Published in final edited form as:

Mol Carcinog. 2015 December ; 54(12): 1734–1747. doi:10.1002/mc.22246.

Grape seed extract targets mitochondrial electron transport chain complex III and induces oxidative and metabolic stress leading to cytoprotective autophagy and apoptotic death in human head and neck cancer cells

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is a major killer worldwide and innovative measures are urgently warranted to lower the morbidity and mortality caused by this malignancy. Aberrant redox and metabolic status in HNSCC cells offer a unique opportunity to specifically target cancer cells. Therefore, we investigated the efficacy of grape seed extract (GSE) to target the redox and bioenergetic alterations in HNSCC cells. GSE treatment decreased the mitochondrial electron transport chain complex III activity, increased the mitochondrial superoxide levels and depleted the levels of cellular antioxidant (glutathione), thus resulting in the loss of mitochondrial membrane potential in human HNSCC Detroit 562 and FaDu cells. Polyethylene glycol-SOD addition reversed the GSE-mediated apoptosis without restoring complex III activity. Along with redox changes, GSE inhibited the extracellular acidification rate (representing glycolysis) and oxygen consumption rate (indicating oxidative phosphorylation) leading to metabolic stress in HNSCC cells. Molecular studies revealed that GSE activated AMP-activated protein kinase (AMPK), and suppressed Akt/mTOR/4E-BP1/S6K signaling in both Detroit 562 and FaDu cells. Interestingly, GSE increased the autophagic load specifically in FaDu cells, and autophagy inhibition significantly augmented the apoptosis in these cells. Consistent with *in vitro* results, *in vivo* analyses also showed that GSE feeding in nude mice activated AMPK and induced-autophagy in FaDu xenograft tumor tissues. Overall, these findings are innovative as we for the first time showed that GSE targets ETC complex III and induces oxidative and metabolic stress, thereby, causing autophagy and apoptotic death in HNSCC cells.

Keywords

Oxidative stress; Bioenergetics; Head and neck squamous cell carcinoma; Grape seed extract; Autophagy

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is a devastating disease worldwide accounting for 650,000 new cases and 350,000 deaths every year [1,2]. In the United States alone, approximately 53,640 new cases and 11,520 deaths were estimated due to HNSCC in the year 2013 [2]. The survival rates of HNSCC patients have remained poor due to diagnosis of the disease at advanced stages, disease recurrence and/or development of secondary primary tumors, and poor response to conventional treatments [3,4]. This underscores the rationale for developing additional/alternative preventive and therapeutic approaches to reduce the morbidity and mortality due to this malignancy [5,6].

Epidemiological studies have suggested that consumption of fruits and vegetables reduces the risk of several malignancies including HNSCC [7,8]. One of the phytochemicals that has been widely investigated for cancer prevention and intervention over the years is grape seed extract (GSE) [9–11]. In the past, extensive preclinical and clinical studies have illustrated the promising anti-cancer and chemopreventive efficacy of GSE in several cancer models [12–15]. GSE has been reported to act as a free radical scavenger reducing oxidative stress by activating Nrf2 (NF-E2 related factor)/ARE (antioxidant response element) mediated expression of antioxidant enzymes [13,16,17]. However, GSE has also been shown to exert prooxidant effects at higher doses by participating in redox cycling and generating reactive oxygen species (ROS) [18]. Recently, we reported that GSE promotes ROS generation in HNSCC cells that play a central role in GSE-mediated DNA damage and apoptosis in HNSCC cells [19]; however, the exact mechanism/s for the prooxidant effects of GSE has remained unknown. In the present study, we examined the role of mitochondrial electron transport chain (ETC) complexes in GSE-mediated induction of oxidative stress and apoptosis in HNSCC cells.

A substantial body of evidence indicates that cancer cells possess an altered metabolic phenotype including an enhanced dependency on aerobic glycolysis [20]. This metabolic shift in cancer cells from oxidative phosphorylation (OXPHOS) to aerobic glycolysis is required to generate macromolecules for rapid proliferation of these cells [21–23]. Clinically, an elevated tumor lactate (a product of glycolysis) concentration has been associated with the development of nodal or distant metastasis in HNSCC patients [24]. Recently, Tripathi et al [25] reported significant alteration in the levels of several metabolites in HNSCC cells suggesting deregulation in multiple metabolic events. Therefore, agents that could effectively target aberrant metabolic pathway/s in HNSCC cells could serve as novel preventive and therapeutic tools to selectively inhibit cancer growth and progression. Accordingly, present study was designed to investigate the effect of GSE on the cellular metabolism, bioenergetics, and metabolism-related signaling pathways (Akt/mTOR/AMPK) in HNSCC cells. Results showed that GSE targets ETC complex III and induces oxidative and metabolic stress in HNSCC cells. Furthermore, GSE activated AMPK, suppressed Akt/mTOR signaling, and induced autophagy and apoptotic death in HNSCC cells.

