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Ebselen sensitizes glioblastoma cells to Tumor Necrosis Factor (TNFα)-induced apoptosis through two distinct pathways involving NF-κB downregulation and Fas-mediated formation of death inducing signaling complex

Vivek Sharma, Richa Tewari, Ugir Hossain Sk, Christy Joseph and Ellora Sen*

National Brain Research Centre, Manesar, Haryana, India

Resistance to tumor necrosis factor (TNFα)-induced apoptosis in various cancer cells has been attributed to the activation of the transcription factor NF-κB. Ebselen (2-phenyl-1,2-benzisoselenazol-3[2H]one)—a selenoorganic compound is known to prevent TNFα-mediated NF-κB activity. As glioblastoma are resistant to the cytotoxic effect of TNFα, we investigated the potential of Ebselen in sensitizing glioma cells to TNFα-induced apoptosis. Although treatment with Ebselen reduced viability of glioma cells, cotreatment with TNFα enhanced apoptosis further through alteration of TNFα-mediated signaling pathways. Sensitization of TNFα activated glioma cells to apoptosis by Ebselen involved 2 pathways: (*i*) abrogation of TNFα induced NF-κB activation and (*ii*) induction of Fas-associated death inducing signaling complex (DISC) formation. Ebselen inhibited the prosurvival pathway mediated by NF-κB by altering the association of TNF receptor associated factor 2 (TRAF2) with TNFα receptor associated death domain (TRADD) in the TNFR1-TRADD-TRAF2 complex —an interaction crucial for mediating NF-κB activity. Ebselen also induced the formation of DISC involving Fas, Fas-associated death domain (FADD) and active caspase 8 to transduce apoptotic signals in situations where NF-κB function was inhibited. Cotreatment with Ebselen and TNFα induced G2/M phase arrest in cell cycle and modulated the expression of molecules involved in cell cycle progression. These results raise the possibility of overcoming resistance to TNFα-induced apoptosis by cotreatment with organoselenium Ebselen as a strategy to kill glioma cells.

Key words: glioblastoma; TNFα; Ebselen; Fas; NF-κB; DISC

Glioblastoma multiforme, the most aggressive malignant brain tumor[1] is largely resistant to current therapeutic regimens. Triggering of apoptosis in tumor cells by targeting death receptors is considered an attractive cancer therapy.[2] Activation of death receptors of the tumor necrosis factor (TNF) receptor family initiates signaling pathways leading either to cell death or survival. TNFα is a proinflammatory cytokine whose signaling pathways are linked to both proapoptotic and antiapoptotic responses.[3,4] Despite TNFαs ability to induce apoptosis, several tumors are resistant to TNFα-mediated apoptosis.[5] Resistance to TNFα-induced apoptosis has been attributed to the activation of the transcription factor NF-κB,[6–9] because blockade of NF-κB sensitizes cells to TNFα induced apoptotic cell death.[6,8] TNFα induces apoptosis and activates NF-κB through signaling cascades originating from TNFR1.[10] Activation of TNFR1 signaling by TNFα triggers recruitment of TNFR-associated death domain (TRADD).[11] TNFR1 utilizes distinct TRADD dependent mechanisms to activate signaling pathways for NF-κB activation and apoptosis.[10,11] Inhibition of TNFα induced NF-κB activity results in apoptosis through recruitment of caspase 8.[12] Thus, TNFα can induce either prosurvival or apoptotic pathway.

Although Fas (a member of the TNFR family death receptors) is expressed in majority of glioblastoma,[13] most glioma cells are resistant to apoptosis induced by Fas ligand.[14] Importantly, Fas has the ability to interact with FADD directly which recruits procaspase 8 to form death-inducing signaling complex (DISC).[15,16] Activation of caspase 8 further activates caspase 3 that initiates apoptosis. Recent studies have indicated that some anticancer chemotherapeutic drugs and TNFα can kill some resistant tumor cells.[17] Not only does inorganic selenium inhibit NF-κB in prostate cancer cells,[18] but it also causes growth inhibition and apoptosis in glioma cells.[19] The selenoorganic drug Ebselen prevents nuclear accumulation of NF-κB in leukocytes.[20] Besides, NF-κB activation has been shown to be critical for protection of leukemic eosinophils from Fas-mediated apoptosis.[21] Selenium-based compounds overcome resistance to TNFα related apoptosis inducing ligand (TRAIL) mediated apoptosis in prostrate cancer cells.[22] We therefore investigated whether Ebselen could sensitize glioma cells to TNFα-induced apoptosis, by modulating the susceptibility of glioblastoma cells to death receptor activation.

Material and methods

Cell culture and treatment

Glioblastoma cell lines U87MG, A172 and T98G were obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% fetal bovine serum. On attaining semiconfluence, cells were switched to serum free media and after 12 hr, cells were treated with different concentration of Ebselen (in Dimethyl sulfoxide, DMSO) in the presence or absence of TNFα (50 ng/ml) in serum free media for 24 hr. Following the treatment, cells were processed for coimmunoprecipitation and Western blot analysis. All reagents were purchased from Sigma unless otherwise stated. DMSO treated cells were used as controls.

Determination of cell viability

Cell viability was assessed using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) (Promega) as described earlier.[23] Following treatment of cells (1×10^3) with different combinations of TNFα and Ebselen, for 24 hr in 96-well plates, 20 μl of MTS solution was added. After 4 hr of incubation, the absorbance reflecting reduction of MTS by viable cells was determined at 490 nm. Values were expressed as a percentage relative to those obtained in controls. To determine the effect of caspase-8 inhibitor Z-IETD-FMK (Tocris) on glioma cell viability, cells were treated with different combinations of TNFα and Ebselen in the presence and absence of caspase-8 inhibitor and MTS assay was performed as described.

