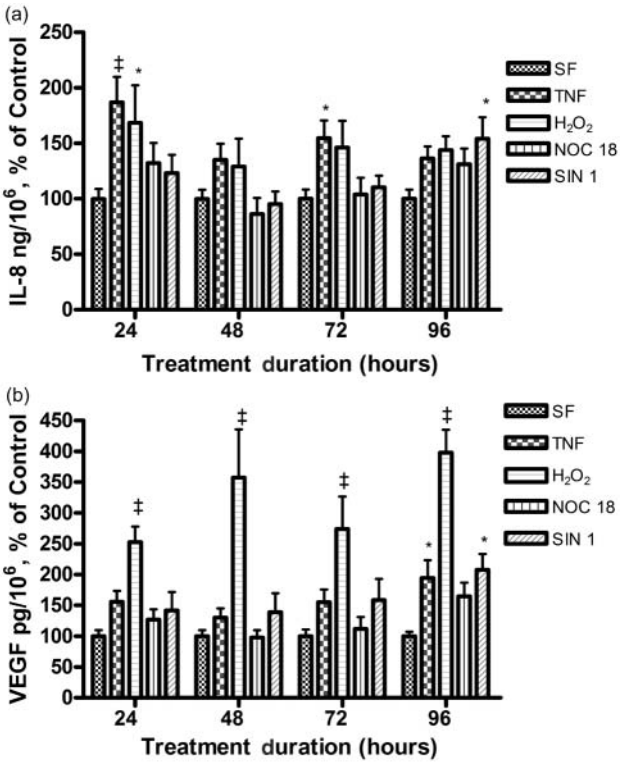

Figure 4. *RS effects on extracellular IL-8 and VEGF protein levels cultured SCC cells' conditioned media were obtained every 24 hours prior to the addition of fresh treatment. IL-8 results (a) show a strong response following TNF treatment at 24 hours and a smaller, yet significant increase in IL-8 release at 72 hours. H_2O_2 increased IL-8 protein levels at 24 hours. Following 96 hours of treatment with SIN1, IL-8 release was significantly increased. VEGF (b) levels were strongly and significantly increased in response to H_2O_2 at all time points. TNF and SIN1 also showed an increase in VEGF levels at 96 hours. $n=12$ for each 24 hour treatment group, with the exception of H_2O_2 ($n=6$), $n=6$ for each treatment group at 48 hours, $n=5$ for each treatment group 72 hours, $n=3$ for each treatment group at 96 hours. *$p<0.05$, ‡$p<0.001$.*

inappropriately sustained intracellular signaling. Accordingly, the majority of HNSCCs demonstrate alterations in intracellular signaling pathways inclusive of overexpression and autocrine activation of EGFR (30, 41, 42), constitutive activation of NF-κB (19, 20) and enhanced NF-κB cross talk with other signaling pathways (43). Furthermore, HNSCCs that show high tumor production of VEGF, IL-8 and EGFR are more clinically aggressive and associated with a worse prognosis (31, 32, 42). HNSCC arises in a milieu that includes both endogenous (expression of Phase I and II enzymes, iNOS and COX-2 in lesional epithelium) as well as exogenous (activated phagocytes and stromal cells) sources for RS generation. Sustained high levels of RS can promote malignant transformation by several mechanisms that include induction of mutations, inactivation of cytoprotective proteins, and modulation of signal transduction pathways (14, 44). In addition, Liss *et al.* demonstrated that HNSCC cells released factors that induced macrophage release of IL-8 and VEGF; thereby establishing a paracrine HNSCC-initiated-macrophage-responsive angiogenic loop (45). This current

