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Activating Transcription Factor-2 (ATF2) in survival mechanisms in head and neck carcinoma cells

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Abstract

Background—Activating transcription factor-2 (ATF2) is associated with tumor progression, but is not well-studied in head and neck squamous cell carcinoma (HNSCC). Its effects in stress and its importance in other survival mechanisms were studied.

Methods—ATF2 expression and nuclear activation were confirmed in HNSCC. Following modulation of ATF2, in vitro effects on proliferation and chemosensitivity were studied. Effects on in vivo tumor growth and Interleukin 8 (IL-8) expression were determined. Tumor Necrosis Factor (TNF) alpha treatment was used to further evaluate cytokine production and chemosensitivity.

Results—Reductions of ATF2 resulted in significant nuclear p-ATF2 activation, cisplatin resistance and augmented IL-8 expression without affecting in vivo tumor growth. In this setting, TNF increases p-p38 phosphorylation and chemosensitivity while further enhancing IL-8 production.

Conclusions—Our data suggest regulatory roles for ATF2 in TNF-related mechanisms of HNSCC. Its perturbation and nuclear activation are associated with significant effects on survival and cytokine production.

Keywords

apoptosis; chemoresistance; IL-8; ATF2; cisplatin

Introduction

Activating Transcription Factor-2 (ATF2), a member of the basic region-leucine zipper transcription factor family, affects the expression of many genes important for regulation of cell functions, including apoptosis.(1, 2) Its effects on regulation of cyclin A and the cell cycle, repair of DNA double strand breaks and apoptosis have been shown in several tumor types.(3) In the absence of appropriate inducers, ATF2 remains transcriptionally inactive due to an inhibitory intramolecular interaction between the activation domain of ATF2 and the bZIP domain.(4) However, when cells are exposed to stress (such as chemotherapy), several kinases, such as Jun-terminal kinase (JNK) and p38, are typically activated to phosphorylate the transcriptional activation domains of ATF2 on amino acid residues Thr-69 and Thr-71.(5–7) The location of ATF2 as an effector target of the Mitogen-activated protein/Stress-activated protein (MAP/SAP) kinase pathway thus increases its importance for cytotoxic treatments like chemotherapy.(8) ATF2 dimerizes preferentially with transcription factor, c-jun, and this dimerization determines its localization within the cell. Dimerization with c-jun is thus permissive for nuclear localization of ATF2 and transcriptional activity.(9, 10) ATF2 phosphorylation per se does not regulate its subcellular localization but is required for full transcriptional activity.(11)

Extensive literature indicates that, depending on the tumor type, ATF2 can facilitate either tumor promotion or tumor suppression.(2, 12, 13) Demonstrating its tumor suppressive effects, transgenic ATF2 deficient mice show impaired tumor suppression and DNA repair.(13) The spontaneous formation of mammary pad tumors is observed in these mice. Concomitant mutation of ATF2 and its closest homologue, ATF7 decreases tissue expression of several members of the dual-specificity phosphatase (DUSP) family, leading to increased p-38 activation and resultant abnormal apoptosis.(14) Human epithelial skin tumors express decreased nuclear ATF2 compared with normal keratinocytes.(12)

Conversely, a tumor promoting role is seen in mouse models of melanoma where inhibition of ATF2 efficiently impairs growth and metastasis while sensitizing tumor cells to radiation and several chemotherapies.(15) A short peptide interfering with phosphorylation of ATF2 also decreases melanoma cell chemoresistance and significantly reduces tumor growth and metastasis.(2, 16) In human melanoma specimens, staining of ATF2 localized in the nucleus correlates with metastases and worse outcome; cytoplasmic staining confers a better prognosis.(17)

In a study of SCC-25, a head and neck cancer cell line, plasmid-induced over expression of ATF2 results in decreased Extracellular Recognition Kinase (ERK) expression and cell cycle arrest, suggesting a tumor suppressive role.(18) However, the role of ATF2 has not been well described in HNSCC treatment response and survival. Cisplatin, currently the most widely-adopted chemotherapy to treat HNSCC, activates the kinases upstream of ATF2. Its mechanism of action creates DNA-platinum adducts followed by up-regulation of p38 and JNK and activation of caspases.(19) The use of cisplatin is, however, frequently associated with chemoresistance and its effectiveness can be improved.(20) We therefore decided to explore the role of ATF2 in the downstream effects of MAP/SAP kinase pathway activation in HNSCC.

Our study aims to determine ATF2 expression in HNSCC, its role (if any) in tumor growth, cisplatin induced cell death, and its effects on other related pro-survival factors such as Interleukin 8 (IL-8) expression. Interleukin 8, an important pro-inflammatory cytokine in HNSCC, is regulated by the Activating Protein-1 (AP-1) family of transcription factors that includes ATF2. Additionally IL-8 is associated not only with the pro-survival functions of Epidermal Growth Factor Receptor (EGFR) and angiogenesis in HNSCC, but also with

chemosensitivity in other tumor models. (21, 22) We report now that ATF2 is expressed in primary human HNSCC tumors and cell lines, and that its activation is strongly enhanced by the stress activators Tumor Necrosis Factor alpha (TNF α) and cisplatin. Our data suggest a regulatory role for ATF2 in TNF-related mechanisms of HNSCC. Its perturbation and nuclear activation are associated with significant effects on survival and cytokine production in HNSCC.

MATERIALS AND METHODS

Cell culture and treatments

UMSCC-9, UMSCC-38 and UMSCC-11B cells were a generous gift of Dr. Thomas Carey at the University of Michigan. HOK16B keratinocyte cells were a gift of Dr. N.K. Park at the University of California at Los Angeles School of Dentistry. Squamous carcinoma cells were incubated at 5% carbon dioxide (CO₂) and 37°C in Dulbecco's modified Eagle Medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA) with 2mM L-Glutamine, 1% non-essential amino acids (NEAA) (10X, Gibco), 10% heat-inactivated fetal bovine serum (FBS) (Gibco); HOK 16B keratinocytes were cultured in serum-free conditions using Keratinocyte growth medium with additives (Cambrex, East Rutherford, NJ). Serum starvation experiments were performed using 10% serum and 0.1% serum.