Western blot analysis

Protein from whole cell lysates and nuclear extracts were isolated as described previously.[23] Twenty to fifty microgram of protein isolated from cells treated with TNFα either in the presence or absence of Ebselen was electrophoresed on 6–10% polyacrylamide gel and Western blotting performed as described.[23] Antibodies were purchased from Santa Cruz Biotechnology unless otherwise mentioned. The following antibodies were used—TNFR1, TRADD, TRAF2, Fas, FasL, FADD (BD Biosciences),

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***Correspondence to:** National Brain Research Centre, Manesar, Gurgaon, Haryana 122050, India. Fax: +91-124-2338910/28.
E-mail: ellora@nbrc.res.in

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caspase 8 (Calbiochem), p65 NF-κB, cMyc and p21. Secondary antibodies were purchased from Vector Laboratories. After addition of chemiluminescence reagent (Amersham), blots were exposed to Chemigenius, Bioimaging System (Syngene) for developing and images were captured using Genesnap software (Syngene). The blots were stripped and reprobed with anti-β-tubulin to determine equivalent loading as described.[23]

Coimmunoprecipitation

For immunoprecipitation of DISC, 50–100 μg protein lysates from glioma cells treated with different combination of TNFα and Ebselen were immunoprecipitated with anti-Fas antibody overnight at 4°C. The lysates were then incubated with 30 μl of protein G Plus-sepharose (Amersham) at room temperature for 2–4 hr. Beads were pelleted, washed 3 times in lysis buffer and resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Western blots were performed to determine the recruitment of FADD, FAS and caspase-8 to the DISC. To investigate the association of TRADD and TRAF2 with TNFR1, 50–75 μg protein lysates from treated cells were immunoprecipitated with anti-TNFR1 antibody and Western blots were performed to determined TRADD and TRAF2 levels in the complex.

Surface staining of TNFR1

Cell surface TNFR1 expression on glioma cells treated with Ebselen or TNFα or both for 24 hr was measured in an immunofluorescence flow cytometry assay. 10^6 cells were collected by centrifugation, washed with PBS and incubated with anti-TNFR1 for 40 min at 4°C. Following incubation cells were washed and incubated with secondary Ab (anti-rabbit conjugated to FITC). After a further incubation for 30 min on ice, cells were washed and resuspended in 500 μl of PBS. Staining was then measured by flow cytometry on FACS Calibur (BD Biosciences) using the CellQuest analysis program (Becton Dickinson, Mountain View, CA).

Assay of caspases 3 and 8 activities

The Colorimetric Assay kits for caspases 3 and 8 (Sigma) were used to determine the enzymatic activity of caspases in glioma cells treated with TNFα in the presence and absence of Ebselen. The assay is based on spectrophotometric detection of the chromophore *p*-nitroaniline (*p*NA) after cleavage from the substrate peptides conjugated to *p*NA. The substrate peptides used for the colorimetric assay of caspase 3 and 8 were Ac-DEVD and Ac-IETD, respectively. Following treatment, cells were lysed with the provided lysis buffer and 100 μg of the protein lysates were incubated with the colorimetric substrate with assay buffer provided at 37°C for 2 hr in a 96-well microplate. The *p*NA released by cleavage of the peptide was quantified spectrophotometrically at 405 nm in a microtiter plate reader.

Luciferase reporter gene assay

The NF-κB luciferase plasmid was obtained from Clontech (was a kind gift from Dr. Nihar Ranjan Jana, NBRC). For the reporter gene assay, transfection with NF-κB luciferase construct was carried using Lipofectamine 2000 (Invitrogen-Life Technologies), according to manufacturer's instruction. After transfection, cells were further incubated for 48 hr. For the last 24 hr, the cells were left either untreated or treated with Ebselen or TNFα or both. Lysates of the transfections were prepared using passive lysis buffer (Promega) and luciferase assays were performed using the Luciferase Assay System (Promega) as recommended by the manufacturer. The results are expressed as fold activity over the vector control.[24]

Flow cytometric analysis of DNA content

To determine the effect of TNFα and Ebselen on cell cycle progression in glioma cell lines, FACS analysis of DNA content was performed as described.[25] Control and cells treated with different combinations of TNFα and Ebselen were fixed in 70% ethanol and stored at −20°C. The fixed cells were washed in PBS, resuspended in propidium iodide solution (BD Biosciences) for 20 min at room temperature and flow cytometric analysis of 10^6 cells were carried out using Cell Quest program on FACS Calibur (Becton Dickinson). The percentages of cells in the G1, S and G2/M phases of the cell cycle were analyzed with the Mod Fit LT program as described.[25]

Statistical analysis

All comparisons between groups were performed using two-tailed Paired Student's *t*-test. All values of p less than 0.05 were taken as significant.

Results

Ebselen sensitizes human glioma cells to TNFα-induced apoptosis

Treatment of glioma cells with increasing concentration of Ebselen indicated that although treatment with 10 and 25 μM of Ebselen had no effect on cell viability, ∼35% reduction in viability was observed in cells treated with 50 μM of Ebselen for 24 hr. In cells treated with 75 μM concentration of Ebselen, the viability was similar to that observed at 50 μM concentration (Fig. 1*a*).

On the basis of these results, we chose the concentration of 50 μM for further characterization of Ebselen induced cell death. Combined use of some anticancer chemotherapeutic drugs and TNFα has been reported to synergistically kill certain resistant tumor cells.[17] As induction of apoptosis by selenium in human glioma has been reported,[26] we investigated whether seleno-organic compound Ebselen could sensitize glioma cells to TNFα-induced apoptosis. To determine the cytotoxic effects of TNFα on human malignant glioma cell lines, MTS assay was performed on A172, T98G and U87MG cells treated with different concentrations (25, 50, 75 and 100 ng/ml) of TNFα for 24 hr (data not shown). As all the glioma cell lines tested were resistant to TNFα irrespective of the treatment concentration, we chose 50 ng/ml concentration of TNFα to study the effect of Ebselen on TNFα treated glioma cells (Fig. 1*b*). Treatment of A172, T98G and U87MG cells with 50 μM Ebselen for 24 hr resulted in ∼30% decrease in viability (Fig. 1*b*). The decreased viability of Ebselen treated glioma cells was further reduced in the presence of TNFα. Approximately 50% decrease in cell viability was observed in TNFα treated A172 and T98G cells treated with Ebselen, viability of U87MG cells was reduced ∼40% when compared with control (Fig. 1*b*). Thus, a significant ∼1.6-fold decrease in cell viability was observed when glioma cells were treated with a combination of Ebselen and TNFα, when compared with Ebselen alone.