MATERIALS AND METHODS

Chemicals and reagents

Antibodies for phosphorylated and total ACC (acetyl-coA carboxylase), AMPK (AMP-activated protein kinase), 4EBP1 (4 eukaryotic binding protein-1), mTOR, and Akt were purchased from Cell Signaling (Beverly, MA). Anti-LC3 antibody was from Novus Biological (Littleton, CO) and anti-Bec1 antibody was from BD Bioscience (San Jose, CA). β -actin antibody, chloroquine, bafilomycin, oligomycin, antimycin A, 2-deoxyglucose (2-DG), polyethylene glycol-superoxide dismutase (PEG-SOD), carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP), and DMSO were from Sigma-Aldrich (St. Louis, MO). GSE (brand name ActiVin) was from San Joaquin Valley Concentrates (Fresno, CA). MitoSox Red, Hoechst 33342, and Vybrant apoptosis assay kit were from Molecular Probes (Eugene, OR). Z-VAD-FMK was from R&D (Minneapolis, MN). Kits for complex I and III activity, and NAD^+/NADH level were from Abcam (Cambridge, MA). GSH and SOD activity kits were from BioVision Inc. (Mountain View, CA). Streptavidin and biotinylated anti-mouse secondary antibody were from Dako (Carpinteria, CA), and biotinylated anti-rabbit secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

FaDu and Detroit 562 cells were obtained from ATCC (Manassas, VA) and maintained in DMEM supplemented with 2 mM/L L-glutamine, 1 mM of sodium pyruvate, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin with 10% FBS. GSE stock solution was prepared in DMSO and diluted in cell culture media to achieve its final concentration (40 $\mu\text{g}/\text{mL}$).

Measurement of mitochondrial superoxide

Mitochondrial superoxide level was measured using MitoSox Red dye. Briefly, HNSCC cells were seeded in 6-well plates, and treated with GSE (40 $\mu\text{g}/\text{mL}$). At the end, cells were washed with Krebs-ringer bicarbonate solution, MitoSox Red solution (final concentration 5 μM) was added and analyzed by flow cytometry using University of Colorado Cancer Center Flow Cytometry Core Facility.

Immunoblotting

Cell lysates were prepared in non-denaturing lysis buffer and immunoblotting was performed as described earlier [19]. Few blots were multiplexed or stripped and re-probed with different antibodies. Immunoblots were scanned with Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA), and as needed, the mean density of each band was analyzed by the Scion Image program (National Institutes of Health, Bethesda, MD).

Determination of mitochondrial membrane potential (MMP)

MMP was assessed by measuring the retention of 3, 3'-dihexyloxacarbocyanine-DiOC₆(3). Briefly, cells were exposed to 40 nM DiOC₆(3) or 1 μM of Hoechst solution for 20 min at 37°C. The fluorescence was visualized at 488 nm using the Operetta High-Content Imaging and Analysis System. The fluorescence means for both DiOC₆(3) and nuclei staining from

several locations in the well were determined with Harmony software and results were normalized by the number of nuclei detected in all planes.

Measurement of mitochondrial complexes I and III activities

Mitochondria were isolated using a QProteome mitochondria isolation kit following the manufacturer's protocol (Qiagen, CA). Freshly isolated 50–100 µg of pure mitochondrial fractions were used to measure the kinetics of complex I and complex III activities using the kits from Abcam (Cambridge, MA). Enzymatic activities were determined as a change in the optical density per minute per µg mitochondrial protein at wavelength 450 nm for complex I and at 550 nm for complex III.

Transmission electron microscopy (TEM)

Cells were initially fixed with a mixture of 2.5% EM grade glutaraldehyde and serum free medium (1:1) for 15 min, followed by replacement with fresh fixative (2.5% glutaraldehyde). Fixed cells were washed with 0.1 M cacodylate buffer, and then post fixed with the mixture of 2% osmium tetroxide containing 1.5% potassium ferricyanide in 0.1M cacodylate buffer. Samples were dehydrated in a graded series of 25% to 100% ethanol, followed by infiltration in a 1:1 mixture of ethanol and epoxy resin. After exposure to 100% resin for over 24 h, samples were embedded. The ultrathin sections were stained with 2% uranyl acetate in 50% methanol followed by staining with 1% lead citrate, and the images were captured using the JEOL JEM 1011 transmission electron microscope (Peabody, MA).