Cisplatin (Acros Chemicals) was diluted in phosphate buffered saline (PBS) at 1mg/ml stock, resuspended and filtered with a 0.22 μ m filter and stored at -20 °C. Recombinant Tumor Necrosis Factor (TNF) alpha (R&D Systems, Minneapolis, MN) was diluted in bovine serum albumin (BSA) as per manufacturer's recommendations and stored at -20 °C.

Knockdown of Activating Transcription Factor-2 and expression of Activating Transcription Factor peptide II

Head and neck squamous cell carcinoma cells were transfected with lentivirus-ATF2-GFP or VIRHD-GFP (control) (generous gift of Dr. Stu Aaronson, Mount Sinai School of Medicine) at multiplicity of infection (MOI) of 10 for 16 hours in 8ug/ml Polybrene (Aldrich, Milwaukee, WI). This vector results in expression of short hairpin RNA (shRNA) targeting ATF2. UMSCC-9 cell lines expressing shRNA were designated A1 or A2, and their GFP controls named G1 and G2. Untreated cell lines were designated C1, C2 or PB (for Polybrene only). Attempts to knock down ATF2 in another cell line (e.g. UMSCC 11B) resulted in complete death, while control vectors survived. After transfection, growth medium was replaced for 48 hours and green fluorescent protein (GFP) expression confirmed with green fluorescence microscopy. Pooled clones were selected using 2ug/ml puromycin (Calbiochem, San Diego, CA) for 2 weeks. Success of ATF2 knockdown was confirmed with western blot 72 hours later (method described below). To test another model of ATF2 interruption employing an interfering short peptide, lentiviral constructs were created with a short 50 amino acid ATF peptide II (gift of Dr. Ze'ev Ronai) along with a GFP expression sequence or GFP expression alone as control. Puromycin selected pooled clones were identified as CA (containing the ATF short peptide II) or G (containing GFP expression alone). Finally, downregulation of ATF2 by transfection with small interfering RNA (siRNA) double stranded 21-mers were designed to target ATF2 within nucleotides 523–541 (Dharmacon, Lafayette, CO) using control scramble sequences from the manufacturer. Different concentrations of siRNA duplex (50–150nM) per 6-well plates were transfected using Lipofectamine 2000 (Invitrogen) per manufacturer's recommendations. SiRNA targeting Cyclophilin B or a scramble sequence (Dharmacon) were used as controls.

In vivo chorioallantoic membrane tumor studies

As previously described (23), chorioallantoic membranes (CAMs) of 10 day old embryos were separated from the egg shell by creation of an artificial air chamber (Ossowski and Reich, 1980). Under aseptic conditions, a 1×1 cm window is created in the shell and removed. In sterile fashion, CAM were inoculated via the window with 5×10^5 cells suspended in sterile PBS per CAM. Groups were UM-SCC9, A1 and G1. Ten eggs were incubated for each group. Extreme care was taken to avoid damage to the CAM during the procedure. The eggs were incubated on their side in a non-rotating incubator at 37°C and 80% relative humidity for 7 days, at which time the tumors were harvested. Viability of the eggs was assessed by light transillumination. Tumors were measured in 2 dimensions and were then subjected to collagenase digestion. Tumor cells were determined under fluorescence microscopy to be positive for GFP, indicating continued expression of the transgene in the CAMs. Statistical analysis was performed using analysis of variance (ANOVA) to compare tumor sizes between groups.

Western immunoblot

Cells were grown to subconfluence, rinsed twice with ice cold PBS, collected with cell scrapers (Fisher) and pelleted at 2600rpm for 5 minutes. Lysis buffer was added in 1:1 volume with pellet and resuspended for 30 minutes on ice. Supernatants were collected after 30 minutes of centrifugation at 14,000 rpm at 4 °C and stored at -80 °C. Protein was quantitated using the Pierce BSA assay kit reading at 570nm on BioTek plate reader. 20–40ug of protein was denatured at 95 °C for 3 minutes and loaded in reducing buffer into an 8–16% Tris-Glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) gel (Invitrogen). Wet transfer was done onto 0.45 µm Polyvinylidene fluoride (PVDF) membranes overnight or for 2 hours with refrigeration. Antibodies used were phospho-ATF2 (p-ATF2) (thr71), total c-jun, p-38, total p38 (Cell Signaling), total ATF2 (Santa Cruz sc-187), total ERK (BD Transduction Laboratories, Lexington, KY), or poly ADP-ribose polymerase (PARP) (R&D). Membranes were blocked in 3% BSA (Sigma) or 5% milk blotto (Cell Signaling) for 1 hour at room temperature followed by primary antibody incubations overnight at 4 °C. Secondary antibody (Amersham, Buckinghamshire, UK) was incubated for 1 hour at room temperature and detected using Lumi-Light PLUS chemiluminescence substrate (Roche, Mannheim, Germany). Images were developed on Kodak BioMax film (Perkin-Elmer, Waltham, MA) and scanned with ScanMaker i900 (Microtek); layout and graphics were performed using Adobe Photoshop. Densitometric evaluation was performed using ChemiDoc imaging with QuantityOne software (BioRad, Hercules, CA).

Immunohistochemical staining

Paraffin-embedded tissue was obtained and evaluated under an institutional review board (IRB)-approved protocol by a head and neck pathologist (Dr. Margaret Brandwein-Gensler). Slides were pre-heated at 50 °C for several hours, then deparaffinized with xylene and rehydrated with ethanol and water. After quenching of endogenous peroxidase with hydrogen peroxide, slides were blocked with 3% normal goat serum for 1 hour. Antigen retrieval was done using buffered citrate (Dako, Glostrup, Denmark) at pH 6.0, boiling for 15 minutes. Primary incubations were done in TRIS-buffered saline-Tween-20 (TBST) overnight at 4 °C for p-ATF2 (Cell Signaling) or ATF2 (Santa Cruz) and secondary horseradish peroxidase antibodies (Dako) used for detection with 3,3'-Diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA).