We next determined the levels of active caspase-3 in cells treated with TNFα in the presence and absence of Ebselen. Although the expression of active caspase-3 in glioma cells was unaffected by TNFα treatment (Fig. 1*c*), a significant 1.8-, 2.0- and 2.1-fold increase in caspase-3 activity was observed in U87MG, A172 and T98G cells, respectively, upon exposure to Ebselen when compared with the control. This enhanced caspase-3 activity were further elevated to ∼2.4, 3.0 and 3.3- fold in U87MG, T98G and A172 cells, respectively, treated with a combination of Ebselen and TNFα when compared with the control (Fig. 1*c*). Because the activation of caspase-3-like proteases is crucial in apoptotic cell death,[27] these results suggest that Ebselen induce apoptosis in glioma cells.

Ebselen affects the expression of molecules associated with TNFα induced signaling event

To understand the mechanism involved in susceptibility of glioma cells to TNFα-induced apoptosis in the presence of Ebselen, we examined the expression of molecules involved in signaling by TNFα. Overexpression of TRADD[11] or TNFR1[15] triggers apoptosis through activation of signaling pathways associated with TNFR1.[10] Activation of TNFR1 signaling by TNFα triggers recruitment of TRADD and TRAF2 into the receptor complex,[12]

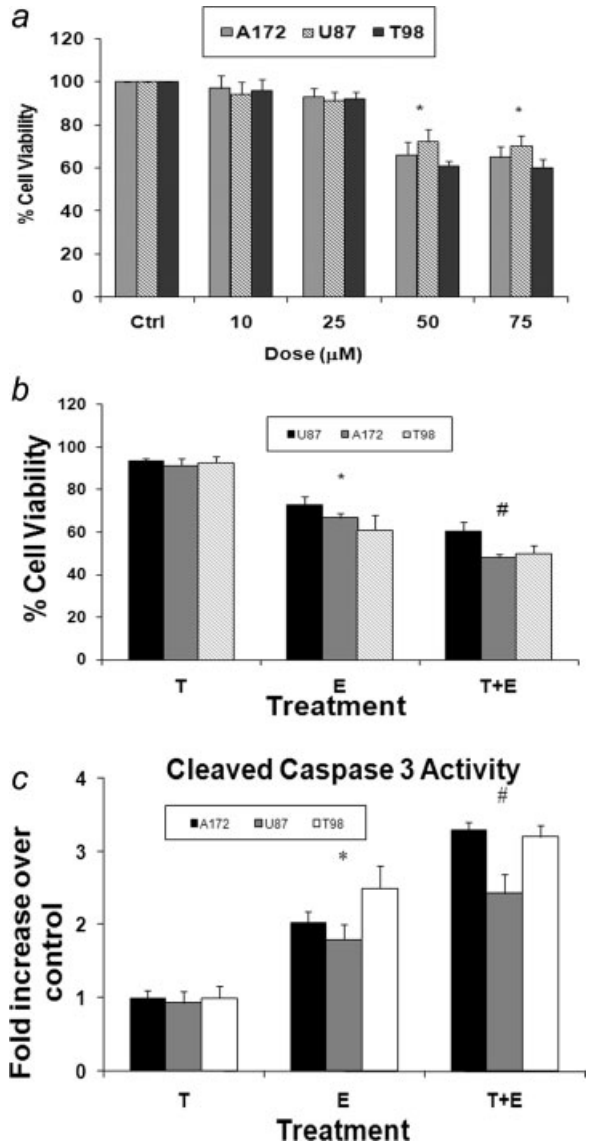

FIGURE 1 – Ebselen sensitizes glioma cells to TNFα-induced apoptosis. (*a*) Viability of glioma cells treated with different concentration of Ebselen was determined by MTS assay. The graph represents the percentage viable cells observed when A172, T98G and U87MG glioma cells were treated with 10, 25, 50 and 75 μM of Ebselen for 24 hr. (*b*) Viability of glioma cells treated with Ebselen in the presence and absence of TNFα was determined by MTS assay. The graph represents the percentage viable cells of control observed when A172, T98G and U87MG cells were treated with either 50 μM of Ebselen or 50 ng/ml TNFα or both for 24 hr. (*c*) Fold increase in caspase-3 activity in A172, T98G and U87MG cells treated with different combination of Ebselen and TNFα for 24 hr, as determined by the Caspase-3 activity assay. Values in (*a*), (*b*) and (*c*) represents the mean ± SEM from 3 independent experiments. *, Significant change from control ($p < 0.05$). T and E denote TNFα and Ebselen, respectively. #, significant change from Ebselen alone.

subsequently eliciting activation of NF-κB.[28] NF-κB activation is involved in mediating resistance to TNFα-induced apoptosis.[6–9] We therefore investigated the expression of TNFR1, TRADD and TRAF2 expression in cells treated with TNFα in the presence and absence of Ebselen. The increase in TNFR1 expression observed in TNFα treated A172, T98G and U87MG cells, was downregulated in the presence of Ebselen (Fig. 2*a*). Treatment with Ebselen alone had no effect on TNFR1 expression in T98G. However, an increase in TNFR1 levels in A172 and decrease in U87MG was observed in the presence of Ebselen alone (Fig. 2*a*). On the other hand, the slight increase in TRADD level observed in A172, T98G and U87MG treated with TNFα was further elevated in the presence of Ebselen. Treatment with Ebselen alone also resulted in increased TRADD expression when compared with the control (Fig. 2*a*). Interestingly, the increase in TRAF2 level observed in TNFα treated A172, T98G and U87MG cells when compared with control, were downregulated in the presence of Ebselen (Fig. 2*a*).

Ebselen decreases the expression of TNFR1 on the surface of TNFα treated glioma cells

As elevated TNFR1 levels observed in TNFα treated glioma cells was decreased in the presence of Ebselen, and because this change represents the amount of TNFR1 in cell lysates independent of its cell surface localization, we analyzed surface expression of TNFR1 by FACS to determine the actual fraction of TNFR1 that interacts with its ligand. The expression of TNFR1 (black) on the surface of untreated glioma cells were elevated in the presence of TNFα (dotted) (Fig. 2*b*). Although treatment with Ebselen alone had no effect on TNFR1 expression (dashed), a decrease in surface expression of TNFR1 was observed in TNFα treated cells in the presence of Ebselen (gray), when compared with those cells treated with TNFα alone (Fig. 2*b*). These results confirmed the Western blot data that, Ebselen downregulates the elevated TNFR1 levels observed in TNFα treated glioma cells.