Measurement of OCR and ECAR

XF24 Extracellular Flux Analyzer from Seahorse Bioscience (North Billerica, MA) was used to detect OCR and ECAR. In brief, Detroit 562 (2×10^4 cells/well) and FaDu (1.5×10^4 cells/well) cells were plated in XF 24-well cell culture micro plates in regular growth medium. Thereafter, cells were treated either with DMSO (control) or GSE (40 µg/mL) for 8, 12, and 24 h. At the end, cells were washed with XF24 running medium and analyzed to get measurement of real-time OCR and ECAR. As indicated, additional measurements were performed after the injection of four compounds through different ports, namely oligomycin (injection A: 1 µg/mL), FCCP (injection B: 1 µM), 2-deoxyglucose (2-DG) (injection C: 10 mM) and antimycin A (injection D: 3 µM), sequentially, in each well. Oligomycin blocks ATP synthase required to determine ATP turnover rates; FCCP, a proton ionophore, induces chemical uncoupling and stimulates maximal respiration; antimycin A inhibits electron transport chain complex III; and 2-DG inhibits glycolysis, which is the first enzyme in the glycolytic pathway. Real-time OCR and ECAR were recorded between the injections of various inhibitors. In general, basal OCR was calculated as respiration before the injection of any compounds minus OCR after antimycin A injection. Similarly, ATP turnover from OCR was determined as basal OCR minus OCR after oligomycin injection. OCR by the proton leak was calculated as OCR in the presence of oligomycin minus non-mitochondrial OCR, and mitochondrial reserve capacity (MRC) was calculated using FCCP minus the basal OCR [26]. Preliminary data analysis was performed using Seahorse XF24 software. The final data calculation was performed after readings were normalized with respective protein concentration evaluated in the wells. ECAR was calculated as recorded by the acidification rate during respiratory conditions described above [26]. OCR and ECAR are

expressed as pico moles per minute per microgram of protein and milli pH unit change per minute per microgram of protein, respectively.

Measurement of intracellular glutathione and NADH/NAD⁺ levels

Intracellular glutathione was measured using ApoGSH Glutathione colorimetric detection kit from BioVision Inc. (Mountain View, CA). Results are expressed as OD/ μ g after normalization with respective protein concentration. NADH/NAD⁺ ratio was determined using a kit from Abcam (Cambridge, MA). The NAD⁺/NADH ratio is expressed as OD values at 450 nm per microgram protein.

Immunohistochemistry (IHC)

We used archived paraffin-embedded FaDu xenograft tumor tissues [19] to determine *in vivo* effect of GSE on the levels of phospho-AMPK and p62 following IHC procedures detailed earlier [19].

Statistical analysis

Statistical analysis was performed using SigmaStat 2.03 software (Jandel Scientific, San Rafael, CA). Data was analyzed using one way ANOVA followed by Bonferroni t-test and differences were considered significant at $p < 0.05$.

RESULTS

GSE inhibits ETC complex III activity, induces mitochondrial superoxide generation, and causes apoptotic death in HNSCC cells

Mitochondrial electron transport chain (mt-ETC) complexes (I and III) are considered the major source of intracellular ROS [27]. Several studies in recent years have shown that compounds inhibiting the activity of mt-ETC complexes promote mitochondrial ROS accumulation [28,29], thus, first we assessed GSE effect on the activity of ETC complexes I and III. As shown in Figure 1A, GSE treatment significantly decreased the complex III activity in Detroit 562 and FaDu cells in a time-dependent manner. However, GSE did not significantly affect the complex I activity in both HNSCC cell lines (data not shown). Under similar treatment conditions, we also analyzed the effect of GSE on mitochondrial superoxide levels using MitoSox Red. As shown in Figure 1A, GSE treatment increased mitochondrial superoxide levels in both Detroit 562 and FaDu cells, which, interestingly, coincided with the observed decrease in complex III activity by GSE. To test whether increased superoxide levels by GSE were responsible for the decrease in complex III activity, HNSCC cells were pre-treated with PEG-SOD (polyethylene glycol-superoxide dismutase), and both superoxide generation and complex III activity were measured. As shown in Figure 1B, PEG-SOD pretreatment significantly reversed GSE-induced mitochondrial superoxide formation, but failed to reverse GSE-mediated inhibition of complex III activity in both Detroit 562 and FaDu cells (Figure 1C), suggesting that an increase in superoxide was not responsible for GSE-mediated decrease in complex III activity and ROS more likely occurred due to reduction of complex III activity. One consequence of increased superoxide and consequent hydrogen peroxide formation and emission is the depletion of cellular glutathione, a key component of cellular antioxidant

defense system. Therefore, we determined whether GSE treatment altered glutathione level in both Detroit 562 and FaDu cells. As shown in Figure 1D, GSE treatment decreased the glutathione level in both the HNSCC cell lines. These results suggest that the increase in mitochondrial superoxide production and glutathione depletion occur following GSE treatment in HNSCC cells.

To further assess whether GSE-induced superoxide generation depolarizes mitochondrial membrane potential (MMP), an activator of apoptosis, we employed DiOC₆(3) staining. GSE exposure for 6 and 12 h significantly decreased DiOC₆(3) fluorescence in both Detroit 562 and FaDu cells (Figure 1E), suggesting the depolarization of MMP. Since an increase in superoxide level preceded MMP depolarization, it suggests that GSE-induced oxidative stress is an earlier event and might be responsible for MMP depolarization in HNSCC cells.

To test the possible involvement of oxidative stress in the biological effects of GSE, HNSCC cells were pre-treated with 100 units/mL of PEG-SOD and GSE effect on apoptosis was analyzed. As shown in Figure 1F, PEG-SOD pretreatment significantly reversed the GSE-mediated apoptotic cell death (at 24 h) in both Detroit 562 and FaDu cells. Together, these results suggested that GSE inhibits complex III activity and increases mitochondrial superoxide level that triggers MMP depolarization and apoptosis induction in HNSCC cells.