Ten thousand cells per well were plated in 8-well slides (Nunc, Rochester, NY) and grown overnight. Cells were fixed using 4% paraformaldehyde buffered with sucrose. After blocking in 3% BSA, primary antibodies were incubated in TBST overnight at 4 °C using p-

ATF2 (Cell Signaling) or ATF2 (Santa Cruz); secondary biotinylated antibody (VectorLabs) was incubated for 1–2 hours at room temperature and detected using the VectaStain ABC kit (Vector Labs) and DAB (Vector) staining. Hematoxylin counterstain was used for 1 minute. Cover slips were applied after Vectashield (Vector Labs) was applied. Microscopy was done using Zeiss Axiophot (Carl Zeiss, Thornwood, NY) microscope.

Measurement of Nuclear Activation of Activating Transcription Factor-2

Nuclear activation and consensus oligonucleotide binding of phosphorylated ATF2 was tested in knockdown and control cell samples, with and without TNF treatment. This was performed using an enzyme-linked immunosorbent assay (ELISA)-based 96-well plate method, the TransAM ATF-2 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA). Nuclear extracts were obtained using the Nuclear Extract Kit (Active Motif, Carlsbad, CA) and 15 μ g of nuclear extract was added to each well containing oligonucleotide for consensus binding sequences and a Complete Binding Buffer as per manufacturer's directions. Competition using either wild type or mutant oligonucleotides was performed in combination with our nuclear extract samples. One-hour incubation was followed by rinsing, then application of a 1:1000 diluted antibody to phosphorylated ATF-2 for one hour at room temperature. Wells were rinsed and secondary horseradish peroxidase (HRP)-conjugated antibody was incubated for one hour at room temperature. A final wash was followed by color development using the Developing Solution with protection from light. Stop solution was then added and the plate was read at 450nm. Statistical analysis was performed to determine a difference between samples using ANOVA and student's t-test.

Cell cycle analysis

Cells were grown to subconfluence in 25 cm² flasks, trypsinized and rinsed in PBS twice. Pellets were fixed in ice cold 70% Ethanol (Sigma) while vortexing and stored at –20 °C. Cells were then pelleted at 1400 rpm for 10 minutes and rinsed twice. Cells were resuspended in RNase A 500 μ g/ml (Roche) and Propidium Iodide 50 μ g/ml (Roche) in 3.8 mM Sodium citrate and incubated for 30 minutes at 25°C. After filtering through a 35 μ m nylon membrane tube cap (Falcon, BD Biosciences, San Jose, CA), cells were then analyzed by FACscan (BD) with gating to eliminate doublets. Data analysis was done using CellQuest (BD). Two-tailed student's t-test or ANOVA was applied.

Cell Proliferation Assay

Cells were plated in 96-well flat-bottomed plates (Falcon) and allowed to adhere overnight. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Kit I (Roche) was used following manufacturer's recommendations. After experimental treatment, 10 μ l of MTT mitochondrial stain was added to culture medium and allowed to incubate at 37 °C for 4 hours. At 4 hours, 100 μ l of solubilizing solution was added and plates incubated at 37 °C overnight per manufacturer's recommendation. Plates were then analyzed at 570 nm for optic densitometry (OD) using the BioTek Plate Reader and analyzed with KCJunior software (BioTek, Winooski, VT). Optic densitometry levels were calculated as a percent of control cells for each cell line and two-tailed student's t-test or ANOVA was applied between groups.

Cell supernatant cytokine measurements

QuantikineTM ELISA kits (R&D) were used according to the manufacturer's instructions. Cells were treated with TNF alpha at 100ng/ml for 48 hours. Cell culture supernatants were collected at subconfluence, spun at 1500 rpm for 10 minutes at 4 °C and stored at –20 °C. Cells were trypsinized and counted at the time of supernatant collection using 0.4% trypan

blue staining (Cellgro). Supernatants were tested as previously described.(24) Samples were assayed in duplicate and two-tailed student's t-test and ANOVA were applied.

Protein Fractionation

Cells were washed at 70–90% confluence with 10 ml of ice cold PBS, then scraped and harvested by centrifuging at 3000 rpm for 3 min at 4 °C. Pellets were rinsed in 400 ul of homogenization buffer [250 ul of 4M sodium phosphate pH 7.2, 15 ul of 1 M Magnesium Chloride, 2 ul of 0.5 M ethylene glycol tetraacetic acid (EGTA) pH 8.0, 100 ul of 1 M potassium chloride, brought to 10 ml volume in water], with one Complete Mini™ ethylenediaminetetraacetic acid (EDTA) free tablet (Roche), and centrifuged for 1 min at 3000 rpm and 4 °C. Cells were resuspended in homogenization buffer (thrice the volume of the cellular pellet), incubated on ice for 30 min, then homogenized for 45 strokes with a bellco glass tissue homogenizer (Fisher). Homogenate was centrifuged at 4000 rpm for 15 min at 4 °C, and the cytoplasmic fraction was collected in a new tube. The pellet was washed with 200 ul of homogenization buffer and centrifuged at 3000 rpm for 1 min at 4 °C. This pellet is then resuspended in nuclear extract buffer with one Complete Mini, EDTA free (250 ul of 0.4 M sodium phosphate pH 7.2, 300 ul of 5M Sodium Chloride, 100 ul of 0.5 M EGTA pH 8.0, brought to 10 ml volume with water), and sonicated at 25°C for 1 minute with a Branson 2210 ultrasonic bath (Danbury, CT). Cells were incubated on ice for 30 min, and centrifuged at 12,000 rpm for 10 min at 4 °C. The chromatin fraction pellet was dissolved with 10 mM Tris pH 8.0. Protein concentrations were determined with BCA Protein Assay Kit (Pierce, Rockford, IL) and optic densitometry. Western blots were done using the following antibodies: phospho-ATF-2 (p-ATF2)(Thr71; Cell Signaling), ATF-2 (C-19; Santa Cruz), β -Actin antibody (Sigma) was used as loading control.