Ebselen increases TRADD and decreases TRAF2 levels in the TNFR1-TRADD-TRAF2 signaling complex in TNFα treated cells

Elevated expression of TRADD and interaction of TRADD with TNFR1 has been shown to trigger apoptosis.[10,11] Besides, TNFα is known to trigger the assembly of a TNFR1-TRADD-TRAF2 signaling complex,[29] that mediates constitutive NF-κB activation.[30] As increase in TRADD and TNFR1 expression was accompanied by changes in TRAF2 levels, we next investigated the status of TNFR1, TRADD and TRAF2 in this complex in TNFα treated cells both in the presence and absence of Ebselen. Lysates from A172 and T98G cells treated with different combinations of Ebselen and TNFα was immunoprecipitated with TNFR1 and the immunoprecipitates were probed with TRADD and TRAF2 antibody. The increased TRADD level observed in the immunoprecipitate of TNFα treated cells when compared with control, was further elevated in the presence of Ebselen (Fig. 2*c*). The amount of TRAF2, which coprecipitated with TRADD, was greater in TNFα treated cells when compared with control (Fig. 2*c*). Treatment with Ebselen decreased TRAF2 levels in the complex slightly. However, TRAF2 level in TNFα treated cells was downregulated in the presence of Ebselen (Fig. 2*c*). In the signaling mediated by the TNFR1-TRADD-TRAF2 complex, TRAF2 is dispensable for apoptosis but it is essential for NF-κB activation.[10,11] An increased expression of TRADD in the complex concurrent with decreased TRAF2 expression could have resulted in apoptosis through decreased activation of antiapoptotic NF-κB in cells receiving Ebselen and TNFα cotreatment.

Ebselen inhibits NF-κB activity in TNFα treated gliomas

TNFα activates NF-κB, and inhibition of NF-κB activity results in apoptosis by recruiting caspase 8.[12] The decrease in TRAF2 in the TNFR1-TRADD-TRAF2 complex indicated reduced NF-κB activity in Ebselen and TNFα treated cells. We therefore determined, NF-κB levels in nuclear extracts from glioma cells treated with TNFα in the presence or absence of Ebselen. The level of p65 (RelA) subunit of NF-κB was elevated in TNFα treated A172, T98G and U87MG cells when compared with the control. However, treatment with TNFα in the presence of Ebselen attenuated p65 expression to levels comparable to that of untreated control (Fig. 3*a*). Interestingly, treatment of glioma cells with Ebselen alone also downregulated p65 expression below the basal level