GSE targets bioenergetic pathways in HNSCC cells

Several studies have clearly demonstrated that the inhibition of ETC complexes lead to compromised energy production [29–31]. To decipher the bioenergetic implications of complex III inhibition following GSE treatment, we next employed Seahorse XF24 extracellular flux analyzer to determine GSE effect on the oxygen consumption rate-OCR (indicative of OXPHOS) and extracellular acidification rate-ECAR (indicative of glycolysis) in both Detroit 562 and FaDu cells [32]. GSE treatment (8–24 h) markedly decreased the baseline OCR in both Detroit 562 and FaDu cells; however, differential effects of GSE were evident depending upon the cell type (Figure 2A and 2B). For example, GSE failed to attenuate OCR at earlier time points in Detroit 562, and a decrease of 45% ($p < 0.001$) was observed only after 24 h (Figure 2B-Left Panel). However, GSE significantly decreased the basal OCR in FaDu cells in a time-dependent manner (Figure 2B-Right Panel).

To understand the effect of GSE on ATP turnover rate, OCR was monitored following oligomycin addition in both HNSCC cell lines (Figure 2A and 2B). Similar to basal OCR, ATP turnover rate was marginally decreased only after 24 h of GSE exposure in Detroit 562 cells (Figure 2B-Left Panel); however, GSE effect on ATP turnover rate was also prominent in FaDu cells and a decrease was observed as early as 8 h (Figure 2B-Right Panel). Similarly, mitochondrial reserve capacity (MRC) was measured in both control and GSE-treated HNSCC cells before and after the introduction of mitochondrial uncoupler FCCP, followed by addition of mt-ETC complex III inhibitor antimycin A. As expected, in response to FCCP, there was a significant increase in OCR followed by a sharp drop in OCR after antimycin A exposure (Figure 2A). Likewise, GSE resulted in a significant decrease in MRC in both Detroit 562 and FaDu cells as compared to vehicle controls (Figure 2C). Similarly, treatment with GSE also significantly reduced the OCR accountable by the proton

leak (H⁺ leak) in both HNSCC cell lines (Figure 2D). Importantly, the proton leak is suggested to dissipate mitochondrial membrane potential [33].

Next, we determined the glycolytic rate (indicated by extracellular acidification rate-ECAR) in control and GSE-treated HNSCC cells. As shown in Figure 2E, GSE treatment (8–24 h) significantly decreased ECAR in both Detroit 562 and FaDu cells. Furthermore, we also determined the ratio of NADH/NAD⁺ which plays a crucial role in maintaining cellular redox homeostasis and energy metabolism [34]. As shown in Figure 2F, GSE treatment for 24 h reduced the intracellular NADH/NAD⁺ ratio, which could be directly correlated with alteration in OXPHOS and glycolysis by GSE in HNSCC cells. Collectively, GSE caused a strong inhibition of OCR and ECAR, thereby compromising the energy production in both HNSCC cell lines.

GSE activates AMPK but inhibits Akt-mTOR signaling in HNSCC cells

To understand the molecular response to the decreased energy status in HNSCC cells, we next evaluated the effect of GSE on the activation of AMPK, which is a master regulator of cellular metabolism. During an acute energy demand in the cells, AMPK is activated to reprogram cellular metabolism enforcing the cells to generate more ATP and to inhibit anabolic processes [35]. GSE treatment led to an increase in the phosphorylation of AMPK (Thr172) along with a slight decrease in total AMPK level in both Detroit 562 and FaDu cells (Figure 3A- blots and the bar diagram). GSE treatment also increased the phosphorylation of acetyl-CoA-carboxylase (ACC), a substrate of AMPK, confirming the increased AMPK activity by GSE (Figure 3A). GSE treatment did not affect the total ACC level in Detroit 562 cells but decreased the total ACC level at later time-points in FaDu cells (Figure 3A).

Activation of AMPK is known to inhibit mTOR as well as its downstream targets that integrate several pathways involved in protein translation, lipogenesis, adipogenesis, etc. [36]. Since Akt is an upstream activator of mTOR signaling, we first analyzed the effect of GSE on Akt phosphorylation. As shown in Figure 3B, GSE strongly suppressed Akt phosphorylation after 8–24 h of its treatment, while only marginally decreased the total Akt level (Figure 3B). Correspondingly, we also observed a strong decrease in mTOR phosphorylation at Ser2448 and Ser2481 sites as well as total mTOR level following GSE treatment. mTOR activates downstream signaling molecules such as eukaryotic initiation factor 4E binding protein (4E-BP1) and S6 ribosomal protein, thereby facilitating both ribosomal- and cap-dependent translation [37]. In both the HNSCC cell lines, GSE caused a downregulation of 4E-BP1 phosphorylation at Thr37/46, Thr70 and Ser65 sites as well as total 4E-BP1 level, while the levels of total and phosphorylated S6 ribosomal protein were not significantly or consistently altered (Figure 3B). Together, these finding suggested that GSE specifically targets cap-dependent translation of protein.