RESULTS

Activating Transcription Factor-2 is constitutively expressed in HNSCC cell lines

In order to assess the expression of ATF2, immunohistochemistry and western blots were performed in HNSCC cell lines. Western blots showed expression in cells at baseline and in conditions of serum starvation to assess response to stress. Threonine-71 phosphorylation was targeted, so that phosphorylation from all upstream kinases would be captured. Western blots of whole cell extracts reveal that total and phosphorylated forms of ATF2 are expressed at baseline in these cells and also in HOK oral keratinocytes (Fig 1A). Varying levels of baseline phosphorylation are observed between cell lines but levels of total ATF2 are similar. In serum starvation conditions, no significant differences were observed when (UM-SCC-9) cells were deprived of serum from 10% (baseline) to 0.1% (deprived) for 24 hours (Fig 1B). Serum starved cells were compared to control cells using densitometry of whole cell lysates for p-ATF2 and levels of total ATF2 (upper inset panel). This finding demonstrates that ATF2 expression and activation is constitutive in these cells and is not dependent on exogenous serum-derived factors. However, when individual cell compartment fractions are evaluated (Fig. 1B, lower inset panel), we observed an increase in p-ATF2 in these same serum starvation conditions. An increase of 1.4 fold over control cells was seen in the nuclear expression of p-ATF2 as shown by densitometry. When cell lines are studied by IHC, staining for total ATF2 revealed the presence of nuclear and cytoplasmic localization, with less obvious cytoplasmic staining seen in the HOK keratinocyte cells. (Fig 1C) In human tumor nests (Fig 1D and 1E), a global staining pattern is seen with ATF2 present in both nucleus and cytoplasm of most cells.

Nuclear activation of phosphorylated Activating Transcription Factor-2 (p-ATF2) is significantly increased in conditions of partial Activating Transcription Factor-2 knockdown

As seen in Figure 2A, stable partial knockdown of total ATF2 was achieved (lentivirus ATF2-GFP, cell lines A1, A2); control vector transduced cells (G1, G2) were compared to untreated UM-SCC-9 (named C1, C2). Densitometry quantitation revealed approximately 50% reduction in total ATF2 protein in A1 and A2 (Fig. 2A, inset panel). Mean expression levels of groups C1 and C2, A1 and A2, and G1 and G2 revealed statistically significant differences between groups ($p < 0.05$) (*). Baseline levels of related transcription factor c-jun and the kinase ERK were not altered as a result of this partial ATF2 knockdown. Similar results were obtained when 21-mer siRNA was introduced with lipofection, using a scramble sequence as a control (data not shown). (B) Nuclear extracts of vector control (G1) and knockdown (A1, A2) cells were compared to control (untreated UM-SCC-9) cells (Fig 2B). 15ug of nuclear extract was evaluated, and results are shown as OD 450nm. Wild type and mutant oligonucleotide competition assays were performed for each cell line (data not shown) and resulted in expected decreases when wild type was present and no change in the presence of mutant oligonucleotide. An almost two-fold increase is observed in A1 consensus binding compared to G1; for cell line A2, a 1.6-fold increase is observed, both of which are statistically significant ($p < 0.025$).

Activating Transcription Factor-2 knockdown does not affect baseline tumorigenesis of UM-SCC-9 in vivo

Given its downstream location in the MAP/SAP kinase pathway, we hypothesized that changes in ATF2 might affect tumorigenicity and the sensitivity of HNSCC cells to cytotoxic treatment. Baseline viability was unaffected and MTT proliferation showed a very small but statistically significant 11% increase induced by the partial knockdown of ATF2 in cell line A1 ($p < 0.05$) (Fig. 3B). These cells were all then introduced into CAM to assess tumor growth effects. Stably transfected ATF2 knockdown cell lines, A1 and A2, or the control cell line G1 developed tumors in CAM after one week's incubation. The size of the tumors (range 23.4 – 36.1mm²) did not differ statistically between the groups and the appearance of the tumors was identical (Fig 3A). These findings were confirmed with duplicate experiments.

Partial inhibition of Activating Transcription Factor-2 affects cisplatin sensitivity

Dose responses were generated for cell lines G1, A1 and A2 using MTT proliferation assay. Based on preliminary experiments, cisplatin doses were chosen from 5uM to 100uM with PBS used as the control treatment. Figure (3C) shows the results at 48 hours. Results are displayed as a proportion of the control group scores and reveal a statistically significant lowering of the proliferation for G1 compared to both A1 and A2 ($p < 0.001$) at doses of cisplatin 25 μ M and below. Our data show that the proliferation for G1 at 10uM is at least 26% lower than either A1 or A2 ($p < 0.001$) (*). Differences of statistical significance ($p < 0.001$) (*) were also observed between control and knockdown groups at 5uM and 25uM.

Activating Transcription Factor-2 modulation alters the apoptotic response to cisplatin

As previously published, variability exists in cisplatin sensitivity between HNSCC cell lines with UM-SCC-9 having sensitivity intermediate to UM-SCC-11B and UM-SCC-38.(25) We confirmed baseline cisplatin sensitivity levels and then chose to impair ATF2 function in UM-SCC-9 using either introduction of short interfering ATF peptide II or using shRNA-targeting ATF2. Baseline viability and proliferation of cells was not significantly affected by expression of the ATF peptide II or by shRNA-mediated knockdown of ATF2. Cells were then subjected to cisplatin treatment at 25uM. ATF2 shRNA knockdown (cell lines A1, A2)

demonstrated significantly less chemosensitivity to cisplatin, demonstrated by sub-G1 localization (Fig 4A). This is in agreement with MTT proliferation studies at this dose. There is a statistically significant reduction in apoptosis in the knockdown cells A1 ($p=0.003$) and A2 ($p=0.002$) compared to controls VIRHD-GFP (G1) and polybrene-treated control (PB) cells in repeated experiments. Significance was roughly the same for both A1 and A2 knockdown cell lines and reflected a nearly threefold reduction in the sub-G1 population. Apoptosis was confirmed by western blot analysis of PARP cleavage products and showed chemoresistance in the ATF2 knockdown cells compared to control cells at this early timepoint (Fig 4B). Because saturation of the PARP enzyme occurred by 24 hours in all groups, the earlier timepoint of 6 hours was studied in order to detect early differences between the groups. When ATF peptide II is introduced, similar results are seen. Less PARP cleavage is seen in cells with ATF peptide II compared to control cells, when subjected to the same cisplatin doses (Fig 4C). These findings demonstrate that multiple methods of silencing ATF2 activity can produce similar effects on cisplatin responsiveness. Cells become less sensitive when either baseline ATF2 levels are decreased by half (fig 4A) or when ATF2 phosphorylation is interrupted (fig 4C).