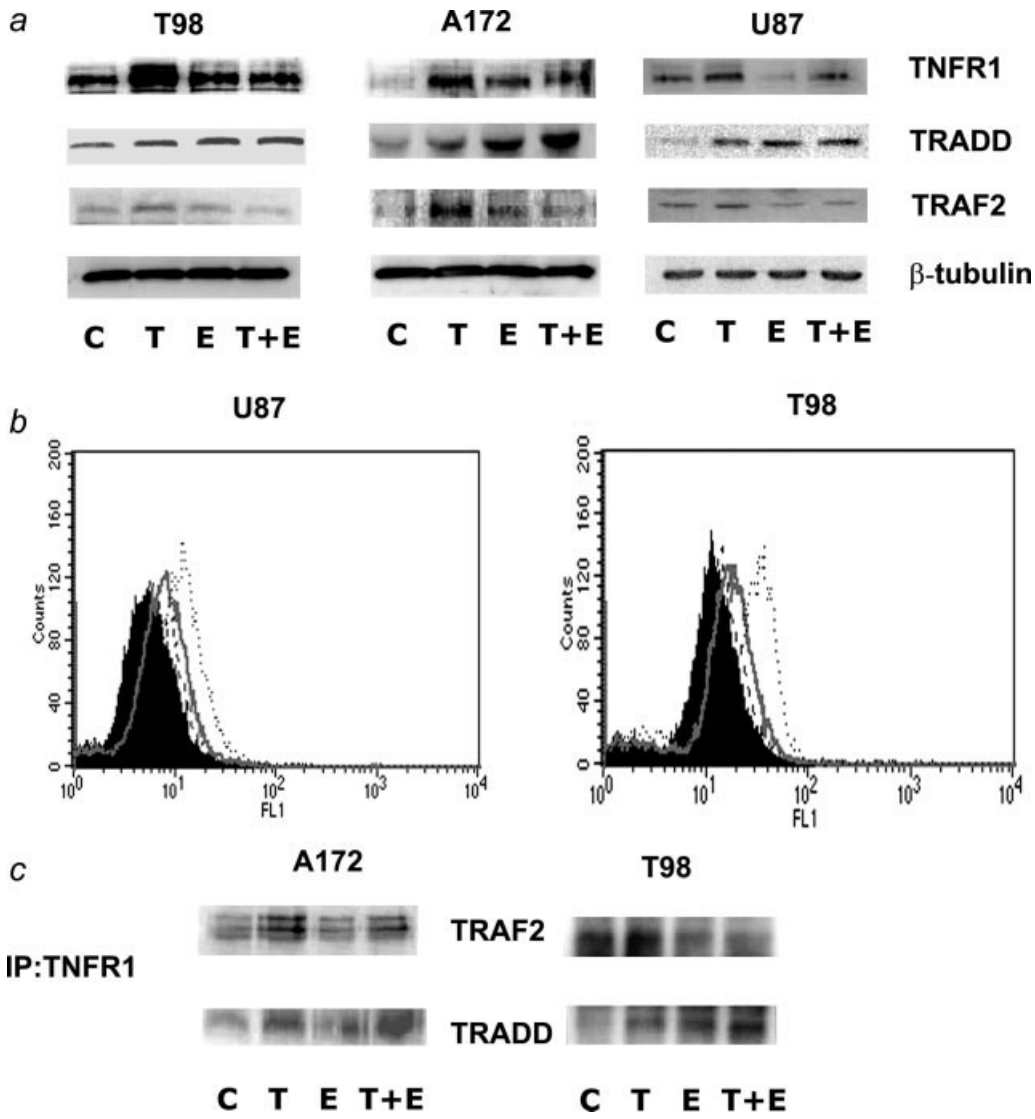

FIGURE 2 – Ebselen alters TNFα induced signaling to decrease association of TRAF2 with TRADD in TNFR1-TRADD-TRAF2 complex in TNFα treated glioma cells. (*a*) Expression of molecules associated with TNFα-mediated signaling events in A172, T98G and U87MG cells treated with different combinations of Ebselen and TNFα. The expressions of TNFR1, TRADD and TRAF2 in cells treated with TNFα or Ebselen or both for 24 hr were analyzed by Western blotting. A representative blot is shown from 3 independent experiments with identical results. Blots were reprobed for β tubulin to establish equivalent loading. (*b*) Ebselen decreases the increased TNFRI expression in TNFα treated glioma cells. Cell surface expression of TNFR1 on glioma cells treated with either TNFα or Ebselen or both for 24 hr, was assayed by FACS as described in Material and methods section. A representative data is shown from 2 independent experiments with identical trend. (*c*) A172 and T98G cells were treated with TNFα in the presence or absence of Ebselen for 24 hr and the lysates were immunoprecipitated (IP) with anti-TNFR1. Western blot analysis was performed and blots were probed with antibodies to TRADD and TRAF2. Increased TRADD and TRAF2 coprecipitated with TNFR1 in TNFα treated cells when compared with control. Further increase in TRADD and abrogation of TRAF2 level in the complex was observed in TNFα treated cells in the presence of Ebselen. A representative blot is shown from 2 independent experiments with identical results. C, T and E denote control, TNFα and Ebselen, respectively.

(Fig. 3*a*). Thus, Ebselen not only abrogated the elevated NF-κB level induced by TNFα (Fig. 3*a*), but was also capable of decreasing the basal level of NF-κB. These observations indicate that TNFα enhances and Ebselen downregulates NF-κB level in TNFα treated gliomas.

Ebselen inhibits TNFα-induced NF-κB reporter gene expression in glioma cells

As Ebselen decreased TNFα treated NF-κB expression in the nucleus, we performed Luciferase reporter assay to determine whether Ebselen increases sensitivity to TNFα-induced apoptosis by decreasing NF-κB activity. Transfection of A172, T98G and U87MG cells with NF-κB luciferase construct followed by activation with TNFα produced a significant ~11, 3 and 2.6-fold increase in luciferase activity over the vector control, respectively (Fig. 3*b*). Not only did treatment with Ebselen result in a significant 50% decrease in NF-κB activity when compared with untreated control, but Ebselen was also able to significantly reduce TNFα induced NF-κB activity in all the glioma cell lines (Fig. 3*b*).

Ebselen increases DISC formation in TNFα treated glioma by enhancing association of Fas with FADD and elevating active caspase 8 expression

The TNFR1-TRADD-TRAF2 signaling complex triggered by TNFα induces prosurvival NF-κB.[29] However, TRADD-TRAF2

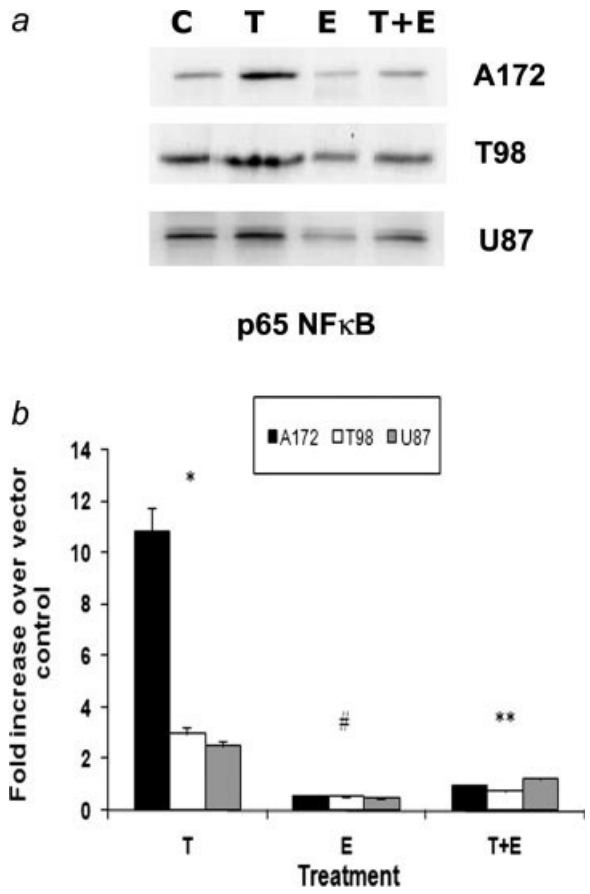

FIGURE 3 – Ebselen downregulates TNFα-mediated increase in NF-κB activity. (*a*) Ebselen decreases nuclear NF-κB p65 level in TNFα treated gliomas. Western blot analysis demonstrates NF-κB p65 expression in the nuclear extracts of A172, T98G and U87MG cells treated with either TNFα or Ebselen or both. A representative blot is shown from 3 independent experiments with identical results. (*b*) Following transient transfection of glioma cells with NF-κB luciferase reporter constructs, cells were treated with either TNFα or Ebselen or both. After 24 hr luciferase reporter assay was performed to determine NF-κB activity. The graph represents fold change in activity over control vector. Values represent the means ± SEM from 3 independent experiments. *, Significant increase from untreated control; #, significant decrease from untreated control; **, significant decrease from TNFα treated cells ($p < 0.05$).

complex can disassociate from TNFR1 to form a second complex with FADD and caspase 8. Apoptosis is activated by this second complex under conditions in which the signal from TNFR1-TRADD-TRAF2 complex fails to activate NF-κB.[31] Moreover, activation of Fas signals caspase-dependent apoptotic cell death in sensitive cells. Stimulation of Fas results in the recruitment of adaptor molecule FADD,[32] that interacts with procaspases-8 to form DISC. This complex stimulates caspase 8 activation, subsequently leading to apoptotic cell death through activation of caspase 3. As caspase 3 levels were elevated in cells treated with Ebselen and TNFα, we performed Western blot analysis to determine the levels of FADD, FasL and Fas in these cells. Treatment with TNFα alone had no effect on the expression of FasL in all the cell lines. Although expression of FasL was elevated in A172 and T98G upon treatment with Ebselen, the levels was unaffected in Ebselen treated U87MG. However, treatment with a combination of both Ebselen and TNFα elevated FasL expression in all the cell lines (Fig. 4*a*). Although treatment with TNFα elevated Fas levels in T98G when compared with the control, no such change was observed in A172 and U87MG cells. However, a marked increase in Fas levels was observed in A172, T98G and U87MG cells treated with a combination of TNFα and Ebselen. Treatment with Ebselen alone had no effect on Fas levels (Fig. 4*a*).The expression of FADD remained comparable between the different treatments and neither Ebselen nor TNFα or a combination of both had any effect on FADD expression in T98G cells (Fig. 4*a*). However, an increase in FADD expression was observed in A172 and U87MG treated with Ebselen either in the presence or absence of TNFα (Fig. 4*a*).