GSE induces autophagy in FaDu cells

One of the consequential biological outcomes of mTOR inhibition is the induction of autophagy in cells under metabolic stress or various other forms of cellular stresses [38,39]. Having observed that GSE inhibited mTOR pathway, we next assessed autophagy in GSE-

treated HNSCC cells. Immunoblot analyses revealed that GSE clearly increased the conversion of cytosolic LC3-I isoform to autophagosomes-associated LC3-II in FaDu cells, but not in Detroit 562 cells where only a modest increase in both LC3 I and II was observed (Figure 4A- blots and the bar diagram). GSE treatment also significantly elevated the expression of Beclin 1 in FaDu cells; however, the expression of Beclin 1 in Detroit 562 cells was not altered by GSE (Figure 4A). Likewise, transmission electron microscopy (TEM) revealed that GSE extensively increased the accumulation of cytoplasmic vacuoles as well as autolysosomes containing cellular debris (marked by red arrows) in FaDu cells, but interestingly these autophagy structures were not observed in Detroit 562 cells (Figure 4B). Together, these findings clearly suggested the existence of different autophagic machineries, leading to differential autophagic response of GSE in these two cell lines.

It has been reported that autophagy might facilitate cell survival under metabolic stress, and inhibition of autophagy may activate stronger apoptosis [39]. We, therefore, determined whether inhibition of autophagy facilitated and/or inhibited apoptosis in FaDu cells after GSE treatment by utilizing the pharmacological inhibitors that act on different stages of the autophagic process. For example, we employed 3-methyladenine (3-MA), a PI3 kinase inhibitor that suppresses autophagosomes formation, and bafilomycin A (Baf A1) and chloroquine [40] that inhibit the fusion of autophagosomes with lysosomes. Pretreatment of FaDu cells with 3-MA followed by GSE, remarkably attenuated the LC3-II formation, sensitized the FaDu cells toward the cytotoxic effect of GSE, and significantly increased the apoptotic cells as revealed by cleavage caspase 3 and annexin V/PI positive cells [~ 5.4 folds in GSE alone ($p < 0.001$) vs. ~ 15 folds in 3-MA + GSE ($p < 0.001$) at 24 h] (Figure 5A). Similarly, co-treatment of FaDu cells with GSE and Baf A1, partially inhibited the autophagic process as observed by accumulation of LC3-I (decreasing the LC3-II/LC3-I ratio), and significantly increased the cleaved caspase 3 level and apoptosis [~ 7 folds in GSE alone ($p < 0.001$) vs. ~ 14 folds in GSE+ Baf A1 ($p < 0.001$) at 24 h] (Figure 5B). Likewise, co-treatment of GSE and CQ remarkably increased the cleaved caspase 3 level and apoptosis in FaDu cells [~ 4.4 folds in GSE alone ($p < 0.001$) vs. ~ 6.3 folds in GSE+ CQ ($p < 0.001$) at 24 h] (Figure 5C). These results clearly showed that GSE-induced autophagy in FaDu cells is a survival mechanism, and autophagy inhibition sensitized the cells towards the cytotoxic effects of GSE.

It has also been reported that apoptosis and autophagy could act in a coordinated and/or cooperative manner to induce cell death [41]. Hence, we next analyzed whether GSE-induced autophagy and apoptosis are directly linked cell death mechanisms, by utilizing the pan caspase inhibitor Z-VAD-FMK. As expected, Z-VAD-FMK significantly reduced GSE-mediated activation of cleaved caspase 3, but failed to modulate LC3-II expression (Figure 5D), indicating that apoptotic pathway does not contribute towards autophagy in FaDu cells.

Effect of GSE on the expression of phospho-AMPK and p62 in FaDu xenograft tissue

To further validate our cell culture findings under *in vivo*, next we examined the effect of GSE on the metabolism and autophagy biomarkers in FaDu xenograft tissues. Earlier, we have reported that GSE feeding (0.2% wt/wt in diet) strongly inhibited the FaDu xenograft growth ($\sim 65\%$ decrease in the tumor volume) in athymic nude mice [19]. FaDu xenograft

tissue analyses revealed that there was a significant increase (1.8 fold, $p<0.001$) in the expression of phospho-AMPK (Thr172) in GSE-fed group (Figure 6A). It has been reported that under low ATP conditions in the cells, AMPK is activated which then promotes autophagy [42,43]. We, therefore, also evaluated the expression of p62/SQSTM1, as a hallmark of autophagic flux, in FaDu xenograft tissues. In comparison to control, FaDu xenograft tissues from GSE-fed group showed a significant decrease (49% decrease, $p<0.001$) in the expression of p62 positive cells (Figure 6B). Together, these results suggested that GSE altered the expression of the sensor of cellular metabolism and induced autophagy *in vivo* as a part of its anti-cancer efficacy against HNSCC.