Activating Transcription Factor-2 is phosphorylated and translocated to the nucleus following treatment with Tumor Necrosis Factor-alpha. Cisplatin also induces Activating Transcription Factor-2 phosphorylation

To determine effects from TNF stimulation on these cells, ATF2 was examined after treatment with recombinant human TNF alpha. As seen in figure 5, phosphorylation of ATF2 is increased following treatment with TNF-alpha in UMSCC-9 and is highest within one hour, with a dropoff seen after 6 hours (Fig 5A). TNF treatment was followed by increased detection of p-ATF2 in both the nuclear and chromatin fractions of cell lysates at 4 and 24 hours (Fig 5B). Using these same cells as a control group in Fig 5C, a statistically significant increase of p-ATF2 in nuclear fractions is clearly demonstrated following 24 hours of TNF treatment in the control UMSCC-9 cells ($p<0.01$). These findings thus demonstrate that TNF is an effective agent to induce both ATF2 phosphorylation and its translocation to the nucleus. ATF2 phosphorylation remains elevated in the presence of TNF when tested at 24 hours (Fig 5B). Treatment with cisplatin likewise resulted in a burst of phospho-ATF2 activation (Fig 6A, lanes 2,6) consistent with the role of ATF2 as a stress response molecule. These data together demonstrate that ATF2 phosphorylation occurs in response to TNF alpha- and stress-activated mechanisms and that sustained phosphorylation can be achieved. At least with TNF-alpha, this activation was associated with significant translocation of ATF2 to the nucleus.

The responsiveness of partial knockdown cells to TNF, as measured by nuclear p-ATF2, remained intact and was increased (TNF treated shown in hatched columns, Figure 5C), even though their baseline activation levels were already significantly elevated ($p<0.01$) over control groups (Fig 5C). These increases did not consistently reach statistical significance in all treatment groups.

Knockdown of Activating Transcription Factor-2 alters expression of Interleukin-8 by head and neck cancer cells

In order to observe downstream effects of ATF2 related to other TNF-mediated functions, Interleukin-8 production by cells was measured using ELISA. In multiple repeated experiments, ATF2 deficient cell lines demonstrate significantly increased secretion of IL-8. Treatment with TNF-alpha resulted in increased stimulation of IL-8 expression by both parental and knockdown cells lines, with an exaggerated IL-8 production response seen in ATF2 knockdown cell lines: A1 alone ($p=0.02$), A1 + TNF ($p=0.001$) (Figure 5D). These data indicate that ATF2 status partly determines the response of TNF-driven IL-8 expression

and confirms that TNF receptor activation and signaling pathways remain intact. Conditions associated with partial knockdown of ATF2 potentiate the cells' response to TNF alpha, suggesting either a regulatory role for ATF2 at IL-8 promoter sites or the existence of a compensatory increase in IL-8 through a parallel pathway [e.g. via Nuclear Factor-kappa B (NFkB) or AP-1]. Increases in TNF-induced IL-8 production were seen following treatment with ATF peptide II (data not shown).

Tumor Necrosis Factor pretreatment increases cisplatin sensitivity in cells

In order to determine if TNF treatment could reverse the observed negative effects of ATF2 modulation on apoptosis, we pretreated both control and knockdown cells with TNF alpha and observed p38 and ATF2 phosphorylation. First, in knockdown cells we confirmed minimal cisplatin-induced apoptosis at this early timepoint (Fig 6A lane 6) that contrasts significantly with the sensitivity seen in control cells (Fig 6A, lane 2). This finding confirms our previous findings and highlights baseline total ATF2 levels as an important factor affecting the early apoptotic response to cisplatin. After 24 hours pretreatment with TNF alpha, we noted that p-ATF2 was activated in both control and knockdown cells, in agreement with previous data (Figure 6A lanes 3,7). Following the addition of cisplatin (lanes 4,8), TNF treated groups had significant enhancement of apoptosis and PARP cleavage compared to no TNF treatment (lanes 2,6). This is observed in both control and knockdown cells. By itself, despite intact and sustained ATF2 phosphorylation, TNF treatment was accompanied by minimal to no cytotoxicity as measured by PARP cleavage (lanes 3 and 7).

Effects on p38

Noting its location as an upstream activator of ATF2 and its status as a known effector molecule of cisplatin toxicity, we studied the activity of p38 (also Fig 6A). We observed that in both knockdown and control cell lines, cisplatin results in activation of both p38 and p-ATF2. (lanes 2,6) However, important differences exist that correlate directly with baseline ATF2 levels. While cisplatin treatment alone results in activation of both phospho-ATF2 and phospho-p38 in both cell lines, (lanes 2,6) such increases were associated with significant apoptosis only in control cells. (lane 2) Markedly significant p38 phosphorylation in the ATF2 knockdown cells is seen following TNF (Fig 6A, lane 7) but this response was not observed in control cells (lane 3). This differential p38 stimulation may be due to disinhibition of upstream activators of p38. In knockdown cells, there was also a more significant increase in p38 phosphorylation when combined TNF and cisplatin are given, coinciding with the recovery of cisplatin-induced apoptosis in the knockdown cells (Lane 8). This further suggests a disinhibition of p38 activation in the ATF2 knockdown setting. A significant synergistic enhancement of apoptosis followed the cotreatment TNF/cisplatin in both cell lines. (lanes 4,8) but a requirement for TNF treatment characterized the knockdown cells' ability to respond with early PARP cleavage to cisplatin (lane 8).

In summary, in control cells, normal baseline levels of ATF2 are associated with cisplatin-induced apoptosis. In controls, there is minimal apoptosis in response to TNF alone, with absence of a p38 response. Combination treatment results in enhanced apoptosis and ATF2 response with no p38 change over cisplatin alone (Figure 6A, lane 4). When control cells respond to cisplatin and TNF co-treatment, higher levels of ATF2 phosphorylation appear to be associated with a higher apoptotic response. In contrast, knockdown cells, with reduced baseline levels of total ATF2, retain the capacity for cisplatin-induced p38 (and p-ATF2) phosphorylation but do not undergo apoptosis in these conditions. Only when TNF pretreatment is provided (sustained increases in p38 and p-ATF2 activation) is cisplatin sensitivity restored. There is an exuberant p38 response in knockdown cells following TNF that may be enhancing the observed responses. Taken together, these findings suggest that 1.

apoptosis induction with cisplatin is associated heavily with baseline ATF2 levels and 2. that dysregulation of p38 activity appears to occur in conditions of ATF2 deficiency and may contribute to the observed cisplatin response.