As FasL and Fas levels were elevated in Ebselen and TNFα treated cells and because increased caspase 8 activity is associated with the formation of DISC involving FADD and Fas, coimmunoprecipitation experiments were performed to determine Fas, FADD and caspase 8 levels. Lysates from A172 treated with TNFα in the presence or absence of Ebselen were immunoprecipitated with anti-Fas antibody, followed by Western blotting with antibodies to FADD and caspase 8. Although the expression of Fas and FADD in DISC from cells treated with TNFα was comparable to the control, an increase in both was observed in cells treated with either Ebselen or TNFα and Ebselen (Fig. 4*b*). Although the expression of cleaved caspase 8 was comparable between control, TNFα and Ebselen treated cells, a dramatic increase in active caspase-8 was observed upon treatment with a combination of TNFα and Ebselen (Fig. 4*b*).

Because increase in active caspase-8 level was observed in the DISC from cells treated with TNFα in the presence of Ebselen, we determined the levels of caspase-8 activity in cells treated with various combinations of TNFα and Ebselen. Although treatment of glioma cells with TNFα alone had no effect on caspase-8 activity, a 3.5, 5 and 5.3-fold increase in caspase-8 activity was observed in U87MG, A172 and T98G cells in the presence of Ebselen (Fig. 4*c*). A further increase in caspase 8 activity by 7, 7.8 and 9.6-fold was observed in U87MG, T98G and A172, respectively, upon treatment with a combination TNFα and Ebselen when compared with control (Fig. 4*c*). Thus, treatment of glioma cells with a combination of TNFα and Ebselen, significantly elevates caspase-8 activity.

To further confirm the involvement of caspase-8 activity in Ebselen induced increased sensitization of TNFα treated glioma cells to apoptosis, the viability of cells treated with TNFα and Ebselen in the presence and absence of caspase-8 inhibitor was investigated. The decreased cell viability observed in the presence of either Ebselen or a combination of Ebselen and TNFα, was abrogated in the presence of caspase-8 inhibitor to the levels of untreated control (Fig. 4*d*).

Ebselen effect molecules associated with cell cycle progression

As TNFα induces the expression of cMyc involved in cell cycle progression,[33] we investigated the effect of Ebselen on the expression of these molecules. Ebselen downregulated cMyc expression both in the presence and absence of TNFα in A172, T98G and U87MG glioma (Fig. 5*a*). Because p21 functions as a transcriptional repressor of the myc,[34] which can consequently arrest cells in G2/M phase of the cell cycle,[35] we investigated p21 levels in cells treated with either TNFα or Ebselen or both. The level of p21 was unaffected by treatment with TNFα, which remained comparable to the control. However, an increase in p21 level was observed in cells treated with Ebselen and TNFα (Fig. 5*a*). In A172 cells an increase in p21 levels was also observed in cells treated with Ebselen alone (Fig. 5*a*).

An increase in p21 expression in cells treated with either Ebselen or Ebselen and TNFα, indicated an alteration in cell cycle progression. We next performed FACS analysis to determine the cell cycle profile of cells treated with different combination of Ebselen and TNFα. Glioma cells were exposed to TNFα or Ebselen or both for 24 hr and subsequently processed for cell cycle analysis. A dramatic 3-fold increase of cells at the G2/M phase in A172 treated with Ebselen and TNFα, whereas treatment with either Ebselen or TNFα had no significant effect on the percentage of