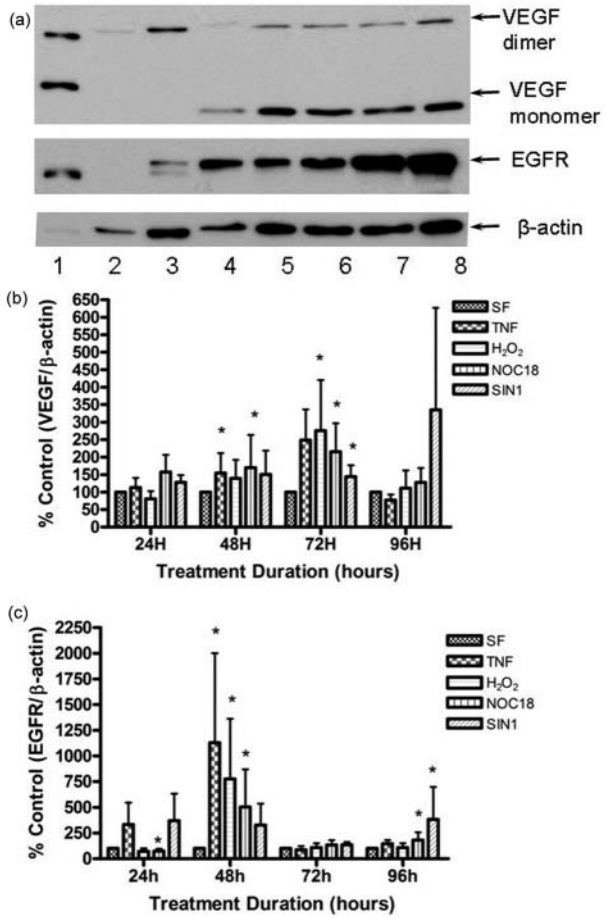

Figure 5. *Effects of RS on cellular protein levels of VEGF and EGFR Western blot analyses to determine cellular levels of VEGF and EGFR were conducted using samples from all 4 HNSCC cell lines. Protein levels were determined by densitometry and represented relative to β-actin levels. A representative immunoblot from a 24 hour harvest is depicted in (a). Lane indications are as follows: 1-marker, 2-NIH/3T3 cell lysates, 3-SKBR3 cell lysates, 4-sera free (SF), 5-TNF (100 U/ml), 6- H_2O_2 (0.1 mM), 7-NOC18 (25 μM) and 8-SIN1 (100 μM). VEGF dimer and monomer are indicated by arrows. EGFR was detected as a doublet consistent with phosphorylation. TNF treatment induced a significant increase in intracellular VEGF protein at the 48 hour time point (b). NOC18 initiated a significant increase in intracellular VEGF after 48 and 72h of treatment (b). H_2O_2 and SIN1 induced significant increases in intracellular VEGF protein levels at the 72 hour time point. While NOC18 decreased levels of EGFR protein at 24 hours, cellular levels were significantly increased at the 48 and 96 hour harvests (c). TNF treatment resulted in a significant cellular EGFR increase at 48 h. n=4 for all treatment groups, at all time points. *$p<0.05$.*

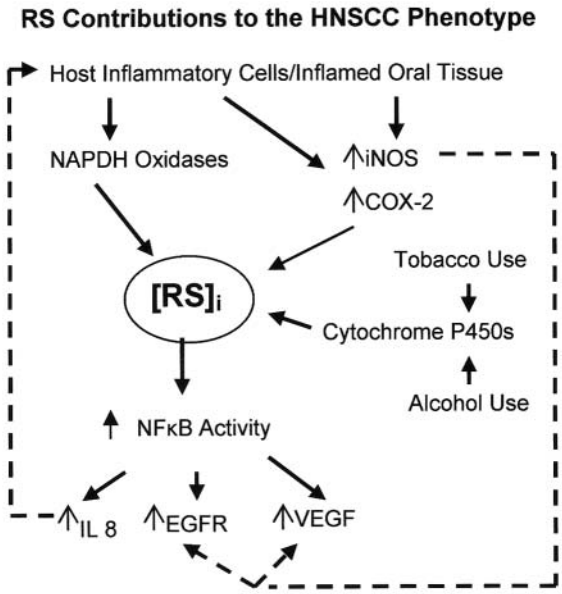

Figure 6. *RS Effects on HNSCC tumorigenesis. Intracellular levels of RS ($[RS]_i$) are increased by a myriad of factors including inflammatory cells and an inflamed local oral environment as well as through induction of cytochrome P450 enzymes by use of tobacco and alcohol. Our data show that reactive species can induce functional consequences on oral tissues via their abilities to activate the pleiotropic transcription activating factor NF-κB. Downstream consequences of NF-κB activation include increased cellular production of VEGF, IL-8 and EGFR. By their abilities to induce all processes necessary for angiogenesis (VEGF), initiate intraepithelial mitogenic signaling (EGFR) and chemoattract a key pro-inflammatory cell (IL-8), increased translation of these proteins could promote the tumorigenic phenotype.*

study investigated the converse *i.e.*, the consequences of RS exposure, such as would be generated by tumor associated macrophages, on HNSCC redox mediated signaling and subsequent downstream production of proangiogenic and promitogenic molecules.

Exposure to RS has been shown to stimulate cell proliferation in a variety of cultured human cells (46, 47). In contrast, our results demonstrate that RS challenge with TNFα, NOC18 or SIN-1 did not affect the cell number or viability up to 72 hours. These disparate results likely reflect differences in experimental design including cell type, duration and dosage. H_2O_2 was the only treatment that resulted in a significant decrease in cell numbers. Although these results may suggest overt toxicity, the dose employed (100 μM) is at the low end concentration released in the vicinity of activated macrophages (36, 37). Furthermore, the H_2O_2 dose utilized activated signaling pathways. We speculate that these cell enumeration data, which imply an ongoing selection for cells more tolerant and/or more responsive to RS, may recapitulate a similar *in vivo* selection process that occurs within cancers that arise within a RS-enriched environment.