Shifts in cisplatin sensitivity occur with Tumor Necrosis Factor pretreatment

Dose response experiments with cisplatin revealed several statistically significant differences resulting from pretreatment with TNF alpha. Correlating with our PARP cleavage findings, these differences were more impressive in the cell lines A1 and A2. Cells were treated with either PBS or TNF 100ng/ml for 24 hours followed by cisplatin or PBS for 48 hours. MTT assay reveals a downward shift in proliferation of TNF treated groups that is significant in A1 ($p<0.002$) and A2 ($p<0.02$) for all doses of cisplatin (Fig 6B). G1 also showed worse proliferation at all cisplatin doses 10uM and higher ($p<0.002$) in the presence of TNF; these differences were minimal above 37.5uM. At doses around 10uM (an accepted cutoff for chemosensitivity to cisplatin) lines A1 and A2 at baseline had significantly worse proliferation in the presence of TNF; a less impressive but statistically significant difference is observed in control G1 cells at the same dose.

DISCUSSION

Transcription factors have been shown to play important roles in tumorigenic functions in HNSCC, such as Signal Transducers and Activators of Transcription (STAT), AP-1 and NFkB.(26, 27) Targeting of these transcription factors reduces oncogenic potential and enhances cytotoxic response in clinical trials via small molecule inhibitors in HNSCC.(28) We demonstrate here that ATF2 can be impaired using conventional gene therapy techniques, resulting in a number of responses that appear to promote survival. In vivo CAM studies demonstrated that the ability of cells to produce tumors was neither significantly impaired nor enhanced by the knockdown of ATF2. Likewise, the time to tumor development was similar between control and knockdown cells. Despite evidence of tumor suppression in other models of ATF2 knockout, our data instead show other important influences of ATF2 on the responses to cisplatin treatment and TNF stimulation. The lentiviral-mediated shRNA targeting of ATF2 results in significantly decreased constitutive ATF2 expression. Attempts to knock down ATF2 in other cell lines resulted in complete death, while control vectors survived. Use of a conditional knockout of ATF2 therefore may prove useful in future studies. Despite incomplete ablation of ATF2, we observed several interesting findings resulting from partial reduction of baseline ATF2. These included a constitutively increased level of nuclear phosphorylated ATF2. The knockdown cells constitutively had 1.6 to 2.0 times the levels of nuclear p-ATF2 as control vector-transfected cells. Furthermore, their responsiveness to TNF, as measured by nuclear p-ATF2, remained intact. Several mechanisms may explain the nuclear localization of p-ATF2 in response to its partial knockdown. Partial knockdown may represent a stressor for these cells, leading to a phenotypic response similar to that seen with serum starvation. Alternatively, auto-regulatory feedback of p-ATF2 on its own upstream kinases or phosphatases may take place. Although this study did not evaluate such upstream regulators, this phenomenon has been described in other systems.

Importantly, we also observed effects of altered sensitivity to cisplatin and a significant increase in IL-8 secretion following partial knockdown of ATF2. These findings suggest that ATF2 affects pathways of cell survival and response to treatment or stress (e.g. stimulation with TNF, treatment with cisplatin). ATF2 may be an important regulatory molecule in these conditions whose effects can be exploited for clinical use.

Apoptosis

Recent clinical trials in recurrent tumors demonstrate efficacy of cisplatin in combination with molecular targeted treatments (e.g. cetuximab). (29) Our data suggest that in HNSCC, baseline ATF2 levels may help determine chemosensitivity to this agent. Treatments with Staurosporine in ATF2 knockdown cells revealed an inverse relationship of chemoresistance to baseline ATF2 expression (data not shown), indicating that various methods of apoptosis induction can be affected by ATF2 status. We show here that a reduction of overall ATF2 and resultant increase in its nuclear activation affords a survival advantage for HNSCC cells subjected to chemotherapy. Exaggerated phosphorylation of p38 is seen in the setting of ATF2 reduction with an enhancement of chemosensitivity after TNF pretreatment. The complexity of MAP kinase signaling is observed in the potentiation of chemosensitivity when cells are exposed to TNF 24 hours in advance of cisplatin. Increasing evidence shows that following cisplatin-induced kinase activation, not only is the nature of the resultant kinase cascade activation important in determining effects on cell fate, but also its duration is important. (30) We suggest that the prolonged TNF stimulation in low baseline ATF2 conditions allows for exaggerated responses of phosphorylated p38, resulting in restoration of early apoptosis induction once the cisplatin is applied. In the control cell model, the TNF mediated stimulation also results in enhanced responses to cisplatin, suggesting that even lower doses of cisplatin might be equally effective when TNF is included in treatment. We observed small but statistically significant deleterious effects on MTT proliferation in these cisplatin treated cell lines in the presence of TNF pretreatment (Fig 6B). The clinical applicability of this finding may thus lead to reductions in cisplatin-related toxicity when TNF is added.

We suggest here the possibility that in HNSCC cells, the baseline levels of total ATF2 are important to maintain the apoptotic responsiveness of these cells to cisplatin. Baseline levels of total ATF2 may also determine its auto-feedback, evidenced by increases in nuclear p-ATF2. ATF2 is a downstream target of p-38, so that baseline increases in p-38 inducibility (or impairment of its inhibition) would be expected to result in increased p-ATF2 activation. When a reduction of baseline total ATF2 exists in our cells, a higher degree of p38 activation is observed in the responses to cisplatin and TNF. We conclude that ATF2 deficient cells exhibit exaggerated p38 activation downstream of TNF, perhaps as an adaptive response. We believe this may occur via disinhibition of upstream inactivators of p38. In other models this mechanism is conducive to apoptosis when ATF2 is knocked out, and is associated with reductions in DUSP.(14) Thus enhancement of p38 activation may be integral to restoring chemosensitivity when total ATF2 levels are low.