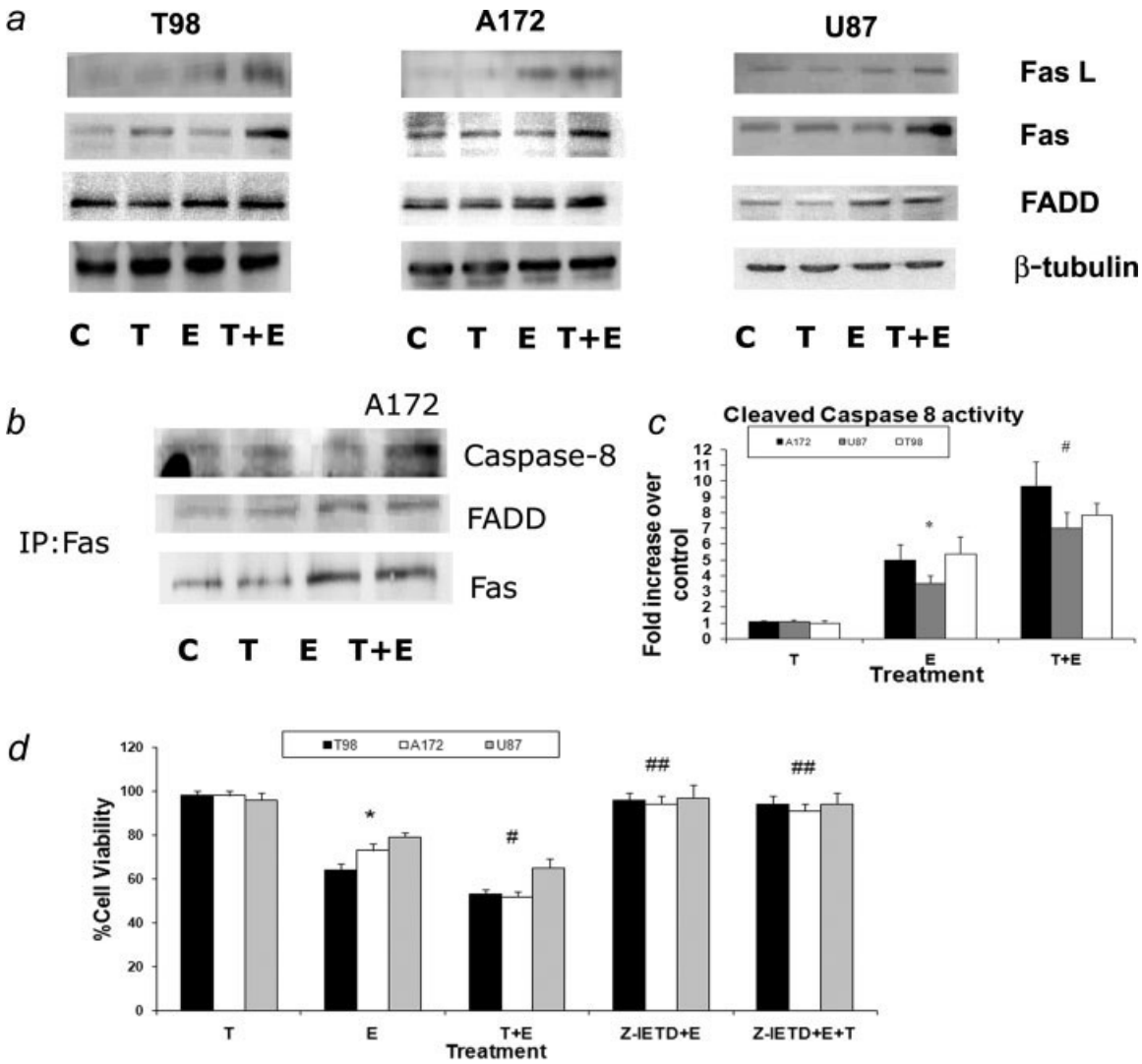

FIGURE 4 – Ebselen increases caspase-8 activity in TNFα treated glioma cells through increased formation of death inducing signaling complex (DISC) involving increased association of FAS with FADD. (*a*) Western blot analysis demonstrating expression of Fas, FasL and FADD in A172, T98G and U87MG cells treated with either TNFα or Ebselen or both. A representative blot is shown from 3 independent experiments with identical results. Blots were reprobed for β tubulin to establish equivalent loading. (*b*) Formation of death inducing signaling complex (DISC) in A172 cells treated with either TNFα or Ebselen or both were analyzed by coimmunoprecipitation. Proteins lysates from glioma treated with TNFα in the presence or absence of Ebselen were immunoprecipitated with anti-Fas antibody, followed by Western blotting with anti FADD and caspase 8. Increased DISC formation associated with elevated active caspase 8 levels was observed in Ebselen and TNFα treated glioma cells. Reprobing with anti-Fas antibody indicated an increase in Fas levels in the complex in Ebselen and TNFα treated glioma cells. A representative blot is shown from 2 independent experiments with identical results. C, T and E denote control, TNFα and Ebselen, respectively. (*c*) Fold increase in caspase-8 activity in A172, T98G and U87MG cells treated with different combination of Ebselen and TNFα for 24 hr, as determined by the Caspase-8 activity assay.(*d*) Cell viability as determined by MTS assay after treatment of cells with different combinations of Ebselen and TNFα in the presence or absence of caspase 8 inhibitor Z-IETD-FMK. Values in (*c*) and (*d*) represent the mean ± SEM from 3 independent experiments. *, Significant change from control ($p < 0.05$). #, Significant change from Ebselen alone; ##, significant change from Ebselen or Ebselen and TNFα treated cells.

cells in the G2/M phase (Fig. 5*b*). These results were not surprising as increased p21 levels in these cells suggested a possible G2/M arrest. Our results indicate that, Ebselen in combination with TNFα causes an enhanced accumulation of cells at G2/Mphase of the cell cycle. Similar trend was observed in T98G and U87MG cells (data not shown).

Discussion

Resistance to apoptosis is one of the main reasons for failure of anticancer treatments in tumor cells. Glioblastoma cells are usually resistant to apoptosis induced by death receptor ligands despite Fas expression. Although Fas a member of the TNFR family death receptors triggers apoptosis, TNFR1 is an inducer of anti-apoptotic NF-κB. Resistance of tumor cells to TNFα-induced apoptosis[5] has been attributed to the activation of NF-κB,[6–9] which plays a pivotal role in tumor progression.[36] Importantly, blockade of NF-κB sensitizes cells to TNFα induced apoptotic cell death.[6,8] Recent studies have indicated that TNFα-TNFR1-TRADD-TRAF2 pathway mediates constitutive NF-κB activation and proliferation in carcinoma, thereby making TNFα a novel target cancer therapy.[30] Combination of chemotherapeutics and TNFα has been reported to synergistically induce apoptosis in resistant tumor cells.[17]