The immunostaining results, which demonstrate RS induce IκB degradation, imply RS activate the IKK kinases as well as other adaptor proteins such as TRADD, TRAF2 and RIP essential for NF-κB activation

(48, 49). These data demonstrate that NOC18 was the most potent inducer of IκB degradation, followed by H_2O_2, TNFα and SIN-1. While H_2O_2 has previously been shown to induce IκB degradation *via* the classic TNFα-mediated pathway in human leukemic T-cells (17), to our knowledge, these data are the first to show NO-mediated IκB degradation. The positive cytosolic staining observed in unstimulated HNSCC cells during culture in serum free medium confirms constitutive NF-κB activation, which is known to occur in HNSCC tumors and cultured HNSCC cells (19, 20, 49).

Reactive nitrogen species' (RNS) diverse biological effects are mediated by reactions with oxygen species and transition metals and depend upon several factors including local protein environment, spacial considerations, relevant targets, rate of production and species generated *e.g.* NOC18-slow, sustained NO release; SIN-1-short term, production of both NO and $O_2^{\cdot -}$ (3, 14). It is therefore not surprising that RNS have been reported to both enhance and suppress NF-κB activation (17, 50). While our data show all RS treatments induced IκB degradation, only H_2O_2 and SIN-1 significantly increased NF-κB-mediated gene expression. TNFα, a known potent inducer of NF-κB activation, did not significantly increase reporter gene activity in our system. Jackson-Bernitsas *et al.* demonstrated that endogenously generated TNFα and its ensuing activation of TNFα signaling induces the constitutive NF-κB activity present in HNSCC cells (49). Therefore, the observed refractory nature of HNSCC cells to exogenous TNFα likely reflects high endogenous TNFα mediated NF-κB activation. Another consideration is that intracellular site-specific redox states can affect NF-κB-mediated increases in gene expression. While an oxidized cytosol augments IκB release, a reduced nuclear environment is necessary for NF-κB-promoter binding. Our NOC18 results imply a pro-oxidative effect of sustained NO release, which would augment cytosolic activation and IκB degradation, while perturbing the reduced nuclear environment necessary for NF-κB promoter binding. Finally, the interactions between the exogenously generated RS and intracellular RS scavengers such as glutathione (GSH) and RS degrading enzymes also affect RS-cell interactions. Studies conducted by Hu *et al.*, which showed a concentration and time-dependent effect of superoxide dismutase (SOD) on the nitrosation reaction, and the ability of GSH to modulate SOD's pronitrosative or antinitrosative effects, clearly delineate the complexity of these intracellular reactions (51). In our HNSCC cell cultures, the acute, concurrent release of $O_2^{\cdot -}$ and NO by SIN-1, and the relatively long lived, membrane permeable H_2O_2, interacted with this complex intracellular environment to induce all aspects, *i.e.* activation and functional activity of NF-κB signaling.

The impact of RS challenge on HNSCC cells was also manifest at the translational level. All RS generating agents increased HNSCC production of the angiogenic and inflammatory cytokines IL-8 and VEGF and/or up-regulated a key mitogenic signaling receptor, EGFR. These data also show agent and duration related effects; findings that likely reflect the fact that the different RS generating agents likely activate NF-κB and associated signaling cascades by distinct mechanisms. Our data also imply that in order to elicit a discernable effect, RS must activate a distinct signaling pathway in order to result in inducible levels that are greater than constitutive production. Notably, there are several, constitutively activated signaling pathways that are associated with HNSCC autologous production of IL-8 and VEGF *i.e.* TNFα, EGFR, and IL-1α (34, 41). "Unstimulated" HNSCC cells produced high levels of both IL-8 and VEGF, which is likely a consequence of these autologously initiated signaling pathways. IL-8 release by nonchallenged HNSCC cells was seven fold greater than normal oral keratinocytes (52), while VEGF production by HNSCC control cultures was twice that released by human ovarian cancer cells (53). Another confounding variable is that IL-8 and VEGF genes contain distinct recognition sites for NF-κB and AP-1; implying co-activation of both transcription factors in cultures and treatment groups that show increased protein production. Despite these nuances that complicate data interpretation, our results show that exogenous RS increase HNSCC production of IL-8, VEGF and EGFR, all of which are proteins that are associated with a more clinically aggressive tumor (15, 42).

As depicted in Figure 6, there are many potential sources, inclusive of carcinogen metabolism, and enzymes associated with oxidative metabolism, inflammation and intracellular signaling, which can contribute to an RS enriched oral microenvironment. Pertinent to this concept, recent studies from our laboratory have demonstrated the efficacy of antioxidants in suppression of the HNSCC tumorigenic phenotype. N-acetylcysteine inhibited activation and function of the basement membrane degrading matrix metalloproteinase-9 and also suppressed HNSCC cellular invasion (54). Further, a phenolic-enriched water ethanol extract of freeze dried black raspberries significantly suppressed HNSCC cell proliferation, iNOS function and VEGF production (25). Provided the many mechanisms by which RS can promote the tumorigenic phenotype, and the constitutive activation of redox regulated transcription factors, such as NF-κB in HNSCC tumorigenesis (55), the potential benefit of antioxidant therapy for suppression of the HNSCC tumorigenic phenotype remains to be investigated.

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