Interleukin-8

With reduced baseline ATF2, sensitivity to TNF alpha signaling remains not only intact, but is in fact enhanced as demonstrated by increased p38 activation and increased nuclear p-ATF2 activation. We also observed that IL-8 expression is increased and is also hyperresponsive to exogenous TNF in the setting of ATF2 knockdown. The IL-8 promoter is known to contain NFkB and AP-1 binding sites.(31) Since AP-1 and ATF2 sites share c-jun as a common transcriptional activator there could be resulting availability of free c-jun in the ATF2 knockdown setting, thus providing increased potential for binding of jun to AP-1. Elevations in IL-8 expression would also be supported by significant increases of transcription of c-jun (a transcriptional target of ATF2) in the knockdown setting, (26) though our data do not support such an explanation.

Effects on IL-8 suggest that ATF2 may be regulatory for IL-8 production in our model. Agelopoulos elegantly showed that ATF2 acts as a transcriptional repressor via interactions with AP-1 binding sites on the IL-8 promoter. This mechanism is dependent upon ATF2

interactions with nuclear MacroH2A that has been recruited to the AP-1 binding site, physically impairing transcription of IL-8 by AP-1. (32) Down-regulation of ATF2 results in a rescue of IL-8 expression in their model. Another potential explanation for the observed increased IL-8 is through cross-talk between the ATF2 and NFkB pathways. Inhibition of activated ATF2 by a p-38 inhibitor is shown to inhibit NFkB activity.(33) Their study identified both ATF2 and c-jun as components of the protein complex bound to IL-8 promoter at AP-1 sites. We therefore propose that enhanced IL-8 production in the setting of ATF2 inhibition may have multiple causes.

We are unable to make conclusions about the role of IL-8 in chemoresistance to cisplatin in our model, but increased IL-8 expression may be important. Other studies in the literature associate IL-8 mechanistically with chemoresistance. IL-8 (or CXCL8), shown in both ovarian and prostate carcinoma to be an important regulator of apoptosis, is associated with poor responses to chemotoxic agents. Prostate carcinomas stimulated with IL-8 show potentiation of NFkB and the antiapoptotic proteins Bcl2 and inhibitor of apoptosis proteins (IAP). IL-8 staining is elevated in prostate carcinoma and is associated with worse disease stage. In ovarian cancer, high serum IL-8 correlates with poor response to taxol therapy(22) IL-8 is responsible for inhibition of TNF-Receptor associated ligand (TRAIL)-induced apoptosis in ovarian carcinoma by p38-mediated pathways.(34) Finally, impaired caspase 8 activation and increased c-FLIP are associated with IL-8 signaling in prostate cancer cells. (35) Our data raise the possibility that elevated IL-8 may be mechanistically implicated in the impaired response to cisplatin and further study in this area is warranted.

CONCLUSION

In summary, we demonstrate that ATF2 appears to be important in chemosensitivity to cisplatin and regulation of IL-8 in HNSCC. Its reduction is associated with impaired chemosensitivity and enhanced IL-8 production. Our study provides evidence that in HNSCC, cisplatin-induced apoptosis is facilitated in the setting of TNF pretreatment and associated p38 hyperactivation. In the conditions of our model, enhanced production of the pro-survival factor IL-8 occurs, suggesting a possible regulatory role for ATF2 in this system. The demonstration of anti-apoptotic functions of IL-8 in other tumor systems supports the hypothesis that ATF2 may thus influence multiple avenues of chemosensitivity in HNSCC. We demonstrate that an enhancement of existing chemosensitivity is attainable using pretreatment with TNF alpha. The restoration to early chemosensitivity occurs simultaneously with activation and translocation of ATF to the nucleus but the observed hyperactivation of inducible p38 suggests that ATF2 may be an important regulator of feedback on p38. We conclude that because of its observed effects on chemosensitivity and TNF related mechanisms, ATF2 may prove useful as a modulator or marker of responsiveness to adjuvant therapy. Its effects on IL-8 production may impact studies where serum cytokines are used to predict disease progression or prognosis. We also suggest that TNF co-treatment may have a role in cisplatin based treatment regimens.

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Fig 1. Baseline Activating Transcription Factor-2 (ATF2) expression in head and neck squamous cell carcinoma (HNSCC) and Oral Keratinocytes

(A) ATF2 and p-ATF2 expression is seen by western immunoblotting of whole cell extracts in HNSCC and human keratinocyte cell lines. HOK-keratinocyte; 38-UMSCC 38; 11B-UMSCC 11B; 9-UMSCC 9

(B) Serum starvation does not affect baseline or phosphorylated ATF2 levels in UMSCC-9. Cells were exposed to growth medium enriched with 10% or 0.1% FBS for 24 hours. Densitometry of total cell lysates reveals no significant differences between groups. Expression of p-ATF2 in nuclear cell fractions was roughly 1.4-fold higher in serum starvation conditions than in 10% serum medium. No significant differences were observed in cytoplasmic fractions; no differences were observed in total ATF2 expression in either cytoplasmic or nuclear fractions.

(C) 40X magnification with 3,3'-Diaminobenzidine (DAB) detection of ATF2 immunostaining. Head and neck squamous cell carcinoma cell lines (UMSCC 9, UM-SCC 38, UMSCC 11B) and human keratinocyte cell line (HOK) are positive for ATF2. Signal is characterized by heavy nuclear and variable cytoplasmic staining in tumor cells. Controls shown are UMSCC 9 and HOK in the absence of primary antibody.

(D), (E). Tumor nests 20X, paraffin-embedded human HNSCC tumor specimens stained with ATF2 antibody and DAB detection.

Fig 2. Knockdown of total Activating Transcription Factor-2 (ATF2) is specific and does not inhibit in vivo tumor production

(A) Knockdown of total ATF2 (cell lines A1, A2, lanes 3,4) is observed. Control untreated cells (C1,C2; lanes 1,2) and green fluorescent protein (GFP)-only cell lines (cell lines G1, G2; lanes 5,6). C-jun and extracellular recognition kinase (ERK) levels are unchanged in response to ATF2 knockdown. Densitometry was done for total ATF2 (inset panel), showing statistically significant (*) differences between groups. A 45%-50% reduction in total ATF2 expression is observed in knockdown cells when compared to transfection controls G1 and G2 ($p < 0.05$).