DISCUSSION

In the present study, we have provided first evidence that GSE targets mitochondrial processes leading to increased accumulation of ROS, subsequently causing loss of mitochondrial membrane potential (MMP) and inducing apoptotic death in HNSCC cells. These conclusions are substantiated by the observations that GSE caused an inhibition of ETC complex III activity and depletion of glutathione, and that pretreatment of HNSCC cells with potent antioxidant PEG-SOD completely abolished GSE-induced apoptotic response in these cells. Only few prior studies have documented the prooxidant effect of GSE, instead, most of the published reports have highlighted the antioxidant properties of GSE, in both cancer and normal cells [44,45]. However, it has been reported earlier that GSE exerts pro-oxidant effect in the presence of transition metals (Fe^{+3} and Cu^{+2}), and these metals serve as important cofactors of many redox enzymes regulating their activity [46]. Similarly, components of GSE undergo oxidation leading to the formation of *o*-quinone that binds covalently to thiol groups in proteins, modifying their activity [44,47]. And these could also be probable mechanisms involved in the prooxidant activity of GSE and for its cytotoxicity specifically against HNSCC cells. Importantly, GSE has shown no or only marginal cytotoxicity against normal human cells (normal human epidermal keratinocytes NHEK cells and normal colon epithelial NCM460 cells) [19,48].

It is important to note that GSE treatment resulted in a decreased complex III activity in HNSCC cells. Deficient mitochondrial complex III activity has been suggested to contribute towards insufficient ATP production during OXPHOS [30]. Previous studies have reported that tumor tissues are highly dependent on glycolysis for energy production, and that an increase in the accumulation of lactate (end product of glycolysis) in tumors is associated with higher incidence of metastasis, recurrence of disease and poor survival in cancer patients including HNSCC [49,50]. Herein, for the first time, we have demonstrated that GSE significantly decreased both OXPHOS and ECAR, providing evidence for mitochondrial and glycolytic dysfunction, and suggesting its ability to limit the bioenergetics of HNSCC cells which is critical for their rapid multiplication. Also, GSE treatment significantly decreased the ratio of NADH/NAD⁺ that might directly correlate with the inhibition of glycolysis [51]. In this regard, NADH/NAD⁺ serves as cofactors to many of the enzymes in glycolytic and OXPHOS pathways, and depletion of NADH/NAD⁺ makes cells vulnerable to carry out the energy dependent functions, thus facilitating cell death or autophagy [52,53]. Collectively, these results suggest that targeting both glycolysis

and OXPHOS by GSE could be a major mechanism of its cell growth inhibitory and death actions against HNSCC.

Cellular energy restriction by pharmacological agents is known to activate master metabolic regulator AMPK [54]. Activated AMPK reprograms cellular metabolism by phosphorylating elongation factor 2 and inhibiting protein synthesis via mTORC1 [35,55]. It has recently been demonstrated that the aberrant activation of phosphatidylinositol 3-kinase (PI3K)/mTOR signaling pathways promote cell growth, survival, differentiation, and metabolism by enhancing translation and protein synthesis via effectors S6K1 and 4E-BP1 [38]. Moreover, inhibition of PI3K/Akt/mTOR signaling is also directly linked to the activation of apoptotic cell death and/or autophagy pathways [56,57]. It has been stated earlier that phosphorylation of Akt (Ser473) is essential to activate mTOR as well as in controlling subcellular localization of pro-apoptotic proteins evading apoptosis [58]. mTOR pathway plays a determining role in amending both resting oxygen consumption and oxidative capacity, and inhibition of mTORC1 by rapamycin lowers the mitochondrial membrane potential, oxygen consumption and cellular ATP levels [59]. Importantly, Akt/mTOR is overexpressed in >80% of head and neck cancer patients and has been investigated as prognostic markers for HNSCC recurrence [60]. Furthermore, much available data support that AMPK/mTOR pathways serve as an attractive chemopreventive and chemotherapeutic targets, and mTOR inhibitors (such as rapamycin and its analogs) are being investigated for their anti-neoplastic effects in various preclinical and clinical studies [60]. In our study, GSE decreased the phospho-Akt (Ser473), phospho-mTOR, and mTOR downstream target 4E-BP1 (in both HNSCC cell lines), which is involved in cap-dependent translation. Generally, cancer cells have a propensity to depend highly on cap-dependent translation compared to adjacent normal tissue, and thus, proteins regulating the initiation of cap-dependent translation are emerging as prospective drug targets in different cancers including HNSCC [61,62].

In the present study, we have also reported that GSE-induced autophagy in FaDu cells antagonizes the induction of apoptosis. Accumulating evidence suggests that mTOR regulate various steps of autophagosomes formation including the functions of autophagy molecules and autophagocytosis via the activation of p70S6K [56], and that abrogating the activation of PI3K/Akt/mTOR pathway stimulates autophagy [63]. In contrast, we also have observed a perplexing discrepancy that despite inhibition of mTOR pathway in both HNSCC cell lines, there was a lack of prominent induction of autophagy in Detroit 562 cells, further suggesting the major differences in the pathways that regulate autophagy in these two cell lines. Evidence from literature suggests that TGF- β plays a major role in activating autophagy [64], nevertheless, whether this genotypic difference between Detroit 562 (wild type Smad 4) and FaDu (Smad 4 null) contributes towards GSE-induced autophagy needs to be examined in future studies.