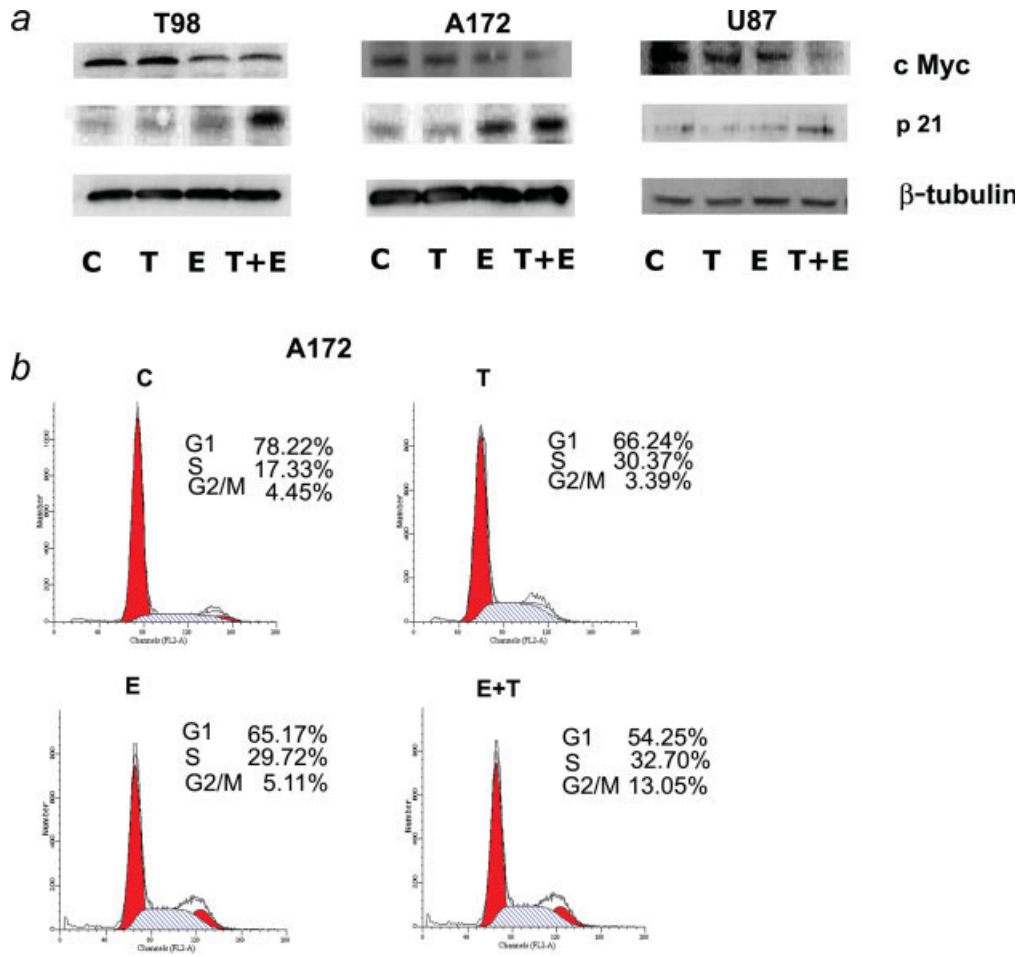

FIGURE 5 – Ebselen effects the expression of cell cycle regulators to induce G2/M arrest (*a*) The expressions of cMyc and p21 in cells treated with TNFα or Ebselen or both for 24 hr were analyzed by Western blotting. A representative blot is shown from 3 independent experiments with identical results. Blots were reprobed for β tubulin to establish equivalent loading. (*b*) Treatment of glioma cells with a combination of Ebselen and TNFα increases accumulation of cells in G2/M arrest in the cell cycle. FACS analysis was performed on cells treated with different combinations of Ebselen and TNFα, and the percentages of cells in the various cell cycle phases were plotted. FACS analysis profiles of cells treated with TNFα or Ebselen or both is shown. Insets indicated percentage cells in G1, S and G2/M phase of the cell cycle in A172 cells. C, T and E denote control, TNFα and Ebselen, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Organoselenium Ebselen inhibits TNFα induced endothelial cell activation through inhibition of TRAF2 signaling and NF-κB activation.[37] Ebselen not only inhibits inflammation induced NF-κB activity, but also suppresses the phosphorylation of JNK.[38] On the other hand, Ebselen renders protection against T cell dependent and TNFα-mediated apoptotic injury, through NF-κB-dependent mechanism.[39] Although these diverse pharmacological ability of Ebselen to modulate TNFα activity can neither be explained in the light of its antioxidant property nor its GSH peroxidase-like catalytic activity,[39,40] it is clearly evident that Ebselen affects NFκB activity to regulate subsequent signaling events. Because resistance of tumor cells to TNFα-induced apoptosis is attributed to NF-κB activation,[6–9] we investigated whether the ability of Ebselen to exert its chemopreventive effect by targeting NF-κB signaling pathway,[18] could sensitize glioma cells to TNFα-induced apoptosis.

TRADD is a crucial signal adaptor in mediating intracellular responses from TNFR1 as interaction of TRADD with TNFR1 not only triggers apoptosis,[10,11] but is also required for TNFR1 to induce NF-κB activation through TRAF2.[41] Also, exogenous TRADD expression sensitizes glioma cells to ionizing radiation and chemotherapy.[42] Our studies indicated that, TRADD and TRAF2 expression and interaction determine the fate of TNFα treated glioma cells between survival and death by triggering either NF-κB activation or apoptosis. The decrease in TRAF2 in the TNFR1-TRADD-TRAF2 complex in Ebselen and TNFα treated cells agrees with previous findings that the apoptotic pathway activated by TNFR1 does not require TRAF2.[10] Although TRAF2 is dispensable for apoptosis it is indispensable for NF-κB activation. TRAF2 coprecipitated with TRADD in TNFα treated cells consistent with elevated NF-κB activity in these cells. The decreased TRAF2 level in TNFR1-TRADD-TRAF2 complex in TNFα and Ebselen treated cells was concomitant with decrease NF-κB activity and increased apoptosis.

Concurrent with the decrease in TRAF2 in this complex, an increase in active caspase-8 level in the DISC was observed in cells treated with a combination of Ebselen and TNFα. This increase in caspase-8 activity was crucial in triggering apoptosis as inhibition of caspase-8 activity abrogated the ability of Ebselen and TNFα to induce apoptosis. Although Ebselen treated cells displayed low levels of TRAF2 in the TNFR1-TRAF2-TRADD when compared with Ebselen and TNFα treated glioma cells, enhanced caspase 8 activation in the later possibly resulted in greater cell death. In addition to downregulating NF-κB activity,

Ebselen also potentiated Fas-mediated apoptosis by enhancing caspase-8 activity in the DISC, events subsequently leading to activation of apoptotic cascades. Taken together, Ebselen sensitized TNFα treated glioma cells to apoptosis by modulating the formation of two independent complexes through separate signaling pathways. Our studies suggest that in TNFα treated cells, Ebselen utilizes Fas to transduce death signal through DISC formation. The formation of DISC was concurrent with increased TRADD and decreased TRAF2 expression that was associated with decreased NF-κB activity and apoptosis. Because increased NF-κB activation is critical for protection of leukemic eosinophils from Fas-mediated apoptosis,[21] it is possible that reduced NF-κB activity in Ebselen and TNFα treated glioma cells could have resulted in increased Fas-mediated apoptosis.

Increased accumulation of cells at the G2/M phase in cells treated with Ebselen and TNFα was concomitant with increased p21 levels. Temozolomide, a DNA-methylating agent introduced into Phase II and III trials for the treatment of GBM, induces G2/M arrest in gliomas.[43] The ability of Ebselen to modulate signaling responses in TNFα treated glioma to impair resistance to TNFα-induced apoptosis as well as to mediate a block in the G2/M phase of the cell cycle, raises the possibility that combination chemotherapy regimen consisting Ebselen and TNFα might serve as an effective therapeutic strategy.

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