(B) p-ATF2 is increased in nuclear extracts of knockdown cells. Nuclear Extracts of control and knockdown cells were compared to control (untreated) cells. 15ug of nuclear extract was evaluated, results are shown as OD 450nm. Replicates were performed; (*) indicates statistically significant difference ($p < 0.05$).

Fig 3. Effects of partial Activating Transcription Factor-2 (ATF2) knockdown on proliferation, cell growth and chemosensitivity

(A) In vivo chorioallantoic membranes (CAM) tumor production is not significantly different between ATF2 knockdown cell lines and controls. G1 is control, A1 and A2 (knockdown). Tumors were similar in appearance and expression of green fluorescent protein (GFP) was confirmed in all tumor specimens with fluorescence microscopy. Each group contained 7–10 tumors, results were replicated in identical experiments. ANOVA (with an alpha of 0.05) was used to compare tumor volumes between the three groups showing no significant differences. (B) Cell proliferation assays (MTT) revealed very small but statistically significant increases ($p < 0.05$) in A1 and A2 when compared to G1 at 24 and 48 hours.

(C) Inhibitory Concentration (IC) 50 for cisplatin is significantly increased in ATF2 knockdown cells. Cells were treated for 48 hours and compared to PBS treated control cells. Results are shown as a proportion of the control [mean treatment group score/mean control group score]. Statistically significant (*) differences ($p < 0.001$) were observed at all doses 25uM and lower.

Fig 4. Activating Transcription Factor-2 (ATF2) deficiency leads to cisplatin chemoresistance in head and neck squamous cell carcinoma (HNSCC)

(A) Flow cytometry cell cycle analysis demonstrates presence of cisplatin sensitivity measured as sub-G1 (“M1”) accumulation at 24 hours in controls (upper 2 panels) and reduced sub-G1 (M1) following cisplatin in ATF2 deficient cell lines A1,A2 (lower 2 panels). Values indicate % of cells seen in M1 (sub-G1) **indicates statistical significance at alpha = 0.05 level.

(B) Response to genotoxic chemotherapy (cisplatin) is altered with ATF2 modulation. Poly-ADP ribose polymerase (PARP) cleavage product (23kD) accumulation shown 6 hours following cisplatin treatment (lanes 3,6). Significant inhibition of apoptosis is seen in knockdown cell lines A1 and A2 (lanes 4,5) while sensitivity is maintained in controls

(C) Response to genotoxic chemotherapy (cisplatin) is altered with ATF2 modulation by peptide II (amino acids 50–100 of ATF2). Poly-ADP ribose polymerase (PARP) cleavage (23kD) in control cells, clones expressing either green fluorescent protein (GFP) (“lenti-GFP”) or expressing GFP with short ATF peptide II (“lenti-ATFII peptide”). Cells were treated for 24 hours with indicated uM dose of cisplatin or with control (PBS).

Fig 5. Treatment with Tumor Necrosis Factor (TNF) alpha stimulates Activating Transcription Factor-2 ATF2 phosphorylation and nuclear localization

(A) Treatment with recombinant TNF alpha (100ng/ml) increases expression of p-ATF2 while total ATF2 remains stable. The most robust increases are observed at very early timepoints but a persistent elevation is observed at later timepoints.

(B) TNF treatment (100ng/ml) results in significant increases in p-ATF2 in nuclear cell compartment fractions. At 4 hours, nuclear staining of p-ATF2 is observed and by 24 hours both nuclear and chromatin fractions stain positive for p-ATF2.

(C) Nuclear activation of p-ATF2 before (first bar) and following 24 hours' treatment with 100ng/ml TNF alpha (darker hatched bar). 15ug of nuclear extract was evaluated, results are shown as OD 450nm. Wild type and mutant oligonucleotide competition assays were performed for each group (data not shown) and resulted in expected decreases when wild type was present and no change in the presence of mutant oligonucleotide. Replicates were performed. Statistical significance was observed for UMSSC-9 controls ($p < 0.01$) and cell line A2 ($p < 0.03$).

(D) Interleukin-8 production is altered in response to ATF2 knockdown. Interleukin-8 concentrations shown in pg/ml per million cells counted at time of supernatant harvest. Cells treated with polybrene alone (PB), GFP expression (G1) or GFP and shRNA targeting ATF2 (A1). TNF indicates 24 hour treatment with 100ng/ml TNF alpha. ** indicate statistical significance observed in replicates compared to controls.

Fig 6. Pretreatment with Tumor Necrosis Factor (TNF) alpha synergizes with cisplatin to enhance apoptosis and reduce proliferation

(A) Following 24 hours pretreatment with TNF alpha (100ng/ml), UMSCC-9 cells were treated with cytotoxic doses of cisplatin (37.5 uM) for 6 hours and poly-ADP ribose polymerase (PARP) cleavage products were detected by western blot. TNF alpha treatment stimulates minimal apoptosis by itself (lane 3) and 6 hours cisplatin produces PARP cleavage (lane 2). An increase in PARP cleavage is detected in the cells receiving cisplatin and TNF pretreatment (lanes 4,8) over those with cisplatin alone. Associated changes in phosphorylation of p38 and pATF2 are also observed.

(B) Proliferation assay of cell lines G1, A2 and A1. Cells were treated in 96-well plates with either PBS or TNF 100ng/ml for 24 hours followed by cisplatin or PBS for 48 hours. Proliferation as measured by OD 570 was reported as proportion of the control [mean treatment group score/mean control group score]. All cell lines showed statistically significant differences ($p < 0.02$ or better) with TNF pretreatment at cisplatin doses 10uM and higher; cell lines A1 and A2 showed statistically significant differences at all cisplatin doses.

Fig 7. Schematic model of Activating Transcription Factor-2 (ATF2)-related signaling in head and neck carcinoma cells

ATF2 transcription factor is phosphorylated by multiple upstream kinases. Activation and localization in the nucleus is determined by its phosphorylation and heterodimerization with other Activator Protein-1 (AP-1) transcription family members. Increased expression of Interleukin 8 (IL-8) can be observed following either Tumor Necrosis Factor (TNF) stimulation or reduction of baseline ATF2, suggesting a regulatory role for ATF2. (NFkB=Nuclear Factor-kappaB; TRAF=TNF Receptor associated factor)