In conclusion, our findings for the first time showed that GSE induces apoptotic death in HNSCC cells through its pro-oxidant effects by inhibiting the mitochondrial ETC complex III activity while reducing the level of intracellular antioxidant glutathione. Furthermore, GSE targeted cellular bioenergetics, thereby enforcing energy restriction in HNSCC cells and activating associated signaling pathways leading to autophagy as well as apoptotic death. Our results also suggest that combining GSE with autophagy inhibitors could further

enhance its anti-cancer efficacy against HNSCC cells. Our proposed molecular mechanisms, involved in pleiotropic effects of GSE that contribute towards its strong anti-cancer and chemopreventive efficacy, are summarized in Figure 7. Overall, the findings from current study provide strong and convincing evidence to define a new mechanism of action of GSE against HNSCC cells, consequently supporting its translational application in the near future.

Acknowledgments

Grant Support: This work was supported by NCI R01 grants CA91883 (C.A.) and CA140368 (R.A.) and NINDS grants R01NS045748 and RO1NS039587 (M.P.).

Authors thank Shane D Rowley for assistance with XF24 analyzer and Dr. Brian Reid, Director of High Throughput and High Content Screening Core Facility, for his help with the Operetta High-Content Imaging and Analysis System. FACS core is supported by CCSG P30CA046934 grant.

List of Abbreviations

HNSCC	head and neck squamous cell carcinoma
GSE	grape seed extract
ROS	reactive oxygen species
3-MA	3-methyladenine
AMPK	AMP-activated protein kinase
mTORC1	mammalian target of rapamycin complex I
ACC	acetyl-coA carboxylase
4EBP-1	4 eukaryotic binding protein-1
LC-3	light chain-3
2-DG	2-deoxyglucose
DMSO	dimethylsulfoxide
MitoRed	MitoSox Red
PI	propidium iodide
NAD	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
GSH	glutathione
PEG-SOD	polyethylene glycol superoxide dismutase
MMP	mitochondrial membrane potential
PMSF	phenylmethylsulfonyl fluoride
DiOC₆	3,3' dihexyloxacarbocyanine
FCCP	carbonyl cyanide p-trifluoromethoxy-phenylhydrazine

OCR	oxygen consumption rate
ECAR	extracellular acidification rate
eIF4E	eukaryotic initiation factor 4E
MRC	mitochondrial reserve capacity
PARP	poly(ADP-ribose) polymerase
TEM	transmission electron microscope
TGF-β	transforming growth factor-beta

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Figure 1.

GSE targets ETC complex III and cellular antioxidants, induces mitochondrial superoxide generation and apoptotic death. Detroit 562 and FaDu cells were treated with either DMSO (untreated control) or GSE (40 $\mu\text{g/mL}$), processed further for different experiments as detailed in 'Material and methods'. (A) Mitochondrial superoxide level was measured by MitoSox red, and complex III activity was assessed using the enzymatic assay kit as described in 'Material and methods'. Data represent the mean fold-induction ($n=3$) $\pm$ SEM for each treatment. (B) HNSCC cells were pretreated with PEG-SOD (100 Units/mL) 2 h prior to GSE (40 $\mu\text{g/mL}$) exposure (4 h), and then analyzed by flow cytometry for MitoSox red fluorescence intensity. The graph represents the mean fold-induction ($n=3$) $\pm$ SEM for

each treatment. **(C)** Following GSE (40 µg/mL) treatments for 4–6 h, mitochondrial fraction was isolated from both HNSCC cells, and complex III activity was analyzed at a wavelength of 550 nm as described in ‘Material and methods’. **(D)** Both HNSCC cell lines were exposed to either DMSO or GSE (40 µg/mL) for 3–6 h and glutathione level was measured. Data are expressed as mean (n=3) ± SEM after normalization with respective protein concentration. **(E)** Mitochondrial membrane potential was measured utilizing DiOC₆(3). DiOC₆(3) fluorescence was visualized at 488 nm using the Operetta High-Content Imaging and Analysis System in both the HNSCC cell lines. The bar diagrams are expressed as percent normalized to control (mean ± SEM, n=3). **(F)** HNSCC cells were pretreated with PEG-SOD (100 Units/mL) 2 h prior to GSE (40 µg/mL) exposure for 24 h, and processed at the end of the treatments, and analyzed by flow cytometry for annexin V/PI positive cells. Data are expressed as of mean ± SEM (n=3) for each treatment. *, p<0.001; \$, p<0.05. Abbreviations: C: untreated control; GSE: Grape seed extract.

Figure 2.

Effect of GSE treatment on oxidative phosphorylation and glycolysis in HNSCC cells. Detroit 562 and FaDu cells were treated with GSE (40 µg/mL) for 8–24 h, and OCR and ECAR were measured using XF24 extracellular flux analyzer as detailed in the ‘Material and methods’. Initially, basal OCR and ECAR of HNSCC cells were measured before the addition of four different metabolic inhibitors namely oligomycin (injection A: 1µg/mL), carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP) (injection B: 1µM), 2-deoxyglucose (2-DG) (injection C: 10 mM) and antimycin A (injection D: 3 µM) at the indicated times. (A) Mitochondrial respiration profile of both Detroit 562 and FaDu cells in

response to different inhibitors expressed as pico moles per minute. **(B)** HNSCC cells were pretreated with GSE (40 µg/mL) for 8–24 h, followed by the real time analysis of basal OCR and ATP turnover, represented as percent of control after normalization with protein concentration. **(C)** The mitochondrial reserve capacity, and **(D)** change in proton leak were determined as described in ‘Material and methods’ and expressed as percent of control. **(E)** HNSCC cells were treated with GSE (40 µg/mL) for 8–24 h, and at the end of the treatments the change in baseline ECAR was calculated before the injection of any compounds. The representative data are presented as mean ± SEM after normalization with respective protein concentration. Each experiment was performed in triplicate or quadruplicate at least twice. **(F)** HNSCC cells were treated with GSE (40 µg/mL) for 12–24 h, and NADH and NAD⁺ were measured as detailed in ‘Material and methods’. The change in NADH/NAD⁺ ratio was expressed as OD values per microgram protein. Each experiment was performed in triplicate or quadruplicate at least twice. *, p<0.001; \$, p<0.05

Figure 3. GSE activates AMPK and inhibits mTOR signaling in HNSCC cells. Detroit 562 and FaDu cells were treated with either DMSO or GSE (40 μ g/mL) for 8–24 h. Whole cell lysate was prepared, and immunoblotting was performed. **(A)** Effect of GSE on phospho-AMPK (Thr172), total AMPK, phospho-ACC (Ser79) and total ACC in Detroit 562 and FaDu cells. Band intensity was measured by densitometry and the ratio of phospho-AMPK and total AMPK is presented in the bar diagram below the blots. **(B)** Effect of GSE treatment on the phospho-Akt (Ser473), total Akt, phospho-mTOR (Ser2448 and Ser2481), total mTOR, phospho-4E-BP1 (Thr37/46), phospho-4E-BP1 (Ser65), phospho-4E-BP1 (Thr70), 4E-BP1,

phospho-S6(Ser235/236), phospho-S6(240/244), and total S6K. Membranes were also stripped and probed for β -actin to check protein loading.

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Figure 4.

GSE treatment induced autophagy selectively in FaDu cells. **(A)** Effect of GSE treatment on the LC3 (I and II) and Beclin1 levels in Detroit 562 and FaDu cells. The ratio of LC3-II/LC3-I was calculated by measuring the density of each bands and normalized with band intensity of respective β -actin and presented as bar diagram below the bands. Membranes were also stripped and probed for β -actin to check protein loading. **(B)** HNSCC cells were treated with GSE for 12 and 24 h and processed for EM as described in the methods. Representative TEM photomicrographs are shown. Arrows in the lower panel marks the vacuoles in the FaDu cells.

Figure 5.

GSE-induced apoptotic cell death was enhanced by autophagic inhibitors in FaDu cells. FaDu cells were either (A) pretreated with 2.5 mM 3-MA or (B) treated with 10 nM Bafilomycin A1-Baf A1 4 h before the termination of the experiment or (C) co-treated with 2.5 µM chloroquine-CQ with GSE (40 µg/mL) for 12–24 h as mentioned in 'Material and methods'. Thereafter, cell lysates were prepared and immunoblotting was performed for LC3 (I and II) and cleaved caspases 3 (Left Panels); and also apoptosis was analyzed by flow cytometry (Right Panels). Data are represented as the mean ± SEM of triplicates. *, $p < 0.001$. (D) FaDu cells were treated with Z-VAD-FMK (20 µM for 2 h) followed by GSE (40 µg/mL) treatment for 12–24 h. Whole cell lysates were prepared at the end of the

treatments, and were subjected to immunoblot analysis for LC3 (I and II) and cleaved caspases 3.

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Figure 6.

Effect of dietary feeding of GSE on phospho-AMPK and p62 in FaDu xenograft tissue. Paraffin-embedded FaDu xenograft tissues from previously completed study were analyzed to determine the effect of GSE administration on the expression of phospho-AMPK (Thr172), and p62 by IHC. Immunoreactivity (represented by brown staining) of these biomarkers was analyzed in 5 random areas for each tumor tissue and scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), 4+ (very strong staining). Representative photographs are presented at 400X; insets are further magnification of a part of the photographs. The data shown in the bar diagram represents mean $\pm$ SEM of 4–5 samples for each group. *, $p < 0.001$.

Figure 7. Schematic diagram of potential signaling mechanisms involved in biological efficacy of GSE against HNSCC cells. GSE targets mitochondrial ETC complex III activity, thereby causing oxidative and metabolic stress in HNSCC cells, resulting in the activation of signaling pathways related to autophagy and apoptotic cell death.